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Ligand-Induced Formation of the Leukotriene B_4 Receptor-G Protein Complex of Human Polymorphonuclear Leukocytes

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Abstract The components of the polymorphonuclear leukocyte (PMNL) receptor for leukotriene B_4 (LTB_4) were examined by Sephacryl S-300 exclusion chromatography of PMNL membrane proteins, which were solubilized before and after the binding of [3H] LTB_4. When the PMNL membranes were solubilized in 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and filtered on Sephacryl S-300 prior to addition of [3H] LTB_4, the binding activity was associated with a 65 kD protein. In contrast, the radioactivity of [3H] LTB_4 bound to PMNL membranes prior to solubilization was recovered predominately with a 140 kD protein. When PMNL membranes had been pretreated with pertussis toxin, but not cholera toxin, before the addition of LTB_4 and subsequent solubilization, radioactivity was recovered predominately with the 65 kD protein. The addition of guanylylimidodiphosphate (GMP-PNP), a nonhydrolyzable derivative of guanosine triphosphate (GTP), to PMNL membrane receptors bearing [3H] LTB_4 either prior to or after CHAPS solubilization reduced the yield of the 140 kD presumed LTB_4 receptor protein-G protein complex. That the maximum specific binding of [35S] guanosine-5′-0-3-thiotriphosphate (GTP-gammaS) to LTB_4-binding proteins in the Sephacryl S-300 effluent corresponded to the 140 kD protein supported the presence of a G protein in the LTB_4 receptor complex.

Key words: inflammation, chemotaxis, signal transduction, cell membrane, mediator, arachidonic acid

The actions of LTB_4 on human blood PMNLs producing chemotaxis, increased adherence, degranulation, and increased oxidative metabolism are mediated by distinct high- and low-affinity subsets of stereospecific cell surface receptors [1,2]. Previous studies have suggested that the LTB_4 receptor may interact with a pertussis toxin-sensitive guanine nucleotide-binding protein or G protein resulting in changes in affinity and activity of the receptor [3,4]. G proteins interact differently with high-affinity LTB_4 receptors than with the peptide chemotactic factor receptors on PMN leukocytes [4], but the distinctive roles of the ligands have not been elucidated.

The results now reported document an LTB_4-induced increase in the apparent molecular size of the solubilized LTB_4 receptor of PMNL. The inclusion of a G protein in the LTB_4-occupied high-affinity LTB_4 receptor complex of PMNL membranes is suggested by the binding of a GTP analogue and by pertussis toxin- and GTP-dependent dissociation of the complex.

MATERIALS AND METHODS

Synthetic LTB_4 (Biomol, Philadelphia, PA), [3H] LTB_4 (200 Ci/mmol, Amersham Corp.), N-formyl-methionyl-leucyl-[3H] phenylalanine ([3H] fMLP) and [35S] GTP-gammaS (NEN-Dupont, Boston, MA), Ficoll-Paque, Dextran, PD-10 columns, aminododecyl-Sepharose (Pharmacia LKB Biotechnology, Inc.), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Pierce, Rockford, IL), GF/F glass fiber filters (Whatman, Inc., Maidstone, England), DNase, N-

Abbreviations: PMNL, polymorphonuclear leukocyte; LTB_4, leukotriene B_4; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; GMP-PNP, guanylylimidodiphosphate; GTP, guanosine triphosphate; GTP-gammaS, guanosine-5′-0-3-thiotriphosphate; DFP, diisopropylfluorophosphate; PC, phosphatidylcholine; EDC, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride.

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formyl-methionyl-leucyl-phenylalanine (fMLP), diethylamine, diisopropyl fluorophosphate (DFP), phosphatidylcholine (PC), phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, polyethyleneimine, fluorescamine (Sigma), polyacrylamide gel electrophoresis (PAGE) chemicals and molecular weight markers (Biorad, Richmond, CA), Hanks' balanced salt solution (HBSS) and HEPES solution (University of California Cell Culture Facility), ovalbumin (ICN Immunochemicals, Costa Mesa, CA), and Suramin (FBA Pharmaceuticals, New York, NY) were obtained from the designated suppliers.

Preparation and Solubilization of Human PMN Leukocyte Membranes

Sodium citrate-anticoagulated venous blood of normal subjects was depleted of erythrocytes by dextran sedimentation, and the mixed leukocytes were resolved by Ficoll-Paque density centrifugation [5,6]. After removing the contaminating red cells by brief hypotonic lysis, the purity of the resulting PMNLs was over 95% [5]. Replicate suspensions of 2–3 $\times$ 10^8 PMNLs were treated with 5 mM DFP, washed, and disrupted by nitrogen cavitation in HBSS containing 2.5 mM $MgCl_2$ and 200 units/ml of DNase as described [7]. Nuclei and intact cells were removed by centrifugation at 850*g* for 10 min at 4°C. Plasma membranes were isolated from the supernatant by 10%–40% discontinuous sucrose gradient sedimentation at 65,000*g* for 30 min at 4°C. The plasma membrane preparation accounted for a mean of 7% (n = 2), 51%, 34%, 13%, and 67%, respectively, of the total protein, alkaline phosphatase, galactosyl transferase, beta-glucuronidase, and [3H] LTB_4-binding activity applied to the sucrose gradient [8]. Membranes were washed once and resuspended in 5 mM sodium phosphate-1 mM EDTA (pH 8.0) and stored at −70°C. Membranes in thawed aliquots of the suspensions were pelleted in a Beckman TL-100 ultracentrifuge at 386,000*g* for 15 min at 4°C and solubilized by the addition of 0.5 g% CHAPS and 0.1 g% PC in phosphate-buffered saline at a final concentration of 5 mg protein/ml. The mixture was stirred carefully to avoid foaming for 1 h at 4°C followed by centrifugation at 386,000*g*. The 386,000*g* pellet was subjected again to the solubilization procedure and the solubilized proteins were combined for assays. The protein concentrations of the membrane preparations were determined by a modified Folin method [9]. CHAPS-solubilized membrane proteins were used immediately.

Binding of [3H] LTB_4 to PMNL Membranes

Replicate aliquots of PMNL membranes containing 0.5 mg of total protein were suspended in binding buffer, consisting of HBSS containing 0.1 g/100 ml of ovalbumin and 10 mM HEPES (pH 7.3), and added to 15 $\times$ 75–mm polypropylene tubes which each had 0.4 nM [3H] LTB_4 in a final volume of 1 ml. After incubation at 4°C for 45 min, the membranes were pelleted by centrifugation at 350,000*g* for 16 min at 4°C in a Beckman TL-100 ultracentrifuge just prior to solubilization.

For the experiments in which the effect of GMP-PNP on size fractionation was examined, the intact membrane preparations were kept on ice until either GMP-PNP or buffer alone was added, followed by incubation with [3H] LTB_4, pelleting, and solubilization as described above. For the experiments in which the binding of [3H] LTB_4 to CHAPS-solubilized membranes was measured, each aliquot of solubilized membranes was added to a polypropylene tube containing 0.4 nM [3H] LTB_4 in a final volume of 1 ml. The mixtures then were incubated at 4°C for 90 min. Each preparation of solubilized membrane proteins then was concentrated tenfold by ultrafiltration through an Amicon P-30 membrane and applied to a Sephacryl S-300 column for size fractionation.

Treatment of PMN Leukocyte Membranes With Activated Pertussis and Cholera Toxins

Toxin-treated membranes were prepared as described by Feltner et al. [10]. Pertussis and cholera toxins first were activated by incubation with 20 mM dithiothreitol for 30 min at 30°C in a buffer consisting of 12.5 mM Tris-HCl, 150 mM sodium phosphate, 5 mM $MgCl_2$, 150μM EGTA, and 1 mM ouabain (pH 7.4). Suspensions of 1 mg/ml of PMNL plasma membranes then were incubated at room temperature for 30 min in 12.5 mM Tris-HCl, 150 mM sodium phosphate, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM dithiothreitol, 1 mM ATP, 10 mM NAD, 10 mM thymidine, 5 units/ml of creatine phosphokinase, and 1 mM phosphocreatine (pH 7.5) with or without 50μg/ml of activated pertussis or cholera toxin. After the addition of a tenfold volume excess of ice-cold buffer consisting of 12.5 mM Tris-HCl, 150 mM sodium phosphate, 5 mM $MgCl_2$, 150 μM EGTA, 1 mM ouabain, and

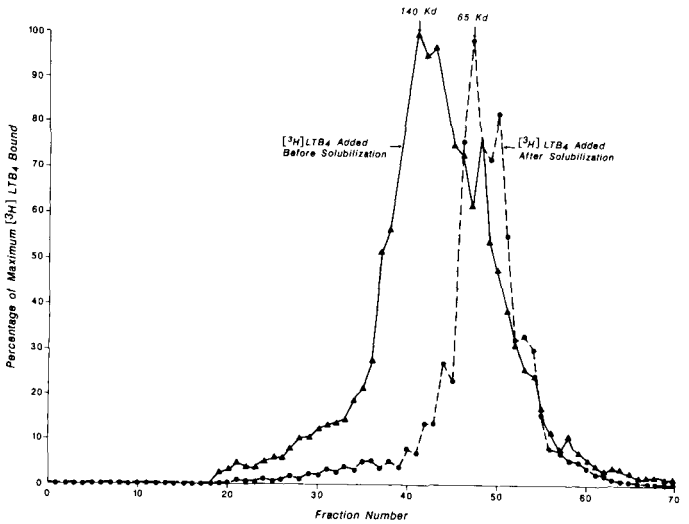

Fig. 1. Filtration on Sephacryl S-300 of CHAPS-solubilized PMNL membrane proteins binding [3H] LTB_4 before or after solubilization. The profiles represent the amount of [3H] LTB_4 in each of the 2% portions of eluate from the separate chromatograms bearing [3H] LTB_4 added either before (—) or after (– – –) CHAPS-solubilization.

1 mM dithiothreitol (pH 7.4), the membranes were pelleted by centrifugation at 350,000*g* for 16 min at 4°C in a Beckman TL-100 ultracentrifuge and then resuspended in buffer for assessment of binding.

Column Chromatography

CHAPS-solubilized membrane preparations were applied to Sephacryl S-300 columns and eluted in the presence of 3 mg of CHAPS and 0.6 mg of PC per ml of phosphate-buffered saline at 4°C. Sephacryl S-300 resin was chosen for gel filtration in these experiments because of its reported resolution in the range of 1×10^4 to 1.5×10^6 D. The dimensions of the chromatography columns were 5×0.7 cm and the elution flow rate was maintained at 3 ml/h with a Rabbit peristaltic pump (Bio-Rad, Richmond, CA).

The radioactivity of [3H] LTB_4 in each of the 2% portions of bed volume collected was quantified in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

Binding of [35S] GTP-GammaS to Portions of the Effluent

Selected portions of eluate from the Sephacryl S-300 filtration of PMNL membrane proteins labeled with [3H] LTB_4 prior to CHAPS-solubilization, as described previously, were added to polypropylene tubes containing [35S] GTP-gammaS and either a 1,000-fold excess of unlabeled GTP-gammaS or buffer in a final volume of 0.25 ml. After incubation at 4°C for 60 min, the mixtures were applied to 1.6-ml columns of Sephadex G-25 and eluted in phosphate-buffered saline containing CHAPS and PC.

RESULTS

Size Fractionation of CHAPS-Solubilized PMNL Membrane Proteins

Solubilized PMNL membrane proteins that bound [3H] LTB_4 before or after CHAPS-solubilization were resolved on a Sephacryl S-300 column developed with a CHAPS solution at a *rate* of 3 ml/h at 4°C (Fig. 1). The PMNL membrane protein that bound [3H] LTB_4 after CHAPS-solubilization eluted with a predominant size of approximately 65 kD. In contrast, the PMNL membrane protein binding [3H] LTB_4 prior to CHAPS-solubilization eluted with a predominant size of 140 kD. The same result was reproduced in chromatograms of three other PMNL membrane preparations. Of the [3H] LTB_4-specific binding activity in portions of each suspension of intact PMNL, a mean of 106% (range = 87%–118%, n = 3) of specific binding was recovered in membranes, 56% (range = 46%–68%) in solubilized membranes, and 4.5% (range = 4.1%–4.7%) in the total of fractions 44–60 of the eluate from Sephacryl S-300. Of the [3H] LTB_4 bound specifically to membranes prior to solubilization, a mean of 35% (range = 32%–39%, n = 3) was recovered in solubilized proteins, and 1.7% (range = 1.6%–2.0%) in the

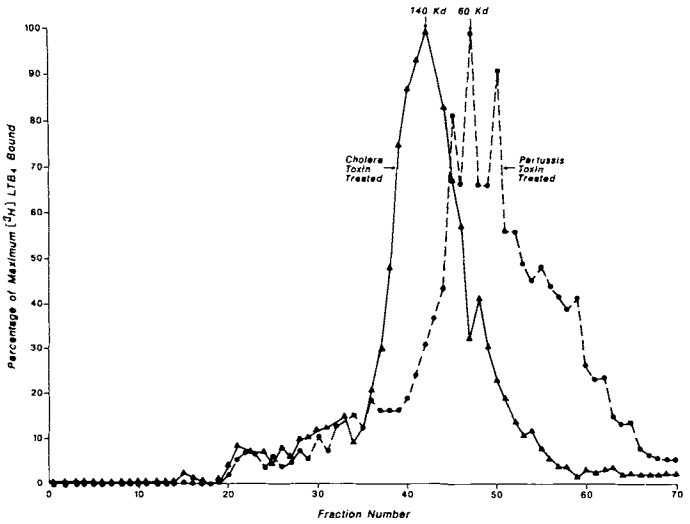

Fig. 2. Effect of toxin treatment on the elution profile of [3H] LTB_4-binding protein from Sephacryl S-300. The amount of [3H] LTB_4 in each fraction is represented as the percentage of maximum [3H] LTB_4 bound for PMNL membranes pretreated with cholera (—) or pertussis (– – –) toxin.

total of fractions 35–46 from Sephacryl S-300. In two of the studies, 10% of fractions 38–42 of prelabeled membrane proteins and 46–50 of proteins binding [3H] LTB_4 after solubilization were also incubated with 2 μM LTB_4, which displaced more than 80% of the radioactivity, as assessed by its passage through GF/F glass fiber filters [4].

Effect of Pertussis and Cholera Toxin Treatment on the Size of [3H] LTB_4-Binding CHAPS-Solubilized PMNL Membrane Proteins

To determine whether a G protein was one component of the 140 kD [3H] LTB_4 binding unit, the effects of pertussis and cholera toxins on the profile of elution from a Sephacryl S-300 column were examined with PMNL membrane proteins solubilized in CHAPS after binding of [3H] LTB_4 (Fig. 2). PMNL membranes were specifically ADP-ribosylated with either pertussis or cholera toxin prior to incubation with [3H] LTB_4 and then solubilization in CHAPS. The pretreatment of membranes with cholera toxin prior to addition of [3H] LTB_4 and solubilization did not prevent formation of the 140 kD LTB_4 receptor complex. However, pertussis toxin treatment prior to the addition of [3H] LTB_4 and CHAPS-solubilization prevented the formation of the 140 kD receptor/G protein complex, so that only a wide peak representing the approximately 65 kD LTB_4 receptor protein was labeled with [3H] LTB_4. This finding was reproduced in two other identical chromatograms. To examine the possible sources of heterogeneity in the 65 kD region of two of the S-300 chromatograms, fractions 45–52 were pooled, and radioactivity was extracted with methanol and acetic acid, and analyzed by high-performance liquid chromatography in parallel with that extracted from membranes binding [3H] LTB_4, as described [11]. The mean percentages of radioactivity recovered as native [3H] LTB_4, 6-trans isomers of [3H] LTB_4, and omega-oxidized [3H] LTB_4, respectively, were 28.5, 57.5, and 14 for membranes and 25.5, 64, and 10.5 for the chromatographic fractions. This suggests that alterations in the receptor protein rather than in LTB_4 accounted for the greater heterogeneity observed after pertussis toxin treatment.

The Effect of GMP-PNP on the Elution Profile of [3H] LTB_4 Binding to CHAPS-Solubilized PMNL Membrane Proteins

To further substantiate the presence of a G protein as a component of the 140 kD LTB_4 receptor complex, PMNL membranes were incubated with [3H] LTB_4 for 40 min at 4°C along with GMP-PNP at a concentration of 0.1 mM, which has a near maximal effect of converting 50% of high-affinity LTB_4 receptors to the low-affinity state [4], prior to CHAPS-solubilization. As assessed by the pattern of resolution of protein-bound [3H] LTB_4, GMP-PNP partially blocks the formation of the 140 kD complex (Fig. 3). Three additional chromatograms showed the same effect of GMP-PNP. The patterns of

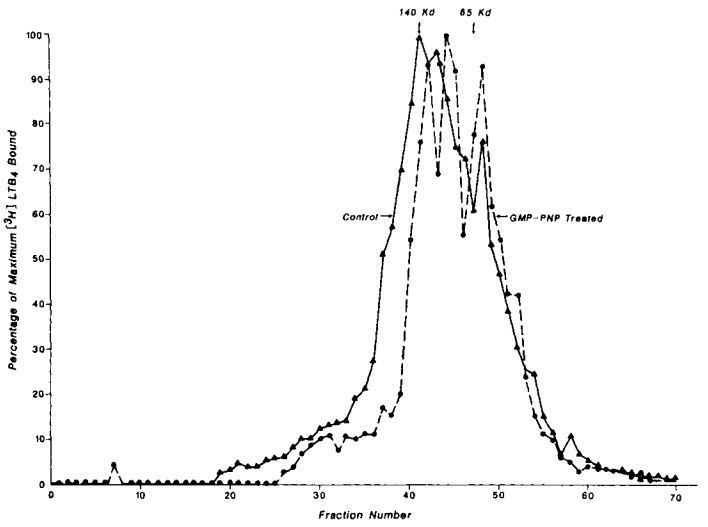

Fig. 3. Effect of GMP-PNP on the elution profile from Sephacryl S-300 of [3H] LTB_4 binding protein of CHAPS-solubilized PMNL membrane proteins. Each point represents the amount of [3H] LTB_4 bound to control (—) and GMP-PNP (– – –) treated membranes.

elution of receptor-bound [3H] LTB_4 added prior to membrane protein solubilization (Figs. 1–3) all show some of the 65 kD form, which is estimated by the relative areas of the peaks to be less than 10% of the total. Small differences in these patterns seen after various treatments cannot be interpreted reliably.

GTP-GammaS Binding to LTB_4-Binding Proteins From CHAPS-Solubilized PMNL Membranes

To identify directly the presence of a G protein in the 140 kD LTB_4 receptor complex, the binding of the nonhydrolyzable [35S] GTP-gammaS analogue of GTP to CHAPS-solubilized LTB_4-binding proteins was examined with portions of the effluent from an S-300 column. The peak of specific binding of [35S] GTP-gammaS to selected portions of effluent from S-300 corresponds to the 140 kD peak exhibiting the maximum percentage of binding of [3H] LTB_4 (Fig. 4). The binding of [3H] LTB_4 and [35S] GTP-gammaS coincided in two additional S-300 chromatograms.

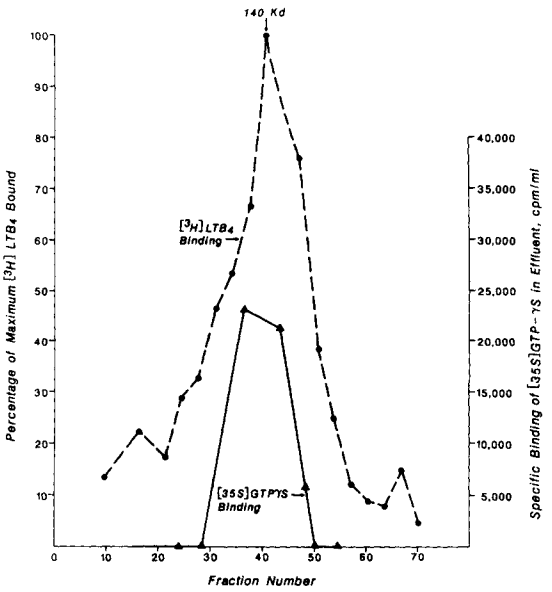

Fig. 4. Gel filtration of [35S] GTP-gammaS-binding and [3H] LTB_4-binding proteins from CHAPS-solubilized PMNL membranes. The profiles represent the binding of [3H] LTB_4 in CHAPS-solubilized PMNL membranes (– – –) and the binding of [35S] GTP-gammaS to selected portions of the eluate (—).

DISCUSSION

LTB_4 is required to stabilize the 140 kD complex (Fig. 1) containing a pertussis toxin-sensitive G protein and the 65 kD PMNL membrane protein which binds LTB_4 specifically. CHAPS solubilization in the absence of LTB_4 (Fig. 1) or in the presence of pertussis toxin (Fig. 2) disrupts the complex. The difference between the apparent size of the complex of 140 kD and that predicted from the sum of the two protein components presumably is attributable to binding of detergent or other proteins. These results are consistent with the previous findings from our laboratory that a pertussis toxin-sensitive G protein modulates the expression of the LTB_4 receptor [3,4].

The altered elution profile observed when [3H] LTB_4 binding is carried out in the presence of GMP-PNP (Fig. 3) is consistent with the natural heterogeneity of the LTB_4 receptor, attributable partially to the effect of guanine nucleotides on the affinity and reversibility of LTB_4 binding to PMNL membranes [4]. That only one protein constitutes the LTB_4-binding unit has been demonstrated by the finding of an identical single band on sodium dodecyl sulfate gels when PMNL membranes were affinity-labeled with and without GTP [8]. At 0.1 mM, guanine nucleotides partially decrease the formation of the LTB_4 receptor-G protein complex. The association of the peak binding activities of both [3H] LTB_4 and GTP-gammaS with the 140 kD receptor-G protein complex (Fig. 4) substantiates that a G protein is a necessary component.

It has been demonstrated that guanine nucleotides regulate a number of receptors [12,13]. The best studied has been the beta-adrenergic receptor, for which agonist also induces an increase in apparent size by inducing the formation of a receptor-G protein complex [14]. There is also evidence for a similar molecular interaction of a G protein with the formyl-peptide chemotactic receptor of PMNLs [15]. A detailed comparison of the roles of the G proteins in ligand binding and signal transduction in the different systems will be needed to provide a better understanding of the molecular mechanisms of the interactions [16].

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