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ACTIVITY OF SUBSTANCE P ANALOGS SUBSTITUTED AT THE GLY^9 AND GLN^6 POSITIONS

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SUMMARY: We have prepared peptide derivatives of Substance P in which Gly^9 or Gln^6 in the C-terminal part of the molecule have been substituted and we have examined the activity of these peptides using the guinea pig ileum bioassay. Gly^9 can be substituted without a significant effect on the activity by Ala or Sar but not with DAla or βAla. Derivatives of the C-terminal pentapeptide were prepared and were almost as potent as the undecapeptide Substance P. The results presented are of particular relevance for the future design of radioactive receptor ligands of high affinity, of Substance P antagonists and of affinity labels.

INTRODUCTION

The undecapeptide Substance P (SP) [1,2] is widely distributed in the peripheral and central nervous systems of vertebrates [3]. It is probably involved in the neurotransmission of pain [4,5] and in sensory axon reflexes [6,7]. Most of the properties of SP can be attributed to the C-terminal heptapeptide portion of the molecule, Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 [8-11]. The basic N-terminal tetrapeptide Arg-Pro-Lys-Pro, however, appears to be responsible for functions which are independent of, and often complementary to, those of the C-terminal heptapeptide. It seems to stabilize SP against proteolytic degradation [12], to modulate the activity of SP on various tissues [13], to exert a stimulatory effect on phagocytes [14] and to release histamine from mast cells [15]. These *in vitro* properties of the N-terminal sequence distinguish SP from other tachykinins, which have a highly conserved C-terminal pentapeptide sequence (Phe-X-Gly-Leu-Met-NH_2 where X is Phe, Tyr,

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Ile or Val) with a variable N-terminal sequence [16] and which display properties similar to those of the C-terminal heptapeptide fragment of SP.

In spite of the increasing interest in the elucidation of the various functions of SP and of its partial segments, only limited chemical work has been carried out so far to characterize the relationship between the structure of SP analogs and their function. As a result, SP derivatives with potent antagonist or affinity label properties have not yet been reported. To progress towards these ends, we have prepared peptide derivatives of SP in which Gly^9 or Gln^6 in the C-terminal part of the molecule have been substituted, and we have examined the activity of these peptides using the guinea pig ileum bioassay. We found that Gly^9 can be replaced with Ala or Sar without a significant effect on the activity while substitution with DAla or βAla results in a great decrease in activity. A variety of substitutions in the Gln^6 position result in compounds which are more active than the free C-terminal pentapeptide, thus stressing the importance of subsite S_6 of the SP receptor [12]. The results presented in this paper are of particular relevance for the future design of SP receptor ligands, SP antagonists and affinity labels.

MATERIALS AND METHODS

Materials

The C-terminal pentapeptide of SP, Phe-Phe-Gly-Leu-Met-NH_2·HCl, was obtained by reacting Boc-Phe-Phe-Gly (Boc=tert-butyloxycarbonyl) via its N-hydroxysuccinimide ester, with Leu-Met-NH_2, followed by removal of the Boc group by HCl/acetic acid. Blocking of the pentapeptide with acetyl, pyroglutamyl or 3-(4-hydroxy-3-iodophenyl)propionyl was performed with acetic anhydride, pyroglutamyl pentachlorophenyl ester or 3-(4-hydroxy-3-iodophenyl)propionyl N-hydroxysuccinimide ester, respectively.

Boc-Gln-Phe-X-Leu-Met-NH_2, where X = Ala, DAla or βAla, were obtained by reacting Boc-Phe-Phe-X via their N-hydroxysuccinimide esters (ONSu) with Leu-Met-NH_2, followed by removal of the Boc with HCl/acetic acid and reaction of the pentapeptide hydrochloride with Boc-glutamine ortho-nitrophenyl ester. Boc-Gln-Phe-Phe-Sar-Leu-Met-NH_2 was obtained by reacting Z-Phe-Sar (Z = carbobenzoxy) with Leu-Met-NH_2 followed by removal of the blocking group with HBr/acetic acid then reaction with Boc-Phe-ONSu, removal of the blocking group and reaction with Boc-glutamine ortho-nitrophenyl ester. The peptides were characterized by elemental (C,H,N,S) and amino acid analyses.

Table I
Activity of Substance P derivatives substituted at Gly^9

Peptide	$p(ED)_{50} \pm 0.2$
Substance P	8.6
Boc-Gln-Phe-Phe-Gly-Leu-Met-NH_2	8.5
Boc-Gln-Phe-Phe-Ala-Leu-Met-NH_2	8.5
Boc-Gln-Phe-Phe-Sar-Leu-Met-NH_2	8.4
Boc-Gln-Phe-Phe-DAla-Leu-Met-NH_2	7.0
Boc-Gln-Phe-Phe-βAla-Leu-Met-NH_2	7.0

Boc, tert-butyloxycarbonyl.

Biological Activity

The activity of the peptides was assayed on the isolated guinea pig ileum as described by Rosell et al. [17]. The ileum was suspended in 10 ml organ bath containing Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.5 mM glucose) thermostated at 37° and bubbled with air. Isotonic contractions were measured with a smooth muscle transducer 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 3 min, to prevent desensitization. Each dose-response curve was composed of at least 7 different peptide concentrations and between 2-5 different dose-response curves were established for each peptide. Each stock solution of peptide was made up daily by dissolving it in dimethylsulfoxide (0.5-5.0 mg/100 µl) and diluting it 1000-fold with 0.16 M NaCl.

RESULTS AND DISCUSSION

Activity of Boc-hexapeptide derivatives substituted at the Gly^9 position

In the structure of SP, the Gly^9 residue separates the molecule into two hydrophobic moieties Phe-Phe and Leu-Met-NH_2 [2]. The importance of this amino acid was investigated by substituting it with residues having slightly different structures. For this purpose we have prepared a series of Boc-hexapeptide analogs and compared their activities with that of the Boc derivative of the native hexapeptide fragment of SP. Table I shows that the Ala^9 and Sar^9 analogs display virtually the same ileum contracting activity as the native Gly^9 derivative. Similar effects on the activity of eledoisin were observed upon substitution of Gly^9 by Ala^9 or Sar^9 [18]. However, the substitution of Gly^9 with $DAla^9$ in Boc-hexapeptide is accompanied by a marked

decrease of activity. These results contrast with those obtained with enkephalins where the DAla^2 analog is actually more active than the native Gly^2 derivative. This phenomenon is probably due to an enhanced resistance of the peptide to degradation [19]. A different situation exists in the case of the luteinizing hormone-releasing hormone where the DAla^6 analog is four-fold more active than the native Gly^6 analog while the Ala^6 analog displays only 4% of the activity of the native derivative [20]. The βAla^9 analog of Boc-hexapeptide, which is longer by one methylene group than the peptide with the native sequence, also shows a marked decrease of activity. The relatively low potencies displayed by the βAla^9 and DAla^9 analogs of Boc-hexapeptide in contracting the ileum can be accounted for by a decrease in their receptor binding affinity and/or by their inability to effectively trigger the biological response. If the latter alternative applies, then βAla^9 and DAla^9 derivatives of SP modified also at other positions might form the basis for a systematic search for SP antagonists.

Activity of hexapeptide derivatives substituted at the Gln^6 position

Studies of the dependence of the guinea pig ileum contracting potency on peptide chain length of SP C-terminal partial sequences, indicate that the SP receptor accommodates optimally the seven amino acid residues of the C-terminal heptapeptide. We have therefore proposed, using the analogy of the active site of proteolytic enzymes such as papain [21] and carboxypeptidase A [22], that the SP receptor is composed of seven subsites denoted S_1 to S_7 [12]. The interactions of each amino acid of the C-terminal heptapeptide sequence with its individual subsite are not equivalent and some subsites appear more important than others. For instance, the importance of the interaction of Gln^6 with S_6 as compared to that of Gln^5 with S_7 is emphasized by the fact that the addition of a Gln^6 residue to the C-terminal pentapeptide increases the potency by almost two orders of magnitude whereas the subsequent addition of a Gln^5 residue improves the potency by only 0.3 log unit [12]. In order to

Table II
Activity of Substance P derivatives substituted at Gln^6

Peptide	$p(ED)_{50} \pm 0.2$
Substance P	8.6
Phe-Phe-Gly-Leu-Met-NH_2	6.6
Gln-Phe-Phe-Gly-Leu-Met-NH_2	8.3
Ac-Phe-Phe-Gly-Leu-Met-NH_2	7.9
Boc-Phe-Phe-Gly-Leu-Met-NH_2	7.7
Glp-Phe-Phe-Gly-Leu-Met-NH_2	8.6
IPP-Phe-Phe-Gly-Leu-Met-NH_2	8.4

Ac, acetyl;
Boc, tert-butyloxycarbonyl;
Glp, pyroglutamyl;
IPP, 3-(4-hydroxy-3-iodophenyl)propionyl.

study more closely the steric requirements of this important subsite S_6, we have synthesized several hexapeptide derivatives substituted at the Gln^6 position and tested their activity on the guinea pig ileum. Table II shows that an effective interaction with subsite S_6 does not require strict structural features. Amino acids or blocking groups, such as acetyl or tert-butyloxycarbonyl, can improve markedly the contracting potency of the pentapeptide derivatives. The acetylpentapeptide has an ED_{50} value of 7.9 ± 0.2 whereas the Boc-pentapeptide has a slightly lower activity, probably due to a steric hindrance of the bulky tert-butyloxycarbonyl group. The most effective interaction with S_6 is achieved by the pyroglutamyl residue: the pyroglutamyl pentapeptide is as active as SP. Almost as effective as SP is the 3-(4-hydroxy-3-iodophenyl)propionyl [23] derivative of the C-terminal pentapeptide. All these results, while showing the functional importance of subsite S_6, stress its weak steric restrictions. They also suggest that affinity and photoaffinity labels of the SP receptor can be prepared by introducing suitable reactive moieties as blocking groups of the C-terminal pentapeptide. Furthermore, in view of the existence of two classes of SP receptors that differ in their interactions with the N-terminal segment of SP [13], derivatives of the C-terminal pentapeptide should be the most appropriate ligands for the study of the various SP receptors.

It is interesting that none of the analogs reported here displays an activity significantly higher than that of the natural sequence of SP. The failure to obtain "superactive" SP analogs with activity much higher than the native sequence suggests that SP possesses the optimal sequence, while it also reflects the fact that only a small number of SP analogs have been synthesized so far. The synthesis of a greater number of SP analogs is needed to further establish the structure-function relationships and to find antagonists of SP. Such antagonists are essential for clarification of the transmitter role of the peptide and could also constitute a new class of compounds with analgesic properties.

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