Characterization of the soluble leukotriene B₄ receptor from sheep lung membranes

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Leukotriene B₄ (LTB₄) is an arachidonate metabolite which elicits a variety of pro-inflammatory responses by activation of a guanine-nucleotide-binding protein-coupled membrane receptor. As a prelude to receptor isolation and purification, we have established assay methods for LTB₄ receptor solubilization and characterization from sheep lung membranes. [³H]LTB₄ binding to the soluble receptor was saturable, specific, protein-concentration- and time-dependent and reversible. Binding of [³H]LTB₄ was enhanced by divalent cations and inhibited by sodium ions in a manner analogous to its binding to the human leukocyte membrane receptor. Saturation binding yielded a dissociation constant (*K*_d) of 0.50 ± 0.05 nM and a receptor density (*B*_{max}) of 330 ± 90 fmol/mg of protein for [³H]LTB₄ binding to detergent-solubilized receptor. In competition experiments, the rank order of binding affinity was LTB₄ > 20-OH-LTB₄ > *trans*-homo-LTB₄ > 6-*trans*-LTB₄ > U-75302. Gel-filtration chromatography showed that the LTB₄ receptor protein in the detergent micellar state has a molecular mass in the range 800–1000 kDa. These results demonstrate that the physiologically and pharmacologically important LTB₄ receptor may be readily solubilized from sheep lung membranes without alteration in binding specificity and characteristics, suggesting that sheep lung membranes represent a rich source with which to pursue receptor isolation and purification.

INTRODUCTION

Leukotriene B₄ (LTB₄) is a 5-lipoxygenase metabolite of arachidonic acid that can induce a variety of pro-inflammatory responses, including cellular aggregation, chemotaxis, lysosomal enzyme release, degranulation and thromboxane synthesis and release in a variety of inflammatory cells and many types of tissues or organs (for reviews, see [1,2]). LTB₄ elicits these responses by activation of an inhibitory guanine-nucleotide-binding protein (G_i)-coupled membrane receptor [3–8]. Radioligand binding studies and functional studies have demonstrated that the G_i-protein-coupled receptor binds [³H]LTB₄ with high affinity [9–12] and the G_i-protein-uncoupled receptor binds [³H]LTB₄ with low affinity [1,13–15]. A molecular model of receptor–G_i-protein interaction was proposed [7] to account for the mechanism of high-affinity state to low-affinity state transition for the ligand receptor binding.

A definitive understanding of the molecular architecture and the mechanism of receptor activation requires purification and structural elucidation of the receptor protein. The very first step toward that goal is to construct efficient methods and assay systems for the solubilization of the membrane-bound receptor protein for characterization and purification. We have recently reported that sheep lung membranes have abundant LTB₄ receptors, and that the specificity of the receptor in sheep lung membranes is equivalent to that in human polymorphonuclear leucocytes (PMN) and lung membranes [8,10]. Removal of the G_i protein from sheep lung membranes reduced the binding affinity of [³H]LTB₄ for its receptor by 3-fold. These results showed that there is only one class of LTB₄ receptors in sheep lung membranes. Thus the sheep lung represents a good model and a rich source for LTB₄ receptor solubilization and future

purification. The results presented in this study demonstrate the feasibility of detergent solubilization and biochemical characterization of the LTB₄ receptor protein(s) from sheep lung membranes.

MATERIALS AND METHODS

Preparation of receptors

Membrane preparation. Sheep lungs were obtained from Vanezia (Conshohocken, PA, U.S.A.). Lung (250 g) in 1.5 litres of ice-cold buffer A (10 mM-Tris, pH 7.4, containing 0.25 M-sucrose, 10 µg of soybean trypsin inhibitor/ml, 100 µM-benzamide, 5 mM-EDTA, 100 µg of bacitracin/ml and 0.5 mM-phenylmethanesulphonyl fluoride) was minced in a Waring blender for 1 min at maximum speed. All procedures were carried out at 4 °C. The tissue was further homogenized using a Polytron at setting 7 for 1 min. This homogenate was then centrifuged at 1300 g for 20 min. The supernatant was transferred to ultracentrifuge tubes, underlayered with buffer A (adjusted to 25% sucrose), and centrifuged at 100 000 g for 60 min. The membranes at the interface were collected, diluted with 2 vol. of distilled water and re-centrifuged at 150 000 g for 60 min. Routinely, 300 mg of partially purified sheep lung membranes were obtained in each preparation.

Alkali treatment was employed to remove non-integral proteins from the lung membranes. Briefly, membranes were resuspended in distilled water, using a Dounce homogenizer, to a protein concentration of 5 mg/ml. Protein concentration was determined according to the method of Bradford [16], using BSA as a standard. One volume of membrane suspension was combined with four volumes of alkaline extraction buffer B (10 mM-K₂HPO₄, adjusted with KOH to a final pH of 12.5) for

Abbreviations used: LTB₄, leukotriene B₄ [(5S,12R)-dihydroxy-(6E,8Z,10Z,14E)-eicosatetraenoic acid]; PMN, polymorphonuclear leukocytes; G_i, protein, inhibitory guanine-nucleotide-binding protein; IC₅₀, concentration that inhibited 50% of specific binding; *B*_{max}, maximum binding capacity; *K*_d, inhibitory binding constant; 20-OH-LTB₄, (5S,12R)-20-trihydroxy-(6E,8Z,10Z,14Z)-eicosatetraenoic acid; 6-*trans*-LTB₄, (5S,12S)- and (5R,12R)-dihydroxy-(6Z,8Z,10Z,14E)-eicosatetraenoic acid; *trans*-homo-LTB₄, (5S,12S)- and (5R,12R)-dihydroxy-(6Z,8Z,10Z,14Z)-heneicosenoic acid; GTP[S], guanosine 5'-[γ-thio]triphosphate; NP-40, Nonidet P40; PEG, poly(ethylene glycol); PEI, polyethyleneimine.

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5 min at 4 °C. This membrane suspension was then centrifuged at 150 000 *g* for 60 min at 4 °C. The membrane pellets were frozen in liquid N₂ and stored at -70 °C for up to 6 months without any appreciable loss of receptor binding activity. Alkali extraction resulted in the removal of 60–70 % of the total protein, whereas more than 95 % of the receptor was retained in the membranes.

Receptor solubilization. Alkali-extracted membranes were resuspended in distilled water and adjusted to 2.5 or 10 mg/ml. Unless otherwise specified, 1 vol. of membrane suspension was combined with 1 vol. of 50 mM-Tris/HCl, containing 2 mM-CaCl₂ and 0.8 % CHAPS. The mixture was incubated at 4 °C for 120 min with constant mixing and then centrifuged at 150 000 *g* for 60 min. The supernatant contained the solubilized LTB₄ receptor. This preparation was used immediately or stored frozen at -70 °C with no detectable loss of binding activity for up to 1 month.

Radioligand binding assays

Unless otherwise specified, all assays were performed in 20 mM-Tris/HCl (pH 7.4) containing 10 mM-CaCl₂ and 10 mM-MgCl₂ in a volume of 0.5 ml, in duplicate, and incubated at 22 °C for 60 min, using Nunc Minisorp polyethylene tubes to minimize non-specific binding (standard conditions). Free ligand was separated from bound ligand by either the polyethyleneimine (PEI) method or the poly(ethylene glycol) (PEG) precipitation method. In the former case, the incubation mixture was filtered through PEI (1 %) pre-soaked Whatman GF/C filter paper and immediately washed with 16 ml of buffer C (10 mM-Tris/HCl, pH 7.4, at 0 °C). Alternatively, at the end of the incubation, the assay tubes were placed in an ice bath. Bovine γ -globulin (1 ml; 0.6 mg/ml in 20 mM-Tris/HCl) and 1 ml of 25 % PEG-8000 (in 20 mM-Tris/HCl) were added, immediately mixed, and left standing at 0 °C for 5 min. The radioligand–receptor complex precipitates were then collected by rapid filtration and washed with 16 ml of ice-cold 8 % PEG in buffer C. The radioactivity retained in the filter was determined by liquid scintillation counting.

In all binding assays, non-specific binding was determined in the presence of a 1000-fold excess of unlabelled LTB₄. The protein dependency of the soluble receptor binding was demonstrated by incubating various amounts of soluble protein (25–200 μ g/tube) with 0.3 nM-[³H]LTB₄ under standard conditions. The association of [³H]LTB₄ with the soluble receptor was determined by incubating 100 μ g of soluble protein with 0.3 nM-[³H]LTB₄ for various lengths of time. Dissociation of [³H]LTB₄ from the soluble receptor was determined by adding either a 1000-fold excess of LTB₄ (0.3 μ M) or EDTA (12 mM) at steady-state binding; the receptor-bound [³H]LTB₄ was determined subsequently using the PEI method. The effects of monovalent and divalent cations on [³H]LTB₄ binding to the soluble receptor were assessed by incubating 100 μ g of protein with 0.3 nM-[³H]LTB₄ in the presence or absence of various concentrations of selected cations under standard conditions. In saturation binding assays, either 50 μ g or 100 μ g of soluble protein was incubated with various concentrations of [³H]LTB₄ in the absence or presence of unlabelled LTB₄. In competition binding assays, 100 μ g of soluble protein and 0.5 nM-[³H]LTB₄ were incubated with various concentrations of LTB₄ or analogues under standard conditions. All receptor binding assays were highly reproducible. The s.d. of duplicate determinations in all cases was < 10 %.

Analysis of receptor binding data

[³H]LTB₄ specific binding data were analysed by using a least-squares best-fit program as described by De Lean *et al.* [17] and

were transformed by the method of Scatchard [18]. The dissociation constant (*K_d*) and maximal number of binding sites (*B_{max}*) were calculated from this method. The inhibition constant (*K_i*) was calculated according to the equation: $K_i = IC_{50}/(1 + [^3H]LTB_4/K_d)$ as described by Cheng & Prusoff [19], where the *IC₅₀* is the concentration of competitor that results in a 50 % reduction in [³H]LTB₄ receptor binding, [³H]LTB₄ is the concentration of radioligand (0.5 nM) and *K_d* is the dissociation constant (0.5 nM), derived from the saturation binding studies.

Gel-filtration chromatography of soluble receptors

A Beckman h.p.l.c. system fitted with TSK-4000 and TSK-3000 gel-filtration columns (in series) was employed to fractionate the CHAPS-solubilized LTB₄ receptor. The column was calibrated with marker proteins and had a void volume of 10.5 ml as determined by Dextran Blue ($\approx$ 2000 kDa) elution in buffer D (20 mM-Tris/HCl, pH 7.4, containing 20 mM-KCl and 0.2 % CHAPS). The soluble receptor (0.2 ml) was injected and eluted with buffer D at a flow rate of 0.35 ml/min, and 1 min fractions were collected. Both the unlabelled and the [³H]LTB₄-prelabelled soluble receptor preparations were chromatographed in this manner. Binding of [³H]LTB₄ to the unlabelled receptor in column fractions was determined by incubating 100 μ l aliquots of the column fractions with 0.3 nM-[³H]LTB₄ under standard conditions. [³H]LTB₄ (0.5 nM) prelabelled soluble receptor was injected and eluted as described above, except that the elution buffer contained 10 mM-CaCl₂ and 10 mM-MgCl₂.

Reagents

LTB₄, *trans*-homo-LTB₄ and 6-*trans*-LTB₄ were synthesized and supplied by K. Erhardt, and 2-(2,6-dihydroxy-*N*-hexyl)-6-(3-hydroxy-2*E*,5*Z*-undecadienyl)pyridine (U-75302) was synthesized and supplied by R. Cousins (Department of Medicinal Chemistry, SmithKline and French Laboratories). 20-OH-LTB₄ was purchased from Biomol Research Labs (Plymouth Meeting, PA, U.S.A.).

Nucleotides (including GTP[S]) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). [³H]LTB₄ (NET-852-, 180–200 Ci/mmol) was purchased from du Pont-NEN (Boston, MA, U.S.A.). PEG 8000, PEI, CaCl₂, MgCl₂, soybean trypsin inhibitor, bacitracin, benzamide, EDTA, phenylmethanesulphonyl fluoride, sucrose, Tris/HCl, Tris base and polyoxyethylene 9-lauryl ether (C₁₂E₉) were purchased from Sigma (St. Louis, MO, U.S.A.). Molecular mass marker proteins and the protein concentration determination reagents were purchased from Bio-Rad (Richmond, CA, U.S.A.). Octyl β -glucoside, CHAPS, Tween 80, Nonidet P-40 (NP-40), Triton X-100 and the Coomassie Blue protein assay reagent were purchased from Pierce (Rockford, IL, U.S.A.). Digitonin was purchased from Gallard-Schlessinger (Carle Place, NY, U.S.A.).

RESULTS

Detergent solubilization of sheep lung LTB₄ receptors

Seven detergents were evaluated to select the most suitable agent and the best conditions to support LTB₄ receptor solubilization. Table 1 summarizes the results obtained with the three best candidates. CHAPS at a concentration of 0.5 % yielded a soluble preparation with the highest amount of [³H]LTB₄ specific binding activity. A CHAPS concentration of < 0.1 % (w/v) resulted in a significant decrease in both yield and specific binding activity. Further studies (results not shown) indicated that the LTB₄ receptor was most efficiently solubilized at a membrane protein concentration of 0.5–1 mg/ml. The other detergents tested, Triton X-100, NP-40, β -octyl glucopyranoside and Tween 80, did not yield soluble preparations with high levels

Table 1. Solubilization of LTB₄ receptor from sheep lung membrane: comparison of detergents

Sheep lung membranes were solubilized by various types of detergent as described. The supernatants were immediately used to determine [³H]LTB₄ specific binding activity under standard conditions, using the PEG precipitation method. C₁₂E₉, polyoxyethylene-9-laurylether.

Detergent	Concn. (% w/v)	Protein solubilized (%)	[³ H]LTB ₄ specific binding (fmol/mg)
Digitonin	0.5	11	46
	1	25	22
	2	31	32
CHAPS	0.5	20	358
	1	24	67
	2	36	4
C ₁₂ E ₉	0.5	42	3.3
	1	45	0.1
	2	103	0.2

of [³H]LTB₄ specific binding activity. We compared two methods in general use for soluble receptor binding. The first method as reported [20,21] involved PEG precipitation of the soluble receptor–radioligand complex prior to rapid filtration. This method gave acceptable results; however, it was relatively inefficient and favoured the dissociation of [³H]LTB₄ from the soluble receptor when the receptor–[³H]LTB₄ complex was precipitated in PEG (results not shown). The second method utilized rapid filtration of the soluble receptor on PEI-pre-treated filters [22]. A direct comparison of these methods in several experiments indicated that the PEI filtration method was superior to the PEG method; e.g. in saturation binding studies, the PEG method yielded $B_{\max}$ values 30–50 % lower than those obtained with the PEI method (results not shown), suggesting that the PEG method underestimated the density of soluble receptors. The following LTB₄ receptor binding assays were carried out using the PEI method.

Kinetics and specificity of [³H]LTB₄ binding to soluble receptor

[³H]LTB₄ binding to soluble receptors from the alkali-extracted sheep lung membranes was protein-concentration-dependent, time-dependent and reversible in a manner equivalent to that described for [³H]LTB₄ binding to sheep lung membranes [8] and to human PMN membranes [9,10]. Binding of [³H]LTB₄ to the soluble receptor was time-dependent, and reached a plateau level at 30 min under standard conditions (Fig. 1). The reversibility of [³H]LTB₄ binding was demonstrated by adding a 1000-fold excess of unlabelled LTB₄ at steady state. [³H]LTB₄ dissociated from the soluble receptor in a time-dependent manner. After 60 min, [³H]LTB₄ was completely dissociated from the soluble receptor. [³H]LTB₄ specific binding was linearly dependent on protein concentration in the range 50–200 µg/ml (Fig. 1, inset). In saturation binding experiments, the specific binding of [³H]LTB₄ to the soluble receptor component increased with increasing concentrations of [³H]LTB₄ and reached a plateau level at 0.75 nM. Non-specific binding was linearly dependent on the concentration of [³H]LTB₄ (Fig. 2). Conversion of the specific binding data by the Scatchard method produced a linear plot (Fig. 2, inset), suggesting a single class of LTB₄ binding sites under these conditions. Analysis of the results yielded a K_d of 0.50 ± 0.05 nM and a $B_{\max}$ of 330 ± 90 fmol/mg of protein ($n = 4$). This K_d is very similar to that obtained using the alkali-extracted lung membranes ($K_d = 0.52 \pm 0.02$ nM) [8] and thus

Fig. 1. Time-dependence and reversibility of [³H]LTB₄ binding to the soluble receptor

Soluble receptor was incubated with [³H]LTB₄ (●) for various times under standard conditions, as described in the Materials and methods section. At 60 min of incubation, a 1000-fold excess of unlabelled LTB₄ (○) was added to a parallel set of tubes. Inset: protein concentration dependence. [³H]LTB₄ was incubated with increasing concentrations of soluble receptor protein. Data (mean values of duplicate determinations) from one representative experiment of three are shown (S.D. < 10 %).

Fig. 2. Saturation binding of [³H]LTB₄ to soluble receptor

Soluble receptor protein (100 µg) was incubated with increasing concentrations of [³H]LTB₄ in the presence (△) or absence (○) of LTB₄ as described in the Materials and methods section. Inset: Scatchard transformation of [³H]LTB₄ specific binding (●) data ($K_d = 0.50$ nM and $B_{\max} = 340$ fmol/mg). B/F = bound/free. Data from one representative experiment of four are shown. Each data point is the mean of duplicate determinations (S.D. < 10 %).

corresponds to the binding affinity of the low-affinity state LTB₄ receptor in sheep lung membranes.

The specificity of the soluble receptors was assessed in radioligand competition assays. LTB₄ and several agonist analogues competed with [³H]LTB₄ binding to the soluble receptor in a concentration-dependent manner. The specificity profile was characterized by a rank-order potency of binding affinity as LTB₄ > 20-OH-LTB₄ > *trans*-homo-LTB₄ > 6-*trans*-LTB₄ > U-75302 (Fig. 3). The binding affinities of these analogues are shown in Table 2. This rank-order potency of soluble LTB₄ receptor binding is equivalent to that observed for the

Fig. 3. Specificity of soluble LTB_4 receptor

$[\text{H}]\text{LTB}_4$ specific binding to soluble receptor was assessed under standard conditions in the presence of various concentrations of agonist analogues LTB_4 (●), 20-OH- LTB_4 (○), *trans*-homo- LTB_4 (□), 6-*trans*- LTB_4 (△) and U-73502 (▲), as described in the Materials and methods section. Data from one representative experiment of three are shown. Each data point is the mean of duplicate determinations (S.D. < 10%).

Table 2. Competition of $[\text{H}]\text{LTB}_4$ binding to soluble receptors

Soluble receptor (100 μg) was incubated with 0.5 nM- $[\text{H}]\text{LTB}_4$ and increasing concentrations of the indicated compounds as described in the Materials and methods section. Data for membrane receptors are taken from [8] for comparison.

Compound	Soluble receptor	K_i (nM)	
		Membrane receptor	
		Regular	Alkali-Extracted
LTB_4	0.20 ± 0.02	0.22 ± 0.1	0.5 ± 0.1
20-OH- LTB_4	2.7 ± 0.2	2.5 ± 0.8	6.1 ± 1.3
<i>trans</i> -Homo- LTB_4	48 ± 5	38 ± 8	71 ± 11
6- <i>trans</i> - LTB_4	270 ± 10	102 ± 20	265 ± 42
U-73502	1800 ± 230	NT	NT

regular and the alkali-extracted sheep lung membrane receptor preparations [8], and to that for human PMN LTB_4 receptors [9,10]. These results indicate that the soluble $[\text{H}]\text{LTB}_4$ receptor from alkali-extracted sheep lung membranes represents the physiologically and pharmacologically relevant LTB_4 receptor.

Effect of GTP[S] on $[\text{H}]\text{LTB}_4$ specific binding to soluble receptors

We have shown previously [8] that alkali extraction of sheep lung membranes removes the G_i protein required for receptor coupling. Removal of the G_i protein was accompanied by a right-shift in receptor affinity of approx. 3-fold and a loss of inhibition of $[\text{H}]\text{LTB}_4$ specific binding by GTP[S]. In regular (non-alkali-extracted) sheep lung membranes, GTP[S] at concentrations greater than 0.1 μM inhibited $[\text{H}]\text{LTB}_4$ binding [8]. Since the receptor preparation described in these studies was solubilized from sheep lung membranes following alkali extraction, it was expected that $[\text{H}]\text{LTB}_4$ binding to the soluble receptor should also be uncoupled from the G_i protein. The results in Fig. 4 show that GTP[S] at concentrations of up to 300 μM did not inhibit $[\text{H}]\text{LTB}_4$ binding to the soluble receptor,

Fig. 4. Regulation of $[\text{H}]\text{LTB}_4$ binding to soluble receptors by GTP[S]

$[\text{H}]\text{LTB}_4$ was incubated with 100 μg of receptor protein solubilized from either alkali-extracted membranes (●) or from regular membranes (○) in the presence of increasing concentrations of GTP[S] as described in the Materials and methods section. These data are from one of two representative experiments. Data represent the means of duplicate determinations (S.D. < 10%).

indicating that the receptor solubilized from alkali-extracted membranes is not coupled to the G_i protein. Furthermore, when regular sheep lung membranes were solubilized with CHAPS, $[\text{H}]\text{LTB}_4$ specific binding to the soluble preparation was inhibited by GTP[S] in a concentration-dependent manner (Fig. 4). This latter observation demonstrates that it is possible to solubilize the high-affinity receptor- G_i protein complex from unextracted sheep lung membranes.

Effect of cations on $[\text{H}]\text{LTB}_4$ binding to soluble receptors

$[\text{H}]\text{LTB}_4$ specific binding to soluble receptors was enhanced in a concentration-dependent fashion by Ca^{2+} and Mg^{2+} (Fig. 5). Ca^{2+} or Mg^{2+} (30 mM) provided maximal enhancement for $[\text{H}]\text{LTB}_4$ specific binding of 30- and 14-fold respectively. Addition of 5 mM- Mg^{2+} plus 5 mM- Ca^{2+} resulted in a response equal

Fig. 5. Effect of CaCl_2 and MgCl_2 on $[\text{H}]\text{LTB}_4$ specific binding

Soluble receptor (100 μg) was incubated with 0.3 nM- $[\text{H}]\text{LTB}_4$ under standard conditions in the presence of increasing concentrations of CaCl_2 (▲) or MgCl_2 (●) as described in the Materials and methods section. These data are from one of three representative experiments. Data represent the means of duplicate determinations (S.D. < 10%).

Fig. 6. Dissociation of [³H]LTB₄ specific binding by EDTA

[³H]LTB₄ was incubated with soluble receptor in Tris buffer containing 5 mM-Ca²⁺ and 5 mM-Mg²⁺ for 30 min. EDTA (12 mM) was added and the [³H]LTB₄ specific binding was determined at various points of time. These data are representative of two experiments, and are the means of duplicate determinations (S.D. < 10 %).

Fig. 7. Gel filtration and post-soluble binding of the soluble receptor

CHAPS-solubilized lung membrane protein (300 µg in 0.2 ml) was fractionated as described in the Materials and methods section. [³H]LTB₄ specific binding (●) and the protein concentration (△) in the column fractions was determined. Data are representative of three experiments. Arrows indicate molecular mass in kDa.

to that of 5 mM-Ca²⁺ alone (results not shown), indicating that the effects of these divalent cations were not additive. Because of the fact that, in the soluble receptors prepared from alkali-extracted lung membranes, the G_i protein had been removed, it appeared that the enhancement of [³H]LTB₄ binding by the divalent cations was effected through the receptor protein rather than through the G_i protein.

In the absence of divalent cations, Na⁺ and K⁺ at concentrations of 100 mM modestly enhanced [³H]LTB₄ specific binding to soluble receptors by 45% and 79% respectively. However, in the presence of divalent cations (10 mM-Ca²⁺ and 10 mM-Mg²⁺), Na⁺ and K⁺ (100 mM) inhibited [³H]LTB₄ specific binding by 50% and 20% respectively (results not shown).

Reversal of [³H]LTB₄ specific binding by EDTA

The importance of divalent cations in [³H]LTB₄ binding to the

Fig. 8. Gel filtration of the [³H]LTB₄-bound soluble receptor

Soluble receptor (300 µg of protein) was prelabelled with [³H]LTB₄ (0.5 nM) either in the absence (●) or presence (□) of a 1000-fold excess of LTB₄ under standard conditions, and chromatographed as described in the Materials and methods section. Data are representatives of three experiments. Arrows indicate molecular mass (kDa)

soluble receptor is further underscored by the effect of EDTA. When 12 mM-EDTA was added to [³H]LTB₄-bound soluble receptor at steady state (10 mM total divalent cations), more than 70% of the specifically bound radioligand was rapidly displaced in less than 1 min (fig. 6). The maximal effect was achieved at 10 min. The remaining (approx. 20%) specifically bound [³H]LTB₄ associated with the soluble receptor was resistant to the effect of EDTA.

H.p.l.c. size-exclusion chromatography of the soluble LTB₄ receptor

The soluble LTB₄ receptor was fractionated by size-exclusion column chromatography. Fig. 7 shows the [³H]LTB₄ specific binding activity in the column fractions under standard conditions. A single broad peak of [³H]LTB₄ binding activity at 33 min retention time was detected. This corresponds to a protein complex with a 1000 kDa molecular mass in the presence of 0.2% CHAPS.

[³H]LTB₄-prelabelled receptor was also analysed by size-exclusion chromatography under similar conditions, with 10 mM-Ca²⁺ and Mg²⁺ added to the elution buffer to minimize the dissociation of [³H]LTB₄ during chromatography. A major broad radioactivity peak (Fig. 8) at 34 min retention time, with an estimated molecular mass of 800–1000 kDa was detected. A trailing broad band of radioactivity which was eluted from 65 to 85 min corresponds to the unbound [³H]LTB₄ (results not shown). When 0.3 µM-LTB₄ was added during the prelabelling of the soluble receptor, the broad peak at 34 min was absent; however, the unbound [³H]LTB₄ at 65–80 min was still detectable. These results suggest that the LTB₄ receptor solubilized by CHAPS is no longer in a membranous or vesicular state. The estimated molecular mass of the radioligand–receptor complex in the micellar state with CHAPS is approx. 800–1000 kDa, far greater than that predicted from the radioligand cross-linking experiments [7]. It is possible that there are oligomeric subunits or aggregates of the receptor proteins preserved by CHAPS for [³H]LTB₄ binding. The actual molecular mass and the oligomeric structure of the protein or subunits cannot be deduced from the current studies.

DISCUSSION

LTB₄ receptors in human PMNs and many other types of cells, organs and tissues have been well characterized. Based on the specificity and the mechanisms of receptor coupling, we report that sheep lung membranes contain a high density of receptors that are equivalent to the human lung and PMN LTB₄ receptors. When the G_i protein was removed from the sheep lung membranes, the LTB₄ receptor was uncoupled and shifted from a high-affinity ($K_{d-H} = 0.18 \pm 0.03$ nM) to a low-affinity ($K_{d-L} = 0.55 \pm 0.02$ nM) state in binding to LTB₄ [8]. These results suggested that sheep lung membranes represent an ideal source for the isolation and purification of the LTB₄ receptors.

A prerequisite for receptor protein isolation and purification is the establishment of assay methods and systems that allow efficient solubilization of the membrane receptor protein while maintaining the ligand-binding functions. Lin *et al.* [20] and Koo *et al.* [21] reported initial results of receptor solubilization and results from the current study confirm and extend the initial solubilization studies. Among the many detergents tested, CHAPS performed best by yielding the highest amount of soluble LTB₄ receptor activity. Binding of [³H]LTB₄ to the CHAPS-solubilized receptor preparation was time-dependent, reversible, saturable and highly specific. The specificity of the soluble receptor is equivalent to that of the membrane-bound state, at either the high- or low-affinity state in the human PMN, human lung and sheep lung membranes. The binding affinity of [³H]LTB₄ to the CHAPS-solubilized receptor was 0.50 ± 0.05 nM, corresponding to the G_i-protein-uncoupled low-affinity-state receptor in the alkali-extracted sheep lung membranes. The maximum number of receptor binding sites was 330 ± 90 fmol/mg, somewhat lower than the density in the alkali-extracted membranes. This may reflect the preferential solubilization of non-receptor-related proteins in the membranes.

Studies of the effects of cations on [³H]LTB₄ binding yielded some interesting observations. Analogously to the previously reported enhancement of [³H]LTB₄ binding to the G_i-protein-coupled receptors in human rat, and rabbit PMN membranes [3,5,7], and guinea pig spleen membranes [6], both Ca²⁺ and Mg²⁺ increased [³H]LTB₄ specific binding to the CHAPS-solubilized LTB₄ receptors from sheep lung membranes. The enhancement of [³H]LTB₄ binding to the membrane receptor by the divalent cations was thought to be indirectly mediated through the G_i protein; however, divalent cations still enhanced [³H]LTB₄ binding in the soluble LTB₄ receptor preparations in which the G_i protein was removed. These results suggest that the divalent cations could directly bind to the receptor protein, rather than to the G_i protein, to enhance agonist binding.

Size-exclusion chromatography demonstrated that a high-molecular-mass receptor-protein-detergent complex with an apparent molecular mass of 800–1000 kDa was isolated. Although the precise molecular mass of the protein cannot be accurately

determined, the retention time and the pattern of the receptor elution profile were highly reproducible, using either the [³H]LTB₄ prelabelling method or the post-soluble binding method. The apparent high molecular mass of the receptor protein in the detergent micelle suggests that there could be multiple protein subunits or aggregates preserved by CHAPS. Alternatively, it could also mean that the receptor-detergent complex has profoundly affected the hydrodynamic properties in the size-exclusion chromatography. Nevertheless, the receptor solubilization studies have established important criteria and several key assay methods for protein purification and structure determination in the future.

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