Chemokine Receptor CXCR2 Activates Distinct Pathways for Chemotaxis and Calcium Mobilization

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Rat cytokine-induced neutrophil chemoattractant-3 (CINC-3) has neutrophil chemotactic activity comparable with that of CINC-1 and CINC-2, but induces calcium mobilization more potently than CINC-1 and CINC-2. However, only one CINC receptor, CXCR2, has been found in rat neutrophils. Therefore we attempted to determine the biochemical basis for the differences in neutrophil responses to CINC-1/2 versus CINC-3. Both chemotactic activity and calcium mobilization induced by CINC-3 were desensitized by a 100-fold excess of CINC-1, which was consistent with our previous results showing that CINC-1 has 70-fold lower affinity to the receptor on rat neutrophils than CINC-3. Desensitization appears to be reflected by the affinity of the ligands to the receptor. CINC-1- and CINC-3-induced chemotaxis was sensitive to inhibition by pertussis toxin, whereas calcium mobilization induced by CINC-1 and CINC-3 was insensitive. These results suggest that CINC-3 induce neutrophil chemotaxis and calcium mobilization through distinct G-proteins with different efficiency.

Key words cytokine-induced neutrophil chemoattractant (CINC); pertussis toxin; chemotaxis; calcium mobilization; macrophage inflammatory protein-2 (MIP-2); chemokine

Cytokine-induced neutrophil chemoattractants (CINC) are potent neutrophil chemotactic factors in rats and major mediators of acute inflammation.^{1–3)} Four members of the CINC family have been identified: CINC-1, CINC-2 α , CINC-2 β , and CINC-3/macrophage inflammatory protein-2 (MIP-2).¹⁾ CINC-2 α and CINC-2 β differ only in the sequence of carboxy-terminal three amino acid residues and are produced by alternative splicing.⁴⁾ CINC-3 shows high degrees of sequence similarity to human GROs, interleukin 8 (IL-8), mouse KC, and mouse MIP-2 and belong to the CXC chemokine family. CINC-1 and CINC-2 show chemotactic activity comparable with that of CINC-3.⁵⁾ Treatment of neutrophils with CINC-1 or CINC-2 inhibited the chemotactic activity of CINC-1 and CINC-2, but not that of CINC-3, whereas treatment with CINC-3 inhibited the activity of all CINC-3s.⁶⁾ On the other hand, CINC-3 induced calcium mobilization more potently than CINC-1 and CINC-2, and desensitized calcium mobilization in neutrophils induced by CINC-1 and CINC-2.⁵⁾ In addition, CINC-3 potently competed with the binding of ¹²⁵I-labeled CINC-3 Tyr, although CINC-1 competed only weakly.⁷⁾ To determine the biochemical basis for the differences in neutrophil responses to CINC-1/2 versus CINC-3, we have cloned the gene encoding the receptor and identified rat CXCR2 as the common receptor for all CINC-3s.⁸⁾ CXCR2 belongs to the family of G-protein-coupled receptors with seven transmembrane-spanning domains. Herein, we report the relationship between the biological activities of CINC-3 and G-protein usage.

MATERIALS AND METHODS

Preparation of Neutrophils Neutrophils were harvested from the peritoneal exudate 16 h after intraperitoneal injection of Krebs/Ringer bicarbonate solution containing 1% casein (120 ml/kg body weight of male Wistar rats), washed twice with Gey's balanced salt solution and twice with the assay buffer and then resuspended at a concentration of 10⁷ cells/ml.⁵⁾

Pertussis Toxin Treatment Neutrophils were incubated with pertussis toxin (PTX, 1 μ g/ml) at 37 °C for 2 h in a humidified 5% CO₂ atmosphere and washed twice with the assay buffer.

Measurement of Intracellular Free Calcium Levels The intracellular free calcium level in neutrophils was measured by the method described previously.⁵⁾ Neutrophils were incubated with the calcium indicator fura-2 acetoxyethyl ester (2.5 μ M) in Ca²⁺/Mg²⁺-free phosphate-buffered saline containing 10 mM HEPES, 0.25% bovine serum albumin, and 10 mM glucose (pH 7.4) for 30 min at 37 °C. After incubation, the cells were washed twice and resuspended at 10⁶ cells/ml with the buffer. In the presence of 1 mM CaCl₂, fluorescence changes were monitored using a Hitachi F4500 fluorescence spectrophotometer at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Fura-2-loaded cells were stimulated with CINC-3, then lysed with 0.1% Triton-X, and finally Ca²⁺ was chelated with 6 mM EGTA. Intracellular calcium levels were calculated as described by Grynkiewicz *et al.*⁹⁾

Chemotaxis Assay *in Vitro* Chemotactic activity for rat neutrophils was assayed in 96-well plates as described previously.⁵⁾ As an index of chemotaxis, the migration rate was expressed as the percentage of the number of neutrophils migrating into the lower wells during an 80-min incubation to that of the neutrophils applied to the upper wells.

RESULTS

Rat CXCR2 on neutrophils is the unique receptor for all three CINC-3s. We determined whether the biological activities of CINC-3, a potent agonist, were desensitized by CINC-1. First, the effects of desensitization of CINC-3 on calcium mobilization were examined in rat neutrophils. Neutrophils exposed first to CINC-1 at 100 nM became unresponsive to subsequent stimulation with CINC-1 at the same concentration, but a second stimulation at a higher concentration (1 μ M) caused reduced calcium mobilization (Figs. 1A, B). CINC-3

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Fig. 1. Effects of Homologous and Heterologous Desensitization of Recombinant CINC-1 and CINC-3 on Calcium Mobilization

Rat neutrophils were loaded with fura-2 and stimulated by each CINC sequentially at the times indicated in the figure.

Fig. 2. Inhibition of CINC-3-Induced Neutrophil Chemotaxis by CINC-1

Rat neutrophils with CINC-1 were added to the upper wells of Boyden chambers and CINC-3 at a concentration of 10 nM was added to the lower wells. Migration of neutrophils was measured as described in Materials and Methods. Each point represents the mean $\pm$ S.E.M. of six determinations.

at 10 nM induced calcium transients in neutrophils treated with CINC-1 100 nM but not 1 μ M (Figs. 1C, D). Next, the effects of desensitization of CINC-1 and CINC-3 on chemotactic activity were examined using Boyden chambers *in vitro*. The addition of CINC-1 to neutrophils in the upper wells of Boyden chambers inhibited the chemotactic activity of CINC-3 dose dependently (Fig. 2).

The effects of pertussis toxin on the biological activities of CINC-1 and CINC-3 were measured to determine the differential regulation of these activities. Pertussis toxin treatment did not affect calcium mobilization of neutrophils induced by CINC-1 and CINC-3, whereas it inhibited the neutrophil chemotactic activity of CINC-1 and CINC-3 (Fig. 3).

DISCUSSION

In previous studies, we demonstrated the differences in neutrophil responses to CINC-1 and CINC-2 versus CINC-3.^{5,6} CINC-3 at a concentration of 10 nM induced calcium mobilization to an extent similar to that induced by CINC-1 and CINC-2 at a concentration of 100 nM. Stimulation of neutrophils with CINC-1 (100 nM) caused a loss of sensitivity to CINC-1 and CINC-2 (100 nM) but not to CINC-3 (10 nM). In contrast, CINC-3 (10 nM) desensitized calcium transients induced by all three types of CINC-1. These responses were essentially identical to those observed in CXCR2-transfected HEK293 cells.⁸ In the present study, we found that the calcium mobilization induced by CINC-3 (10 nM) was inhibited completely by CINC-1 at a 100-fold excess concentration (1 μ M) (Fig. 1). This result seems to reflect a 70-fold lower affinity of CINC-1 to the receptor than CINC-3.⁷ On the other hand, CINC-3 showed chemotactic activity comparable with that of CINC-1 and CINC-2.⁵ The chemotactic activity of CINC-1 was inhibited completely by a 3-fold excess of CINC-3, although the chemotactic activity of CINC-3 was not inhibited by a 3-fold excess of CINC-1.⁶ This also appears to reflect the low affinity of CINC-1, because a 100-fold excess of CINC-1 desensitized the chemotaxis induced by CINC-3 (Fig. 2). Umland *et al.* reported that the relationship between the increase in intracellular calcium and the fraction of eotaxin receptor (CCR3) occupancy was linear in eosinophils, whereas a hyperbolic nonlinear relationship was observed between eosinophil chemotaxis and the receptor occupancy, suggesting that CCR3 interacted with G-proteins that were poorly coupled to the calcium response and effi-

Fig. 3. Effect of Pertussis Toxin on Biological Activities of CINC-1 and CINC-3

Rat neutrophils were treated with pertussis toxin (PTX, 1 µg/ml) at 37°C for 2 h and A) neutrophil chemotactic activity of CINC-1 and CINC-3, and B) CINC-1 and CINC-3-induced calcium transients were measured. Each column represents the mean ± S.E.M. of four to six determinations.

ciently coupled to chemotaxis.¹⁰ A similar explanation may be possible in the case of the CINC receptor CXCR2.

Formyl-methionyl-leucyl-phenylalanine, platelet-activating factor, and leukotriene B₄ induced chemotaxis and calcium mobilization *via* pertussis toxin-sensitive G-protein in neutrophils.^{11–15} However, others reported that platelet-activating factor and leukotriene B₄ utilized an additional pertussis toxin-resistant mechanism for calcium mobilization.¹⁶ Human neutrophils express two receptors, CXCR1 that binds IL-8 with high affinity and GROα with low affinity, and CXCR2 showing high affinity to IL-8 and GROα.^{17–20} Although both receptors have been shown to be able to couple to both pertussis toxin-sensitive and -resistant G-proteins,^{21,22} they mediated IL-8-induced neutrophil chemotaxis *via* pertussis toxin-sensitive G-protein.^{23,24} On the contrary, the calcium mobilization induced by IL-8 at CXCR2 was inhibited by pertussis toxin, but that induced by IL-8 at CXCR1 was not completely inhibited, suggesting the involvement of both pertussis toxin-sensitive and -resistant pathways in the signaling of CXCR1-elicited calcium mobilization.²⁵ CINC-1 and CINC-3-induced chemotaxis, but not calcium mobilization, was sensitive to inhibition by pertussis toxin (Fig. 3). The results were reproducible even when the same neutrophils treated with pertussis toxin were used for both assays. The different usage of G-proteins may come from the fact that rodent neutrophils have only one receptor, CXCR2. The results presented in this report suggest that CINC-1 and CINC-3 induced neutrophil chemotaxis and calcium mobilization through distinct G-proteins with different efficiency.

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