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SUBSTANCE P BINDS TO THE FORMYLPEPTIDE CHEMOTAXIS RECEPTOR ON THE RABBIT NEUTROPHIL

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SUMMARY: Substance P, a potent vasodilatory and smooth muscle contracting agent, binds specificially to the formyl peptide receptor on the rabbit neutrophil. Substance P stimulates chemotaxis and induces lysosomal enzyme secretion in concentrations which similarily inhibit f Met-Leu-(^{3}H)Phe receptor binding. Competitive antagonists of the formyl peptide receptor also inhibit the activity of Substance P. The finding of a naturally occurring eukaryotic peptide interacting with the neutrophil formyl peptide receptor is of importance.

INTRODUCTION

The synthetic N-formylpeptides bind specifically to polymorphonuclear leukocytes and macrophages and activate them to perform multiple functions, chemotaxis, granule enzyme secretion (in the presence of cytochalasin B), aggregation, triggering of the respiratory burst etc. (1,2). The most active synthetic tripeptide presently known is N-formylmethionyl-leucyl-phenylalanine, f Met-Leu-Phe with an ED_{50} for chemotaxis of 7×10^{-11}M (3,4). The naturally occuring bacterial chemotactic factors reacting with the neutrophil formylpeptide receptor are believed to be N-formylated signal peptides involved in the initiation of prokaryotic protein synthesis (5-8). To date, however, no eukaryotic peptide capable of binding to the formylpeptide receptor has been found. Such a finding would broaden our understanding of the multifunctional nature of this receptor in mediating events of the PMN i.e. O_2 metabolism, enzyme secretion etc. at sites of inflammation not associated with bacterial invasion.

We have identified an amino acid sequence homology between a biologically active family of peptides, the tachykinins (9,10), and the N-formyl-

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lated chemotactic peptide, f Met-Leu-Phe. One such tachykinin, Substance P, has widespread distribution in the animal kingdom from vertebrates to invertebrates (11) and is a hypotensive, vasodilatory and smooth muscle contracting agent (12,13) present both in the peripheral and central nervous system (14). Substance P has also been implicated in the neurotransmission of pain (15,16). The amino acid sequence of this undecapeptide is Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2. The four carboxyl terminal amino acids, Phe-Gly-Leu-Met-NH_2 resemble, in reverse, the amino acid sequence of f Met-Leu-Phe with the carboxyl-terminal free acid of methionine modified by amidation. We therefore investigated the possibility that Substance P is sufficiently similar in conformation to the formylpeptide to bind to the same receptor site on the neutrophil and elicit the subsequent biological responses.

MATERIALS AND METHODS

Substance P and formyl-methionyl-leucyl-phenylalanine (f Met-Leu-Phe) were obtained from Sigma Chemical Co., St. Louis, MO. Dr. A. Day (Medical College of Virginia, Richmond, VA.) analyzed Substance P for purity by thin layer chromatography (TLC) using cellulose plates. Homogeneity was observed using butanol: acetic acid: water (4:1:1) and benezene: acetic acid: water(9:9:1) solvent systems as well as by amino acid analysis.

Binding studies were carried out at 5 x 10^6 cell/100ul and were incubated at 4^o C in Hanks buffer plus (1.6mM) calcium containing one p mole of f Met-Leu-(^{3}H)Phe (46.0 Ci/m mole) (New England Nuclear, Boston MA) and varying concentrations of nonradiolabelled f Met-Leu-Phe or Substance P. After 20 min. incubation, the cells were harvested by a glass fiber vacuum filtration method (17) and analyzed for specific binding to the formyl peptide receptor.

Enzyme release was performed at 1 x 10^7 cells/ml in the presence of 5ug cytochalasin B and was terminated after 3 min. at 37^o C. by immersing the tubes in an ice bath, followed by centrifugation (3). Lysozyme was assayed in the supernatent by measuring turbidometrically the lysis of Micrococcus Lysodeikticus after 3 min. at 37^o C. B-glucuronidase was assayed by measuring the release of phenolphthalein fron its B-glucuronate after overnight incubation at pH 4.5 (3).

Chemotaxis was performed with rabbit PMN at 2 x 10^6 cells/ml in a modified Boyden chamber (18). For analysis of cell orientation, the Zigmond chemotaxis chamber was used (19,20). Rabbit PMN were suspended in Hanks buffer plus 0.7 mM Mg++ supplemented with 1 mg/ml bovine serum albumin. 25ul of a cell suspension (3 x 10^6 cell/ml) was incubated on a glass coverslip for 15 min. at 37^o C. in a humidity chamber. After an add-

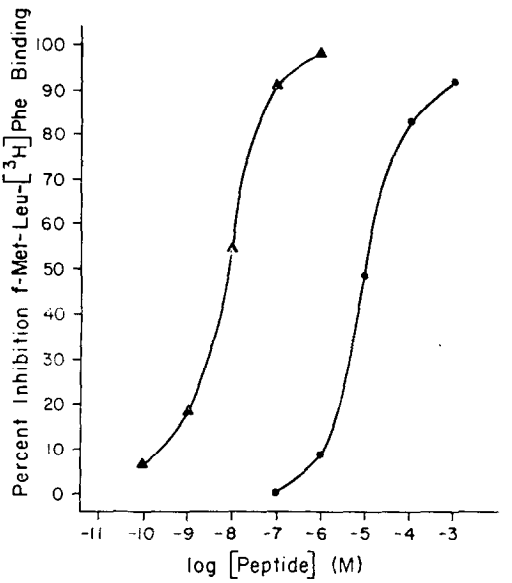

Figure 1. Ability of Substance P to compete for f Met-Leu-(^{3}H)Phe binding to rabbit neutrophils. (●——●) Substance P: (▲——▲) f Met-Leu-Phe.

itional 15 min. preincubation in a gradient of chemotactic peptide versus buffer, the chambers were placed under a phase microscope and a typical field was photographed (20). For kinetic measurements of cell migration, motile cells were traced on a stencil taped over a video screen calibrated in microns. This was linked to a time lapse videorecorder and a videocamera mounted on a phase microscope.

RESULTS AND DISCUSSION

Substance P can compete with f Met-Leu-(^{3}H)Phe for binding to the neutrophil formyl peptide receptor (Fig 1). The molar concentration rerquired to cause half maximal inhibition of specific f Met-Leu-(^{3}H)Phe binding i.e. ID_{50} for Substance P (1×10^{-5}M) is 1400 fold greater than that for f Met-Leu-Phe (7×10^{-9}M).

We employed direct visual analysis to analyze whether Substance P was able to chemotactically attract PMN, in the Zigmond chemotaxis chamber. In the absence of a chemotactic gradient, the cells appeared rounded and non-oriented. However, in an optimal concentration of either f Met-Leu-Phe (10^{-8}M) or Substane P (10^{-5}M), the cells become accurately oriented with respect to the chemotactic gradient. The dose response curves of the two

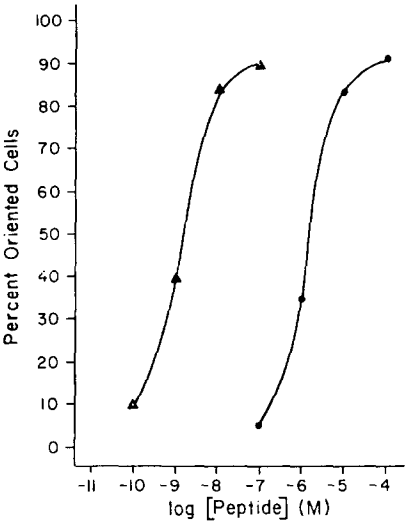

Figure 2. Orientation response of rabbit PMN to Substance P and f-Met-Leu-Phe. Cells were allowed to incubate in the Zigmond chamber with buffer in one well and varying concentrations of peptide in the other for 15 minutes before being placed on the phase microscope (40x) and scored for orientation (12). (●——●) Substance P: (▲——▲) f Met-Leu-Phe.

peptides for chemotaxis in the Zigmond chamber (Fig 2) show the same maximal orientation response. In addition, at high concentrations, chemotactic deactivation occurs for both f Met-Leu-Phe and Substance P (data not shown). Using time lapse cinematography at these optimal concentration of the two peptides, the rates of locomotion were identical (Table 1).

Peptide-induced inhibition of binding correlates very strongly (r=0.97) with formyl peptide-induced chemotaxis, lysosomal enzyme release and inhibition of binding (3,4). We therefore tested the ability of Substance P also to mediate these related events. Table 2 shows the concentrations of both peptides required to cause half maximal biological response. Substance P induced release of the lysosomal granule enzymes, lysozyme and B-glucuronidase in the same concentration range required to cause chemotaxis, stimulate locomotion and inhibit f Met-Leu-(^{3}H)Phe binding.

Competitive antagonists of the formylpeptide receptor inhibit both *in vivo* and *in vitro* neutrophil responses to the formyl peptides (1,21). These

Table 1

Peptide	(Molar)	Rate of PMN movement (um/min ± S.E.M)
Substance P	(10^{-5}M)	18.63 * ± 0.94 (10) #
		17.21 ± 0.86 (10)
f Met-Leu-Phe	(10^{-8}M)	18.14 ± 1.04 (10)
		16.94 ± 0.76 (10)

* Duplicate chambers were run and then compared to rates obtained with f Met-Leu-Phe. Results not significantly different at $P < .05$ (Student t test).

\# Number of cells measured per chamber.

same antagonists inhibit the neutrophil response to Substance P. Table 3 compares the K_D of two of these antagonists, tert-butoxy-phenylalanyl-leucyl-phenylalanyl-leucyl-phenylalanine (t-Boc-Phe-Leu-Phe-Leu-Phe) and carbobenzoxy-phenylalanyl-norleucine (CBZ-Phe-Nle) calculated from their ability to inhibit to release of lysozyme and B-glucuronidase by either f Met-Leu-Phe

Table 2

ED50 for Chemotaxis, Enzyme Secretion and Inhibition of Binding (M)

	Chemotaxis (Boyden chamber)	Enzyme release * Lysozyme	Enzyme release * β-glucuronidase	Inhibition of binding f Met-Leu-(^{3}H)Phe
Substance P	6.4×10^{-6}	6.1×10^{-6}	7.7×10^{-6}	1×10^{-5}
	3.5×10^{-6}	3.0×10^{-6}		
f Met-Leu-Phe	7.5×10^{-11}	2.1×10^{-10}	2.0×10^{-10}	7×10^{-9}
	6.8×10^{-11}	1.3×10^{-10}		

* Lactate dehydrogenase measurements indicated a greater than 95% cell viability.

Table 3

Inhibition of lysosomal enzyme secretion by competitive antagonists*

	K_D for lysozyme (M)		K_D for β-glucuronidase (M)	
	CBZ-Phe-Nle	BOC-Phe-Leu-Phe-Leu-Phe	CBZ-Phe-Nle	BOC-Phe-Leu-Phe-Leu-Phe
Substance P	6.4×10^{-6}	4.3×10^{-7}	1.1×10^{-5}	5.4×10^{-7}
	8.8×10^{-6}	4.4×10^{-7}		
f Met-Leu-Phe	6.4×10^{-6}	2.2×10^{-7}	3.3×10^{-6}	2.2×10^{-7}
	2.6×10^{-6}	1.4×10^{-7}		

* Dose- response curves obtained from the indicated chemotactic peptides in the absence of, or in the presence of varying concentrations of competitive antagonist. From a plot of $\frac{A}{a} - 1$ verses concentration of antagonist, where A and a are the concentrations of agonist which induce equal responses in the presence (A) and absence (a) of a given concentration of antagonist (B), the dissociation constant (K_D) was calculated with the formula $K_D = \frac{aB}{A-a}$.

or Substance P. As evident, the two competitive antagonists of the formyl peptide receptor inhibit the response to Substance P and f Met-Leu-Phe equally well.

Substance P has been shown to enhance phagocytosis by mouse macrophages and human neutrophils (22). This activity resides in its N-terminal tetrapeptide portion which resembles in its structure the phagocytosis stimulating peptide, tuftsin (23). The activity we report i.e. binding to the formyl peptide receptor, is novel and this activity is presumably due to amino acid sequence homology in its C-terminal tetrapeptide portion. Indeed from various structure - function relationships among synthetic Substance P analogues, it appears that the region of effective interaction with the substance P receptor also resides in the C-terminal portion of this undecapeptide (9).

Substance P is the first naturally occuring peptide of eukaryotic origin shown to act on the neutrophil formyl peptide receptor. It suggests that other eukaryotic peptides i.e. tachykinins and bombesin-related peptides, may be found which are much more active. In addition, the ability of Substance P to react with the neutrophil receptor throws further light on the specificity of the receptor. Whereas, the synthetic chemotactic formyl peptides fit into the neutrophil receptor via insertion of Met, Leu, Phe from the amino terminal end, Substance P may fit into the site in reverse with the amide (NH_2) of Substance P possibily substituting for the NH_2-terminal formyl group. It is of interest and possibly significant in this regard that similar to the dramatic loss of activity for the neutrophil following the removal of the formyl group (3), removal of the amide group in Substance P results in severe loss of its biological activity(24).

The ED_{50} for the spasmogenic activity of Substance P is approximately $1 \times 10^{-9}M$ (12,13,25). This makes it unlikely that its actions mediated through the formyl peptide receptor on the neutrophil with an ED_{50} of $1 \times 10^{-5}M$ (Table 2) has any physiological significance. Much lower concentrations of Substance P were required to stimulate phagocytosis by human PMN (22). However the findings of this paper may have significance for the pharmacology of Substance P since despite extensive search (25), no inhibitors of Substance P have been found. Thus, the ability of competitive antagonists to inhibit the actions of Substance P on the formyl peptide receptor of the neutrophil will have profound implications if it can be shown that these same agents will inhibit the pharmacologic actions of Substance P on its more physiologic receptor.

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