PERSISTENT ACTION OF *N*-METHYLATED ANALOGS OF SUBSTANCE P ON RAT PAROTID SLICES

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1. Introduction

Substance P, a hypotensive and smooth muscle contracting agent, was first found in equine brain and intestinal extracts [1]. The active compound was isolated in pure form from bovine hypothalami and was found to be an undecapeptide of the structure: Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2 [2,3]. The highest concentrations of SP have been found in the hypothalamus, the spinal cord and the nervous system supplying the gastro-intestinal tract. Preferential localization of SP in the dorsal column of the spinal cord and its ability to excite spinal motoneurons suggest that SP is involved in sensory transmission [4,5]. The presence of SP in the peripheral plasma raised the possibility that the peptide can be released into the blood stream and act like a hormone on distal organs [6–8]. However, intravenous application of SP results in short-lived effects that hamper detailed analysis of its function. Proteolytic degradation of SP was observed in various smooth muscle [9] and brain [10–12] preparations and in plasma [7,9,13]. Although metabolically stable analogs of SP could be useful in studies of SP action, to data no such analog has been reported. In an effort to prepare metabolically stable SP analogs we have chosen the hexapeptide [pGlu^6]SP_{6-11} as the parent compound and synthesized peptide analogs in which specific amino acid residues were substituted by the corresponding *N*-methyl derivatives. Peptides containing *N*-methyl amino acid residues are resistant to the action of various proteolytic enzymes [14–17] and produce protracted biological activities in vivo [18,19].

2. Materials and methods

2.1. *Synthesis*

Boc-*N*-methyl phenylalanine (Boc *N*-MePhe) and Boc-*N*-methyl leucine (Boc *N*-MeLeu) were prepared according to [20]. Peptides were synthesized in solution by stepwise coupling of Boc amino acids from the C-terminal end. Coupling was effected by the excess mixed anhydride method, using isobutylchloroformate [21]. Deprotection was achieved by 4 N HCl in acetic acid. All peptides as well as intermediate compounds were characterized by elemental analysis, melting point, optical rotation and amino acid analysis. Purity was assessed by TLC and reverse-phase HPLC.

2.2. *In vitro assays of SP analogs*

Parotid slice system: Rat parotid slices were prepared and incubated as in [22]. To prevent adsorption of the hydrophobic peptides to glass, vials were pretreated with 1% trimethylchlorosilane in toluene, and gelatin (0.1%) was added to the incubation medium. Stock solutions and appropriate dilutions of SP analogs were prepared in *N*,*N*-dimethylformamide (DMF). Aliquots of 10–20 μM of the agonist solution were added to the slice system. In control systems, 1% DMF had no deleterious effect on K^+ release or its reuptake into the slices. ED_{50} is the concentration of analog required to produce half-maximal K^+ release within 2 min from its addition to the slice system. Relative potency is the ED_{50} ratio of the analog and that of [pGlu^6]SP_{6-11}, expressed as %. The figures show the results of typical experiments, each repeated for at least 4 times. A control system containing the parent compound [pGlu^6]SP_{6-11} was included in each experiment.

Isolated guinea pig ileum assay was done as in [23].

3. Results

Studies of SP degradation by chymotrypsin [3] and papain [24] as well as by a partially purified brain neutral protease [11] indicated that the peptide is cleaved at the Gln^6–Phe^7 or Phe^7–Phe^8 and at the Gly^9–Leu^{10} bonds. We report here on structural analogs of SP which were designed to resist proteolytic cleavage of these specific peptide bonds. The hexapeptide pGlu–Phe–Phe–Gly–Leu–Met–NH_2 ([$pGlu^6$]SP_{6-11}) was chosen as the parent compound, as this hexapeptide is as potent as the whole undecapeptide both in the guinea pig ileum and in the rat parotid assays. Furthermore, the presence of blocked amino- and carboxy-terminals protects the hexapeptide against amino and carboxy exopeptidases. To prevent hydrolysis of the Gln^6–Phe^7 and Gly^9–Leu^{10} bonds the analogs [$pGlu^6$, *N*-MePhe^7]SP_{6-11} and [$pGlu^6$, *N*-MeLeu^{19}]SP_{6-11} were prepared. The anaogs and the unmodified parent compound were tested on rat parotid slices. Addition of [$pGlu^6$]SP_{6-11} to the parotid slice system initiated a rapid and transient release of K^+ from the cells. Maximal K^+ release was achieved within 2 min, after which time the amount of K^+ in the medium decreased due to reuptake of K^+ into the cells (fig.1). We have shown that the transient effect of SP on the parotid slice system is due to inactivation of the peptide and not due to a decline in the response of the tissue [22]. The inactivation of the peptide in this system is probably caused by proteolytic cleavage, as addition of bacitracin (1 mg/ml) caused a delay in reuptake of K^+ into the slices (not shown). The peak amount of K^+ release into the medium was used to assay the potency of the analogs and the delay in reuptake of the released K^+ was used to assess their metabolic stability. It should be pointed out that in order to test the metabolic stability of an analog, the peptide has to be applied at concentrations that do not saturate the receptor. At supersaturating concentrations, considerable degradation of the peptide can take place without effect on receptor occupancy.

Addition of the analog [$pGlu^6$, *N*-MeLeu^{10}]SP_{6-11} to the parotid slice system caused a rapid K^+ release like that elicited by the unmodified hexapeptide, but net reuptake of the released K^+ was observed only after a considerable delay and proceeded at a slow rate. The effect of [$pGlu^6$, *N*-MeLeu^{10}]SP_{6-11} on K^+ release decreased to its half-maximal extent in 60 min (fig.2). The relative potency of this analog was 45 ± 5%. Application of the analog [$pGlu^6$, *N*-MePhe^7]-

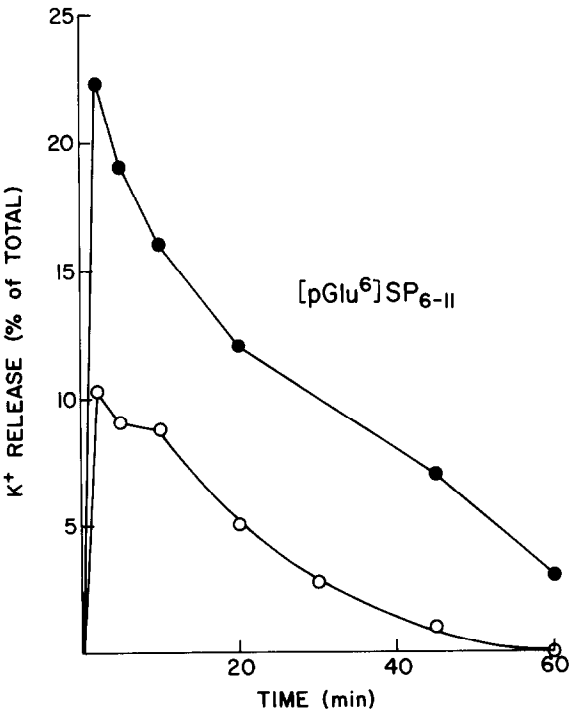

Fig.1. Time course of K^+ release from rat parotid slices caused by the addition of [$pGlu^6$]SP_{6-11} (○) 40 nM, (●) 5 μM at zero time.

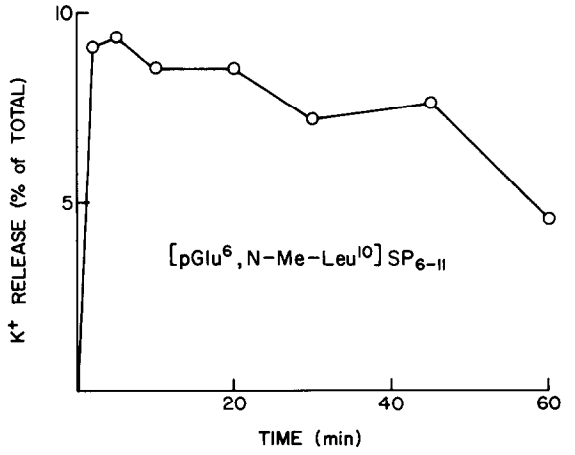

Fig.2. Time course of K^+ release from rat parotid slices caused by the addition of [$pGlu^6$, *N*-MeLeu^{10}]SP_{6-11} 80 nM at zero time.

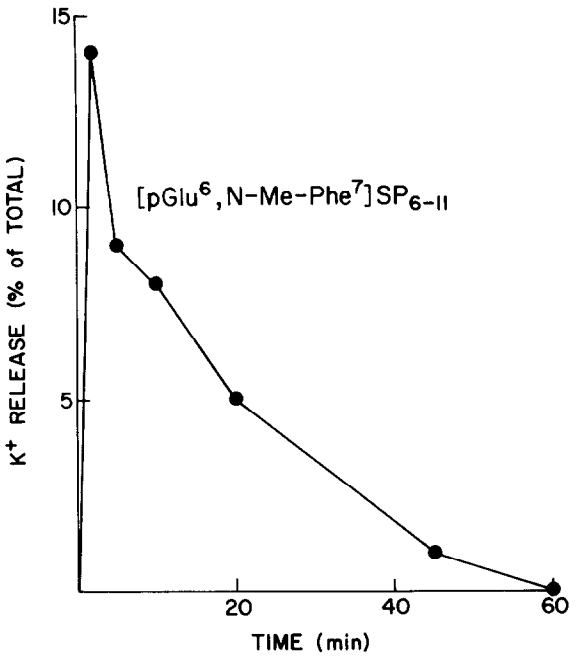

Fig.3. Time course of K^+ release from rat parotid slices caused by the addition of [pGlu^6, *N*-MePhe^7]SP_{6-11} 1.5 μM at zero time.

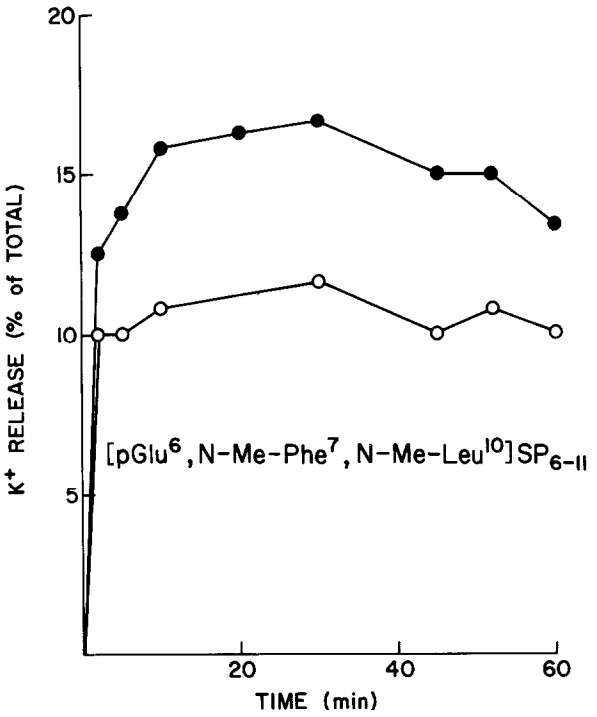

Fig.4. Time course of K^+ release from rat parotid slices caused by the addition of [pGlu^6, *N*-MePhe^7, *N*-MeLeu^{10}]SP_{6-11} (○) 1 μM), (●) 3 μM at zero time.

SP_{6-11} to the parotid slice system gave a transient K^+ release similar in its kinetics to that of [pGlu^6]-SP_{6-11} (fig.3). The potency of [pGlu^6, *N*-MePhe^7]-SP_{6-11} was decreased to 5 ± 1% of that of the unmodified parent compound.

Despite the fact that a single substitution of Phe^7 by *N*-MePhe did not confer metabolic stability and yielded an analog of low potency we have substituted both Leu^{10} and Phe^7 by their corresponding *N*-methyl derivatives. The analog [pGlu^6, *N*-MePhe^7, N-MeLeu^{10}]-SP_{6-11} was found to have a relative potency of 4 ± 1% but its effect on K^+ release did not significantly decrease within 60 min of incubation (fig.4). Apparently the introduction into the hexapeptide of *N*-MePhe together with *N*-MeLeu further increases the metabolic stability contributed by a single substitution with *N*-MeLeu. When tested at saturating concentrations, the 3 *N*-methylated analogs were full agonists of SP and have similar activity both in the parotid and in the isolated guinea pig ileum assay.

4. Discussion

The transient K^+ release evoked by SP action on rat parotid slices has been shown to be due to inactivation of the peptide rather than due to a decline in the response of the tissue [22]. The apparent lack of desensitization of the SP response in the parotid slices makes it a suitable preparation to test the metabolic stability of SP analogs. This is in contrast to cells of other tissues in which desensitization of the response to SP rapidly occurs [25]. Another advantage of the test system is the rather homogenous population of acinar cells which comprise ~90% of the cell mass of the parotid. It is thus likely that the same cells which respond to SP are also involved in the inactivation of the peptide.

The approach used in the present study was to stabilize particular peptide bonds in the C-terminal hexapeptide sequence of SP that were found susceptible to cleavage by proteolytic enzymes. The Gly^9–Leu^{10} and pGlu^6–Phe^7 peptide bonds were stabilized by substituting Leu^{10} or Phe^7 by their *N*-methyl- derivatives. The effects of the analogs on the parotid system indicate that stabilization of the Gly^9–Leu^{10} bond yields a potent analog which demonstrates a considerably more persistent action on the parotid slices than the parent compound. On the other hand,

stabilization of the pGlu[6]–Phe[7] bond by incorporation of *N*-MePhe[7] greatly reduces the potency of the analog and by itself does not produce a persistently acting analog. However, substitution of both Leu[10] and Phe[7] by their *N*-methyl derivatives resulted in an analog which was much more stable than the analog containing only *N*-MeLeu[10]. These results could be explained by assuming that the Gly[9]–Leu[10] bond is cleaved by the parotid slice system at a much faster rate than the pGlu[6]–Phe[7] bond. The small amounts of analogs which are used in the parotid assay makes it difficult to demonstrate a direct correlation between persistent action of the analogs and their resistance to proteolytic cleavage without the use of radioactively labeled compounds. We have, however, tested the stability of the analogs to proteolytic cleavage by several endopeptidases. These studies showed that the analog [pGlu[6], *N*-MePhe[7], *N*-MeLeu[10]]SP_{6-11} was resistant to degradation by pepsin, chymotrypsin, papain and thermolysin, using concentrations of these enzymes which resulted in rapid breakdown of the unmodified parent compound. It is hoped that this analog which combines full agonistic activity with resistance to proteolytic cleavage will prove useful in further studies of SP action.

Acknowledgement

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