be postulated that the presence of GM1 ganglioside on the surface of the presynaptic membrane is involved in the rapid uptake of the B-subunit of cholera toxin, since it binds specifically to GM1 ganglioside[6]. It has been shown that tetanus toxin, conjugated to colloidal gold, is selectively taken up in nerve endings in the iris of the rat[12]. Like cholera toxin, tetanus toxin binds specifically to gangliosides[11]. Thus, the binding of macromolecules to gangliosides at the surface of the presynaptic membrane may greatly enhance endocytosis. This may be an important function of gangliosides which occur in high concentrations at the synaptic regions[13].

According to the recycling model of synaptic vesicles of Heuser et al.[2, 14, 15] which is based on studies on frog neuromuscular junction, synaptic vesicles coalesce with the axolemma during transmittor release. Membrane is then retrieved by coated vesicles which coalesce to form cisternae from which new synaptic vesicles are formed. During this recycling, exogenous macromolecules may be endocytosed[2, 14]. It has been shown in the neuromuscular junction of the frog that efficient uptake of horseradish peroxidase into synaptic vesicles is a process dependent on stimulation of the nerve for a considerable period[16]. It has not been established whether the uptake of macromolecules which is related to retrograde axonal transport is dependent on stimulation of the nerve, although synaptic activity may to some extent influence the amount of material retrogradely transported[17]. The finding in the present study of numerous colloidal gold particles within the terminal axon as early as 5 min after administration into an inactive muscle may indicate occurrence of endocytosis which is independent of recycling of vesicles during transmitter release. This is further supported by the fact that macromolecules are also taken up in sensory nerve terminals[18, 19]. Since the endocytosed particles appeared in both synaptic and coated vesicles the type of vesicle involved in the endocytosis could not be determined in this study.

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Hypotensive activity of histidine-containing analogues of C-terminal hexapeptide of Substance P

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Summary. Four new hexapeptide analogues of C-terminal Substance P fragment with increased solubility in aqueous solutions are described. The peptides contain histidine in positions 6, 8, 9 and 10, respectively. The effect of the structural changes on the hypotensive activity and antigenic properties of analogues was compared. It was found that substitution of amino acid residues in various positions in the C-terminal hexapeptide of Substance P resulted in different effects on the hypotensive and antigenic properties, respectively. Only the [His^6] SP_{6-11} analogue had an unchanged antigenic structure when compared with the C-terminal region of Substance P, but it showed an almost total loss of hypotensive activity. The [His^9] SP_{6-11} analogue retained 50% of the hypotensive activity of the C-terminal hexapeptide but showed a markedly reduced expression of the antigenic epitope localized in this region of Substance P.

Key words. SP_{6-11} analogues; hypotensive activity; antigenic properties.

Substance P, discovered in horse brain by von Euler and Gaddum[1], is an undecapeptide with sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH_2. The C-terminal hexapeptide and its pyroglutamyl-analogue [pGlu^6] SP_{6-11} show the full activity of the native Substance P in most biological tests[2] and are a very useful model to study the structure-function relationships of this neuropeptide. Previous studies showed that the substitution of the C-terminal MetNH_2 and Phe^7 by other amino acids leads to a complete loss of biological activity[3].

Owing to the poor solubility of the C-terminal hexapeptide in aqueous medium the C-terminal hexapeptide of Substance P is not very convenient to use. Therefore we attempted to synthetize analogues of the C-terminal hexapeptide with improved solubility. For this purpose, analogues containing histidine in positions known from previous studies not to be involved in the biological function of SP_{6-11} were prepared and their biological activity tested.

Material and methods. Peptides. Four hexapeptides were synthesized by a classical solution method[4]. The purity of the peptides was checked routinely by thin-layer chromathography (TLC) on Merck silica gel plates. In addition, melting points and specific rotations were taken for all peptides and compared with those from the literature when available (table 1). Final products of synthesis, i.e. all hexapeptides, were characterized by amino acid

composition analysis. The intermediate peptides Leu-$MetNH_2 \cdot HCl$, Gly-Leu-$MetNH_2 \cdot HCl$, and Phe-Phe-Gly-Leu-$MetNH_2 \cdot 2HBr$ were prepared according to Lübke et al.[5]. The hexapeptide His-Phe-Phe-Gly-Leu-$MetNH_2 \cdot 2HBr$ was obtained by condensation of Di-Z-His with Phe-Phe-Gly-Leu-$MetNH_2 \cdot HCl$ by the DCC/HOBT method followed by HBr/CH_3COOH deprotection. In the same way the condensation of Di-Z-His with Gly-Leu-$MetNH_2 \cdot HCl$, Leu-$MetNH_2 \cdot HCl$, and $MetNH_2 \cdot HCl$ was performed and the resulting intermediates were elongated stepwise up to hexapeptides by the mixed anhydride method[6].

Bioassay. Experiments in vivo were performed on female Wistar rats weighing from 200 to 250 g. The animals were anesthetized with urethane (1.4 g/kg) and each peptide was injected into the jugular vein of six rats. Arterial blood pressure was recorded directly with a mercury manometer, through a cannula inserted into the right carotid artery, on a smoked drum driven by a Palmer kymograph[7]. 200 µl of peptide solution at a concentration of 5×10^{-7} moles/l were rapidly injected in regular intervals of 10 min. The maximal fall of arterial blood pressure was expressed in mm Hg. The high and prolonged vasodepressor response was calculated according to the formula:

$Vd.r. = hx\ (t_{1/3}+t_{2/3})$,

where Vd.r. is vasodepressor response; h, maximal fall of blood pressure in mm Hg; $t_{1/3}$ and $t_{2/3}$, time in seconds during which blood pressure remained below 1/3 and 2/3 of its maximal fall, respectively.

Radioimmunoassay. N-succimidil 3-(hydroxyphenyl) propionate (Tagit) was conjugated with [Lys^6] SP_{6-11} by reaction with both the α- and ε-amino groups of the N-terminal Lys. The ability of the hexapeptide to lead to competitive inhibition of the binding of specific antibodies to SP_{6-11} with [Lys^6] SP_{6-11} containing Tagit and radioiodinated [^{125}I-T-Lys^6] SP_{6-11} was examined by an equilibrium competitive radioimmunoassay[8]. The analytic parameters CI_{50}, CI_{max}, and CI_s were calculated from the concentration required for 50% competitive inhibition, the maximum attainable inhibition, and the competitive inhibition slope, respectively[9].

Results and discussion. The first question to be asked in these studies was whether the histidine-containing analogues of SP_{6-11} preserved the native conformation of the C-terminal region of Substance P. In previous studies[8] we described the antigenic epitope localized in this region of Substance P. Therefore, using a radioimmunoassay system permitting us to analyze the expression of this epitope, we could draw conclusions about structural and conformational changes within the peptide region bearing the epitope. For this reason four new hexapeptide analogues of [$pGlu^6$] SP_{6-11} were synthesized. Each peptide contained one histidine residue, in the position 6, 8, 9 or 10. Phe^7 and $MetNH_2$ are considered to be indispensable for binding with receptors[3], and therefore were not substituted. Histidine with its polar side chain was expected to increase solubility of the analogues in aqueous solutions. As it is seen in table 1, the solubility of all four hexapeptides increased 100- to 200-fold when compared with [$pGlu^6$] SP_{6-11}. The new synthetic analogues with histidine in the position 8, 9 or 10 showed significantly reduced expression of the SP_{6-11} antigenic epitope as judged by changes in values of competitive inhibition parameters such as CI_{50}, CI_s, and CI_{max} (table 2). The analogue containing histidine in the position 6 had an almost unchanged antigenic structure when compared with the C-terminal region of Substance P. However, its biological activity was reduced to 5% of that shown by SP_{6-11}. In contrast to that, the analogue with histidine in position 9 showed a critical loss of expression of the SP_{6-11} epitope, but it retained 50% of the original biological activity. The remaining three analogues had no effect on the arterial blood pressure.

In summary, it appears that only the [His^9] SP_{6-11} may be of potential value as the analogue which can be used in place of SP_{6-11}. These studies further support the concept that the biologically active region of the C-terminal hexapeptide of Substance P is different from the antigenic epitope.

Table 1. Physicochemical data of [$pGlu^6$] SP_{6-11} and its His-containing analogues

Peptide	Melting point (°C)	$[\alpha]_D^{20}$	Water solubility (g/l)
pGlu-Phe-Phe-Gly-Leu-$MetNH_2$	200–203	−39° (c = 1, DMF)	0.01
His-Phe-Phe-Gly-Leu-$MetNH_2$	159–162	−9.5° (c = 1, MeOH)	1.4
pGlu-Phe-His-Gly-Leu-$MetNH_2$	185–188	4.5 (c = 1, MeOH)	1.2
pGlu-Phe-Phe-His-Leu-$MetNH_2$	191–193	4.6° (c = 1, MeOH)	2.0
pGlu-Phe-Phe-Gly-His-$MetNH_2$	decomp.	2.0° (c = 1, MeOH)	1.0

Table 2. Biological activity and antigenic expression of the SP_{6-11} epitope showed by histidine-containing analogues of the C-terminal hexapeptide of Substance P

Analogue	Rat blood pressure Vd.r (%)	n	Competitive inhibition parameters CI_{50} (nmoles)	CI_s	CI_{max} (at 10^{-5} M)
pGlu-Phe-Phe-Gly-Leu-$MetNH_2$	100	10	20	0.315	1.0
His-Phe-Phe-Gly-Leu-$MetNH_2$	5	6	50	0.240	1.0
pGlu-Phe-His-Gly-Leu-$MetNH_2$	0	6	44,500	0.470	0.17
pGlu-Phe-Phe-His-Leu-$MetNH_2$	50	6	15,800	0.470	0.41
pGlu-Phe-Phe-Gly-His-$MetNH_2$	0	6	22,300	0.230	0.26

Vd.r = vasodepressor response (calculated from the formula written in 'methods'; n = number of determinations; CI_{50} = concentration required for 50% competitive inhibition; CI_s = competitive inhibition slope; CI_{max} = maximum attainable inhibition.

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