Transition of affinity states for leukotriene B₄ receptors in sheep lung membranes

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Leukotriene B₄ (LTB₄) is a pro-inflammatory arachidonate metabolite. We have characterized the LTB₄ receptors in sheep lung membranes and have assessed the contribution of the guanine-nucleotide-binding (G) protein in the regulation of receptor affinity states. Saturation isotherms have demonstrated a single class of LTB₄ receptor with a K_d of 0.18 ± 0.03 nM and a density (B_{max}) of 410 ± 84 fmol/mg of protein in sheep lung membranes. The effect of the G-protein on receptor affinity was assessed in the presence of non-hydrolysable GTP analogues (e.g. GTP[S]) and in membranes following alkali treatment (pH 12.1) to remove the G-protein. Saturation isotherms produced either in the presence of GTP[S] ($K_{d,GTP[S]} = 0.51 \pm 0.02$ nM) or with alkali-treated membranes ($K_{d,alk.} = 0.52 \pm 0.02$ nM) demonstrated a 3-fold shift in receptor affinity for [³H]LTB₄ binding. In competition experiments, the rank order of affinity of LTB₄ analogues was LTB₄ > 20-OH-LTB₄ > *trans*-homo-LTB₄ > 6-*trans*-LTB₄ > 20-COOH-LTB₄, using either untreated or alkali-treated membranes, both in the presence and absence of GTP[S]. These findings demonstrate that, in sheep lung membranes, there is only one class of LTB₄ receptor. Removal of the G-protein or uncoupling of the receptor from the G-protein shifted the agonist-binding affinity of the receptor by 3–4-fold, without affecting the specificity of the LTB₄ receptor in either the high- or the low-affinity state.

INTRODUCTION

Leukotriene B₄ (LTB₄) is a pro-inflammatory mediator that induces chemotaxis, chemokinesis, degranulation, enzyme secretion and cellular aggregation in polymorphonuclear leucocytes (PMNs), monocytes and potentially many other types of inflammatory cells (for reviews, see [1,2]).

The LTB₄ receptor was initially identified in PMNs [3–14], and was later demonstrated in many types of cells and tissues [12,13]. Using whole cells or cellular membrane preparations, many laboratories [3–15] have demonstrated that an inhibitory guanine-nucleotide-binding protein (G_i-protein)-coupled plasma-membrane-bound receptor is the critical component in the recognition, propagation and amplification of the signal for LTB₄-mediated inflammatory responses. Radioligand binding studies [3–9,12–14] have shown that binding of [³H]LTB₄ to the PMN receptor can exist in a high-affinity or a low-affinity state [3,16]. A molecular model of receptor–G_i-protein interaction was proposed [15,16] to account for the mechanism of high-affinity to low-affinity transition.

When human PMNs were exposed to LTB₄, the chemotaxis response, but not the degranulation response, of the PMNs, was selectively deactivated [3,15]. This deactivation was coincidental with a disappearance of the high-affinity, but not the low-affinity, [³H]LTB₄ binding sites. This observation led to the ‘functional

assignment hypothesis’ for LTB₄ receptor biology, i.e. the chemotaxis and chemokinesis responses are mediated via the high-affinity LTB₄ receptor, whereas the enzyme secretion and lysosomal degranulation responses are mediated via the low-affinity LTB₄ receptor [3,4]. Studies with rat PMNs appear to support this speculation: LTB₄ could induce enzyme secretion and degranulation but not chemotaxis and chemokinesis responses [4] in rat PMNs, and only low-affinity ($K_d = 10$ nM) LTB₄ receptors could be identified in rat PMNs. This hypothesis would predicate that there are two functionally different, or even structurally different, high- and low-affinity LTB₄ receptors in human PMNs, and perhaps in other types of cells or tissues.

Because of the various types of cells, tissues, species and experimental approaches utilized by the many investigators, the characteristics and the specificity profiles of the high-affinity-state and the low-affinity-state LTB₄ receptor are variable and undefined. Thus there is no clear quantitative evidence reported in the literature to substantiate the receptor affinity transition model.

In the current study, we seek quantitative experimental evidence to substantiate the high- and low-affinity-state LTB₄ receptor model. In contrast with some of the studies mentioned above, we were able to demonstrate only a single class of LTB₄ receptors in sheep lung membranes. We also demonstrate that a G_i-protein regulates the conversion of the LTB₄ receptor-binding-affinity states. The observed affinity shift of LTB₄ binding

Abbreviations used: LTB₄, leukotriene B₄ (5S,12R-dihydroxy-6E,8Z,10Z,14E-eicosatetraenoic acid); PMN, polymorphonuclear leucocytes; G_i-protein, inhibitory guanine-nucleotide-binding protein; IC₅₀, concentration that causes 50% inhibition of specific binding; B_{max} , maximum binding capacity; 20-OH-LTB₄, 5S,12R-20-trihydroxy-6E,8Z,10Z,14Z-eicosatetraenoic acid; 20-COOH-LTB₄, 5S,12R-dihydroxy-20-carboxy-6E,8Z,10Z,14E-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; p[NH]ppG, guanosine 5'-[β-γ-imido]triphosphate; IAP, islet-activating protein.

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is much less dramatic than originally proposed. Thus we offer an alternative model to account for the high-affinity-state and low-affinity-state receptor transition.

MATERIALS AND METHODS

Membrane preparation

Sheep lung was obtained from the Vanezas (Conshohocken, PA, U.S.A.). Lung (250 g) in 1.5 litres of ice-cold buffer A (0.25 M-sucrose, 10 mM-Tris/HCl, pH 7.4, 10 μ g of soybean trypsin inhibitor/ml, 100 μ M-benzamidine, 5 mM-EDTA, 100 μ g 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 0–4 °C. The tissue was further homogenized using a Polytron homogenizer at setting 7 for 1 min to disrupt the cells. This homogenate was then centrifuged at 1300 *g* for 20 min. The resulting supernatant was transferred to ultracentrifuge tubes, underlaid with buffer B (buffer A adjusted to 40% sucrose) and centrifuged at 100 000 *g* for 60 min. The membrane at the interface was collected, diluted with 2 vol. of ice-cold distilled water and re-centrifuged at 150 000 *g* for 60 min. The membrane pellets were then frozen in liquid N₂ and stored at –70 °C for up to 3 months with no loss of binding activity. Routinely, 300 mg of partially purified sheep lung membrane was obtained per preparation. Protein concentration was determined using the method of Bradford [17] with bovine serum albumin as standard.

Alkaline extraction 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. Membrane suspension (1 vol.) was combined with 4 vol. of alkaline extraction buffer C (10 mM-K₂HPO₄, pH 12.5) for 5 min at 4 °C. The final pH was 12.1; a pH of less than 11.5 did not give an optimal result. This membrane suspension was then centrifuged at 150 000 *g* for 60 min at 4 °C. The alkali-extracted pellets were then frozen in liquid N₂ and stored at –70 °C until use. Alkali extraction resulted in the removal of 60–70% of the total protein, whereas >95% of the receptor was retained in the membrane. The alkali-extracted membranes were neutralized when used for the receptor-binding assays. Conc. KOH solution was used to adjust the pH of the buffers. It should be noted that neutralization of the alkaline membrane suspension before centrifugation did not result in the removal of the G-protein(s).

Radioligand binding assays

All assays were performed in 10 mM-Tris/HCl (pH 7.4) buffer containing 5 mM-CaCl₂ and 5 mM-MgCl₂ in a total volume of 0.5 ml, at 22 °C for 40 min, in duplicate, using Nunc 'Minisorp' polyethylene tubes to minimize non-specific binding (standard conditions). In saturation binding assays, membrane protein (20 μ g of regular or 10 μ g of alkali-extracted) was incubated with increasing concentrations of [³H]LTB₄, in the absence or presence of a 1000-fold excess of unlabelled LTB₄. GTP[S] (20 μ M) was included in some experiments as indicated. In competition binding assays, membrane protein (100 μ g of regular or 50 μ g of alkali-extracted) and 3 nM-[³H]LTB₄ (32 Ci/mmol) were incubated in the presence of increasing concentrations of unlabelled LTB₄ or LTB₄

analogues. In assays to study guanine nucleotide effects, membrane protein (100 μ g of regular or 50 μ g of alkali-extracted) and 0.3 nM-[³H]LTB₄ (200 Ci/mmol) were incubated with various concentrations of guanine nucleotides or analogues under standard conditions.

Free ligand was separated from membrane-bound ligand by rapid filtration at 0 °C using Whatman GF/C filters. Filters were washed with 4 × 4 ml of ice-cold washing buffer D (10 mM-Tris/HCl, pH 7.4) and the radioactivity retained was determined by liquid scintillation counting.

Analysis of binding data

[³H]LTB₄ specific binding was calculated by subtracting the non-specific binding from the total [³H]LTB₄ bound. [³H]LTB₄ specific binding results, from saturation binding experiments, were transformed by the method of Scatchard [18]. The dissociation constant (*K*_d) and maximal number of binding sites (*B*_{max}) were determined by using a least-square best-fit program [15]. The inhibition constant (*K*_i), derived from competition binding assays, was calculated according to Cheng & Prusoff [19], using the equation: $K_i = IC_{50} / (1 + [^3H]LTB_4 / K_d)$, where the *IC*₅₀ is the concentration of competitor that results in a 50% reduction in [³H]LTB₄ specific binding, the [³H]LTB₄ concentration is 0.3 or 3 nM and the *K*_d is the dissociation constant (0.18 or 0.55 nM) derived from the saturation binding studies.

[³²P]ADP-ribosylation of lung membrane G-proteins

Pertussis toxin (islet-activating protein, IAP) was activated by incubating for 10 min at 30 °C in 50 mM-glycine (pH 8.0), 20 mM-dithiothreitol and 1 mg of ovalbumin/ml. Unextracted and alkali-extracted lung membranes (1 mg/ml, final concn.) were incubated with the activated IAP (4 μ g/ml) and 5 μ M-[³²P]NAD⁺ (total vol. 100 μ l, 5 μ Ci/assay) at 30 °C for 60 min as reported previously [20], except that Lubrol PX was added to a final concentration of 0.05 mM.

Gel electrophoresis

Native or [³²P]ADP-ribosylated proteins were precipitated with cold 10% trichloroacetic acid. Precipitated proteins were solubilized with SDS sample buffer and subjected to electrophoresis on SDS/polyacrylamide gels by the method of Laemmli [21].

Reagents

Chemicals, reagents and radioligands were obtained from the following sources. LTB₄, *trans*-homo-LTB₄ and 6-*trans*-LTB₄ were synthesized and supplied by K. Erhardt (Department of Medicinal Chemistry, SK&F Laboratories). 20-OH-LTB₄ and 20-COOH-LTB₄ were purchased from Biomol Research Labs (Plymouth Meeting, PA, U.S.A.). Nucleotides (including GTP[S] and p[NH]ppG) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). [³H]LTB₄ (NET-852, 180–200 Ci/mmol and NET-772, 32 Ci/mmol) was purchased from Dupont-NEN (Boston, MA, U.S.A.). CaCl₂, MgCl₂, soybean trypsin inhibitor, bacitracin, benzamidine, EDTA, phenylmethanesulphonyl fluoride, sucrose and Tris/HCl were purchased from Sigma (St. Louis, MO, U.S.A.). A Bradford protein determination kit was purchased from Bio-Rad (Richmond, CA, U.S.A.).

RESULTS

[³H]LTB₄ binding to receptors in sheep lung membranes

Specific binding of [³H]LTB₄ to sheep lung membranes (either regular or alkali-extracted) was temperature-dependent, protein concentration-dependent and time-dependent (results not shown) in a manner equivalent to that described for [³H]LTB₄ binding to human PMN membranes [3–6,8]. Under standard conditions, the specific binding reached a stable steady state after 20 min, and the [³H]LTB₄ binding was reversible upon addition of an excess of unlabelled LTB₄ (results not shown). Fig. 1 shows a representative experiment of [³H]LTB₄ binding to regular sheep lung membranes. Non-specific binding was linearly dependent on the concentration of [³H]LTB₄. The specific binding component increased with increasing concentrations of [³H]LTB₄ and reached a plateau at 1 nM-[³H]LTB₄. Conversion of these data by the Scatchard method (Fig. 1, inset) yielded a linear plot, suggesting a single class of LTB₄ specific binding sites under these experimental conditions. The saturation binding data were analysed

by using a least-square best-fit program [14]. The results (Table 1) indicated that the data were best described by a 'single class of binding site' model, with $K_d = 0.18 \pm 0.03$ nM and $B_{max} = 410 \pm 80$ fmol/mg ($n = 4$). These values correlated well with those from human PMN membranes reported from other laboratories [3,6–10]. The [³H]LTB₄ specific binding was selectively but incompletely inhibited by guanine nucleotides (Fig. 2). Both GTP[S] and p[NH]ppG inhibited [³H]LTB₄ specific binding, and 50 % of maximal inhibition occurred at a concentration of 0.2 ± 0.1 μ M. Maximal inhibition of [³H]LTB₄ binding was achieved at 1 μ M and did not exceed more than 40 ± 5 % (from six experiments). Adenine nucleotides, ATP, p[NH]ppA, ADP and AMP, as well as CTP, GMP and GDP at concentrations less than 100 μ M, did not inhibit significantly [³H]LTB₄ specific binding (results not shown). GTP also inhibited [³H]LTB₄ binding; however, the IC_{50} cannot be determined accurately because of hydrolysis by GTPase under the current experimental conditions.

[³H]LTB₄ specific binding to sheep lung membranes was highly selective; LTB₄ analogues, such as 20-OH-LTB₄, 20-COOH-LTB₄ and 6-*trans*-LTB₄, can specifically compete with [³H]LTB₄ binding to the membrane receptors (Table 2), whereas the peptidyl leukotrienes did not

Fig. 1. Saturation analysis of [³H]LTB₄ specific binding sites

Regular sheep lung membranes were incubated with [³H]LTB₄ (200 Ci/mmol) in the absence (□) or presence (○) of unlabelled LTB₄ as described in the Materials and methods section. Inset, Scatchard transformation of [³H]LTB₄ specific binding (●) data ($K_d = 0.14$ nM and $B_{max} = 393$ fmol/mg). These data are from one experiment representative of four.

Fig. 2. Effect of guanine nucleotides on [³H]LTB₄ specific binding

Regular sheep lung membrane (●, ▲) and alkali-extracted sheep lung membrane (○, △) were incubated with 0.3 nM-[³H]LTB₄ in the presence of increasing concentrations of either GTP[S] (●, ○) or p[NH]ppG (▲, △) as described in the Materials and methods section.

Table 1. Regulation of sheep lung membrane LTB₄ receptor affinity by guanine nucleotides

Mean $\pm$ S.E.M. results from four experiments are shown.

	Regular membranes		Alkali-extracted membranes	
	Control	+GTP[S]	Control	+GTP[S]
K_d (nM)	0.18 ± 0.03	0.51 ± 0.02	0.52 ± 0.02	0.55 ± 0.02
B_{max} (fmol/mg of protein)	410 ± 84	448 ± 220	1050 ± 120	1560 ± 380

Table 2. Effect of GTP[S] on binding of LTB₄ and agonist analogues to sheep lung membrane receptors

A 100 μ g portion of sheep lung membranes was incubated with 3.0 nM-[³H]LTB₄ and increasing concentrations of the indicated compounds as described in the Materials and methods section. $K_i = IC_{50}/(1 + [^3H]LTB_4/K_d)$, where $K_d = 0.18$ nM and [³H]LTB₄ = 3.0 nM. $K_{i-GTP[S]} = IC_{50}/(1 + [^3H]LTB_4/K_{d-GTP[S]})$, where $K_{d-GTP[S]} = 0.51$ nM and [³H]LTB₄ = 3.0 nM.

Compound	K_i (control) (nM)	$K_{i-GTP[S]}$ (nM)	$\frac{K_{i-GTP[S]}}{K_i}$
LTB ₄	0.22 ± 0.1	1.1 ± 0.1	5.0
20-OH-LTB ₄	2.5 ± 0.8	13 ± 3	5.2
<i>trans</i> -Homo-LTB ₄	38 ± 8	130 ± 20	3.4
6- <i>trans</i> -LTB ₄	102 ± 20	225 ± 70	2.2
20-COOH-LTB ₄	678 ± 200	1450 ± 400	2.1

compete (results not shown). These results show that [³H]LTB₄ can bind to the specific sites in sheep lung membranes with high affinity and with specificity. The specificity of binding correlated directly with that derived from chemotaxis, chemokinesis and aggregation studies using human or rabbit PMNs [2,3,5–10], and rat PMN aggregation studies [13,15]. These results, taken collectively, strongly indicate that the [³H]LTB₄ specific binding sites in sheep lung membranes represent the physiologically and pharmacologically important LTB₄ receptors.

Alkali extraction removes G_i-protein

We noted that [³H]LTB₄ specific binding to sheep lung membranes was only incompletely inhibited in the presence of high concentrations of p[NH]ppG and GTP[S]. According to the proposed model, [³H]LTB₄ specific binding in the presence of GTP[S] or p[NH]ppG should represent the low-affinity-state receptor [4,16]. Alternatively, if the G_i-protein could be completely inactivated (e.g. by extensive treatment with IAP) or removed (e.g. by alkali extraction), [³H]LTB₄ specific binding should no longer be regulated by GTP[S] or p[NH]ppG and should also represent low-affinity-state receptor binding.

Fig. 3 shows the functional state of the G_i-protein in sheep lung membranes. In the regular membrane preparation, an IAP-induced [³²P]ADP-ribosylated α -subunit of a G_i-protein (41 kDa) can be identified. After the alkali treatment, >98% of the [³²P]ADP-ribosylable α -subunit of the G_i-protein was removed (quantified by densitometric evaluation of the autoradiogram). The [³H]LTB₄ specific binding to the alkali-extracted sheep lung membranes was no longer inhibited by GTP[S] or p[NH]ppG (Fig. 2).

Functional characteristics of the low-affinity-state LTB₄ receptor

To assess the characteristics of the low-affinity LTB₄ receptor, we performed [³H]LTB₄ saturation binding experiments in the presence of 20 μ M-GTP[S]. As a control, binding of [³H]LTB₄ to regular sheep lung membranes, in the absence of GTP[S], was saturable with high affinity (Fig. 4a). The K_d and B_{max} were 0.18 ± 0.03 nM and 410 ± 84 fmol/mg ($n = 4$) respectively. Under the current conditions, there are no data to indicate that the LTB₄ specific binding sites in the regular membranes could be fitted with a low-affinity ($K_d = 0.5$ nM) model. In the presence of 20 μ M-GTP[S], specific binding of [³H]LTB₄ was also saturable (Fig. 4a). The

Fig. 3. [³²P]ADP-ribosylation of regular and alkali-extracted sheep lung membranes

Regular lung membrane protein (lanes a–c) or alkali-extracted membrane protein (lanes d–f) was incubated with the activated IAP and [³²P]NAD⁺ as described. The proteins were separated by SDS/PAGE and displayed by autoradiography.

dissociation constant (designated as $K_{d-GTP[S]}$) and the density (designated as $B_{max-GTP[S]}$) were 0.51 ± 0.02 nM and 448 ± 220 fmol/mg respectively ($n = 4$). A more useful way to evaluate the data is to transform the saturation binding data by the Scatchard method (Fig. 5a). An alteration of the slope, but not of the apparent x-axis intercept, indicated that the binding affinity of [³H]LTB₄ binding is shifted downward by 3-fold in the presence of GTP[S]. The density of the receptors, either in the presence or absence of GTP[S], was not significantly affected. Because the density of [³H]LTB₄ specific binding remained unchanged, the result suggested that the LTB₄ receptors in the regular membranes could exist in either a high- or a low-affinity state in binding to [³H]LTB₄. Under the present experimental conditions, LTB₄ receptors in regular lung membranes were coupled to the G_i-protein and existed predominantly in the high-affinity state ($K_d = 0.18$ nM). [³H]LTB₄ binding to the low-affinity-state receptor in the regular lung membranes was not detectable.

Another way to assess the low-affinity-state LTB₄ receptors is to study [³H]LTB₄ binding to the alkali-

Fig. 4. Saturation binding of $[^3\text{H}]\text{LTB}_4$ to the receptors in regular and alkali-extracted membranes

$[^3\text{H}]\text{LTB}_4$ specific binding using regular lung membranes (a) or alkali-extracted lung membranes (b) in the absence (●) or presence (■) of $20\ \mu\text{M-GTP[S]}$ is shown. Non-specific binding of $[^3\text{H}]\text{LTB}_4$ in the absence (○) or presence (□) of GTP[S] was also determined. The data shown were from an experiment representative of four.

Fig. 5. Conversion of the saturation binding data

The results of $[^3\text{H}]\text{LTB}_4$ specific binding data using the regular or alkali-extracted lung membranes (from Fig. 4) were recalculated and plotted by the Scatchard method. (a) Regular lung membrane $[^3\text{H}]\text{LTB}_4$ specific binding in the absence (●) or in the presence (■) of $20\ \mu\text{M-GTP[S]}$. (b) Alkali-extracted membrane specific binding of $[^3\text{H}]\text{LTB}_4$ in the absence (●) or in the presence (■) of GTP[S] .

extracted membranes. The result of a representative $[^3\text{H}]\text{LTB}_4$ saturation binding experiment is shown in Fig. 4(b). Specific binding of $[^3\text{H}]\text{LTB}_4$ to the alkali-extracted membrane was saturable. The dissociation constant (designated as $K_{\text{d,alk.}}$) and the density (designated as $B_{\text{max,alk.}}$) were determined as $0.52 \pm 0.02\ \text{nM}$ and $1050 \pm 120\ \text{fmol/mg}$ of protein respectively ($n = 4$). The effects of GTP[S] on LTB_4 binding to the alkali-extracted membranes were also investigated. Fig. 4(b) shows that $[^3\text{H}]\text{LTB}_4$ binding to the specific site in the alkali-extracted membranes in the presence of $20\ \mu\text{M-GTP[S]}$ was also saturable. The dissociation constant (designated as $K_{\text{d,alk.,GTP[S]}}$) and density (designated as $B_{\text{max,alk.,GTP[S]}}$) were $0.55 \pm 0.02\ \text{nM}$ and $1560 \pm 380\ \text{fmol/mg}$ respectively ($n = 4$). The saturation binding data were converted by the Scatchard method and are shown in Fig. 5(b). It is apparent that the slopes of the plots are not significantly different, suggesting that the affinity of $[^3\text{H}]\text{LTB}_4$ specific

binding ($K_{\text{d,alk.}}$ versus $K_{\text{d,alk.,GTP[S]}}$) was not significantly altered by GTP[S] . On the other hand, $[^3\text{H}]\text{LTB}_4$ binding affinity to the regular lung membrane receptors ($K_{\text{d}} = 0.18 \pm 0.03\ \text{nM}$) was significantly downward-shifted by alkali treatment ($K_{\text{d,alk.}} = 0.52 \pm 0.02\ \text{nM}$). Furthermore, the $[^3\text{H}]\text{LTB}_4$ binding affinity to the GTP[S] -shifted regular lung membrane receptors ($K_{\text{d,GTP[S]}} = 0.51 \pm 0.02\ \text{nM}$) is almost identical to that in the alkali-extracted lung membrane receptors, either in the presence ($K_{\text{d,alk.,GTP[S]}} = 0.55 \pm 0.02\ \text{nM}$) or the absence ($K_{\text{d,alk.}} = 0.52 \pm 0.02\ \text{nM}$) of GTP[S] . The difference in $[^3\text{H}]\text{LTB}_4$ binding density in the regular or alkali-extracted membranes is difficult to compare, because alkali extraction removes at least 60–70 % of the non-integral proteins, in addition to the G_i -protein. Thus the apparent 2.5-fold increase in LTB_4 receptor density may be the result of the removal of unrelated proteins. Table 1 summarizes these parameters for comparison.

Table 3. Effect of GTP[S] on LTB₄ and agonist binding to receptors in alkali-extracted membranes

A 50 µg portion of alkali-extracted sheep lung membranes was incubated with 3.0 nM-[³H]LTB₄ in the presence of increasing concentrations of the indicated compounds as described in the Materials and methods section. $K_{i\cdot alk.} = IC_{50}/(1 + [^3H]LTB_4/K_{d\cdot alk.})$, where $K_{d\cdot alk.} = 0.52$ nM and [³H]LTB₄ = 3.0 nM. $K_{i\cdot alk. \cdot GTP[S]} = IC_{50}/(1 + [^3H]LTB_4/K_{d\cdot alk. \cdot GTP[S]})$, where $K_{d\cdot alk. \cdot GTP[S]} = 0.53$ nM and [³H]LTB₄ = 3.0 nM.

Compound	$K_{i\cdot alk.}$ (nM)	$K_{i\cdot alk. \cdot GTP[S]}$ (nM)	$\frac{K_{i\cdot alk. \cdot GTP[S]}}{K_{i\cdot alk.}}$
LTB ₄	0.5 ± 0.1	0.4 ± 0.2	0.8
20-OH-LTB ₄	6.1 ± 1.3	8.1 ± 0.6	1.3
<i>trans</i> -Homo-LTB ₄	71 ± 11	84.0 ± 20	1.2
6- <i>trans</i> -LTB ₄	265 ± 42	280 ± 50	1.1
20-COOH-LTB ₄	4400 ± 927	4200 ± 960	1.0

Specificity of the LTB₄ receptors in sheep lung membranes

The question of the specificities of the LTB₄ receptors, in either the high- or the low-affinity state, was addressed by using radioligand competition assays. The high-affinity-state LTB₄ receptor was characterized by using regular sheep lung membranes labelled with 3 nM-[³H]LTB₄. Since, in the absence of GTP[S] or alkali treatment, the majority of the LTB₄ receptors in the regular lung membranes are in the high-affinity state ($K_d = 0.18$ nM), we assume that more than 95 % of the high-affinity LTB₄ receptors are labelled by 3 nM-[³H]LTB₄. The specificity profile is characterized by the rank order potency of binding affinity as: LTB₄ > 20-OH-LTB₄ > *trans*-homo-LTB₄ > 6-*trans*-LTB₄ > 20-COOH-LTB₄. The binding affinities of these LTB₄ analogues are shown in Table 2. The rank order potency is equivalent to that described in the human PMN membrane binding study [3,6–8] or in the human PMN chemotaxis study [2,3,6–8,10], thus attesting to the identity of the LTB₄ receptors in sheep lung. The specificity profile of the low-affinity-state LTB₄ receptor was characterized using either an alkali-extracted sheep lung membrane preparation or a regular lung membrane preparation in the presence of 100 µM-GTP[S]. Since the majority of the LTB₄ receptors, under these conditions, are in the low-affinity state ($K_d = 0.52$ – 0.55 nM), we have used 3 nM-[³H]LTB₄ to label more than 90 % of the low-affinity LTB₄ receptors. Tables 2 and 3 show that, under either set of conditions, the rank order potency was LTB₄ > 20-OH-LTB₄ > *trans*-homo-LTB₄ > 6-*trans*-LTB₄ > 20-COOH-LTB₄, identical to that of the high-affinity LTB₄ receptors in regular lung membranes. Thus these results suggest that, although the binding affinity of LTB₄ to the receptor may be high (0.18 nM) or low (0.52–0.55 nM), the specificity of the receptors both in the high- and the low-affinity state appears to be identical.

Frame-shift of agonist binding affinity constants

According to the proposed molecular model [16], LTB₄ binding to the receptor recruits the G_i-protein to form the high-affinity-state receptor-G_i-protein-ligand complex. The ability of ligands to form the high-affinity complex is a hallmark of LTB₄ and partial agonist analogues. A corollary is that the affinities of agonist analogues in binding to the alkali-extracted membrane receptors should also be affected, because the receptor is

no longer able to form the high-affinity ternary complex. Table 3 shows that the ratio of $K_{i\cdot GTP[S]}/K_i$ for LTB₄ and agonist analogues was close to unity (0.8–1.3) in the alkali-extracted membranes, indicating that the low-affinity receptor in binding to the agonist analogues is no longer regulated by the G-protein and, hence, is not capable for forming the high-affinity ternary complex. The ratio of $K_{i\cdot GTP[S]}/K_i$ for LTB₄ and agonist analogues determined from regular membranes was greater than 2 (2.1–5.2). These results show that binding of LTB₄ and the agonist analogues is regulated by the G_i-protein. A downward frame-shift of the binding affinity for the agonist compounds is observed when G_i-protein function is inactivated.

DISCUSSION

Using the radioligand binding method, the results from this study have shown that in sheep lung membranes, [³H]LTB₄ can bind to the G_i-protein-coupled specific receptors. The rank order potency of the LTB₄ agonist analogues in binding to the sheep lung membrane receptors directly correlated with that determined for rat PMN membranes [5,8], guinea pig spleen membranes [13] and human lung membranes (B. Votta & S. Mong unpublished work), and those determined from the human and rabbit PMN chemotaxis studies [2,3,6,7,10–12]. Thus the LTB₄ receptor in sheep lung is equivalent to those identified in PMNs and in other cells, tissues and species. [³H]LTB₄ specific binding sites in sheep lung membranes represent the biochemically and physiologically important receptors [22].

Several previous studies have shown that binding of [³H]LTB₄ to the PMN receptors could occur in a high-affinity state ($K_d = 0.08$ – 10 nM) or a low-affinity state ($K_d = 50$ – 500 nM) [3,5–9,13–15]. Because the methods and sources of membranes or cells used are highly variable from one laboratory to another, it has been difficult to carry out a quantitative generic assessment of the high- and low-affinity binding constants. Furthermore, it is possible that there could be other factors or proteins, in addition to G_i-protein, that could regulate the binding affinity of the receptor to LTB₄. Thus the radioligand binding characteristics in the intact cells may be very different from those described in this study. By using alkali extraction and the non-hydrolysable GTP analogues, we have confirmed that the LTB₄ receptor in

sheep lung membranes could exist in two affinity states and have provided, for the first time, a direct measurement of the high-affinity ($K_d = 0.18$ nM) and the low-affinity ($K_{d-GTP[S]} = 0.51$ nM) binding constants. Quite unexpected was the finding that only a 3-fold shift of affinity for [3 H]LTB₄ binding was observed. In comparison with many other G-protein-coupled receptor systems, the shift of LTB₄ receptor binding affinity is far less dramatic than anticipated. In the adrenergic, dopaminergic or muscarinic receptor systems, for example, a greater than 50-fold affinity shift is usually observed [23–25].

The results from saturation binding experiments showed that although the binding affinity of [3 H]LTB₄ to the receptors in the regular lung membranes was shifted 3-fold by GTP[S], the density of [3 H]LTB₄ binding to the high- or low-affinity receptors was unchanged. In addition, the saturation binding studies showed that in the regular lung membranes, LTB₄ receptors exist predominantly in the G_i-protein-coupled high-affinity state. Data derived from saturation binding studies did not support the conclusion that low-affinity-state LTB₄ receptors ($K_d = 0.52$ – 0.55 nM) could be detected or identified in the regular membranes. On the other hand, in alkali-extracted membranes or in the regular membranes treated with GTP[S], the results derived from saturation binding experiments showed that nearly all LTB₄ receptors were in the low-affinity state. It is possible that alkali extraction may remove other proteins or factors that could regulate the LTB₄ receptor binding affinity. Thus the transition of the affinity states of LTB₄ receptor may not reflect accurately the biological functional states of the LTB₄ receptor in intact cells (e.g. PMN). Nevertheless, the experimental data provided in this study suggest that the LTB₄ receptor in sheep lung membrane could exist in two interconverting affinity states dependent on the functional status of the G_i-protein. There is one, and only one, class of LTB₄ receptor in sheep lung membranes, and whereas the [3 H]LTB₄ receptor binding affinity may be high or low, the total number of the receptors remains constant.

An important finding in the LTB₄ receptor-affinity transition studies was that the specificity pattern of the high-affinity-state receptor was almost identical with that of the low-affinity-state receptor. This observation would argue against the functional assignment hypothesis of the LTB₄ receptor model, and support the idea that there is one and only one class of LTB₄ receptor, the biological consequences of LTB₄ receptor inactivation being mediated through the same population of the LTB₄ receptors.

Preliminary experimental results (not shown) using normal and alkali-extracted human monocyte membranes support these findings, in that we observed a single-class affinity-transition LTB₄ receptor model qualitatively similar to the sheep lung LTB₄ receptor model described in the current study. Based on these observations, we would predict that the LTB₄ receptor antagonists will be effective in binding to LTB₄ receptor at either the high- or the low-affinity state, and will be able to block all of the LTB₄-receptor-mediated cellular, physiological and pharmacological responses in several

species. The ramifications of this prediction will await the future development of specific and potent LTB₄ receptor antagonists.

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