Chemoattractant Receptors Activate Distinct Pathways for Chemotaxis and Secretion

ROLE OF G-PROTEIN USAGE*

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Human leukocyte chemoattractant receptors activate chemotactic and cytotoxic pathways to varying degrees and also activate different G-proteins depending on the receptor and the cell-type. To determine the relationship between G-protein usage and the biological and biochemical responses activated, receptors for the chemoattractants formyl peptides (FR), platelet-activating factor (PAFR), and leukotriene B4 (BLTR) were transfected into RBL-2H3 cells. Pertussis toxin (Ptx) served as a Gαi inhibitor. These receptors were chosen to represent the spectrum of Gα usage as Ptx had differential effects on their ability to induce calcium mobilization, phosphoinositide hydrolysis, and exocytosis with complete inhibition of all responses by FR, intermediate effects on BLTR, and little effect on PAFR. Ptx did not affect ligand-induced phosphorylation of PAFR and BLTR but inhibited phosphorylation of FR. In contrast, chemotaxis to formylmethionylleucylphenylalanine, leukotriene B4, and platelet-activating factor was completely blocked by Ptx. Wortmannin, a phosphotyrosine 3-kinase inhibitor, also completely blocked ligand-induced chemotaxis by all receptors but did not affect calcium mobilization or phosphoinositide hydrolysis; however, it partially blocked the exocytosis response to formylmethionylleucylphenylalanine and the platelet-activating factor. Membrane ruffling and pseudopod extension via the BLTR was also completely inhibited by both Ptx and wortmannin. These data suggest that the chemoattractant receptors studied, G-protein usage varies with FR being totally dependent on Gαi, whereas BLTR and PAFR utilize both Gαi and a Ptx-insensitive G-protein. Both Ptx-sensitive and -insensitive G-protein usage can mediate the activation of phospholipase C, mobilization of intracellular calcium, and exocytosis by chemoattractant receptors. Chemotaxis, however, had an absolute requirement for a Gαi-mediated pathway.

Migration of leukocytes to sites of inflammation is mediated via the activation of G-protein-coupled chemoattractant receptors (1, 2). Chemoattractants at low concentrations elicit shape change, pseudopod extension, and chemotaxis, and at higher doses; many of them also trigger degranulation and generation of superoxide anions (1, 3). Pathways leading to these activities have been shown to have different dose requirements, kinetics and regulation (4, 5), but the role of G-protein usage remains unknown.

Formylpeptides (fMLP),1 platelet-activating factor (PAF), and leukotriene B4 (LTB4) are potent chemoattractants for neutrophils and to varying degrees also activate exocytosis and generation of superoxide anions (3, 4, 6). These activities are mediated through G-protein-coupled receptors (FR, PAFR, and BLTR) (2, 7). G-protein usage of chemoattractant receptors was known to be different depending on cell types (1, 8–10). Previous studies in RBL cells indicated that FR activated Gαi, whereas PAFR utilized both Gαi and a Ptx-insensitive G-protein to activate phosphoinositide hydrolysis, calcium mobilization, and exocytosis (11, 12). However, the G-protein usage requirements for activating chemotaxis versus exocytosis in these cells were unknown. Therefore, we sought to determine the relationship between G-protein usage versus the subsequent responses activated by these receptors in a single cell line viz. RBL-2H3 cells. Epitope-tagged BLTR, FR, and PAFR were expressed in RBL-2H3 cells, and inhibitors of signaling through Gα proteins (Ptx) and the PI3 kinase pathway (wortmannin) were used to determine the role of these pathways in pseudopod extension, chemotaxis, phospholipase C activation, calcium mobilization, and exocytosis. These data demonstrate distinct G-protein usage among chemoattractant receptors and suggest that a Gαi-mediated pathway, presumably involving βγ and PI3 kinase, is required for motility-related functions. On the other hand, stimulation of phospholipase C activity, calcium mobilization, and exocytosis can be mediated through activation of a Ptx-insensitive G-protein as well as through Gαi.

EXPERIMENTAL PROCEDURES

Materials—[32P]Orthophosphate (8500–9120 Ci/mmol), myo-[2-3H]inositol (24.4 Ci/mmol), and [3H]leukotriene B4 (200 Ci/mmol) were purchased from NEN Life Science Products. LTB4 was obtained from Cayman Chemicals. Rat tail collagen was obtained from Collaborative Biomedicals, 25 × 80 mm 8 μm pore size polycarbonate filters are from Neuroprobe, and the Leukostat staining kit was obtained from Fisher. All other materials were obtained from sources previously described (12).

Construction of Epitope-tagged BLTR—Nucleotides encoding a nine amino acid hemaglutinin-epitope sequence (YPYDVPDYA) are in-
serty between the N-terminal initiator methionine and the second amino acid of human BLTR by polymerase chain reaction methods as described previously for other chemoattractant receptors with minor changes in polymerase chain reaction conditions (12, 13). Because of high GC content of the DNA encoding the carboxyl-terminal third of the BLTR, cDNA polymerase chain reaction amplification occurred only in reactions containing 10% dimethyl sulfoxide (Me2SO). The integrity of the epitope tag as well as the rest of the molecule was verified by dideoxy sequencing after cloning into eukaryotic expression vector PRK-5. In BLTR a single nucleotide change resulted in a conservative substitution of amino acid 87 (Leu→Met). Competition ligand binding experiments and calcium mobilization studies indicated an affinity for LTB4 (Kd 4.4 ± 0.63 nM) comparable to that seen in neutrophils and in other BLTR-transfected cell lines (7). In some experiments, i.e. calcium mobilization, RBL cells expressing a clone without this point mutation showed identical results.

Cell Culture, Transfection, Phosphoinositide Hydrolysis, Ca2+ Mobilization, and Phosphorylation—We previously isolated RBL cells stably expressing FR and PAFR, and cells expressing BLTR were prepared following essentially the same procedures (11, 12). Methods for RBL-2H3 culture, transfection and clonal selection, phosphoinositide hydrolysis, calcium mobilization, 32P labeling, and immunoprecipitation of epitope-tagged receptors were essentially as described previously (12).

Chemotaxis—Migration of RBL stable transfectants was measured by a 48-well micro-chemotaxis chamber technique as described (14). Polycarbonate filters (25 × 80 mm 8 μ pore size Neuprobe) were coated with 50 μg/ml of rat type I collagen (Collaborative Biomedicals) in HEPES-buffered RPMI 1640 medium for 2 h at 37 °C. A dry coated filter was placed on a 48-well chamber containing different amounts of LTB4, FMLP, or PAF. RBL cells expressing the corresponding receptors (5 × 105/well) were added to the top wells, and the chamber was incubated at 37 °C in 5% CO2 for 4 h. Cells migrating to the underside of the filter were fixed using a leukostat staining kit. Cells from four high power (400X) fields representing at least two independent wells for each concentration of ligand were counted. Chemotaxis, measured with ligand at same concentration in both chambers, was less than 15% of the directed migration.

Micromanipulation—Cell micromanipulation was performed as described elsewhere (15). Briefly, the cells were added to a thermostated chamber, which was 2 mm thick and open from both sides to allow micromanipulation. The experiments were performed at 30 °C 15 min after removing the cells from the culture dishes. To prevent nonspecific cell activation, the bottom of the chamber was coated with collagen. After adding the cells to the experimental chamber a single cell was chosen and held with a collagen-coated pipette. A local region of the membrane of the chosen cell was exposed for 5 min to a solution containing 10 nM LTB4, which was delivered from another pipette. The cell was observed with an inverted microscope Nikon 200 with 60X oil immersion objective. The microscope images were recorded using a COHU CCD camera.

RESULTS

Functional Expression of LTB4 Receptor in RBL-2H3 Cells—Several chemoattractant receptors including FR and PAFR were previously expressed in RBL-2H3 cells for functional and regulation studies (11, 12). Native RBL cells did not express any LTB4 receptors as determined by lack of calcium mobilization to 1.0 μM LTB4. To determine the signal transduction pathways activated by LTB4 receptors, they were stably expressed in transfected clonal lines of RBL cells (BLTR). These receptors induced calcium mobilization, inositol phosphate production, exocytosis, and chemotaxis (see below) in RBL cells. Competition ligand binding experiments indicated an affinity for LTB4 (Kd 4.4 ± 0.63 nM) comparable to that seen in neutrophils and in other BLTR-transfected cell lines (7).

Phosphorylation of Chemoattractant Receptors—LTB4 and phorbol 12-myristate 13-acetate stimulated the phosphorylation of BLTR by severalfold (Fig. 1A, lanes 1–3). As the cDNA is predicted to encode a 35-kDa protein, the two phosphoprotein bands at 40 and 60 kDa likely represent distinct glycosylation forms. Pertussis toxin (16), an inhibitor of signaling through Gi and Go family of G-proteins, did not have any effect on ligand or phorbol 12-myristate 13-acetate-induced phosphorylation of BLTR (Fig. 1A) or PAFR (Fig. 1C). In contrast, FMLP-induced phosphorylation of FR was significantly inhibited by Ptx (Fig. 1B).

Distinct Pathways for Chemotaxis and Exocytosis—The ef-
FIG. 3. Effect of pertussis toxin and wortmannin on generation of total inositol phosphates (IP) and exocytosis. RBL cells expressing BLTR (A), FR (B), or PAFR (C) cultured overnight in the presence of [3H]inositol (1.0 μCi/ml) were left untreated (open circles), pretreated overnight with 100 ng/ml Ptx (closed squares), or pretreated for 3 h with 100 nM wortmannin (closed triangles) and stimulated with different concentrations of LTB₄, fMLP, or PAF as indicated. Total inositol phosphate generation was determined as described previously (12). The experiment was repeated three times with similar results. Ligand-induced secretion of β-hexoseaminidase (D, E, and F) was determined in cells left untreated (open circles), pretreated overnight with 100 ng/ml Ptx (closed squares), or pretreated for 3 h with 100 nM wortmannin (closed triangles) as described previously (12). The data are expressed as percentage of total β-hexoseaminidase present in cells. Data are mean ± S.E. of a single representative of three experiments performed in triplicate.
fects of Ptx and the PI3-kinase inhibitor wortmannin (17) on ligand-induced calcium mobilization (Fig. 2), phosphoinositide hydrolysis, exocytosis (Fig. 3), and chemotaxis (Fig. 4) were measured to determine the differential regulation of these responses. For BLTR, Ptx did not have a significant effect on the initial spike of calcium release but the length of the response was diminished (Fig. 2A). Whereas FR-induced calcium mobilization was completely inhibited by Ptx, it did not affect the initial spike or sustained calcium mobilization by PAFR (Fig. 2, B and C). A similar differential effect of Ptx was also seen in phosphotidylinositol hydrolysis and exocytosis responses of these three receptors. Whereas the responses of FR were completely inhibited, BLTR showed some resistance to inhibition by Ptx and about 50% of the PAFR responses were Ptx-resistant (Fig. 3). Wortmannin had no effect on calcium mobilization by BLTR, FR, or PAFR (Fig. 2) and also did not inhibit phosphotidylinositol hydrolysis (Fig. 3). Wortmannin did not affect the exocytosis by BLTR but partially inhibited these responses to FR and to low concentrations of PAF. In contrast to these differences, chemotaxis to fMLP, PAF, and LT B4 was completely inhibited by Ptx (Fig. 4). Wortmannin also inhibited chemotaxis to a similar extent (~90%) for all three receptors (Fig. 4).

Micromanipulation of RBL-BLTR Cells—Cytoskeletal alterations and pseudopod formation in the direction of the source of chemoattractant are essential events in leukocyte migration (18). To better define the role of distinct signaling events in leukocyte chemotaxis, ligand-induced membrane ruffling and pseudopod formation were studied using the micromanipulation assay (Fig. 5) (15, 19). A pipette held a single cell, and a region of its body was exposed to 10 nM LT B4 blown continuously from a second pipette. The local exposure of the cell surface to chemoattractant provided a condition for the formation of a single pseudopod-like structure in suspension (Fig. 5a). Incubation of cells with Ptx (Fig. 5b) or wortmannin (Fig. 5c) resulted in complete inhibition of LT B4-induced pseudopod formation. However, Ptx-treated cells spread on culture dishes when replated (data not shown) indicating that they retain their ability to extend lamellipodia during physical attachment but were unable to respond to LT B4 activation. They also displayed spontaneous activation and membrane ruffles (6 of 10 cells) at other sites on the cell body distinct from the area of stimulation by the second pipette. Cells treated with wortmannin also failed to extend a pseudopod-like structure. However, many of these cells showed a partial response and extended several membrane ruffles at the site of stimulation (7 of 10 cells) as shown in Fig. 5c.

DISCUSSION

Expression of chemoattractant receptors that activate different G-proteins in a single cell line allowed the determination of the role of specific G-proteins in activating distinct biological responses. The data show that chemoattractant receptors may activate phospholipase C, calcium mobilization, and exocytosis through multiple G-proteins, but ligand-stimulated pseudopod extension and chemotaxis require the activation of a Ptx-sensitive G-protein and PI3 kinase.

Comparison of biochemical responses activated by FR, BLTR, and PAFR, all of which activate G i, but couple to varying degrees to a Ptx-insensitive G-protein, allowed clear distinctions to be made for the role of G-proteins in different activities. Previous studies have indicated that receptor occupancy, but not signaling through G-proteins, is essential for receptor phosphorylation (20, 21). Whereas the PAFR and BLTR were phosphorylated to similar levels in Ptx-treated cells, FR phosphorylation was inhibited. These data suggest that FR is likely phosphorylated by G-protein-coupled receptor kinases GRK2 or GRK3, because both require free βγ for translocation to membrane and activation (20). Studies on in vitro phosphorylation of the C terminus of FR also support this contention (22). Lack of any affect, by Ptx, on ligand-induced

FIG. 4. Effect of pertussis toxin and wortmannin on chemotaxis. Ligand-dependent chemotaxis was measured in control cells (open circles), cells treated overnight with 100 ng/ml Ptx (closed squares), or cells treated with 100 nM wortmannin (closed triangles) 10 min prior to the start of chemotaxis assay as described under “Experimental Procedures.” Data are the mean ± S.E. from four individual fields for each concentration from a representative experiment from at least three repetitions.
The unique biochemical pathways activated by the βγ sub-units of G_i proteins that mediate chemotaxis are at present unknown. The βγ induced PI3 kinase activity, and its related signaling pathway could be one of these events (28–30). Other studies also indicated that GTPγS could activate actin polymerization in soluble cell extracts of leukocytes in the presence of the low molecular weight G-protein CDC42 but not Rhô or Rac (31, 32). Therefore it is possible that βγ dimers released from G_i, but not the G_q family of G-proteins, activate one or more low molecular weight G-proteins that may participate in leukocyte migration. In any case, the data clearly demonstrate that chemotactant receptors may couple to individual or multiple G-proteins. To stimulate chemotaxis however, they must activate G_i. Moreover, distinct signaling pathways among G-proteins are likely to determine other cellular responses initiated by chemotactant receptors. These data may explain numerous observations in neutrophils and other cells that different chemotactant receptors display different abilities to trigger nonmotility-related functions (i.e. respiratory burst, mitogenesis) despite exhibiting similar chemotactic responses (1, 23).

The availability of a model system in which migration responses may be measured both at population and single cell levels will allow further investigation of the divergent pathways for motility and cytotoxic functions.

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