Vol. 175, No. 1, 1991
February 28, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 165-172

CHEMOTACTIC PEPTIDE FROM ROPALIDIAN WASP AS WELL AS THE AUTHENTIC CHEMOTACTIC TRIPEPTIDE STIMULATES TWO DISTINCT PATHWAYS IN NEUTROPHILS, BUT THE [LYS^7] ANALOG DOES ONLY ONE OF THEM

Masamichi Koike, Ken Nagashima, Kazuki Saito, Mamoru Nakanishi,[¶] Masahiko Fujino[§] and Terumi Nakajima*

Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

[§]Tsukuba Research Laboratories, Takeda Chemical Industries, Ltd., Wadai, Tsukuba, Ibaraki 300-42, Japan

Received January 17, 1991

SUMMARY: In our previous study on chemotactic peptide isolated from Ropalidian wasp, it was found to induce not only chemotaxis but also other cellular responses, such as superoxide generation and lysosomal enzyme release, but [Lys^7] analog was found to induce only chemotaxis but not others. Here, studies on intracellular Ca^{2+} changes and receptor-binding revealed that the wasp chemotactic peptide and the authentic tripeptide stimulated two distinct receptors, but that the [Lys^7] peptide could have access to only one of them.

Icaria chemotactic peptide (I-CP) is a tridecapeptide amide isolated from the venom of Ropalidian wasp, *Icaria sp.*, and the primary structure has been determined as Ile-Val-Pro-Phe-Leu-Gly-Pro-Leu-Leu-Gly-Leu-Leu-Thr-NH_2 (1). It has a unique structure of lacking basic amino acid residues, while other chemotactic peptides from wasp venoms have lysine in the sequences (2). Just like the authentic artificial chemotactic peptide, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), I-CP has been found to induce not only chemotaxis of neutrophils but also other cellular responses, such as superoxide generation and lysosomal enzyme release (2-4).

[¶]Present address: Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan.

*To whom correspondence should be addressed.

Abbreviations: EGTA, ethyleneglycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; G protein, GTP-binding regulatory protein; I-CP, the chemotactic peptide isolated from *Icaria sp.*

0006-291X/91 $1.50

In our previous study (2), two distinct signal transduction systems (or receptors) for the chemoattractants were found in neutrophils. One provides for chemotaxis, and the other provides for superoxide generation and lysosomal enzyme release. Although I-CP and FMLP which have no basic residues activated both systems, [Lys^7]I-CP induced only chemotaxis but neither superoxide production nor lysozyme release. Since this finding suggests the presence of two different modes for stimulation of intracellular metabolisms in the cells, calcium mobilization and receptor-binding of [Lys^7]I-CP were observed and compared with those of the other chemotactic peptides.

MATERIALS AND METHODS

FMLP was purchased from Peptide Institute, Inc. (Osaka, Japan). I-CP and the analog were chemically synthesized by the conventional solution method. Another wasp venom peptide mastoparan, whose sequence is Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2 (5), was also synthesized by the solution method according to the previous paper (6). Tritiated FMLP (formyl-Met-Leu-[^{3}H]Phe) with specific activity 57 Ci/mmol was obtained from New England Nuclear Research Products (Boston, MA).

Preparation of neutrophils and the membranes: Polymorphonuclear leukocytes (neutrophils) were induced by intraperitoneal injection (15 ml) of 3% casein sodium in sterile saline into a female Hartley guinea pig (500-700 g). After 16 h, the cells were collected by centrifugation in Hepes-buffered Hanks' balanced salt solution (137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.1 mM $MgCl_2$, 0.17 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.1% glucose, 10 mM Hepes, pH 7.4) containing 0.1% bovine serum albumin.

Guinea pig neutrophil plasma membranes were prepared by the method of Lohr and Synderman (7). Briefly, the cells in ice-chilled buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.6) were homogenized and sonicated. After centrifugation at 500,000×g for 20 min at 4 °C, the pellets were resuspended. Protein concentration was determined by Bio-Rad Protein Assay reagent (Richmond, CA) using bovine serum albumin as a standard.

Measurements of intracellular calcium mobilization: Cells (10^7 cells/ml) were incubated with 1 μM Fura 2/acetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan) at 37 °C for 15 min. The cells were washed twice with Hanks' solution or Ca^{2+}-free Hanks' solution containing 1.0 mM EGTA. Changes of the fluorescence intensity at 500 nm (ex. 334 nm) induced by the peptides at indicated final concentrations were measured with a Hitachi 650-10(S) fluorescence spectrophotometer or a Union-giken RA-401 stopped-flow system (dead time: 0.5 ms).

Formyl-Met-Leu-[^{3}H]Phe binding assay: Neutrophils (3×10^7 cells/ml) were incubated with 50 nM (or 0.5-500 nM for Scatchard analyses) [^{3}H]FMLP in a total volume of 0.1 ml in the presence of indicated amount of the peptides at 20 °C for 10 min. Assays were terminated by the addition of 4 ml of ice-cold buffer followed by vacuum filtration through Whatman GF/C glass fiber filters (Maidstone, England). After washed twice with 4 ml of the ice-cold buffer, filters were placed in 10 ml of toluene

scintillator and radioactivities were quantified by liquid scintillation spectrophotometry. Binding to crude neutrophil membranes were also measured as above.

RESULTS

Figure 1 shows the changes of intracellular free calcium concentration in neutrophils induced by the chemotactic peptides and mastoparan. The peptide concentrations used were 0.1 μM for FMLP and 10 μM for the wasp venom peptides, which had been known to be optimum to exhibit the maximal activities in various cellular responses (2). All the peptides examined here caused rapid increase of the concentration within 20 s even if the cells were suspended in Ca^{2+}-free medium. Mastoparan is another wasp toxic peptide isolated from *Vespula lewisii* and causes degranulation and histamine release from mast cells (5), but induces neither chemotaxis nor superoxide generation in neutrophils (unpublished data). Furthermore, it has been known to increase the internal Ca^{2+} concentration in rat hepatocytes (8). As shown in Fig. 1A-d, changes in cytoplasmic free calcium

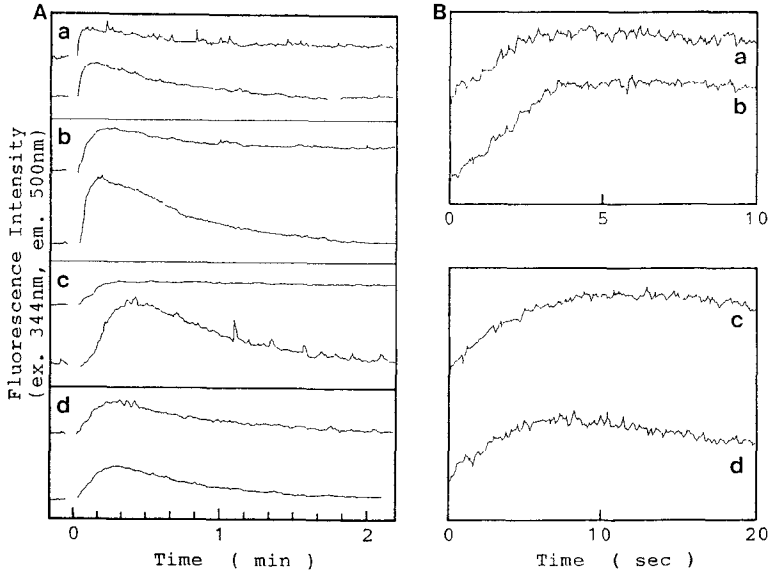

Figure 1. Changes in Cytoplasmic Calcium Concentration Measured in Fura 2-loaded Neutrophils Stimulated by 0.1 μM FMLP (a), 10 μM I-CP (b), 10 μM [Lys^7]I-CP (c), and 10 μM Mastoparan (d).

A; Changes of fluorescence intensity induced by the peptides in the presence (upper trace in each box) and absence (lower trace) of Ca^{2+} in the external medium. Intensities were not normalized among the experiments recorded.

B; Stopped-flow measurements of the fluorescence changes induced by the peptides in the presence of Ca^{2+} in the external medium.

concentration induced by mastoparan were regardless of the calcium in the extracellular medium, which was consistent with the fact that mastoparan does not require any external Ca^{2+} when it induces the histamine release from mast cells. In contrast, changes induced by the chemotactic peptides, FMLP, I-CP and [Lys^7]I-CP, were affected by the external Ca^{2+} concentration (Figs. 1A-a, 1A-b and 1A-c, respectively). Although initial elevation of the internal Ca^{2+} concentration was not affected, following progressive decrease to basal level was made much slower by the addition of Ca^{2+} to the external medium. These results indicate that both Ca^{2+} mobilization from intracellular store(s) and Ca^{2+} influx from extracellular medium contribute to the elevation in the cytosolic free Ca^{2+} in neutrophils stimulated with the chemotactic peptides. In the Quin 2-loaded neutrophils, Hamachi *et al.* (9) had already shown that FMLP induced the intracellular Ca^{2+} mobilization from the two distinct origins.

Figure 1A also shows the different initial rates of elevation in the internal Ca^{2+} concentration. FMLP and I-CP induced very rapid increase both in the presence and absence of the external Ca^{2+}, but [Lys^7]I-CP and mastoparan induced slower increase. This difference was much clearly observed in stopped-flow experiments (Fig. 1B). A rise of the cytosolic free Ca^{2+} concentration induced by FMLP or I-CP reached a maximal detectable level at about 4 s, while one induced by [Lys^7]I-CP or mastoparan reached a maximum at 8 s. Considering that [Lys^7]I-CP and mastoparan have no activities to stimulate the O_2^- production nor granule exocytosis, the rapid phase of the elevation of internal Ca^{2+} which was accomplished in 4 s after stimuli is thought to be associated with these activities.

Figure 2 shows reductions of [^{3}H]FMLP binding when the radioactive peptide was incubated with 10 μM I-CP or 10 μM [Lys^7]I-CP for 10 min. Time course of [^{3}H]FMLP binding to neutrophils revealed that 10 min was enough for the binding to reach equilibrium (data not shown). Although [Lys^7]I-CP which is distinct from I-CP itself stimulates neither superoxide anion release nor lysosomal enzyme secretion, 10 μM [Lys^7]I-CP as well as 10 μM I-CP reduced the [^{3}H]FMLP binding to approximately 60%. Since the inhibition of [^{3}H]FMLP binding is thought to reflect the affinity of a peptide for the putative FMLP receptor, substitution of Lys^7 for Pro^7 in the sequence does not affect the global affinity. Figure 2 also shows that the reducing ability

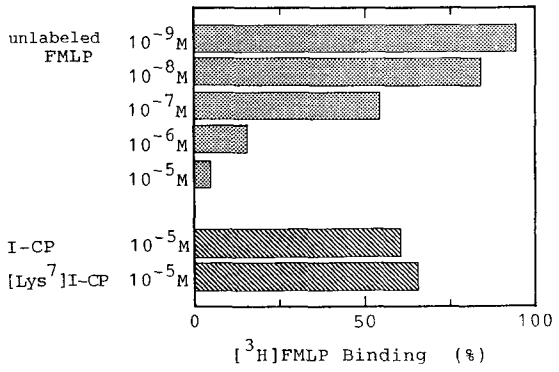

Figure 2. Reductions of [^{3}H]FMLP Binding to Neutrophils by Wasp Chemotactic Peptides.
The cells (3×10^7 cells/ml) were incubated with 50 nM [^{3}H]FMLP in the presence of indicated amount of I-CP, [Lys^7]I-CP or cold FMLP at 20 ^{o}C for 10 min.

of 10 μM wasp chemotactic peptides corresponded to that of 0.1 μM unlabeled FMLP, which comes to a good explanation with the differences in ability to promote the cellular responses that FMLP was 100-fold more effective than the wasp chemotactic peptides (2). Synderman and Fudman (10) had also shown the correlation of affinity of *N*-formylated peptides for [^{3}H]FMLP binding sites with chemotactic potency of the peptides.

In Fig. 3, Scatchard plots of [^{3}H]FMLP binding to guinea pig neutrophils (Fig. 3A) and the membranes (Fig. 3B) are shown. It is clearly observed that the Scatchard plots obtained for both the cells and the membranes are curvilinear. Non-linear regression analysis of [^{3}H]FMLP binding data to the cells using the least squares method (Fig. 3A) indicates the presence of two populations of binding sites: a higher affinity site (670 ± 280 sites per cell) with an apparent K_d value of 0.15 ± 0.21 nM, and a lower affinity site (27,000 ± 3,700 sites per cell) with a K_d value of 56 ± 16 nM, respectively. In the binding to neutrophil membranes (Fig. 3B), the two affinities of binding sites were also observed: a higher affinity site with K_d of 2.2 ± 0.42 nM, and a lower one with 74 ± 17 nM.

In the presence of I-CP or [Lys^7]I-CP, the binding of [^{3}H]FMLP to the cells was reduced almost to the background level. On the other hand, the binding to the membranes was not completely reduced by them. Only the higher affinity binding of FMLP was reduced in the presence of I-CP, and a curvilinear plot was still obtained in the presence of the [Lys^7] analog. This difference between the sites in the cells and in the membranes

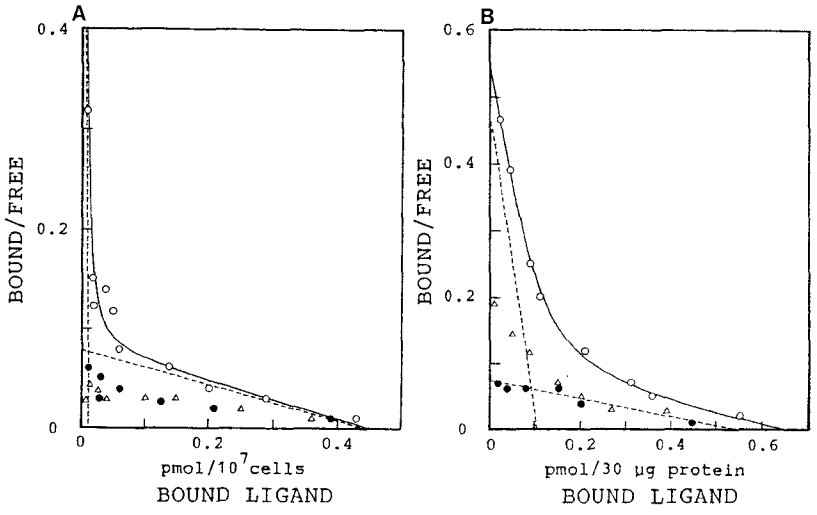

Figure 3. Scatchard Plots of [^{3}H]FMLP Binding to Neutrophils (A) and to the Membranes (B).

[^{3}H]FMLP binding to 3 × 10^6 neutrophils or 30 µg protein in the membranes was determined at peptide concentrations between 0.5 to 500 nM in the absence (○) or presence of 10 µM I-CP (●) or 10 µM [Lys^7]I-CP (△). The two components of the curvilinear plots in the absence of the wasp peptides were reconstructed with data from regression analysis and are shown as the dotted lines.

were thought to be due to the cellular metabolic responses of the cells. Subsequent to the initial reversible binding, bound peptides must become progressively non-dissociable and be internalized into the cells, while they may not into the cell-free membranes. In sum, these data demonstrate that I-CP bound to the higher affinity population of FMLP receptors at least, but [Lys^7]I-CP bound to only a part of the receptors.

DISCUSSION

The elevation of intracellular Ca^{2+} concentration has been thought to be required for many leukocyte responses to FMLP, because it is one of the earliest measurable events after exposure of neutrophils to the chemoattractant (11,12). However, the direct correlation between the changes in internal Ca^{2+} concentration and various cellular responses has been always suspected, because increase of intracellular Ca^{2+} concentration like that generated by ionomycin was found not to be sufficient for O_2^- generation in neutrophils (14). In this study, mastoparan was also observed to induce transient elevation of intracellular Ca^{2+} concentration although it causes neither chemotaxis nor superoxide production.

The rise in the cytosolic free Ca^{2+} in neutrophils stimulated with FMLP had been already observed to be composed of two phases. Hamachi *et al.* (9) and Lew *et al.* (11) concluded that the rapid phase had to be attributed to release of Ca^{2+} from the cytosolic non-mitochondrial stores, and that the other slower phase had to be attributed to Ca^{2+} influx through the surface membrane. In the elevation of internal free Ca^{2+} concentration, two components were also observed here, but they could not be identified with ones observed in the previous works. The slow phase of Ca^{2+} rise in the works lasted for >4 min, while the rise induced by [Lys^7]I-CP in this study reached a maximum in 8 s. Our findings revealed that the rapid phase in those works could be further divided into two components: a rapid one which is induced by FMLP and I-CP but not by [Lys^7]I-CP, and another semi-rapid one which is induced by any chemotactic peptide.

Although it could be argued yet how the following cellular reactions would be induced by the increased internal free Ca^{2+}, the intracellular Ca^{2+} concentration must be increased via activation of *pertussis* toxin-sensitive GTP-binding regulatory proteins (G proteins) in FMLP-stimulated neutrophils, because the treatment with *pertussis* toxin has been found to reduced the changes of the internal free Ca^{2+} in neutrophils (14). Considering that [Lys^7]I-CP as well as mastoparan has an ability to activate the G proteins directly (3), it apparently seems to induce the cellular responses via direct stimulation of the G proteins in neutrophils. But it might not do directly *in vivo*, because more than 0.5 mM of [Lys^7]I-CP was required for the stimulation.

The study on FMLP-receptor binding revealed the presence of two distinct populations of binding sites. Mackin *et al.* (15) who had also found the presence of such two kinds of binding sites in rabbit neutrophils using silicone oil centrifugation assays suggested that the higher affinity sites might be biological relevant. In the study on [^{3}H]FMLP binding to the membrane, I-CP completely reduced the higher affinity binding, while [Lys^7]I-CP reduced it only partially. Such properties of the higher affinity binding looks very similar to those of the rapid phase of internal Ca^{2+} elevation. Both could be further divided into two components at least. I-CP stimulates both of the components, but [Lys^7]I-CP does only one of them.

In conclusion, even though more detailed analysis is now under way on the action mechanism of the chemotactic peptides, we

could propose the presence of two distinct pathways for the chemoattractants in neutrophils using the wasp chemotactic peptide and the analog.

REFERENCES

1. Yasuhara, T., Itokawa, H., Suzuki, N., Nakamura, H., and Nakajima, T. (1985) Peptide Chemistry 1984 (N.Izumiya, Ed.), pp.177-182. Protein Research Foundation, Osaka.
2. Nagashima, K., Koike, M., Saito, K., Yasuhara, T., Tsukamoto, Y., Mori, M., Fujino, M., and Nakajima, T. (1990) Biochem. Biophys. Res. Commun. 168, 844-849.
3. Koike, M., Yasuhara, T., Nakajima, T., Higashijima, T., Miyazawa, T., Kitada, C., Fujino, M., and Tsukamoto, Y. (1987) Peptide Chemistry 1986 (T.Miyazawa, Ed.), pp.63-68. Protein Research Foundation, Osaka.
4. Nagashima, K., Koike, M., Saito, K., Yasuhara, T., Tsukamoto, Y., Mori, M., Fujino, M., and Nakajima, T. (1989) Peptide Chemistry 1988 (M.Ueki, Ed.), pp.327-332. Protein Research Foundation, Osaka.
5. Hirai, Y., Yasuhara, T., Yoshida, H., Nakajima, T., Fujino, M., and Kitada, C. (1979) Chem. Pharm. Bull. 27, 1942-1944.
6. Saito, K., Higashijima, T., Miyazawa, T., Wakimasu, M., and Fujino, M. (1984) Chem. Pharm. Bull. 32, 2187-2193.
7. Lohr, K.M., and Synderman, R. (1982) J. Immunol. 129, 1594-1599.
8. Tohkin, M., Yagami, T., and Matsubara, T. (1990) FEBS Lett. 260, 179-182.
9. Hamachi, T., Hirata, M., and Koga, T. (1986) Biochim. Biophys. Acta 889, 136-148.
10. Synderman, R., and Fudman, E.J. (1980) J. Immunol. 124, 2754-2757.
11. Lew, P.D., Wollheim, C.B., Waldvogel, F.A., and Pozzan, T. (1984) J. Cell Biol. 99, 1212-1220.
12. Lazzari, K.G., Proto, P.J., and Simons, E.R. (1986) J. Biol. Chem. 261, 9710-9713.
13. Pozzan, T., Lew, D.P., Wollheim, C.B., and Tsien, R.Y. (1983) Science, 221, 1413-1415.
14. Verghese, M.W., Smith, C.D., and Synderman, R. (1985) Biochem. Biophys. Res. Commun., 127, 450-457.
15. Mackin, W.M., Huang, C.-K., and Becker, E.L. (1982) J. Immunol. 129, 1608-1611.