Evidence that activation of a common G-protein by receptors for leukotriene B₄ and *N*-formylmethionyl-leucyl-phenylalanine in HL-60 cells occurs by different mechanisms

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Differentiated HL-60 cells were found to respond to the chemoattractants leukotriene B₄ (LTB₄) and *N*-formylmethionyl-leucyl-phenylalanine (FMLP), in a manner similar to neutrophils. Membranes of myeloid differentiated HL-60 cells were used (a) to examine the ability of LTB₄ receptors to interact with a guanine-nucleotide-binding protein (G-protein), and (b) to compare this G-protein with that which is coupled to the FMLP receptor. LTB₄ stimulated a dose-dependent increase in GTP hydrolysis and guanosine 5'-[γ-thio]triphosphate (GTP[S]) binding, demonstrating that LTB₄ receptors on HL-60 cells are coupled to a G-protein. Both pertussis toxin and cholera toxin inhibited stimulation of GTPase activity and GTP[S] binding by either LTB₄ or FMLP, indicating that both receptors are coupled to a G-protein containing a 40 kDa α-subunit. That the two receptors share a common G-protein was shown by FMLP enhancement of cholera-toxin-induced inhibition of GTPase activity stimulated by either FMLP or LTB₄. However, LTB₄ did not enhance cholera-toxin-induced inhibition of GTPase activity, suggesting that the receptors interacted differently with this G-protein. This difference was confirmed by showing that FMLP, but not LTB₄, stimulated receptor-specific [³²P]ADP-ribosylation of the 40 kDa α-subunit. Concentrations of LTB₄ and FMLP which produced maximal responses produced enhanced stimulation in both assays. This additive effect was not abolished by inactivation of up to 80 % of G-protein activity by *N*-ethylmaleimide or cholera toxin. We conclude that LTB₄ and FMLP receptors in HL-60 cells are coupled to a common G-protein. The receptor–G-protein interaction is different for the two receptors, and G-proteins not coupled to both receptors may account for the additive response.

INTRODUCTION

Leukotriene B₄ (LTB₄) is a 5-lipoxygenase metabolite of arachidonic acid which is produced by polymorphonuclear leucocytes (PMNs) in response to specific stimuli [1–3]. LTB₄ shares many pro-inflammatory properties with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), including the ability to stimulate PMN adherence, chemotaxis, release of lysosomal enzymes and production of superoxide (O₂^{•-}) [4–8]. In addition to shared functional activities, LTB₄ and FMLP appear to share a similar signal-transduction pathway. Specific receptors for both compounds mediate stimulation of phospholipase C, leading to the breakdown of phosphatidylinositol 4,5-bisphosphate and the formation of inositol 1,4,5-trisphosphate and diacylglycerol [9–17]. Despite the similarities of PMN responses to LTB₄ and FMLP, significant differences in the magnitude and time course of these responses have been described. Synderman and colleagues [18,19] have reported that LTB₄ and FMLP differ in their ability to stimulate sustained inositol trisphosphate generation, diacylglycerol production, and a sustained increase in

intracellular Ca²⁺. Similarly, LTB₄ is a poor stimulant for neutrophil O₂^{•-} release and lysosomal-enzyme secretion compared with FMLP [7,18–20].

Substantial evidence supports the conclusion that a guanine-nucleotide-binding protein (G-protein) couples the FMLP–receptor interaction with subsequent biochemical and functional responses (reviewed in [21]). The LTB₄ receptor may also be coupled to a G-protein, since LTB₄-induced mobilization of intracellular Ca²⁺, stimulation of inositol trisphosphate formation and stimulation of PMN functional responses are inhibited by pertussis toxin [22–26]. However, no direct examination of the ability of LTB₄ to activate G-proteins in human cells has been reported.

The present study was initiated to examine the LTB₄–receptor–G-protein interaction and to compare the characteristics of the G-proteins coupled to LTB₄ and FMLP receptors. The human promyelocytic cell line HL-60, which can be induced to differentiate into cells possessing functionally active LTB₄ and FMLP receptors [10,28,29], was used for these studies. The ability of differentiated HL-60 cells to simulate PMN function and

Abbreviations used: G-protein, guanine-nucleotide-binding proteins; GTP[S], guanosine 5'-[γ-thio]triphosphate; p[NH]ppG, guanosine 5'-[β-γ-imido]triphosphate; LTB₄, leukotriene B₄; NEM, *N*-ethylmaleimide; PMNs, polymorphonuclear leucocytes; FMLP, *N*-formylmethionyl-leucyl-phenylalanine.

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provide a model for the disparate PMN responses to FMLP and LTB₄ was demonstrated. We show that LTB₄ receptors are coupled to a pertussis-toxin- and cholera-toxin-sensitive G-protein. Both LTB₄ and FMLP receptors appear to be coupled to a common G-protein, but interact differently with this G-protein.

MATERIALS AND METHODS

Reagents

Pertussis toxin was purified from the supernatant of *Bordetella pertussis* suspensions kindly provided by Dr. F. Blackkolb, Behringwerke (Marburg, Germany), as previously described [30]. Cholera toxin was obtained from List (Campbell, CA, U.S.A.). FMLP was from Sigma (Deisenhofen, Germany), and LTB₄ was generously supplied by Dr. W. Bartmann and Dr. M. Schorr (Hoechst, Frankfurt, Germany). GTP[S] was from Boehringer (Mannheim, Germany). GTP[³⁵S] (1200–1300 Ci/mol) was from New England Nuclear (Dreieich, Germany). [γ -³²P]GTP and [³²P]NAD were prepared as previously described [31].

Cell cultures and membrane preparation

HL-60 cells were grown in suspension culture in RPMI 1640 supplemented with 10% (v/v) horse serum, 1% (v/v) non-essential amino acids, 2 mM-L-glutamine, 50 units of penicillin/ml and 50 μ g of streptomycin/ml in a humidified atmosphere (8% CO₂) at 37 °C. To induce myeloid differentiation, cells were seeded at a density of 10⁶ cells/ml and were cultivated for 5 days in medium containing 1.25% (v/v) dimethyl sulfoxide [32]. HL-60 cell membranes were prepared as previously described, except that centrifugation through Percoll gradients was omitted [33]. To obtain pertussis-toxin-treated membranes HL-60 cells were cultivated with the toxin at 50 ng/ml for 24 h before membrane preparation.

Chemotaxis and superoxide (O₂⁻) assays

HL-60-cell chemotaxis was assayed by a modification of the method described by Nelson *et al.* [34]. A series of three concentric rings of wells were cut in 0.6% agarose layers in Petri dishes. The outer ring of wells was filled with the chemoattractant, the wells of the middle ring were each filled with 2.5 $\times$ 10⁵ differentiated HL-60 cells, and the inner ring of wells was filled with buffer to determine random migration. Plates were incubated for 2 h at 37 °C in 5% CO₂ in air, after which the agarose layer was removed and the cells were fixed with methanol and stained with Giemsa stain. Chemotaxis was measured under light microscopy (magnification $\times$ 100) by using a micrometer. The distance of random migration in mm was subtracted from the distance of directed migration toward the chemoattractant to derive the chemotaxis index.

Superoxide release was measured as the reduction of cytochrome *c* by using a spectroscopic assay as previously described [35]. HL-60 cells were suspended at 4.4 $\times$ 10⁶/ml in Phenol-Red-free Hanks balanced salt solution. Cytochrome *c* (80 μ M; 100 μ l) and cells (900 μ l) were prewarmed at 37 °C for 5 min. FMLP, LTB₄ or control buffer (10 μ l) was added to the reaction mixture to give the desired final concentration, vortex-mixed, and incubated for 10 min at 37 °C. After incubation, the cells were separated from the supernatant by centrifugation,

and the reduced cytochrome *c* was determined by spectroscopic analysis of the supernatant at 550 nm. The quantity of reduced cytochrome *c* was calculated by using the absorption coefficient of 2.1 $\times$ 10⁴ M⁻¹·cm⁻¹, and the results are expressed as nmol of reduced cytochrome *c*/4 $\times$ 10⁶ cells.

GTPase assay

Hydrolysis of [γ -³²P]GTP (0.02–0.05 μ Ci per tube) was determined in a reaction mixture (100 μ l) containing 2–10 μ g of membrane protein, 50 mM-triethanolamine/HCl, pH 7.4, 1 mM-dithiothreitol, 0.1 mM-ATP, 5 mM-MgCl₂, 1 mM-EDTA, 100 mM-NaCl, 0.1 μ M-GTP, 5 mM-phosphocreatine, 0.4 mg of creatine kinase/ml, and 0.2% (w/v) bovine serum albumin. Reactions were carried out for 10 min at 30 °C in the absence or presence of LTB₄ and/or FMLP. Reactions were terminated and high-affinity GTPase activity was determined as previously described [36]. Membrane protein was determined as described by Bradford [37], with bovine IgG as the standard. Results were expressed as pmol of free P_i generated/min per mg of protein.

GTP[S]-binding assay

This was done in a reaction mixture (100 μ l) containing 50 mM-triethanolamine/HCl, pH 7.4, 1 mM-dithiothreitol, 1 mM-EDTA, 5 mM-MgCl₂, 150 mM-NaCl, 0.5 μ M-GDP and GTP[³⁵S] (0.02–0.04 μ Ci per tube). The reaction was initiated by addition of 2–10 μ g of membrane protein to each tube. Reactions were terminated after 20 min at 30 °C by filtration through GF/C Whatman filters, followed by washing with 10 ml of buffer containing 50 mM-triethanolamine/HCl and 5 mM-MgCl₂. Specific binding was calculated by subtracting the amount of GTP[³⁵S] bound in the presence of 10 μ M-GTP[S] from total GTP[³⁵S] bound, and expressed as pmol of GTP[S] bound/mg of membrane protein.

[³²P]ADP-ribosylation by cholera toxin

Cholera toxin (2 mg/ml) was activated by dilution with an equal volume of 40 mM-dithiothreitol and incubated for 10 min at 30 °C. Membranes (50–100 μ g of protein) were incubated for 60 min at 37 °C in a volume of 50 μ l containing 100 mM-potassium phosphate buffer, pH 7.5, 2.5 mM-MgCl₂, 1 mM-ATP, 10 mM-thymidine, 10 mM-arginine, 100 μ g of activated cholera toxin/ml, 1 μ M-[³²P]NAD⁺, and guanosine 5'-[β -imido]triphosphate (p[NH]ppG), FMLP, and/or LTB₄ at concentrations indicated in the Figure legends. SDS/polyacrylamide gel electrophoresis and autoradiography were performed as previously described [38,39].

Cholera-toxin pretreatment

For treatment of membranes with cholera toxin and NAD, membranes (1 mg/ml) were incubated in a buffer containing 50 mM-triethanolamine/HCl, pH 7.4, 2 mM-ATP, 10 mM-NAD, 2.5 mM-MgCl₂, and 100 μ g of dithiothreitol-activated cholera toxin/ml or an equal volume of the carrier solution, as previously described [40]. After a 20 min incubation at 37 °C, membranes were washed by centrifugation, resuspended in 10 mM-triethanolamine/HCl, and used directly in the appropriate assay.

N-Ethylmaleimide (NEM) pretreatment

Membranes (2–10 μ g/tube) were incubated with NEM

Fig. 1. Dose-response of superoxide release stimulated by FMLP or LTB₄

HL-60 cells (4×10^6) were incubated with 80 mM-cytochrome *c* and the appropriate concentration of FMLP or LTB₄ for 10 min at 37 °C. Thereafter, cells were separated from supernatant, which was assayed for reduced cytochrome *c* by spectroscopy. Results are expressed as means $\pm$ S.D. for five separate experiments each performed in duplicate, in nmol of reduced cytochrome *c* per 4×10^6 cells.

(10–100 μ M) for 15 min at 25 °C in a reaction mixture containing 50 mM-triethanolamine/HCl, pH 7.4, 0.1 mM-ATP, 5 mM-MgCl₂, 1 mM-EDTA, 100 mM-NaCl, 5 mM-phosphocreatine, 0.4 mg of creatine kinase/ml and 0.2 % bovine serum albumin. Thereafter, 5 mM-dithiothreitol was added for another 5 min period. The membranes were then used directly in the GTPase assay.

RESULTS

The ability of differentiated HL-60 cells to undergo chemotaxis and to produce a respiratory burst in response to FMLP and LTB₄ was examined. Both LTB₄ and

FMLP at 0.1 μ M stimulated chemotaxis. The chemotaxis index, determined by subtracting random migration from directed migration, was 28.9 ± 3.7 mm for FMLP ($n = 17$) and 12.0 ± 4.8 mm for LTB₄ ($n = 13$). The ability of various concentrations of FMLP and LTB₄ to stimulate superoxide release is shown in Fig. 1. FMLP stimulated a dose-dependent increase in superoxide release, but LTB₄ failed to stimulate its release. Thus HL-60 cells not only provide a good model of PMN function [41], but also exhibit the disparate functional responses to FMLP and LTB₄ [7,18–20].

To determine if LTB₄ activated a G-protein, the dose-dependent responses of GTP hydrolysis and GTP[S] binding to LTB₄ in membranes isolated from differentiated HL-60 cells were determined (Fig. 2). A similar response was found in both assays, with the earliest effect seen at 1 nM- and the maximal response at 1 μ M-LTB₄. To determine if LTB₄ and FMLP produce similar degrees of GTP-GDP exchange and GTP hydrolysis, the abilities of optimal concentrations of LTB₄ and FMLP to stimulate GTP[S] binding and GTPase activity in different membrane preparations were compared (Table 1). The maximal increase in GTPase activity varied from 35 to 100 %, and that in GTP[S] binding from 43 to 250 %, in different membrane preparations. The level of response to LTB₄ (1 μ M) was, in general, equivalent to or slightly less than that produced by a 10-fold greater concentration of FMLP (10 μ M).

To characterize the G-proteins coupled to LTB₄ and FMLP receptors, the ability of pertussis toxin and cholera toxin to inhibit G-protein activation by LTB₄ and FMLP was examined. Fig. 3 demonstrates that treatment of HL-60 cells with 50 ng of pertussis toxin/ml for 24 h completely blocked the ability of LTB₄ and FMLP to stimulate either GTP hydrolysis or GTP[S] binding in isolated membranes. The effect of cholera toxin on LTB₄-stimulated GTPase activity and GTP[S] binding was examined by pretreating isolated membranes with 100 μ g of activated cholera toxin/ml (Fig. 4). Both activities were markedly inhibited, as compared with membranes

Fig. 2. Dose-response of GTP hydrolysis (a) and GTP[S] binding (b) to LTB₄

Results are averages of triplicate determinations in a single experiment representative of four experiments. Hydrolysis of [γ -³²P]GTP was determined after a 10 min incubation at 30 °C of 2–10 μ g of membrane protein with the appropriate concentrations of LTB₄, by the release of free [³²P]P_i. Results are expressed as pmol of P_i generated/min per mg of membrane protein. GTP[S] specific binding was determined after a 20 min incubation of 2–10 μ g of membrane protein with LTB₄ and GTP[³⁵S] at 25 °C. Results are expressed as pmol of GTP[³⁵S] bound/mg of membrane protein.

Table 1. Comparison of maximal GTP[S] binding and GTPase activity stimulated by FMLP and LTB₄

Results of a single experiment for each membrane are shown, expressed as means $\pm$ s.d. for triplicate determinations of GTP[S] binding and GTPase activity stimulated by 10 μ M-FMLP or 1 μ M-LTB₄.

Membrane no.	GTP[S] bound (pmol/mg of protein)		GTPase activity (pmol/min per mg of protein)	
	FMLP	LTB ₄	FMLP	LTB ₄
8	0.959 $\pm$ 0.018	0.725 $\pm$ 0.017	61.2 $\pm$ 5.1	73.5 $\pm$ 8.0
10	0.762 $\pm$ 0.022	0.708 $\pm$ 0.018	54.6 $\pm$ 9.3	62.3 $\pm$ 1.9
12	0.808 $\pm$ 0.020	0.660 $\pm$ 0.008	54.9 $\pm$ 1.2	56.2 $\pm$ 0.5
14	1.226 $\pm$ 0.025	1.196 $\pm$ 0.090	31.8 $\pm$ 6.6	22.7 $\pm$ 3.2
20	0.638 $\pm$ 0.009	0.405 $\pm$ 0.018	27.8 $\pm$ 1.6	20.7 $\pm$ 0.3
21	1.009 $\pm$ 0.049	0.559 $\pm$ 0.064	26.6 $\pm$ 1.7	20.2 $\pm$ 1.0
241	0.787 $\pm$ 0.030	0.628 $\pm$ 0.009	66.3 $\pm$ 1.8	47.2 $\pm$ 1.6

treated identically except for the omission of cholera toxin. It has previously been shown that cholera toxin inhibits GTPase activity in HL-60 cell membranes stimulated by FMLP [40]. Fig. 4 demonstrates that cholera toxin also prevents FMLP-induced GTP[S] binding.

The ability of both pertussis toxin and cholera toxin to inhibit LTB₄- and FMLP-stimulated G-protein activation suggested to us that LTB₄ and FMLP receptors may be coupled to the same G-protein. To examine the hypothesis that the two receptors share a common G-protein, isolated membranes were pretreated under conditions which result in receptor-specific cholera-toxin-catalysed ADP-ribosylation [40]. Membranes were pretreated with activated cholera toxin, or cholera toxin plus FMLP or LTB₄, or treated identically except for the omission of cholera toxin. These membranes were then separated from the reaction mixture, and GTPase activity was stimulated by LTB₄ (1 μ M) or FMLP (10 μ M). Fig. 5(a) demonstrates that inclusion of FMLP during pretreatment enhances cholera-toxin-mediated inhibition of GTPase activity stimulated by either FMLP or LTB₄.

In the absence of cholera toxin, FMLP had no effect on subsequent GTPase activity. These results indicate that the G-proteins inactivated by FMLP-specific cholera-toxin activity are also coupled to LTB₄ receptors. On the other hand, inclusion of LTB₄ during pretreatment did not alter cholera-toxin-mediated inhibition of GTPase activity stimulated by either LTB₄ or FMLP (Fig. 5b).

To determine the reason for the inability of LTB₄ to enhance cholera-toxin-mediated inhibition, the ability of FMLP and LTB₄ to stimulate cholera-toxin-catalysed ADP-ribosylation of the 40 kDa α -subunit was examined. Fig. 6 demonstrates that FMLP stimulates a receptor-specific dose-dependent increase in [³²P]ADP-ribosylation in the presence of the guanine nucleotide p[NH]ppG, as previously reported [40]. On the other hand, LTB₄ failed to stimulate [³²P]ADP-ribosylation and failed to produce an additive effect when added simultaneously with FMLP (10 nM). Therefore, LTB₄ failed to enhance cholera-toxin-mediated inhibition of GTPase activity because it does not stimulate receptor-specific cholera-toxin-catalysed ADP-ribosylation.

Fig. 3. Effect of pertussis toxin (PT) on LTB₄- (○, ●) and FMLP- (□, ■) stimulated GTPase activity (a) and GTP[S] binding (b)

The FMLP concentration was 10 μ M. Results are averages of triplicate determinations from a single experiment representative of three experiments. ○, □, Response of membranes prepared from cells cultivated with pertussis toxin (50 ng/ml) for 24 h as described in the Materials and methods section; ●, ■, response of membranes prepared from the same cell culture without exposure to pertussis toxin.

Fig. 4. Effect of cholera toxin (CT) on LTB₄ (●, ○) and FMLP- (■, □) stimulated GTPase activity (a) and GTP[S] binding (b)

The FMLP concentration was 10 μ M. Results are the average of triplicate determinations from a single experiment representative of three experiments. ○, □, Response of membranes pre-exposed to cholera toxin as described in the Materials and methods section; ●, ■, response of membranes treated identically except that the cholera toxin was omitted.

If LTB₄ and FMLP receptors share all their G-proteins, we expected that addition of FMLP and LTB₄ at concentrations which produced a maximal response would not produce an effect greater than that of the same concentrations of FMLP or LTB₄ alone. The ability of these two compounds to stimulate GTP hydrolysis and GTP[S] binding, either alone or in combination, is shown in Fig. 7. In both assays LTB₄ and FMLP produced an enhanced effect at concentrations which evoked a maximal response. One possible explanation for this result is that the quantity of membrane G-protein exceeds that which can be activated by either receptor alone. To test this possibility, the ability of LTB₄ and

FMLP to produce an additive effect on GTPase activity was measured after pretreatment of membranes with cholera toxin or various concentrations of NEM, which alkylates pertussis-toxin-sensitive G-proteins, rendering them non-functional [42]. The results of pretreatment with 30 μ M-NEM are shown in Fig. 8. Despite an 80% decrease in basal GTPase activity and a 70–75% decrease in stimulated activity, optimal concentrations of LTB₄ and FMLP produced an increased effect above either compound alone. Similar results were seen at

Fig. 5. Effect of pretreatment of membranes with cholera toxin in the absence or presence of FMLP (10 μ M) or LTB₄ (1 μ M) on subsequent GTPase activity stimulated by FMLP (10 μ M) or LTB₄ (1 μ M)

For details see the Materials and methods section. (a) Inhibition of GTPase activity by pretreatment with cholera toxin alone (▨) and cholera toxin plus FMLP (▩) compared with control (■). (b) Inhibition of GTPase activity by pretreatment with cholera toxin (▨) or cholera toxin plus LTB₄ (▩) compared with control (■). Results are expressed in pmol/min per mg of protein, as means $\pm$ S.D. for three separate experiments each performed in triplicate.

Lane	1	2	3	4	5	6	7	8	9	10
p[NH]ppG ...	—	+	+	+	+	+	+	+	+	+
[FMLP] (M) ...	—	—	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	—	—	—	10 ⁻⁸
[LTB ₄] (M) ...	—	—	—	—	—	—	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁶

Fig. 6. Effect of FMLP and LTB₄ on ADP-ribosylation by cholera toxin

Membranes (30 μ g of protein) were incubated with [³²P]NAD and cholera toxin alone (lane 1) or cholera toxin plus 100 μ M-p[NH]ppG (lanes 1–10) as described in the Materials and methods section. To the reaction mixture various concentrations of FMLP, LTB₄, or LTB₄ (1 μ M) plus FMLP (10 nM) were added and incubated at 37 $^{\circ}$ C for 60 min. The proteins were subjected to SDS/PAGE, and the dried gel was autoradiographed.

Fig. 7. Response to addition of 10 μ M-FMLP with various concentrations of LTB_4 on GTPase activity and GTP[S] binding

●, Response to LTB_4 alone; ■, response to FMLP plus the indicated LTB_4 concentration. Results are averages of triplicate determinations from a single experiment representative of three experiments.

Fig. 8. Effect of G-protein inactivation by pretreatment of membranes with NEM on the additive stimulation of GTPase activity by FMLP and LTB_4

Membranes were preincubated with 30 μ M-NEM, then the NEM was inactivated with dithiothreitol, and basal (C) GTPase activity and that stimulated by LTB_4 (1 μ M), FMLP (10 μ M), or both LTB_4 and FMLP, were determined. Results are expressed as averages of triplicate determinations from a single experiment. The control represents the response of membranes treated identically except that NEM was omitted.

NEM concentrations of 10, 50 and 100 μ M (results not shown). Pretreatment of membranes with cholera toxin for 20 min produced a 40–50% inhibition of both LTB_4 - and FMLP-stimulated GTPase activity (Fig. 9). Similarly to the results with NEM, cholera-toxin-induced decrease in the number of G-proteins coupling to the two receptors resulted in an enhanced stimulation of GTPase activity by FMLP and LTB_4 .

Fig. 9. Effect of G-protein inactivation with cholera toxin on the additive stimulation of GTPase activity by FMLP and LTB_4

Membranes were preincubated with cholera toxin or the carrier solution (Control) as indicated in the Materials and methods section. Membranes were then stimulated with FMLP (10 μ M), LTB_4 (1 μ M), or the two together, and the hydrolysis of [γ - ^{32}P]GTP was compared with basal activity (C). Results are expressed in pmol/min per mg of protein, as means $\pm$ s.d. for three separate experiments each performed in triplicate.

DISCUSSION

The results indicate that HL-60 cells not only mimic PMN functional responses [41], but also serve as a model for the disparate PMN responses to FMLP and LTB_4 [7, 18–20]. Both FMLP and LTB_4 stimulate PMN chemotaxis and adherence [4, 7, 8], whereas LTB_4 is an ineffective stimulator of lysosomal-enzyme secretion and the respiratory burst compared with FMLP [7, 20]. In our studies, both LTB_4 and FMLP stimulated a significant chemotactic response by HL-60 cells, whereas FMLP, but not LTB_4 , stimulated a dose-dependent release of O_2^- .

The present study provides direct evidence that LTB₄ receptors are coupled to G-proteins in human cells. LTB₄ stimulated the binding of the hydrolysis-resistant GTP analogue, GTP[S], and stimulated GTP hydrolysis in plasma membranes isolated from myeloid-differentiated HL-60 cells. The dose-response curves indicate that the LTB₄-receptor-G-protein interaction was detectable at concentrations of LTB₄ as low as 1 nM, and the maximal response occurred at 1 μ M. These results confirm the LTB₄-receptor-G-protein interaction suggested in reports demonstrating that pertussis toxin inhibited PMN biochemical and functional responses to LTB₄ [15,16,22–26] and that LTB₄ stimulated GTPase activity in rabbit neutrophil membranes [27].

Substantial evidence indicates that a G-protein couples FMLP receptors to effector enzymes in human PMNs and HL-60 cells. This evidence includes pertussis-toxin inhibition of biochemical and functional responses to FMLP [22,43–48], FMLP stimulation of a high-affinity GTPase in plasma membranes of PMNs and HL-60 cells [21,27,48], regulation of FMLP receptor affinity by guanine nucleotides [49], and requirement of guanine nucleotides for FMLP-stimulated phospholipase C activation [50,51]. Because both chemotactic receptors are coupled to G-proteins, but the functional responses they elicit are different, we compared the characteristics of G-proteins coupled to FMLP and LTB₄ receptors. Our aim was to determine if FMLP and LTB₄ receptors are equally capable of activating G-proteins and if these two receptors are coupled to the same or different G-proteins.

To determine if FMLP and LTB₄ receptors have different abilities to stimulate activation or inactivation of G-proteins, the maximal stimulation of GTP[S] binding and GTPase activity by FMLP and LTB₄ was compared in different membrane preparations (Table 1). Although the maximal responses varied in different membrane preparations, in most preparations the responses to FMLP and LTB₄ were similar. Therefore, differences in the ability of FMLP and LTB₄ to activate G-proteins by stimulating GTP-GDP exchange and to inactivate G-proteins by GTP hydrolysis are an unlikely explanation for the marked disparity in functional responses.

The characterization of the G-proteins coupled to FMLP and LTB₄ receptors suggests that the two receptors are coupled to a common G-protein. Both FMLP and LTB₄ receptors are uncoupled from their G-proteins by pertussis toxin and cholera toxin, as both toxins inhibited LTB₄- and FMLP-stimulated GTP[S] binding and GTPase activity. Feltner *et al.* [27] also reported inhibition of FMLP- and LTB₄-stimulated GTPase activity after pertussis-toxin treatment, but in rabbit neutrophils. On the other hand, cholera toxin failed to inhibit LTB₄-stimulated GTP hydrolysis in these cells. The discrepancy between our results with cholera toxin and those of Feltner *et al.* [27] may be due to their treatment of intact cells with cholera toxin, their use of sonicated cell preparations, or to differences between rabbit neutrophils and human HL-60 cells. Pertussis toxin catalyses ADP-ribosylation of a 40 kDa protein in HL-60 cell membranes [33], whereas cholera toxin catalyses ADP-ribosylation of two proteins with molecular sizes of 40 and 43 kDa (Fig. 6 and ref. [40]). Therefore our data indicate that a G-protein containing a 40 kDa α -subunit ($G\alpha_{40}$) is coupled to receptors for both chemoattractants. This conclusion is consistent with the recent finding that a G-protein containing a

40 kDa α -subunit co-purifies with the FMLP receptor [52].

More direct evidence that FMLP and LTB₄ receptors share a common G-protein was provided by measuring the ability of FMLP to enhance cholera-toxin activity. Our laboratory has previously reported that, in the presence of guanine nucleotides, FMLP stimulates enhanced cholera-toxin-catalysed ADP-ribosylation of $G\alpha_{40}$ and uncouples this G-protein from the FMLP receptor [40]. We used this response to determine if the FMLP-specific uncoupling of $G\alpha_{40}$ from FMLP receptors also uncouples this G-protein from LTB₄ receptors. Membranes were pretreated with cholera toxin alone or in combination with LTB₄ (1 μ M) or FMLP (10 μ M) for periods of time which produced sub-optimal inhibition of basal and stimulated GTPase activity. The presence of FMLP with cholera toxin enhanced the inhibition of GTPase activity stimulated by either FMLP or LTB₄ (Fig. 5). We interpret these results to indicate that the G-proteins inactivated by FMLP-specific ADP-ribosylation are coupled to both FMLP receptors and LTB₄ receptors. However, inclusion of LTB₄ during pretreatment did not enhance cholera-toxin-mediated inhibition of GTPase activity stimulated by either LTB₄ or FMLP. This was shown to result from the inability of LTB₄ to stimulate cholera-toxin-catalysed ADP-ribosylation of $G\alpha_{40}$. Although FMLP stimulated dose-dependent cholera-toxin-catalysed labelling of $G\alpha_{40}$ in the presence of [³²P]NAD and p[NH]ppG, LTB₄ at concentrations of 10 nM to 1 μ M failed to stimulate this labelling. LTB₄ (1 μ M) also failed to produce an additive effect when added simultaneously with 10 nM-FMLP (Fig. 6). Thus FMLP and LTB₄ receptors interact differently with $G\alpha_{40}$, despite the ability of both receptors to activate this G-protein. The mechanism and functional implications of this difference in receptor-G-protein interaction remain to be determined.

To determine if this common G-protein comprised all of the G-protein coupled to the two receptors, the additive effect of FMLP and LTB₄ was examined. We reasoned that, if the G-proteins used by FMLP and LTB₄ receptors were totally shared, simultaneous addition of FMLP and LTB₄ would not result in additive stimulation of GTP[S] binding or GTPase activity. FMLP and LTB₄ at concentrations which, alone, stimulated an optimal response produced an enhanced response in both assays (Fig. 7). Two hypotheses which might explain this additive effect are the presence of non-limiting quantities of the shared G-protein in the membrane or the presence of G-proteins coupled to one, but not both, receptors. To examine the first hypothesis, the ability of cholera toxin or NEM to uncouple G-proteins functionally from their receptors was utilized. We expected that inactivating a large proportion of G-protein available to the receptors would abrogate any additive effect resulting from excess G-protein. Despite a 70–80 % decrease in basal and stimulated GTPase activity with NEM and a 40–50 % decrease with cholera toxin, a partially additive effect remained. Thus there may be G-proteins coupled to one of the chemotactic receptors that are not shared with the other.

In summary, we have shown that LTB₄ receptors on human cells are coupled to a pertussis-toxin- and cholera-toxin-sensitive G protein. At least some of the G-proteins coupled to LTB₄ receptors also couple to FMLP receptors. The receptor-G-protein interaction appears to differ from FMLP and LTB₄ receptors. The

role of these different interactions in the regulation of the disparate functional responses to FMLP and LTB₄ remains to be determined.

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