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SUBSTANCE P: *IN VITRO* INACTIVATION BY RAT BRAIN FRACTIONS AND HUMAN PLASMA

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Abstract—The *in vitro* degradation of substance P (SP) by rat brain fractions and human plasma was studied by means of polyacrylamide gel electrophoresis and ^{3}H-[Nle^{11}]SP. Substance P was degraded by neutral metalloendopeptidase systems of the crude synaptosomal fraction and plasma with K_m values of 3.6×10^{-5} and 8×10^{-6} M, respectively. The peptide was also degraded by other subcellular brain fractions. The degradation rates corresponded with the biological inactivation as assayed on guinea pig ileum. The basic N-terminal sequence of the peptide was found to be important for this inactivation. No specific uptake of the peptide in the synaptosomal brain fraction could be observed.

It is concluded that *in vivo* substance P is degraded with first order dependence and that this proteolytic breakdown is responsible for the termination of the biological activity of substance P as a putative neurotransmitter or neuromodulator.

The presence of the undecapeptide substance P in the mammalian central nervous system has focused attention on its putative neurotransmitter or neuromodulator function. Substance P was found in various nerve fibres [1–5] and was localized in subcellular organelles containing nerve ending particles [6–8]. Morphological structures for storage and transport of the peptide were observed [9]. Substance P was released from synaptosomal preparations [7,8], rat hypothalamus slices [10] and the spinal cord [11] by depolarizing stimuli. Synthetic substance P showed depolarising actions on certain neurons [12–14]. There were first reports on specific binding of substance P to presumable receptor [15] and storage sites [16] in subcellular mammalian brain fractions.

These results strongly support the suggestion that substance P acts as a central neurotransmitter or neuromodulator. Another criterion for such a role is the mechanism of inactivation in the synaptic cleft. For peptides the inactivation by proteolytic degradation seems to be more likely than a reuptake system. Benuck and Marks [17] purified an enzyme from the soluble rat brain fraction degrading substance P. However, the inactivation of substance P by this fraction would require an intracellular uptake of the peptide.

The present study was undertaken to assess the biological significance of the degradation of substance P. For this purpose we have studied the *in vitro* degradation of the peptide by rat brain fractions under physiological conditions in connection with binding and uptake studies. The degradation studies also included human plasma where substance P is present [18].

As our results show, substance P is rapidly degraded by brain fractions and plasma. This degradation is responsible for the biological inactivation of the peptide. A preliminary report has been presented [19].

MATERIALS AND METHODS

Peptides. [Phe-^{3}H, Nle^{11}]substance P (^{3}H-[Nle^{11}]SP; sp. act. 27 Ci/mmole) with full biological activity on guinea pig ileum was used as labelled peptide. It was obtained by tritiation of the [L-*p*-chlorophenylalanine^{7,8}, Nle^{11}]-substance P. The synthesis of this peptide, of its unlabelled analogue [Nle^{11}]SP, and of all partial sequences of substance P is published elsewhere [20, 21]. [Asn^1, Val^5]angiotensin II was from VEB Berlin-Chemie (GDR); bradykinin was from Reanal (Hungary).

Other substances. Bacitracin from AIS Apothekernes Lab. (Norway); aprotinine (Contrykal®) from Arzneimittelwerk Dresden (GDR); soybean trypsin inhibitor from Reanal (Hungary); 1.4-dithioerithritole from Ferak (FRG).

Human plasma. Human plasma was prepared from heparinized blood. The plasma from 40 healthy donors was pooled and stored frozen.

Subcellular rat brain fractions. The whole brains (without cerebellum) and hypothalami, respectively, were isolated from Wistar rats of both sexes and homogenized in 0.32 M sucrose 1mM EDTA 10mM imidazole buffer (pH 7.4) by 5–10 strokes with a Teflon–glass homogenizer (800 rev/min). The following subcellular fractions were prepared by differential centrifugation according to Whittaker [22]: nuclear (P_1, 1000 *g* for 10 min; this fraction was discarded); crude mitochondrial (P_2, 10 000 *g* for 20 min); microsomal (P_3, 100,000 *g* for 60 min); and soluble (S_3, supernatant above the P_3 pellet). The P_2 fraction was further fractionated by centrifugation of the resuspended (in homogenization medium) pellet into a discontinuous density gradient (0.8 and 1.2 M sucrose) to obtain the following fractions: myelin (A), synaptosomal (B) and mitochondrial (C) [22]. All pellets were rehomogenized by hand and washed with incubation medium.

Binding studies were performed directly after preparation. The proteolytic activity was stable at 4° for several weeks (as tested for P_2).

The protein concentration was determined by the method of Lowry *et al.* [23] using human serum albumin (HSA) as standard.

Incubation medium. Unless otherwise stated, for the

brain fractions a solution with 140 mM NaCl, 4 mM KCl, 1.2 mM $MgCl_2$, 2.3 mM $CaCl_2$, 0.1% HSA and 20 mM imidazole buffer with pH 7.25 at 37° (BF-medium) was used. The HSA was added to the BF-medium and to all stock peptide solutions to avoid surface adsorption of the peptides. Sodium phosphate buffer (10mM, pH 7.4 at 37°) in 154 mM NaCl (PBS) was used for plasma.

Degradation studies. Whereas the intact ^{3}H-[Nle^{11}]SP is positively charged, the breakdown of the peptide has to produce products of both charges. We have established that during the degradation of the peptide by brain and plasma products are formed which contain phenylalanine and which are negatively charged at pH 8.4. Because these products in our case contain the ^{3}H-label as [^{3}H]phenylalanine, an electrophoretic assay was looked for to separate the sum of the labelled negatively charged degradation products from the labelled positively charged intact peptide (no labelled products with a positive charge could be observed). This separation was achieved best by polyacrylamide gel electrophoresis (PAG electrophoresis) in which the degradation products with the ^{3}H-label migrated into the gel contrary to the intact peptide.

Twenty per cent (v/v) plasma and the brain fractions (<0.8 mg protein/ml incubation mixture), respectively, were incubated with the labelled peptide ^{3}H-[Nle^{11}]SP and the indicated additions. The reaction was stopped by transferring 400 μl incubation solution into 50 μl 5 M HCl under ice. After centrifugation 50–100 μl (for the lowest concentrations 1000 μl) supernatant made to 10% sucrose were applied to the PAG electrophoresis. A 15% crosslinked acrylamide gel was used. The gels were polymerized with *N, N, N′, N′*-tetramethylethylenediamine and ammonium persulfate in the presence of 0.1% HSA in polyethylene tubes (diameter 5 mm, height of gel 70 mm). The buffer for electrophoresis was 0.05 M Tris –0.038 M glycine –0.1% HSA (pH 8.4). The sample was layered over 100 μl 25% sucrose in incubation medium above the gel. The electrophoresis was performed at 3 mA per tube until the front had migrated about 6 cm into the gel (toward the plus pole). The gel tubes were cut into discs of about 5 mm and the gel was separated from the tube material. The gel discs and the tube material from one gel tube were placed in a total of four scintillation vials. For the study of the ^{3}H-activity profile in the gel about 2 mm discs were used. The vials were shaken with 1.5 ml 5% sodium dodecyl sulfate at about 40° for 2 days. Ten millilitres of a toluene–Triton X-100 (2:1) scintillator were added and the samples were monitored for ^{3}H in an LKB-Wallac S1000 counter.

The percentage of degradation in the samples was calculated according to

$$\text{degradation} = \frac{A_t - A_o}{A_\infty - A_o} \times 100$$

where A_o, A_t and A_∞ are the activities migrating into the gel from samples taken at the time 0, t and a time sufficient for complete degradation, respectively. The activities were expressed as percentages of the total activity applied to the gels. A_o as a blank value was about 5 per cent; A_∞ as the recovery value was about 90 per cent.

At least three samples were taken from one incubation at different times. From the degradation ratios obtained the degradation velocity at $t = 0$ was calculated according to the first order dependence in terms of the peptide concentration (see Results).

Binding and uptake studies. ^{3}H-[Nle^{11}]SP was incubated with the P_2 fraction of the whole rat brain or hypothalamus. Two hundred and fifty microlitre samples were layered over two layers consisting of 100 μl 6% sucrose in incubation medium and 10 μl 40% dioctylphthalate–60% dihexylphthalate in 400 μl polyethylene microtubes. The thin phthalate layer reduced the 'trapping' of radioactivity into the tips of the tubes which was controlled by means of [^{14}C]inulin. After centrifugation the supernatants were removed by suction and the tips with the pellets were cut off. The pellets were solubilized with Soluene-350. A toluene scintillation cocktail was added and the samples were counted for ^{3}H. Furthermore, binding studies of the labelled peptide to plasma and HSA were performed by means of equilibrium dialysis.

RESULTS

Method of degradation studies. The present assay for degradation of substance P using the labelled analogue ^{3}H–[Nle^{11}]SP was compared with the degradation in terms of biological inactivation. As seen in Fig. 1 the degradation rates by the P_2 fraction and plasma obtained by the PAG electrophoresis correlated directly with the biological inactivation assayed by the isolated guinea pig ileum [24]. The electrophoretic assay, however, was simpler and allowed determinations at peptide concentrations as low as 10^{-10} M (Fig. 4) (corresponding to 10 fmoles per gel tube). The degradation velocity increased linearly with the protein concentration of the P_2 fraction but not with increasing plasma concentration (Fig. 2). Incubations within 3 hr of both the P_2

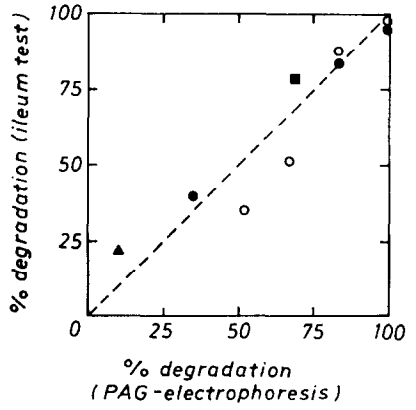

Fig. 1. Comparison of the degradation rates of [Nle^{11}]SP assayed by PAG-electrophoresis and contraction test on guinea pig ileum 1.5×10^{-5} M [Nle^{11}]SP with a tracer of ^{3}H-[Nle^{11}]SP was incubated with 20% (v/v) human plasma (●), plasma plus 0.1% bacitracin (■), plasma plus 5 mM *o*-phenanthroline (▲) and rat brain P_2 fraction (0.54 mg protein/ml (○) at 37°. Different incubation times were chosen to obtain appropriated degradation values also in the presence of inhibitors. The percentage of degradation was determined by the two methods. The dotted line is the theoretical one for direct correspondence.

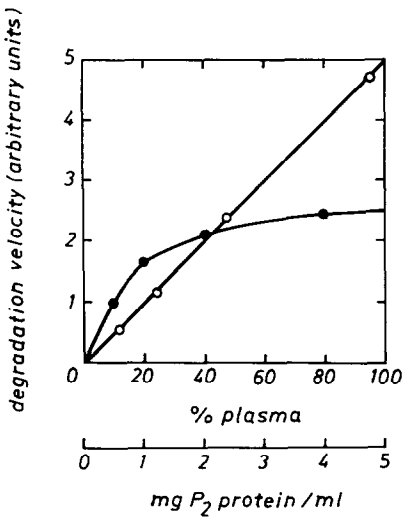

Fig. 2. Dependence of ^{3}H-[Nle^{11}]SP degradation velocity on the concentration of rat brain P_2 fraction (○) and human plasma (●). 3×10^{-8} M peptide, 37°.

fraction and plasma at 37° did not change the degradative activity.

The gel radioactivity profile of the degradation products showed one broad peak near the start and a second at the front. The latter was identified as phenylalanine from an incubation of the P_2 fraction with 5×10^{-4} M [Nle^{11}]SP and tracer of ^{3}H-[Nle^{11}]SP by amino acid analysis (Amino Acid Analyzer 881, Mikrotechna, Czechoslovakia). The percentage of this peak increased with the progress of degradation (Fig. 3), but not strictly linearly, probably due to the interplay of the consecutive breakdown steps of the peptide and its fragments.

Peptide concentration dependence of degradation. The degradation velocity of [Nle^{11}]SP and ^{3}H-[Nle^{11}]SP as a function of the peptide concentration was studied in the wide range from 10^{-10} (nearly physiological) to 10^{-4} M to clarify if there are different enzyme systems responsible for the degradation at physiological and higher concentrations. Figure 4 shows that the P_2 fraction of the rat brain and human plasma degraded [Nle^{11}]SP with only one K_m value of 3.6×10^{-5} and 8×10^{-6} M, respectively (maximum activity 2.7 nmoles/min per mg P_2-protein and 0.16 nmoles/min per 1 ml 20% plasma, respectively). At concentrations below 10^{-7} M the *in vitro* half-life of the peptide at 37° was found to be 9.3 min per mg P_2-protein/ml and 24 min for 100% plasma (by taking into account the dependence on plasma concentration; Fig. 2).

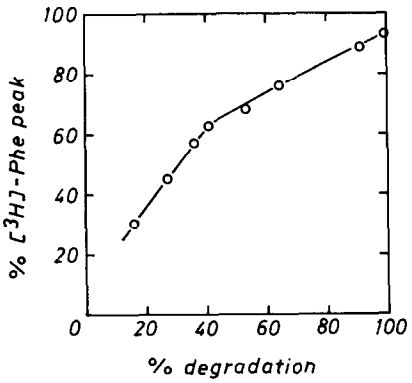

Fig. 3. The percentage of the [^{3}H]Phe peak within the labelled degradation products of ^{3}H-[Nle^{11}]SP in the gel. 5.4×10^{-7} M peptide was incubated with rat brain P_2 fraction at 37°.

Influence of temperature, pH and substances on degradation. Decrease of temperature to 0° strongly decreased the degradation velocity of ^{3}H-[Nle^{11}]SP by rat brain P_2 and human plasma to only about 4% of that at 37° (Fig. 5). The enzyme activity at 37° had a broad optimum between pH 7.0 and 8.0 and was decreased at pH 6.0 to 30 and 48% for P_2 and plasma, respectively (in comparison to pH 7.5). The omission of Ca^{2+} and Mg^{2+} in the BF-medium, the use of PBS as medium and the presence of 1 mM phenylalanine did not change the degradation velocity by the P_2 fraction, whereas 20 mM Tris diminished the velocity by 24%. The influence of some potential inhibitors is shown in Table 1. Bacitracin (K_i 0.11 mg/ml) and particularly *o*-phenanthroline (K_i 0.1 mM) were strong inhibitors for the enzymes in P_2 and plasma, whereas aprotinine had only weak inhibitory activity.

The specifity of the degradative enzyme activity was studied by the inhibition of the degradation of ^{3}H-[Nle^{11}]SP by several unlabelled peptides (Fig. 6). The native substance P inhibited the degradation of the labelled Nle-analogue to the same extent as the unlabelled [Nle^{11}]SP. The inhibition by shorter C-terminal sequences of substance P was substantially lower. On the other hand, bradykinin, angiotensin II and the N-terminal sequence of substance P Arg-Pro-Lys-Pro also inhibited the degradation.

Localization of proteolytic activity in subcellular brain fractions. Besides the crude mitochondrial fraction P_2, other particulate subcellular fractions, such as the microsomal fraction (P_3) and subfractions of P_2, also contained degradative enzyme activity (Table 2). High activity was found in the soluble supernatant of the 100,000 *g* pellet of the brain homogenate. Therefore, it is possible that the activity of the other fractions were artefacts due to adhering soluble fraction. However, the enzyme activity did not dissociate from the P_2 pellet (i) after two additional washings with incubation medium, (ii) during 2 hr incubation at 0 or 37° and (iii) during the density gradient centrifugation. Therefore, it was assumed that the hydrolysing activity of the particulate fractions is intrinsic.

Uptake and binding studies. These were performed to evaluate the significance of the degradation in comparison with a possible uptake mechanism.

The [^{3}H]phenylalanine formed quickly during incubation of ^{3}H-[Nle^{11}]SP with P_2 at 37° (Fig. 3) was bound to the P_2 fraction and displaced by unlabelled phenylalanine (K_{50} about 1.5×10^{-4} M) and by other amino acids, preferentially by hydrophobic ones (Fig. 7). From the Scatchard plot it was concluded that phenylalanine was bound to a heterogenous population of binding sites for amino acids which might be related to the low affinity amino acid transports [25]. This binding influenced the binding and uptake studies with

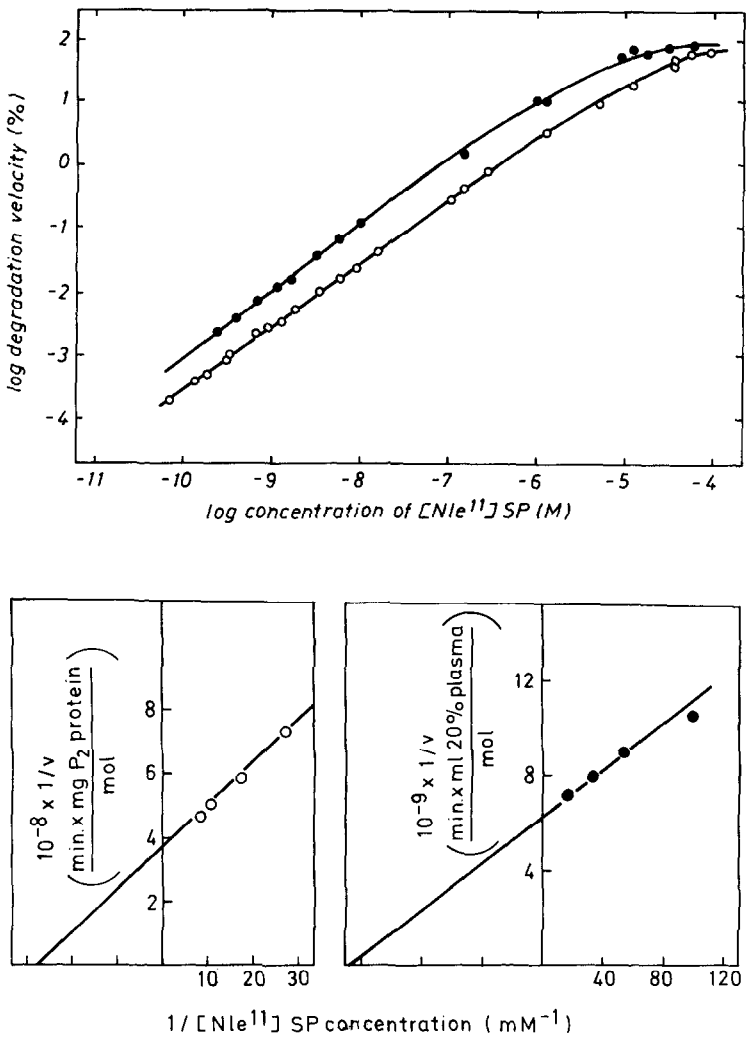

Fig. 4. (a) Plot of log degradation velocity (as percentage of the maximum activity) of [Nle^{11}]SP vs log peptide concentration. Mixtures of labelled and unlabelled peptide were incubated at 37° with 0.2–0.5 mg protein/ml of the rat brain P_2 fraction (○) and 20% (v/v) human plasma (●). (b,c) Lineweaver–Burk plot with the high peptide concentrations obtained from the data shown in Fig. 4a.

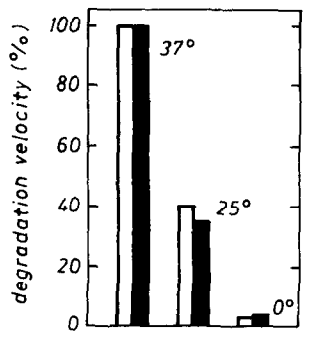

Fig. 5. Influence of temperature on degradation velocity of 2.1×10^{-8} M ^{3}H-[Nle^{11}]SP by the P_2 fraction of rat brain (□) and by human plasma (■). One hundred per cent corresponded to 1.55 pmoles/per min per mg P_2 protein and 0.42 pmoles per min per ml 20% plasma, respectively.

^{3}H-[Nle^{11}]SP at low physiological concentrations as an aspecific component. The amount of the intact peptide bound to the P_2 fraction was only 25% in comparison with that of [^{3}H]phenylalanine and already little degradation contributed largely to the overall binding. Therefore, binding and uptake studies were performed at 37° in the presence of inhibitors or at 0°. Under these conditions the degradation could be neglected. Uptake studies showed that a small amount of ^{3}H-[Nle^{11}]SP was bound. However, this binding did not show the character of a specific uptake system [26], due to (i) the small temperature dependence, (ii) the increase of binding without Na^+ and (iii) its aspecific character (Fig. 8). In further binding studies with the P_2 fraction at 0° a displacement by the unlabelled peptide of the bound ^{3}H-[Nle^{11}]SP was detected (Fig. 9). Using 1.4×10^{-8} M labelled peptide the displacement was observed over a concentration range up to nearly

Table 1. Influence of some potential inhibitors on the degradation velocity of 5.4×10^{-8} M [^{3}H-Nle^{11}]SP

Inhibitor	Inhibitor concentration	Degradation velocity (%) P_a	Plasma
None (control)	—	100*	100†
Tryspin inhibitor (from soybean)	1 mg/ml	83	108
Aprotinine (Contrykal)	1 mg/ml	73	80
Phenylmethylsulfonyl fluoride (PMSF)	0.2 mM	74	69
HSA	17 mg/ml‡	70	
	30 mg/ml‡		100
EDTA	10 mM	53	47
EDTA–(without Ca^{2+}–Mg^{2+})	1 mM	45	
Dithioerithritole	5 mM	53	34
Fusaric acid	1 mM	27	
Bacitracin	1 mg/ml	19	28
o-Phenanthroline	5.0 mM	5	4
	0.2 mM	23	
	0.04 mM	83	

Incubations were performed with the P_2 fraction of rat brain (0.26 mg protein/ml BF-medium) and 20% (v/v) human plasma in PBS at 37°.

* 4.2 pmoles per min per mg protein.

† 1.1 pmole per min per ml 20% plasma.

‡ In addition to 1 mg/ml HSA in the BF-medium (P_2) and about 9 mg/ml HSA in 20% plasma, respectively.

10^{-4} M unlabelled peptide. It can be suggested that the displacement at these high concentrations reflects the degradative enzyme system because the activity of the latter was saturated in the same concentration range. However, the labelled peptide was also displaced to some extent by unlabelled peptide concentrations below 10^{-7} M where the enzyme acts with first order dependence. It is possible that this part reflects binding to receptor sites which are predominantly seen at lower labelled peptide concentrations (1.5×10^{-9} M; Fig. 9) because in this case the amount of low affinity binding is low in comparison to that of the high affinity binding.

No binding of ^{3}H-[Nle^{11}]SP to either HSA or whole human plasma could be detected.

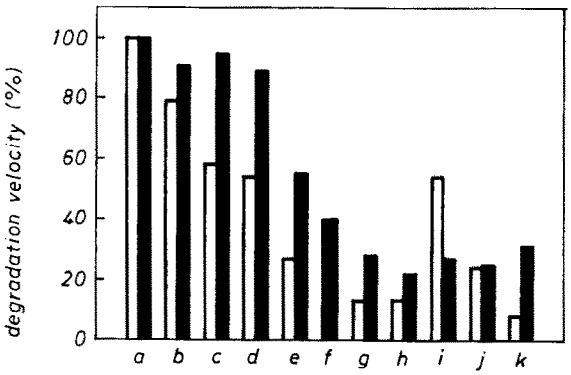

Fig. 6. Influence of unlabelled peptides on the degradation velocity of ^{3}H-[Nle^{11}]SP. Incubations: 8.9×10^{-5} M unlabelled peptides, 2.3×10^{-8} M labelled peptide and 0.5 mg protein/ml of the rat brain P_2 fraction (□), 37°, pH 7.25; 6.7×10^{-5} M unlabelled peptides, 2.3×10^{-8} M labelled peptide and 20% (v/v) human plasma (■), 37°, pH 7.4. The following unlabelled peptides were used: (a) without (control); the sequences of native substance P (SP): (b) SP$^{10-11}$; (c) SP$^{8-11}$; (d) SP$^{6-11}$; (e) SP$^{3-11}$; (f) SP$^{2-11}$; (g) SP$^{1-11}$; (h) [Nle^{11}]SP; (i) SP$^{1-4}$; (j) bradykinin; (k) [Asn^1, Val^5]angiotensin II. One hundred per cent corresponded to 2.0 nmoles per min per mg P_2 protein and 0.13 nmoles per min per ml 20% plasma, respectively.

Table 2. [^{3}H-Nle^{11}]SP hydrolysing activity in rat brain subfractions

Fraction*		Enzyme specific activity (%)†
Crude mitochondrial	(P_2)	100‡
Myelin	(A)	25
Synaptosomal	(B)	107
Mitochondrial	(C)	231
Microsomal	(P_3)	136
Soluble	(S_3)	827

Incubations were done with 1.8×10^{-8} M peptide at 37° in BF-medium.

*Letters in brackets refer to the classification of subcellular fractions as used by Whittaker [22].

†Activity was calculated per mg protein and compared in per cent.

‡ 1.26 pmole per min per mg protein.

DISCUSSION

Substance P is assumed to be a central neurotransmitter or neuromodulator. Starting from the current hypothesis that peptidases involved in the control of the concentration of such a peptide in the synaptic cleft

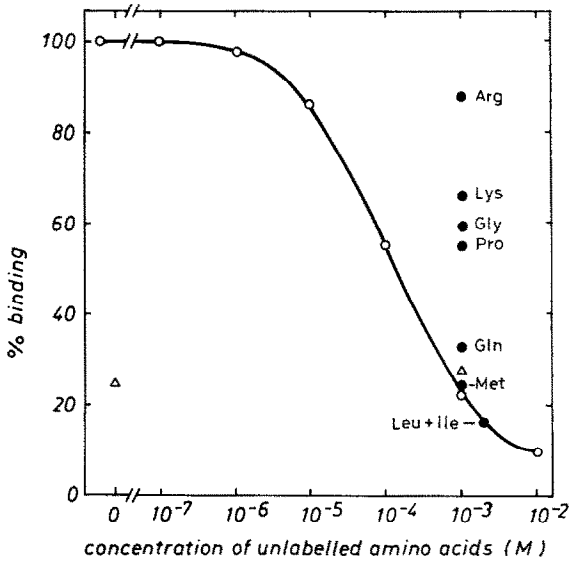

Fig. 7. Displacement of the binding of [^{3}H]phenylalanine to the rat brain P_2 fraction by unlabelled phenylalanine (○) and other amino acids (● and indicated). ^{3}H-[Nle^{11}]SP was degraded by the P_2 fraction and the resulting labelled 2.9×10^{-8} M phenylalanine was bound to fresh P_2 (10 min at 25°). One hundred per cent corresponded to 0.96 pmoles [^{3}H]phenylalanine/mg protein. As a comparison, the binding of the same concentration of intact labelled peptide is also shown (△).

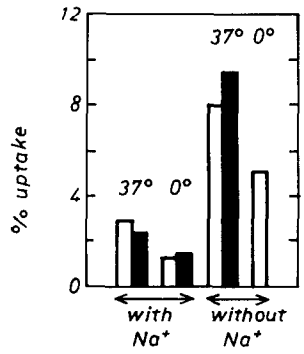

Fig. 8. Uptake experiments with the rat hypothalamus P_2 fraction and ^{3}H-[Nle^{11}]SP. 2×10^{-9} M labelled peptide was incubated without (□) and in the presence (■) of 200-fold excess of unlabelled peptide (■) (10 min, 0 and 37°, 1.3 mg P_2 protein/ml). Medium: 4 mMKCl, 1.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, 1 mM EDTA, 15 mM Tris–HCl with pH 7.4, 5 mM *o*-phenanthroline, 0.1% HSA and, in addition, 170 mM NaCl ('with Na^+') and 0.32 M sucrose ('without Na^+'), respectively.

should be membrane-bound we have used the synaptosomal fraction for degradation studies. To elucidate the *in vitro* degradation of substance P an electrophoretic assay was developed, based on the separation of the negatively charged degradation products containing the [^{3}H]phenylalanine residue from the positively charged intact peptide. Using ^{3}H-[Nle^{11}]SP this method also allowed degradation studies in the physiological concentration range. This peptide, differing from the native substance P by exchange of the C-terminal methionine residue by norleucine, had nearly the same biological activity on guinea pig ileum as the native form [20]. Both peptides inhibited the degradation of the labelled norleucine analogue to the same extent (Fig. 6). The degradation assay used reflects the biological inactivation rate (Fig. 1). From these facts it is concluded that the present results reflect physiological conditions and

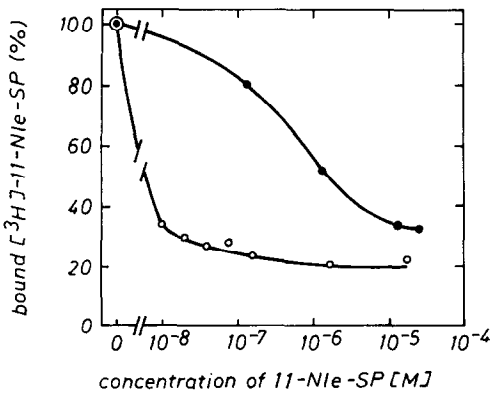

Fig. 9. Displacement by unlabelled [Nle^{11}]SP of the binding of ^{3}H-[Nle^{11}]SP to the rat brain P_2 fraction. Incubations: labelled peptide 1.4×10^{-8} M (●) and 1.5×10^{-9} M (○), 1.6 mg P_2 protein/ml BF-medium, 20 min at 0°. 100% binding corresponded to 6.8 and 10.9% binding per mg protein of the labelled peptide added for 1.4×10^{-8} and 1.5×10^{-9} M labelled peptide, respectively.

permit conclusions for the *in vivo* inactivation of substance P.

Peptide degradation studies including substance P [17, 27, 28] were often performed with high peptide concentrations. The question remains open as to whether the enzymes, observed under such conditions, are of physiological importance. For the degradation of substance P by the rat brain P_2 fraction and human plasma the present study has revealed only one enzyme system with K_m values of 3.6×10^{-5} and 8×10^{-6} M, respectively (Fig. 4). This allows conclusions for physiological conditions from studies with higher substance P concentrations. For this reason we have generally used about 3×10^{-8} M peptide, which was methodically easily applicable.

A second conclusion is that, *in vivo*, the inactivation of the peptide proceeds with first order dependence under all conditions. This could be of regulatory importance in so far as an increase of peptide concentration is answered by a proportional increase of degradation velocity. The single first order dependence also confirmed in progression curves at peptide concentrations below 10^{-7} M (not shown) is not in accordance with the biphasic pattern for the degradation of substance P by human plasma as found by Bury [28] with bioassay and by Skrabanek [29] with radioimmunoassay.

In the present study, substance P was exposed to the whole proteolytic capacity of plasma and brain fractions. It can be assumed that among all possible degradation steps by various enzymes those which are responsible for the biological inactivation were observed. This approach differed from investigations done with partially purified brain enzymes by Benuck and Marks [17] and Akopyan *et al.* [30]. Such a purified enzyme need not necessarily be of great importance for the biological inactivation of the peptide. Nevertheless, the enzyme system detected as physiologically essential seems to be identical with the endopeptidase system found in the soluble brain fraction by Benuck and Marks [17]. In both cases phenylalanine was rapidly split off (Fig. 3). Furthermore, a splitting by endopeptidases in our system was assumed because negatively charged degradation products (containing the [^{3}H]phenylalanine residue as assayed by the method used) required internal cleavage steps. Due to the rapid cleavage in the C-terminal region which is responsible for the biological activity [31] no shorter sequences with substance P-like activity were intermediately accumulated. It was shown that the X-Pro dipeptidylaminopeptidase can hydrolyze N-terminal dipeptide Arg^1—Pro^2 and subsequent dipeptide Lys^3—Pro^4 from substance P [32, 33]. However, with the P_2 fraction we did not observe inhibition by four chromogenic substrates (concentrations 6 times the K_m value) for this enzyme of the degradation of ^{3}H-[Nle^{11}]SP. Therefore, it is assumed that this enzyme is not involved in the inactivation of substance P in the brain.

From the inhibition by chelating agents (Table 1), a metalloenzyme character of the enzyme system is concluded. Other peptides such as bradykinin and angiotensin II also seem to be substrates (Fig. 6). However, among the sequences of substance P, only the undecapeptide had the full substrate activity. Furthermore, the N-terminal basic sequence Arg-Pro-Lys-Pro also de-

creased the degradation of ^{3}H-[Nle^{11}]SP although it could not be a substrate for the enzyme system under study (Fig. 6). This part of the peptide can be assumed to be essential for substrate enzyme binding. The N-terminal sequence alone is probably a competitive inhibitor. These facts do not exclude the possibility that there are other enzymes which can split shorter substance P sequences more effectively.

In consideration of the similar properties of the degradation by plasma and brain, the enzymes in both tissues can be assumed to be very similar. However, it remains open as to why the specific plasma activity decreased with increasing plasma concentrations (Fig. 2). The higher albumin concentration in the case of higher plasma concentration was obviously not responsible for this effect. First, albumin did not decrease the degradation by plasma (Table 1) and second, no binding of the ^{3}H-[Nle^{11}]SP to albumin and the whole plasma was detectable. It has to be assumed that the total amount of substance P in plasma is in the free form.

Substance P is a neurotransmitter or neuromodulator candidate. Therefore, the main point was to clarify whether the degradation or a reuptake is responsible for the biological inactivation in the synaptic region. We have observed binding of ^{3}H-[Nle^{11}]SP to the crude synaptosomal brain fraction which could be partially displaced by unlabelled peptide (Fig. 9). No binding, however, with the characteristic of a specific uptake system was detected (Fig. 8). The binding is suggested to be composed of enzyme binding (at high peptide concentrations) and receptor binding (at low peptide concentrations). Nakata *et al.* [15] have characterized the specific binding of substance P to high affinity sites assumed to be receptors but they, too did not find an uptake [34]. Quantitative estimations from our data show that 1 mg P_2 protein/ml binds only about 3% of the peptide from the medium whereas about 50% was degraded within 10 min at 37°.

From all these facts the conclusion is drawn that the enzymatic degradation is responsible for the biological inactivation in the central nervous system. Such a conclusion was also drawn by Iversen *et al.* [10] from the decline of substance P-like immunoreactivity in suspensions of rat hypothalamic slices. Furthermore, it must be assumed that the enzyme is localized in the membrane, being active towards the synaptic cleft. However, high proteolytic activity was also found intracellularly (Table 2). Due to the lack of an uptake system, this activity should not substantially participate in the inactivation of substance P delivered into the synaptic cleft. It is possible that it has a function in the regulation of the intracellular level of substance P at the presynaptic side.

Up to the present it is not clear if there is any function for substance P in circulation. Therefore, the physiological significance of the degrading system in plasma is not clear.

In summary, the results provide strong evidence that a neutral metalloendopeptidase system in the synaptic region terminates the biological activity of substance P. This enzymatic activity also found in plasma is proportional to the peptide concentration. Whereas the C-terminal sequence of substance P is responsible for the biological activity, the N-terminal one is important for the termination of activity.

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