Differential cholera-toxin- and pertussis-toxin-catalysed ADP-ribosylation of G-proteins coupled to formyl-peptide and leukotriene B₄ receptors

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N-Formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) and leukotriene B₄ (LTB₄) induce disparate second-messenger generation and functional responses in neutrophils and HL-60 granulocytes. Receptors for these chemoattractants couple to a common pool of G-proteins which are substrates for both pertussis-toxin- and cholera-toxin-catalysed ADP-ribosylation. The hypothesis that formyl-peptide and LTB₄ receptors induce different receptor-specific conformations of activated G-proteins was tested. The ability of pertussis toxin and cholera toxin to ADP-ribosylate G_i proteins coupled to formyl-peptide or LTB₄ receptors in membranes isolated from HL-60 granulocytes was used to assess the conformational state of the α subunits. Cholera-toxin-catalysed ADP-ribosylation of α_{40} (40 kDa α subunit) was inhibited by guanosine 5'-[β -

imido]triphosphate and GDP in a concentration-dependent manner. Addition of fMet-Leu-Phe, but not LTB₄, re-established cholera-toxin labelling of α_{40} in the presence of either guanine nucleotide. In the absence of guanine nucleotides, fMet-Leu-Phe and C5a enhanced cholera-toxin-catalysed labelling of α_{40} , whereas LTB₄ and platelet-activating factor had no effect. Preincubation with fMet-Leu-Phe, but not LTB₄, inhibited pertussis-toxin labelling of α_{40} in the presence of guanosine 5'-[γ -thio]triphosphate and in the absence of guanine nucleotides. Preincubation with fMet-Leu-Phe or LTB₄ enhanced pertussis-toxin labelling of α_{40} in the presence of GDP. These data suggest that activated G_i proteins coupled to formyl-peptide and LTB₄ receptors exist in different conformations determined by the receptor with which they interact.

INTRODUCTION

Polymorphonuclear leucocytes (PMNs) are capable of migrating to a site of inflammation and then releasing lysosomal enzymes or undergoing a respiratory burst when stimulated by a heterogeneous group of agonists termed chemoattractants. This group of agonists consists of peptide chemoattractants, including formylated peptides, C5a and interleukin 8, and lipid chemoattractants, including leukotriene B₄ (LTB₄) and platelet-activating factor (PAF). Chemoattractants differ in their ability to stimulate PMN responses. Formylated peptides and C5a are potent stimuli of the respiratory burst, whereas LTB₄ is relatively impotent [1–7]. Disparate functional responses are associated with differences in second-messenger generation. Specifically, phospholipase D generation of diacylglycerol is more transient and quantitatively smaller after LTB₄ and PAF stimulation compared with that resulting from *N*-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) stimulation [3,8,9]. The molecular basis for disparate PMN second-messenger generation and functional responses to different chemoattractants is not known.

Current evidence indicates that chemoattractants stimulate PMN activation through G-protein-coupled signalling pathways [10–18]. We have shown previously that formyl-peptide and LTB₄ receptors stimulate GTP hydrolysis and guanosine 5'-[γ -thio]triphosphate (GTP[S]) binding in membranes prepared from HL-60 granulocytes [6]. G-protein activation by fMet-Leu-Phe and LTB₄ in these membranes was inhibited by pretreatment with either pertussis toxin or cholera toxin. We used receptor-specific cholera-toxin-catalysed ADP-ribosylation, described by Gierschik and Jakobs [19], to show that fMet-Leu-Phe and LTB₄ receptors coupled to common G-proteins. However, whereas

fMet-Leu-Phe stimulated receptor-specific cholera-toxin labelling, LTB₄ lacked this activity [6].

One hypothesis which could explain these findings is the ability of activated G-proteins to exist in different receptor-specific conformations. In the present study, the ability of pertussis toxin and cholera toxin to ADP-ribosylate G-proteins coupled to formyl-peptide or LTB₄ receptors in membranes isolated from HL-60 granulocytes was used to assess the conformational state of these G-proteins [20–23]. The ability of G_i to act as a substrate for these toxins was modulated by the presence of guanine nucleotides and fMet-Leu-Phe or LTB₄. The data support the hypothesis that formyl-peptide and LTB₄ receptors induce different conformations of activated α_i (α subunit of G_i).

MATERIALS AND METHODS

Reagents

fMet-Leu-Phe, C5a and PAF were obtained from Sigma (St. Louis, MO, U.S.A.). GDP, GTP, guanosine 5'-[β -imido]triphosphate (p[NH]ppG) and GTP[S] were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). LTB₄ was purchased from Calbiochem (San Diego, CA, U.S.A.). [³²P]NAD⁺ was from DuPont–New England Nuclear (Boston, MA, U.S.A.). Pertussis toxin and cholera toxin were from List Biological Laboratories (Campbell, CA, U.S.A.).

Cell culture and membrane preparation

HL-60 cells were grown in suspension culture in RPMI 1640 supplemented with 10% horse serum, 1% non-essential amino acids, 2 mM L-glutamine, 50 units/ml penicillin and 50 μ g/ml

Abbreviations used: fMet-Leu-Phe, *N*-formylmethionyl-leucyl-phenylalanine; LTB₄, leukotriene B₄; PAF, platelet-activating factor; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; PMNs, polymorphonuclear leucocytes.

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streptomycin in a humidified atmosphere of 8% CO₂ in air 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% dimethyl sulphoxide. HL-60 cell membranes were prepared as previously described [24].

[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. Equal quantities of membrane protein were used in each experiment with protein added ranging from 50 to 100 µg. Membranes were incubated for 60 min at 37 °C in a volume of 50 µl containing 100 mM potassium phosphate buffer, pH 7.5, 2.5 mM MgCl₂, 1 mM ATP, 10 mM thymidine, 10 mM arginine, 100 µg/ml activated cholera toxin, [³²P]NAD⁺ and guanine nucleotides and/or agonists at concentrations indicated in the Figure legends. SDS/PAGE (10%-acrylamide gels) and autoradiography were performed as previously described [24].

[³²P]ADP-ribosylation by pertussis toxin

Pertussis toxin (100 µg/ml) was activated by dilution with an equal volume of 100 mM dithiothreitol and incubated for 10 min at 30 °C. Equal quantities of membrane protein were incubated for 15 min at 37 °C in a volume of 50 µl containing 200 mM Tris/HCl, pH 7.5, 6.3 mM MgCl₂, 2 mM ATP, 2 mM thymidine, 2 mM arginine and the indicated concentrations of agonist and guanine nucleotide. Subsequently, 5 µg/ml activated toxin and [³²P]NAD⁺ were added, and the reaction mixture was incubated for another 60 min at 37 °C. SDS/PAGE and autoradiography were performed.

Miscellaneous

Membrane protein was determined as described by Bradford [25], with bovine IgG as the standard. All experiments were performed at least twice with two different membrane preparations.

RESULTS

Cholera-toxin-catalysed ADP-ribosylation of HL-60 granulocyte membranes results in labelling of two α subunits. One is an α_s of 43 kDa, and the other (α_{40}) is composed of α_2 and α_3 of about 40 kDa on SDS/PAGE (10% gels) [19,26]. Gierschik and Jakobs [19] reported that labelling of the 40 kDa subunit was inhibited by 100 µM p[NH]ppG, but labelling was re-established in the presence of fMet-Leu-Phe [19]. We utilized this receptor-specific cholera-toxin-catalysed ADP-ribosylation to compare the ability of fMet-Leu-Phe and LTB₄ to enhance labelling of α_{40} . As shown in Figure 1 and reported previously [6], fMet-Leu-Phe re-established labelling of α_{40} in a concentration-dependent manner, whereas LTB₄ failed to re-establish cholera-toxin labelling of α_{40} .

One possible explanation for the failure of LTB₄ to re-establish cholera-toxin labelling is that LTB₄ receptors are less effective in stimulating guanine nucleotide release. We have shown that G-proteins activated by fMet-Leu-Phe receptors demonstrate a significantly lower affinity for GDP than those activated by LTB₄ receptors [27]. Thus, in the presence of 100 µM p[NH]ppG, LTB₄ may not sufficiently alter affinity for guanine nucleotides to allow

G_i to serve as a substrate for cholera toxin. This possibility was addressed in two separate experiments. First, the ability of fMet-Leu-Phe and LTB₄ to re-establish cholera-toxin labelling of α_{40} was determined in the presence of threshold concentrations of guanine nucleotides. Figure 2(a) shows the effect of 2, 5 and 10 µM p[NH]ppG on fMet-Leu-Phe- and LTB₄-receptor-specific cholera-toxin labelling of α_{40} . In each case, fMet-Leu-Phe, but not LTB₄, stimulated receptor-specific labelling of α_{40} . Similar results were seen in the presence of 0.1 or 1 µM GDP (Figure 2b). Second, the ability of fMet-Leu-Phe and LTB₄, as well as C5a and PAF, to enhance cholera-toxin-catalysed ADP-ribosylation of α_{40} in the absence of added guanine nucleotides was examined. Figure 3 demonstrates that fMet-Leu-Phe and C5a stimulate enhanced labelling, whereas cholera-toxin labelling in the presence of LTB₄ and PAF is unchanged.

Receptor-specific alterations in pertussis-toxin labelling of G_i have been reported for rhodopsin, thyrotropin and insulin receptors [20,21,28,29]. Therefore we examined the ability of fMet-Leu-Phe and LTB₄ to affect pertussis-toxin labelling of α_{40}

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

Figure 1 Receptor-specific cholera-toxin-catalysed ADP-ribosylation of HL-60 membranes by fMet-Leu-Phe and LTB₄

HL-60 granulocyte membranes were incubated with [³²P]NAD⁺ and cholera toxin alone (lane 2) or cholera toxin plus p[NH]ppG (100 µM) (lanes 1 and 3–9) as described in the Materials and methods section. The indicated concentrations of fMet-Leu-Phe (FMLP) or LTB₄ were added to the reaction mixture and incubated for 60 min at 37 °C. The proteins were subjected to SDS/PAGE, and the dried gel was autoradiographed. Only the ~35–50 kDa region of the autoradiogram from a single experiment representative of five separate experiments is shown.

Figure 2 Effect of threshold concentrations of p[NH]ppG and GDP on receptor-specific cholera-toxin labelling

Cholera-toxin-catalysed [³²P]ADP-ribosylation was performed as indicated in Figure 1 in the presence or absence of 10 µM fMet-Leu-Phe (FMLP) or 1 µM LTB₄ and with various concentrations of p[NH]ppG (a) or GDP (b). Autoradiograms of the 35–50 kDa regions from single experiments representative of two separate experiments are shown.

Figure 3 Ability of fMet-Leu-Phe, LTB₄, C5a and PAF to enhance cholera-toxin labelling

Membranes were incubated with [³²P]NAD⁺, cholera toxin and 10 μ M fMet-Leu-Phe (FMLP), 1 μ M LTB₄, 1 nM C5a or 1 μ M PAF. After incubation at 37 °C for 60 min, proteins were separated by SDS/PAGE and autoradiography was performed. Only the ~35–50 kDa region of the autoradiogram from a single experiment representative of two separate experiments is shown.

Figure 4 Ability of fMet-Leu-Phe and LTB₄ to alter pertussis-toxin labelling in the presence of GTP[S]

Membranes were incubated with 10 μ M GTP[S] and 10 μ M fMet-Leu-Phe (FMLP) and/or 1 μ M LTB₄ for 15 min at 37 °C before addition of pertussis toxin and [³²P]NAD⁺. After another incubation for 60 min at 37 °C, proteins were separated by SDS/PAGE and autoradiography was performed. Densitometric scanning determined the integrated area of the labelled bands to be 4.31 for control, 2.80 for fMet-Leu-Phe, 4.66 for LTB₄ and 2.64 for fMet-Leu-Phe plus LTB₄.

Figure 5 Alteration of pertussis-toxin labelling by fMet-Leu-Phe and LTB₄ in the presence or absence of GDP

Membranes were incubated with 10 μ M fMet-Leu-Phe (FMLP) and/or 1 μ M LTB₄ in the presence or absence of 5 μ M GDP for 15 min at 37 °C before addition of pertussis toxin and [³²P]NAD⁺. After another incubation for 60 min, proteins were separated by SDS/PAGE and autoradiography was performed.

Table 1 Densitometric analysis of the alterations in pertussis-toxin labelling by fMet-Leu-Phe and LTB₄ in the presence or absence of GDP

Results are presented as the means $\pm$ S.D. of the integrated area of pertussis-toxin-labelled bands at 40 kDa in the presence of 10 μ M fMet-Leu-Phe, 1 μ M LTB₄, or both agonists. Percentage change from control is shown in parentheses.

[GDP] (μ M)	<i>n</i>	Control	fMet-Leu-Phe	LTB ₄	fMet-Leu-Phe + LTB ₄
0	3	4.73 $\pm$ 0.37	3.12 $\pm$ 0.68 (34%)	4.33 $\pm$ 0.77 (9%)	3.40 $\pm$ 0.80 (28%)
5	2	4.18	5.37 (28%)	5.84 (40%)	5.82 (39%)

in the presence or absence of various guanine nucleotides. Membranes were incubated with 10 μ M fMet-Leu-Phe or 1 μ M LTB₄ for 15 min before addition of pertussis toxin and [³²P]NAD⁺. Figure 4 demonstrates that fMet-Leu-Phe inhibited pertussis-toxin labelling by 35% in the presence of 10 μ M GTP[S], whereas LTB₄ had no effect. The combination of fMet-Leu-Phe and LTB₄ resulted in a decrease in labelling, similar to that seen with fMet-Leu-Phe alone. The ability of fMet-Leu-Phe and LTB₄ to alter pertussis-toxin labelling of α_{40} in the presence or absence of GDP is shown in Figure 5, and the computer-assisted analysis of densitometric scanning of the autoradiograms is shown in Table 1. In the absence of GDP, there was about a 35% decrease in labelling by fMet-Leu-Phe (lane 2 versus lane 1), but no decrease by LTB₄ (lane 6 versus lane 1). The combination of fMet-Leu-Phe and LTB₄ inhibited pertussis-toxin-induced labelling by about 30% in the absence of added guanine nucleotides. In the presence of 5 μ M GDP, both fMet-Leu-Phe (lane 3 versus lane 8) and LTB₄ (lane 5 versus lane 8) enhanced the ability of pertussis toxin to label α_{40} by about 30% and 40% respectively. The combination of LTB₄ and fMet-Leu-Phe enhanced labelling by about 40%.

DISCUSSION

Pertussis toxin and cholera toxin covalently label some G-protein α subunits. Pertussis toxin catalyses ADP-ribosylation of a cysteine residue four amino acids from the C-terminus of α_i , α_o and α_1 (reviewed in [30]). The site of cholera-toxin-catalysed ADP-ribosylation is an arginine residue at position 179 and 178 on α_{i2} and α_{i3} respectively [31]. In HL-60 granulocyte membranes, pertussis toxin and cholera toxin ADP-ribosylate α subunits which migrate at about 40 kDa [6,19,24,32]. The two toxins label the same α subunits, as exposure of intact HL-60 granulocytes to pertussis toxin prevents subsequent ADP-ribosylation of isolated membranes by cholera toxin [19,26]. Thus the two toxins require access to two different sites on the same G-proteins in HL-60 granulocytes for ADP-ribosylation to occur.

The mechanism by which G-proteins relay signals from receptors to effectors depends on cyclic conformational changes in the α subunit related to the binding of receptors and different guanine nucleotides [33–35]. Alterations in G-protein conformation by guanine nucleotides lead to altered susceptibility of G_i to ADP-ribosylation by pertussis and cholera toxins [19–23,35–37]. The present study confirms that, in the absence of guanine nucleotides, cholera toxin catalyses ADP-ribosylation of α_i in membranes from HL-60 granulocytes, whereas p[NH]ppG inhibits cholera-toxin labelling of α_i [19,26]. We show that GDP also inhibits cholera-toxin-catalysed ADP-ribosylation

of α_i in a dose-dependent manner. Thus the optimal state for G_i to serve as substrate for cholera-toxin labelling appears to be with an empty guanine-nucleotide-binding site. Previous reports showed that GDP enhances, whereas GTP[S] inhibits, pertussis-toxin-catalysed ADP-ribosylation of α_i [21–23,36]. The ability of guanine nucleotides to alter pertussis-toxin- and cholera-toxin-induced ADP-ribosylation of α_i indicates that various conformations of these α subunits can be detected in HL-60 granulocyte membranes. We used alterations in toxin-induced labelling to examine the conformations of α_i coupled to ligand-bound formyl-peptide and LTB_4 receptors.

The ability of fMet-Leu-Phe to alter pertussis-toxin- and cholera-toxin-induced labelling of α_i indicates that receptor-specific conformational changes can be detected in HL-60 granulocyte membranes. Receptor-specific cholera-toxin labelling has been reported for formyl peptides in HL-60 cell membranes by Gierschik and co-workers [19,26] and for opioid peptides in NG108-15 cells by Milligan and McKenzie [37]. Preincubation of membranes with fMet-Leu-Phe in our study decreased pertussis-toxin-catalysed ADP-ribosylation in the absence of guanine nucleotides or in the presence of GTP[S] and enhanced pertussis-toxin labelling of α_i in the presence of GDP. Rothenberg and Kahn [20] showed that insulin decreased pertussis-toxin labelling of G-proteins on liver cell membranes in the absence of guanine nucleotides. Tsai et al. [21] reported that photolysed rhodopsin decreased pertussis-toxin labelling of α_i in the absence of guanine nucleotides and enhanced labelling in the presence of GDP. Preincubation of thyroid cell membranes with thyrotropin has been shown to decrease pertussis-toxin labelling of G_i in the absence of guanine nucleotides [28,29].

Differences in the ability of fMet-Leu-Phe and LTB_4 to alter pertussis-toxin- and cholera-toxin-induced labelling of α_i indicate that formyl-peptide and LTB_4 receptors induce different conformations of receptor-coupled G_i proteins. LTB_4 failed to restore cholera-toxin labelling in the presence of p[NH]ppG or GDP, and LTB_4 failed to enhance cholera-toxin labelling of α_i in the absence of guanine nucleotides. Receptor-specific cholera-toxin-catalysed labelling of α_i was not unique to fMet-Leu-Phe, as C5a also enhanced α_i labelling. Interestingly, PAF failed to enhance cholera-toxin-induced labelling, suggesting that there may be a fundamental difference between receptors for peptide and lipid chemoattractants. Similarly to the differences between fMet-Leu-Phe and LTB_4 in the presence of cholera toxin, LTB_4 did not decrease pertussis-toxin labelling of α_i in the absence of guanine nucleotides or in the presence of GTP[S].

We have reported previously that fMet-Leu-Phe receptors induce a lower affinity of guanine-nucleotide-binding sites for GDP than do LTB_4 receptors [27]. This finding suggests that differences in the ability of fMet-Leu-Phe and LTB_4 to alter ADP-ribosylation may be due to differences in the rate of release of p[NH]ppG or GDP from guanine-nucleotide-binding sites. However, the ability of fMet-Leu-Phe, but not LTB_4 , to alter toxin-catalysed labelling of G_i in the absence of guanine nucleotides indicates that differences in receptor-specific G-protein conformations induced by fMet-Leu-Phe and LTB_4 are not likely to be due only to differences in guanine-nucleotide-binding site affinity. Our interpretation of these results is that both the difference in guanine-nucleotide-binding site affinity and the difference in receptor-specific toxin labelling are due to different receptor-specific conformations of G_i . Additionally, the ability of LTB_4 and fMet-Leu-Phe to enhance pertussis-toxin labelling in the presence of GDP shows that LTB_4 -receptor-induced conformational changes in α_i are detectable by differences in toxin labelling under the appropriate conditions.

We conclude that formyl-peptide and LTB_4 receptors induce

different conformations of G_i after ligand binding. Gierschik et al. [26] reported that formyl-peptide receptors are coupled to both G_2 and G_3 proteins in HL-60 granulocyte membranes. Our data do not address the question of whether formyl-peptide and LTB_4 receptors induce different conformations of both α_2 and α_3 . Our results suggest that activated G-proteins can exist in multiple conformations determined by the receptors with which they interact. The functional consequences of receptor-specific conformations of activated G-proteins are unknown. The possibility exists that these differences may provide one explanation for the different capabilities of formyl-peptide and LTB_4 receptors to activate effector enzymes.

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