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BIOLOGICAL ACTIVITY AND ENZYMIC DEGRADATION OF SUBSTANCE P ANALOGS: IMPLICATIONS FOR STUDIES OF THE SUBSTANCE P RECEPTOR

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SUMMARY: Partial sequences of Substance P, either free or blocked at their amino terminal, have been examined for their stability towards inactivation by homogenate or particulate fractions of rat brain and for their relative potencies as smooth muscle contractors. The C-terminal hexapeptide in both the free and blocked forms displays activity comparable to that of the longer C-terminal peptides as well as to that of the native undecapeptide. The blocked peptides, however, are much more stable than their corresponding free peptides. Among the free peptides Substance P is degraded slower than the free hexa- and hepta-peptides, suggesting that the N-terminal tetrapeptide part may play a role in stabilizing the molecule. Blocked hepta- and octapeptide analogs, carrying probe properties, may be useful for studies of the Substance P receptor.

INTRODUCTION

Substance P (SP), a hypotensive and smooth muscle contracting agent first isolated from equine brain and intestine (1), is an undecapeptide of the structure Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (2,3). This peptide is widely distributed in peripheral and central nervous systems of vertebrates, where it is thought to act as a transmitter (4-6). Methods for determining the concentration and distribution of SP have improved greatly since it was first synthesized (7). However, attempts to characterize the receptor of SP have been hampered by the presence of tissue proteases which rapidly degrade SP as well as several of its radioactive analogs. To overcome this handicap, we have undertaken a search for peptide derivatives that bind tightly to the receptor and are less susceptible to proteolytic digestion. Having examined the degradation of amino terminal-free and blocked partial sequences of SP by rat brain preparations, we found that the blocked peptides are far more resistant to proteolysis than the corresponding free peptides. The free hexa- and hepta-

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peptides are hydrolyzed faster than SP itself, indicating that the amino terminal tetrapeptide part may be important for stabilization of the peptide. The blocked hepta- and octapeptides, which display virtually the same smooth muscle contracting potency as SP, are significantly more resistant to proteolysis than the native peptide, suggesting that similarly blocked derivatives with appropriate probe properties could be used to study the SP receptor.

MATERIALS AND METHODS

SP-peptides. SP and C-terminal partial sequences of SP, either free at the amino terminal or blocked with Boc, were synthesized by conventional methods of peptide synthesis. Amino terminal-free peptides, isolated as hydrochlorides, were recrystallized from ethanol or ethanol/ether. Boc-peptides were recrystallized from ethanol or ethanol/water. The purity of the peptides was checked by thin layer chromatography (Silica; *n*-butanol/acetic acid/water, 4:1:1 or 2:1:1) and high performance liquid chromatography (Lichrosorb RP-8; 4 mM triethylammonium phosphate, pH 3.0/AcCN, 60:40). The compounds were characterized by their melting point, optical rotation, nitrogen and sulfur content and amino acid analysis.

Assay of SP-peptides. Activity of SP-peptides was assayed on isolated guinea pig ileum as described by Rossel *et al.* (8), employing some minor modifications. The ileum was suspended in 10 ml organ bath containing Tyrode solution (149 mM NaCl, 2.7 mM KCl, 3.6 mM $CaCl_2$, 2.1 mM $MgCl_2$, 0.4 mM KH_2PO_4, 11.9 mM $NaHCO_3$, 5 mM glucose), thermostated at 37°C and bubbled with air. Isotonic concentrations were measured with a smooth muscle transducer and recorded on a chart mover, both from Harvard Apparatus. Dose-response curves were obtained by adding the peptide to the bath and washing with Tyrode solution as soon as a steady response was reached. The time between peptide addition and washing was shorter than 30 sec and between two consecutive additions of peptide longer than 2 min to avoid desensitization. Fresh stock solutions of the free or blocked peptides were made up daily by dissolving the peptides in small amounts of dimethylsulfoxide and then diluting 1000-fold with 0.16 M NaCl. The concentration of the organic solvent in the assay mixture did not exceed 0.01%. Each peptide was characterized by an ED_{50} value corresponding to a peptide concentration which causes 50% of the maximal response. No hydrolysis of SP and its analogs was observed during the time of exposure to the ileum.

Preparation of rat brain homogenates. Male Sprague-Dawley rats of 50 days were decapitated, their brains rapidly removed and homogenized (1:10, w/v) with ice cold buffer A [136 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 1 mM ethylene glycol-bis(ß-aminoethyl ether)-N,N'-tetraacetic acid, 1 mM dithioerythritol (DTE), pH 7.4] at 1000 rpm, using 8 up and down strokes of a teflon pestle in a glass homogenizer. The homogenate was filtered through cheese cloth and diluted with ice cold buffer A to a final concentration of 1% or 5%. The SP degrading activity of these homogenates was stable for at least 4 h.

Preparation of particulate fraction. The rat brains were homogenized (1:10, w/v) with ice cold buffer B (20 mM sodium phosphate, 0.32 M sucrose, pH 7.4) at 1400 rpm. After centrifugation at 1000 *g* for 10 min the pellet was discarded and the supernatant centrifuged at 20000 *g* for 20 min. The twice washed pellet was suspended in buffer B and kept frozen at -80°C. The SP degrading activity of the particulate fraction was stable for at least 10 days. Prior to use it was suspended in buffer A. Protein concentration was estimated by the method of Lowry *et al.* (9).

Degradation of SP-peptides. The method for monitoring the degradation of SP analogs by brain homogenate and particulate fraction was similar to that described by Krivoy (10), using the guinea pig ileum assay. The peptide ($\sim$2 x 10^{-7} M) was incubated with the homogenate or particulate fraction at 24°C with gentle magnetic stirring. Samples were removed and assayed at intervals. The sample size was selected to be equivalent to a volume that contained 2 to 3 times the ED_{50} of the peptide at the beginning of the experiment and was determined from the dose-response curve measured on the same ileum prior to the degradation experiment. The half-life of each peptide, defined as the time required to decrease its concentration 2-fold, was found to be the time taken to reduce concentration from 80% to 50% of the full response.

RESULTS AND DISCUSSION

Since a reliable binding assay for SP and its analogs is not yet available, the relative affinities of SP fragments to the SP receptor can only be inferred, e.g. by using a guinea pig ileum contraction bioassay. The dose-response curves obtained for different peptides were parallel and the maximal response reached by them was virtually the same. The shortest peptide fragment displaying contracting activity was the C-terminal tripeptide Gly-Leu-Met-NH_2 with an ED_{50} = 5 x 10^{-4} M (Table I), while the dipeptide Leu-Met-NH_2 failed to elicit any response even at a concentration as high as 10 mM. The contracting potency increased as a function of the peptide chain length until an ED_{50} value of $\sim$2 x 10^{-9} M was reached at the heptapeptide level. The octapeptide and SP had about the same potency as the heptapeptide (Table I). The observations that even the

Table I

Relative potencies of Substance P sequences in contracting the guinea pig ileum

C-Terminal peptide	*Peptide with free amino terminal* $p(ED_{50}) \pm 0.2$[a]	*Peptide blocked with Boc* $p(ED_{50}) \pm 0.2$[a]
(9-11) Tripeptide	3.3	N.D.
(8-11) Tetrapeptide	5.0	N.D.
(7-11) Pentapeptide	6.6	7.7
(6-11) Hexapeptide	8.3	8.5
(5-11) Heptapeptide	8.6	8.6
(4-11) Octapeptide	8.6	8.6
Substance P	8.6	N.D.

[a] Represents averages of 3-8 experiments. N.D. = Not determined.

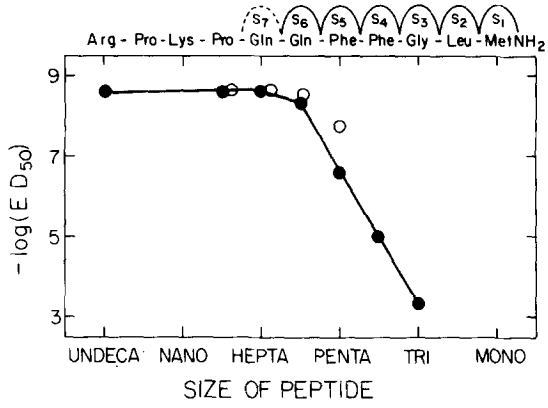

Figure 1. Effect of peptide chain length on the guinea pig ileum contracting potency of C-terminal partial sequences of Substance P. The ED_{50} values of the free (•) and Boc (o) peptides imply that the Substance P receptor can accomodate 6-7 amino acid residues in sites designated S_1, S_2, *etc.*

small tri-, tetra- and pentapeptides can display full contracting response, albeit at higher peptide concentrations than SP, is rather unusual when compared with other biologically active peptides, where larger segments of the native peptide are needed for the expression of the biological response.

Blocking of the N-terminal residue with Boc resulted in a substantial increase in the potency of the pentapeptide and had only a slight effect on the potency of the hexa-, hepta- and octapeptides (Table I). These observations, taken together with previous structure-function studies of SP carried out with various bioassays (11-14), indicate that the SP-receptor interacts only with six or seven amino acid residues corresponding to the C-terminal part of the eleven amino acid SP molecule (Fig.1).

On the basis of their activity, the hexa- to octapeptides and their N-blocked derivatives seem to be the simplest compounds for probing the receptor of SP. However, the stability of such peptides has also to be considered since SP is known to be degraded by various tissues including brain (15-19). In the case of the opioid peptides, successful stabilization of the molecule was achieved by substituting a Gly residue with DAla. Using a similar approach we have synthesized SP analogs in which Gly^9 was replaced with DAla or βAla, but these substitutions resulted in a substantial loss of potency. However,

Table II

Half life of Substance P sequences in rat brain preparations[a]

C-Terminal peptide	Homogenate t/2(min) free	Homogenate Stability ratio blocked/free	Particulate fraction t/2(min) free	Particulate fraction Stability ratio blocked/free
(6-11) Hexapeptide	1 ± 0.5	20	1.5 ± 0.5	8
(5-11) Heptapeptide	1 ± 0.5	45	2 ± 0.5	12
(4-11) Octapeptide	2 ± 1	22	5 ± 2	6
Substance P	5 ± 2	-	5 ± 2	-
IPP-Heptapeptide[b]	-	40	-	15

[a] Peptide concentration was 2 x 10^{-7} M. Both amino terminal-free (free) and blocked with Boc (blocked) peptides were used. The peptides were incubated with 1% homogenate or 1 mg particulate protein/ml.

[b] IPP-Heptapeptide is the heptapeptide blocked with Bolton-Hunter N-blocking group (4-hydroxy-5-iodophenyl)propionyl (20).

the use of other amino acids, e.g. *N*-methylamino acids, may prove useful in the future. Since the blocked derivatives of the natural partial sequences of SP display high potency, we studied their stability and compared it to that of the free peptides and to SP itself. Table II shows that the rate of inactivation of the free heptapeptide by rat brain homogenate is ∿40-fold greater than the rate of inactivation of the Boc-heptapeptide. The same relationship also pertains to the hexa- and octapeptides where the blocked peptides are ∿20-fold more stable than the free peptides (Table II). Interestingly, among the free peptides the hexa- and heptapeptides are more susceptible to inactivation than SP itself, suggesting that the amino terminal tetrapeptide segment of the molecule may be important for the stability of the peptide.

The particulate fraction of rat brain also degrades SP and its partial sequences very effectively (Table II), raising the possibility that the observed enzyme activity may be related to a specific process of SP inactivation since the latter is expected to be carried out by membrane-bound enzymes. Again, SP is more stable than the free hexa- or heptapeptides and the blocked peptides are more stable than the free peptides (Table II, Fig.2). These results indicate that SP and its analogs are substrates of both brain aminopeptidase(s) and endopeptidase(s). The stabilizing effect of the blocking group seems, to a

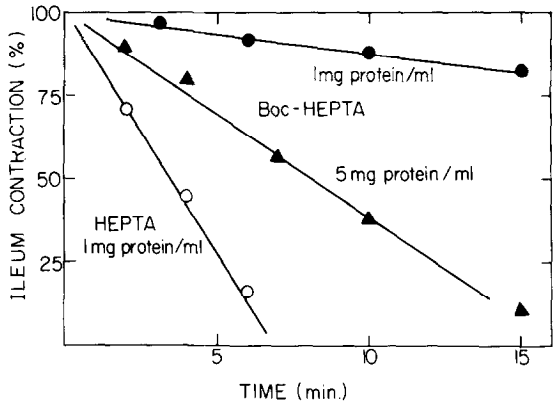

Figure 2. Effect of blocking group on the rate of degradation of the C-terminal heptapeptide of Substance P, by rat brain particulate fraction. Free heptapeptide was hydrolyzed by 1 mg particulate protein/ml at 24°C (o); Boc-heptapeptide was hydrolyzed by 1 mg protein/ml (●) and by 5 mg protein/ml (▲). Peptide concentration was 2 x 10^{-7} M. Activity was assayed by the guinea pig ileum contraction bioassay. Ileum contraction at time zero was taken as 100% (see Materials and Methods).

great extent, to be due to the prevention of aminopeptidase(s) action. Such enzymes are metalloenzymes and, indeed, the presence of the transition metal ion-chelator 1,10-phenanthroline (10^{-3} M) reduces the rate of inactivation of the free hexa- or heptapeptides 3 to 5-fold, but not the rate of inactivation of the corresponding blocked peptides. The blocking group may also interfere with the recognition of the peptide by endopeptidase(s). This effect is more pronounced for the hepta- and octapeptides than for the hexapeptide (Table II).

The stabilizing effect of the blocking group is not limited to Boc but applies to the other blocking groups examined, e.g. the heptapeptide blocked with 3-(4-hydroxy-5-iodophenyl)propionyl (20) displays stability similar to the Boc-heptapeptide (Table II). This suggests that similarly radioactively labeled or fluorescent derivatives could be employed in studies of the SP receptor in preference to the whole molecule (21).

The conservation of the relative potencies of the different derivatives as measured by various bioassays (11-14), suggests that the receptor molecules involved share similar structural characteristics, all recognizing the hexa- or heptapeptide C-terminal part of the molecule. Yet a different effect of SP, the induction of neurite extension of neuroblastoma cells in culture, has

recently been reported and attributed to the N-terminal tetrapeptide part of the molecule (22). It has also been observed that an enzyme from bovine brain cleaves SP into an N-terminal tetrapeptide and a C-terminal heptapeptide (19) raising the possibility that such a post-proline cleaving enzyme may be involved not only in the turnover of SP but also in its conversion into two active peptides. The search for a distinct receptor for the N-terminal tetrapeptide part of SP would require an approach complementary to the one adopted here.

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