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Binding of Radiolabeled High Affinity Antagonist to Leukotriene D_4 Receptor in Guinea Pig Lung Membranes: Interconversion of Agonist-Receptor Binding Affinity States

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SUMMARY

The kinetic parameters and the pharmacological specificity of a high affinity leukotriene D_4 (LTD_4) receptor antagonist, ICI-198615, binding to guinea pig lung membranes were characterized. Binding of [^{3}H]ICI-198615 to the membranes was rapid and displaceable with excess ICI-198615. The specific binding of [^{3}H]ICI-198615 was dependent upon the concentration of membrane protein. Monovalent cations (Na^+, Li^+, and Cs^+), divalent cations (Mg^{2+}, Ca^{2+}, and Mn^{2+}), and guanine nucleotides did not significantly affect the specific binding of [^{3}H]ICI-198615 to guinea pig lung LTD_4 receptors. The specific binding of [^{3}H]ICI-198615 to guinea pig lung membranes was saturable and the equilibrium saturation binding was best approximated by a single-site model. The dissociation constant (K_D) and the density (B_{max}) were 0.08 ± 0.04 nM and 1030 ± 180 fmol/mg, respectively. In competition studies, LTD_4, stereoisomer (5*R*,6*S*)-LTD_4, and leukotriene E_4 (LTE_4) competed with [^{3}H]ICI-198615 binding to specific sites, with a stereoselectivity and a rank order of potency equivalent to those described in [^{3}H]LTD_4 binding studies and in functional studies. LTD_4 and LTE_4 displaced maximally 70 and 40%, respectively, of the [^{3}H]ICI-198615 specific binding component defined by ICI-198615. Several LTD_4 receptor antagonists (ICI-198615, WY-48252, WY-49511, FPL-55712, and LY-171883) displaced [^{3}H]ICI-198615 specific binding, with a rank order of potency equivalent to that described in the guinea pig tracheal smooth muscle contraction system. The leukotriene structure-like receptor antagonists, e.g., SK&F 104353 and SK&F 104373, also competed with the [^{3}H]ICI-198615 specific binding, with binding affinities comparable to those expected from the functional studies. However, SK&F 104353 and SK&F 104373 displaced maximally 70% of the specific binding component of [^{3}H]ICI-198615, equivalent to that displaced by LTD_4. Guanosine-5′-3-thiotriphosphate (GTPγS), EDTA, and Na^+ shifted the LTD_4 displacement curve to the right, indicating that these agents regulated the binding of LTD_4 to the receptor. In the absence of GTPγS or cations, the LTD_4 displacement curve was heterogeneous. The LTD_4 displacement curve was resolved into a higher affinity component (K_{D_H} = 0.5 ± 0.2 nM; percentage of receptor density at high affinity = 24 ± 3%) and a low affinity component (K_{D_L} = 60 ± 7 nM; percentage of receptor density at low affinity = 76 ± 3%). When GTPγS, EDTA, and Na^+ were used in combination, the LTD_4 displacement curve was significantly shifted to the right and a single low affinity component (K_{D_L} = 200 ± 30 nM; percentage of receptor density at low affinity = 98 ± 5%) for LTD_4 binding was observed. These results show that [^{3}H]ICI-198615 specific binding components are selective for the LTD_4 receptor antagonists. A major portion of the [^{3}H]ICI-198615 specific binding sites (≈70%) represent the LTD_4 receptors in guinea pig lung membranes and GTPγS, Na^+, and divalent cations regulate agonist-receptor binding by interconversion of the affinity states.

Peptidoleukotrienes C_4, D_4, and E_4 constitute the slow reacting substance of anaphylaxis (1, 2). LTD_4 and LTE_4 cause a vast array of pathophysiological responses, including smooth muscle contraction, bronchoconstriction, mucus hypersecretion, plasma exudation, and decrease of cardiac contractility (1–3), in the target cells, tissues, or organs of several species. These pharmacological effects induced by LTD_4 and other agonists are highly stereoselective and specific (3, 4), indicating that they are mediated via receptors. Supporting this hypothesis, a G protein-regulated, high affinity, specific, saturable membrane-bound receptor for [^{3}H]LTD_4 has been identified (5, 6) and characterized (6–10). Studies also demonstrated that LTD_4 and agonist analogs induced rapid hydrolysis of phosphoinositides in guinea pig lung (11) and intracellular calcium ($[Ca^{2+}]_i$) mobilization in leukotriene target cells in culture (12, 13). These results suggested that diacylglycerol and inositol-

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ABBREVIATIONS: LTD_4, leukotriene D_4; LTC_4, leukotriene C_4; LTE_4, leukotriene E_4; [^{3}H]ICI-198615, [1-(2-methoxy-4-[[(phenylsulfonyl)amino]carbonyl)phenylmethyl)-1*H*-indazole-6-yl]-carbamic acid 2,3-ditritiumcyclopentylester; GTPγS, guanosine-5′-3-thiotriphosphate; WY-48252, 1,1,1-trifluoro-*N*-[3-(2-quinolinyl-methoxy)phenyl]methane sulfonamide; LY-171883, 1-[2-hydroxy-3-propyl-4-[4-(1*H*-tetrazol-5-yl)butoxy]phenyl]ethanone; SK&F 104353, (2*S*)-hydroxy-(3*R*)-carboxyethylthio-3-[2-(8-phenyloctyl)phenyl]propanoic acid; G protein, guanine nucleotide-binding protein; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid].

1,4,5-trisphosphate could function as intracellular messengers for LTD_4 receptor in the target cells or tissues. The specificity of agonist-induced pharmacological effects, either dependent on or independent of cyclooxygenase activation, directly correlated with those derived from [^{3}H]LTD_4 receptor binding studies, smooth muscle contraction studies, and phosphoinositide hydrolysis studies. Thus, these results indicate that most of the pharmacological effects induced by LTD_4 and LTE_4 are mediated via the LTD_4 membrane receptors (4, 10, 14, 15). Furthermore, recent evidence indicates high affinity receptor antagonists can prevent and/or reverse most, if not all, of the pharmacological processes induced by LTD_4 or LTE_4 in guinea pig lung and other target cells (13, 15–19), thus further supporting the hypothesis that most of the LTD_4-induced pharmacological effects are initiated via agonist binding to the receptor and the formation of intracellular messengers.

A molecular model has been proposed (9, 11) to describe the regulatory mechanisms of LTD_4 binding and signal transduction. Binding of agonist to the receptor is regulated by Na^+, divalent cations (Ca^{2+} and Mg^{2+}), and a G protein (5–7, 9, 20). Agonist binding to the receptor promotes a ligand-receptor-G protein complex. Divalent cations increase the density of [^{3}H]LTD_4 binding in guinea pig lung membranes. Na^+ and GTP increase the association and dissociation rates of [^{3}H]LTD_4 binding to and from the ternary complex and, thus, decrease the dissociation constant of [^{3}H]LTD_4 binding to the receptor (9, 20). Activation of phosphoinositide-specific phospholipase C in many types of receptors requires GTP and Na^+ and is independent from, but facilitated by, the presence of Ca^{2+} (21), thus suggesting that it is a consequence of the dissociation of the high affinity ternary complex. In this study, we utilized a novel radiolabeled high affinity LTD_4 receptor antagonist, [^{3}H]ICI-198615 (16, 17), to study guanine nucleotide and cation regulation of agonist-induced LTD_4 receptor affinity states and the potential molecular mechanism of signal transduction for the LTD_4 receptor.

Materials and Methods

Reagents. [^{3}H]LTD_4 (specific activity, 39 Ci/mmol) and [^{3}H]ICI-198615 (specific activity, 60 Ci/mmol) were obtained from NEN-Dupont (Boston, MA). They were greater than 95% pure by high pressure liquid chromatography analysis. LTD_4, (5*R*,6*S*)-LTD_4, LTE_4, SK&F 104353, SK&F 104373, LY-171883, and FPL-55712 were obtained from the Medicinal Chemistry Department, Smith Kline & French Laboratories. WY-48252 and WY-45911 were gifts from Dr. J. Chang, Ayerest-Wyeth Laboratories (Princeton, NJ). ICI-198615 was obtained from Dr. R. Krell at Stuart/ICI Pharmaceutical (Wilmington, DE). The structures of these chemicals are illustrated in Fig. 1.

Guinea pig lung membrane preparation. Guinea pig lung membranes were prepared as described previously (6, 7, 18). Briefly, cryopreserved guinea pig lungs (50 g) were thawed, pooled, and broken by homogenization in 500 ml of buffer A (20 mM Tris·HCl, pH 7.5, containing 0.25 M sucrose, 100 μM benzamidine, 100 μM soybean trypsin inhibitor, and 0.5 mM phenylmethylsulfonyl flouride). The homogenate was centrifuged at 2000 × g · min and the supernatant was layered on a 40% sucrose cushion and centrifuged at 9,000,000 × g · min. The membranes between the 0.25 M and 40% sucrose layers were collected, diluted with an equal volume of water, and centrifuged at 9,000,000 × g · min. The membrane pellets were frozen with liquid nitrogen and stored at −70° and were used within 3 months. No deterioration of [^{3}H]LTD_4 receptor binding activity was observed. EDTA (acid form) and the buffers were prepared in distilled water and titrated with KOH to the desirable pH. NaOH or sodium salts were avoided unless indicated.

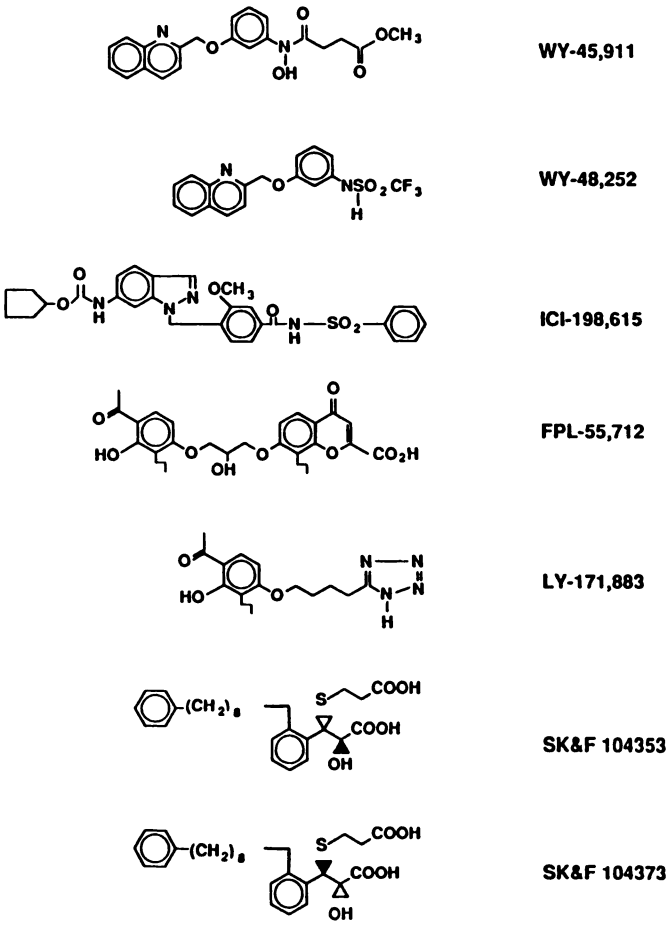

Fig. 1. Structures of leukotriene receptor antagonists.

[^{3}H]LTD_4 binding to guinea pig lung receptors. Guinea pig lung membranes (150 μg/ml) were incubated with [^{3}H]LTD_4 (0.5 nM), in the presence of 5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM glycine, 5 mM cysteine, 20 mM PIPES (pH 6.5), and varying concentrations of competing agonists or antagonists, in a final volume of 0.5 ml, in triplicate, for 40 min at 22°. The unbound ligands were separated from the membranes by filtration through GF/C Filters and washing with 20 ml of ice-cold washing buffer (10 mM Tris·HCl, pH 7.4), as described previously (6, 7). The radioactivity retained on the filters was quantitated by liquid scintillation counting, with efficiency of 40 to 45%.

Binding of [^{3}H]ICI-198615 to guinea pig lung membranes. Guinea pig lung membranes (100 μg/ml) were incubated with 0.3 nM [^{3}H]ICI-198615 in 10 mM PIPES buffer (pH 6.5) that contained 1 mM $CaCl_2$ and 1 mM $MgCl_4$, at 22° in 1 ml (standard conditions), and varying concentrations of drugs and other additional agents, in triplicate, as indicated in the figure legends. Nonspecific binding of [^{3}H]ICI-198615 was determined in the presence of 1000-fold excess ICI-198615. In saturation binding experiments, guinea pig lung membrane protein (20 μg/ml) was incubated with varying concentrations of [^{3}H]ICI-198615 (0.02 to 2 nM) in the presence or absence of 1000-fold excess ICI-198615 (20 nM to 2 μM), in 10 mM PIPES buffer that contained 1 mM $CaCl_2$ and 1 mM $MgCl_2$, in a volume of 1 ml, in triplicate, for 60 min at 22°. In kinetic experiments, lung membrane (1000 μg/ml) was added to incubation mixtures that contained 0.2 nM [^{3}H]ICI-198615, 10 mM PIPES buffer (pH 6.5), 1 mM $CaCl_2$, and 1 mM $MgCl_2$, in the presence or absence of 200 nM ICI-198615, in a volume of 2 ml. The reaction mixture was incubated at 22°, and duplicate aliquots (100 μl) were retrieved at varying times. The radioactivity bound to membranes in these aliquots was analyzed by filtration and washing as described

above. [^{3}H]ICI-198615 dissociation studies were performed under identical conditions, except that the binding of radioligand was allowed to proceed to equilibrium at 35 min, and the mixture was then added to test tubes that contained 300 nM ICI-198615; duplicate aliquots (150 μl) were retrieved at varying time points and analyzed as described above. Each experiment has been repeated two or three times and averaged results from three experiments, indicated as mean ± standard error, are shown. The conditions employed in this study are similar, but not identical, to those utilized by Aharony *et al.* in a recent report (22).

The membrane-bound [^{3}H]ICI-198615 was extracted with methanol and analyzed by reverse phase high pressure liquid chromatography. Briefly, using ICI-198615 as an internal standard, the methanol extract and the standard were mixed, injected into a C_{18}-Ultrasphere Zorbax reverse phase column, and eluted with a solvent mixture of acetonitrile/water/acetic acid (75:25:0.1; pH 7.3) isocratically. The bulk of the radioactivity (99%) coeluted with the internal standard (7.5 min). Less than 0.5% of the injected radioactivity eluted at 13 min.

Data calculation. [^{3}H]ICI-198615 saturation binding data were analyzed using a nonlinear least squares curve-fitting modeling method, as described by Delean *et al.* (23), to estimate the dissociation constant (K_D) and maximum binding density (B_{max}). The agonist and antagonist competition data were analyzed by using the SCATFIT program (23), as described by DeLean and co-workers, to determine the ligand dissociation constants and the proportion of receptor high and low affinity states. Each experiment was repeated at least two times. The results shown are averaged means ± standard errors from these experiments.

Results

Competition of ICI-198615 with [^{3}H]LTD_4 binding to guinea pig lung receptors. Many laboratories, including ours, have established saturable, specific, high affinity receptors for [^{3}H]LTD_4 binding in guinea pig lung membranes. The affinity and density of [^{3}H]LTD_4 binding to guinea pig lung membranes were 0.4 ± 0.05 nM and 890 ± 150 fmol/mg, respectively. ICI-198615 is a potent LTD_4 receptor antagonist (16, 17), structurally unrelated to the natural leukotrienes. This compound completely displaced [^{3}H]LTD_4 binding to guinea pig lung receptors in a dose-dependent manner (Fig. 2). The K_i was 0.05 ± 0.008 nM. This confirms the recent report by Aharony *et al.* (16). Two other recently reported novel receptor antagonists, WY-48252 and WY-45911 (24, 25), also displaced [^{3}H]LTD_4 binding in a dose-dependent manner. These results establish the rank order of potency of a series of structurally unrelated receptor antagonists as ICI-198615 > SK&F 104353 > WY-48252 > SK&F 104373 > FPL-55712 ≥ LY-171883 ≥ WY-45911, in competition with [^{3}H]LTD_4 binding to the guinea pig lung membrane receptor (Table 1).

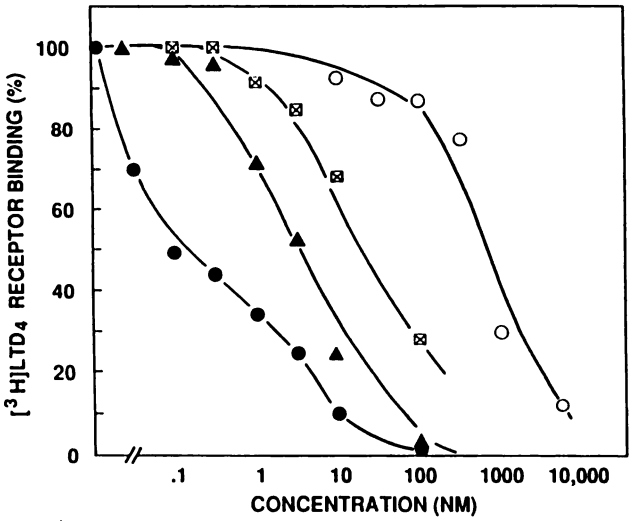

Fig. 2. Displacement of [^{3}H]LTD_4 binding to receptors by antagonists. Guinea pig lung membranes (150 μg/ml) were incubated with 0.5 mM [^{3}H]LTD_4 in 20 mM PIPES (pH 6.5) that contained 5 mM glycine, 5 mM cysteine, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and varying concentrations of ICI-198615 (●), WY-48252 (⊠), WY-49511 (○), or SKF 104353 (▲), as described in Materials and Methods.

Binding of [^{3}H]ICI-198615 to guinea pig lung membranes. Binding of [^{3}H]ICI-198615 to guinea pig lung membranes was rapid and time dependent in the first 5 min and then reached a plateau. In the presence of excess unlabeled compound, the specific binding of radiolabeled antagonist was substantially reduced. The nonspecific binding was approximately 10 to 15% of total binding under the current experimental conditions (Fig. 3A). The specific binding was linearly dependent upon the concentration of membrane protein from 10 to 250 μg/ml (result not shown). The specific binding was not significantly altered in the presence of $CaCl_2$ (1 to 20 mM), $MgCl_2$ (1 to 20 mM), NaCl (1 to 1000 mM), CsCl (1 to 1000 mM), KCl (1 to 1000 mM), GTP (1 to 1000 μM), Gpp(NH)p (1 to 1000 μM), GTPγS (1 to 1000 μM), or ATP (1 to 1000 μM) (results not shown). These results confirm and extend those reported recently (22) and indicate that [^{3}H]ICI-198615 binding to guinea pig lung membranes is dependent on the concentration of membrane protein, is displaceable, and is insensitive to monovalent cations, divalent cations, or guanine nucleotides (see additional results in Table 2).

Specific binding of [^{3}H]ICI-198615 was also reversible when excess unlabeled antagonist was added to the incubation mixture, after steady state was established (Fig. 3B). The dissociation rate (K_{off}) was determined to be $-0.406\ min^{-1}$. The association rate (K_{on}) was calculated as $1.24 \times 10^9\ M^{-1} \cdot min^{-1}$. A kinetically determined dissociation constant (K_d = 0.32 nM) was close but not identical to that determined from saturation binding assays (see Fig. 4). Displacement of specifically bound [^{3}H]ICI-198615 was relatively slow and complete within 20 min, indicating that binding of [^{3}H]ICI-198615 was fully reversible.

Equilibrium saturation binding of [^{3}H]ICI-198615 to guinea pig lung membranes. Varying concentrations of [^{3}H]ICI-198615 (0.02 to 2 nM) were incubated with guinea pig lung membranes in the presence of 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM PIPES buffer (pH 6.5) for 60 min. The results (Fig. 4) show that specific binding of [^{3}H]ICI-198615 was dependent on the concentrations of radioligand from 0.02 to 0.3 nM and

TABLE 1

Comparative properties of LTD_4 receptor antagonists

Smooth muscle contraction antagonist activities were excerpted from previous publications (8, 13–16, 21, 22), derived from guinea pig tracheal smooth muscle contraction assay systems. The LTD_4 receptor binding activities were determined by using [^{3}H]LTD_4 binding and competition as described in Fig. 2.

Compound	LTD_4 Receptor Affinity	Antagonist Activity
	nM	$-log[K_B]$
ICI-198615	0.05	9.3
SK&F 104353	2.5	8.4
WY-48252	35	7.6
SK&F 104373	370	6.8
FPL-55712	890	6.7
LY-171883	980	6.5
WY-45911	820	6.7

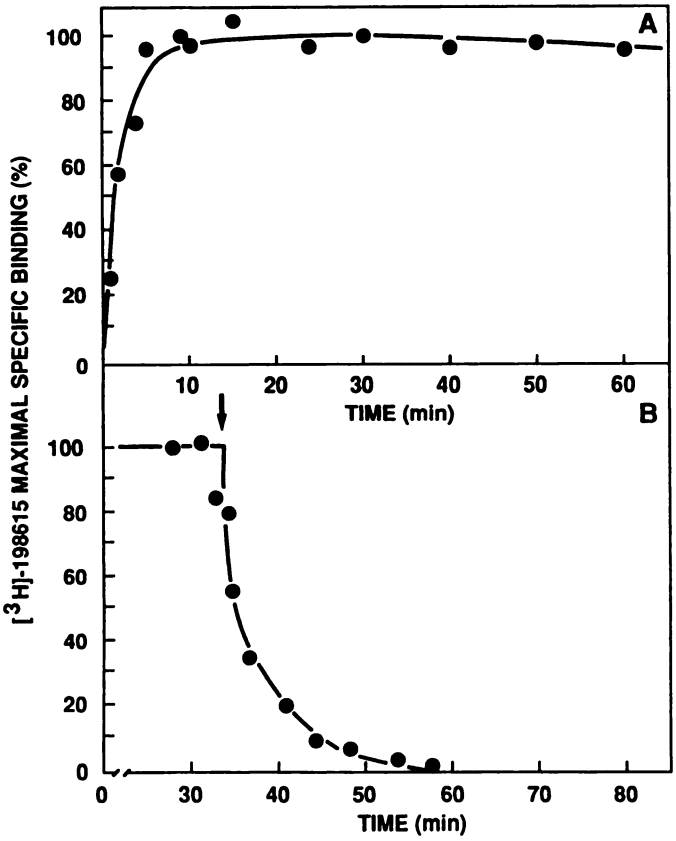

Fig. 3. Binding kinetics of [^{3}H]ICI-198615 to guinea pig lung membranes. A, Association of [^{3}H]ICI-198615 specific binding. Guinea pig lung membranes (150 μg/ml) were incubated with 0.2 nM [^{3}H]ICI-198615, under standard conditions, for 0 to 60 min, and duplicate 100-μl aliquots were removed at varying times and processed to determine total binding. Nonspecific binding at each point was determined similarly except 200 nM ICI-198615 was included in the incubation mixture. The specific binding (●) of [^{3}H]ICI-198615 to guinea pig lung membranes was calculated by subtraction and averaged from the results of three separate experiments. The specific binding after 40-min incubation was taken as 100% for each experiment. B, Dissociation of [^{3}H]ICI-198615 specific binding. Guinea pig lung membranes were incubated with [^{3}H]ICI-198615, as described above, for 35 min to achieve equilibrium state. ICI-198615 (200 nM) was added to the incubation mixture, and the membrane-bound [^{3}H]ICI-198615, in duplicate 150-μl aliquots, was retrieved at indicated points of time and determined as described in Materials and Methods.

TABLE 2

Effects of cations and GTPγS on [^{3}H]ICI-198615 binding to LTD_4 receptor

K_D and B_{max} were calculated based on three averaged experiments and are expressed as mean ± standard error. Additions were 3 mM EDTA, 100 mM NaCl, or 100 μM GTPγS.

	Binding Affinity, K_D	Receptor Density, B_{max}
	pM	*fmol/mg*
Standard conditions[a]	80 ± 36	1030 ± 180
Standard conditions + EDTA	34 ± 16	900 ± 310
Standard conditions + NaCl + EDTA	50 ± 25	880 ± 130
Standard conditions + GTPγS	44 ± 30	990 ± 100
Standard conditions + GTPγS + NaCl	35 ± 27	910 ± 150

[a] Standard conditions, 1 mM $MgCl_2$; 1 mM $CaCl_2$; 10 mM PIPES (pH 6.5); and guinea pig lung membrane, 15 μg/ml.

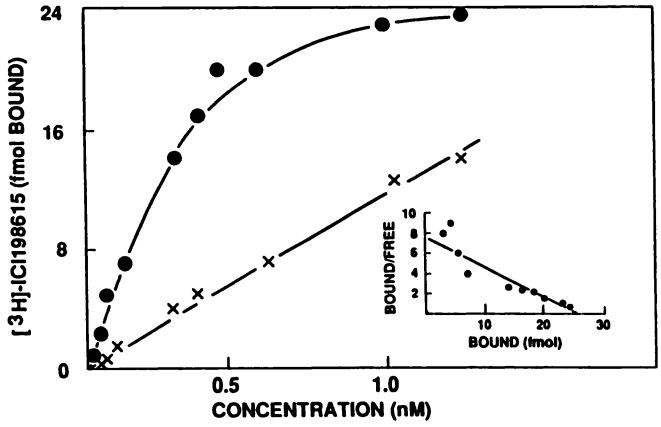

Fig. 4. Saturation binding of [^{3}H]ICI-198615 to guinea pig lung membranes. Guinea pig lung membranes (20 μg/ml protein) were incubated with increasing concentrations (0.02 to 2 nM) of [^{3}H]ICI-198615, under standard conditions, as described in Materials and Methods. Nonspecific binding (×) of [^{3}H]ICI-198615 was determined similarly, in the presence of 1000-fold excess, i.e., 20 nM to 2 μM, ICI-198615. *Inset*, the conversion of the [^{3}H]ICI-198615 specific binding (●) by the Scatchard method (26).

then reached a plateau level. Conversion of the saturation binding data by the Scatchard method (26) showed a linear plot (Fig. 4, *inset*), suggesting the existence of a single class of specific binding sites. Using a SCATFIT program (23), we have further analyzed the saturation binding data. The results indicate that the binding data could best be described by a single-site model. The dissociation constant (K_D) and binding density (B_{max}) predicted from the single-site model were 0.08 ± 0.04 nM and 1030 ± 180 fmol/mg, respectively. These results showed that the specific binding of [^{3}H]ICI-198615 was saturable and of high affinity. It is entirely possible that, at higher concentrations, [^{3}H]ICI-198615 could bind to other specific sites with lower affinity (see Figs. 5 and 6). It is also possible that, extending the concentrations of [^{3}H]ICI-198615 to the 100 nM or 1 μM range, one might be able to detect other low affinity sites of [^{3}H]ICI-198615 binding. The pharmacological function of these potential low affinity sites, however, is not understood. The high affinity sites labeled by [^{3}H]ICI-198615 appear most relevant to LTD_4 receptors. Under the present conditions, concentrations are used in the range that is relevant to the receptor antagonist activity (i.e., less than 10 nM). Thus, these results show that, under the current conditions, [^{3}H]ICI-198615 specifically binds to a single class of receptor sites. NaCl (100 mM), EDTA (3 mM), or GTPγS (100 μM) did not significantly alter the affinity or density of [^{3}H]ICI-198615 specific binding to guinea pig lung membranes (Table 2).

Specificity of [^{3}H]ICI-198615 binding to guinea pig lung membranes. The specificity of radioligand binding was characterized by using several classes of pharmacological agents in competition with [^{3}H]ICI-198615. Under standard conditions, at a concentration 20 μM or less, the following agents did not compete with [^{3}H]ICI-198615 binding to its specific sites in guinea pig lung membranes. These are platelet-activating factor, platelet-activating factor antagonist CV-3988, adenosine, ADP, ATP, epinephrine, norepinephrine, prazosin, propanolol, histamine, vasopressin, prostagland E, prostaglandin D_2; thromboxane antagonists SQ29548 and BM104505; dopamine, serotonin, hydrocortisone, dexamethasone, betamethasone, ibuprofen, indomethacin, neomycin, streptomycin, verapamil, amiloride, NADPH, and yohimbine (results not shown).

Thus, binding of [^{3}H]ICI-198615 is highly selective and is not shared by these agents tested.

The specificity of [^{3}H]ICI-198615 was also evaluated by using several leukotriene agonists and three classes of structurally distinct receptor antagonists. ICI-198615 displaced 100% of [^{3}H]ICI-198615, under standard conditions. The competition curve appeared to be biphasic (Fig. 5), suggesting heterogeneity of the specific binding sites. We used the SCATFIT analysis program to further resolve these data. The results indicated that the competition curve was best described with a two-site model ($p < 0.05$). The dissociation constants for the high affinity (K_{D_H}) and low affinity (K_{D_L}) states were 0.2 ± 0.08 and 110 ± 30 nM (three experiments), respectively. The high affinity site represented 60 ± 10% of all the specific sites detected under the current conditions. These results show that ICI-198615 can bind to a high affinity and a low affinity component in guinea pig lung membranes. Judging from the potency of the compound in antagonizing [^{3}H]LTD_4 binding (K_i = 0.05 ± 0.008 nM) and its pharmacological function (Table 1), we believe that the high affinity component labeled by [^{3}H]ICI-198615 most likely represents the LTD_4 receptors. The low affinity binding component (K_{D_L} = 110 ± 30 nM) does not appear to be related to the LTD_4 receptors and was not further studied.

Agonist, LTD_4 in this system, also displaced [^{3}H]ICI-198615 specific binding. The competition curve was shallow and, at the highest tolerated concentration (20 μM), displaced 70 ± 10% of the specific binding sites labeled with [^{3}H]ICI-198615. These results indicate that LTD_4 binding to the [^{3}H]ICI-198615-labeled specific sites is heterogeneous. The LTD_4-displaceable fraction remained constant when LTD_4 (10–30 μM) was incubated with 0.3 nM [^{3}H]ICI-198615 in the presence of 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM cysteine, and 10 mM glycine to favor agonist binding (9) to the receptor (results not shown). These results suggest that 70% of [^{3}H]ICI-198615 specific binding represents an LTD_4 receptor component and thus can be maximally competed with agonist (LTD_4) and receptor antagonists that are structurally related to leukotrienes (see below). The LTD_4 competition curve was also further analyzed using the SCATFIT program. The results show that LTD_4 binding (to 70% of the [^{3}H]ICI-198615 specific sites) could be resolved into a high affinity and a low affinity component. The high affinity and the low affinity dissociation constants were 2.2 ± 1.0 and 140 ± 30 nM, respectively. Under the current conditions, the high affinity LTD_4 binding component represented 52 ± 3% and the low affinity LTD_4 binding component represented 48 ± 3% of the LTD_4 receptors in guinea pig lung membranes labeled with [^{3}H]ICI-198615. LTE_4 also competed with and displaced [^{3}H]ICI-198615 specific binding. Similar to that exhibited by LTD_4, the competition curve was shallow and incomplete at 20 to 30 μM. (5*R*,6*S*)-LTD_4, the unnatural form of LTD_4, also competed with [^{3}H]ICI-198615 specific binding. The rank order of potency of LTD_4, LTE_4, and (5*R*,6*S*)-LTD_4 in displacement of [^{3}H]ICI-198615 binding to specific sites was equivalent to those observed in [^{3}H]LTD_4 binding studies and in smooth muscle contraction studies (10).

Another relatively new class of LTD_4 receptor antagonists, SK&F 104353 and its stereoisomer SK&F 104373, are structurally unrelated to ICI-198615 or acetophenone-type (FPL55712) antagonists. SK&F 104353 and SK&F 104373 also competed with [^{3}H]ICI-198615 specific binding to guinea pig lung membranes in a concentration-dependent and stereoselective manner (Fig. 6). The maximal level of displacement for SK&F 104353 and SK&F 104373 (at 20–30 μM) was 70 ± 8%, similar to that displaced by the agonist LTD_4. Unlike the shallow competition curves characteristic of the agonists, the dose-response competition curves were steep, suggesting that these compounds could bind to the LTD_4 receptor labeled with [^{3}H]ICI-198615 in a nonheterogeneous manner. The binding affinities of SK&F 104353 and SK&F 104373 were estimated as 4.0 ± 1.5 and 120 ± 36 nM, respectively. Two quinoline derivatives of REV-1901, WY-48252 and WY-45911, structurally related to ICI-198615 but without its hydrophobic cyclopentane portion, competed with the binding of [^{3}H]ICI-198615 to the specific site, with K_i values of 60 ± 15 and 1300 ± 210 nM, respectively. The acetophenone class antagonists, FPL-55712 and LY-171883, completely inhibited [^{3}H]ICI-198615 binding to the specific site, with K_i values of 0.4 and 0.6 μM, respectively. The binding affinities of these three classes of antagonists differed slightly from those determined using [^{3}H]LTD_4, especially for LY-171883, FPL-55712, and WY-49511. However, the general rank order of binding affinities, determined using [^{3}H]ICI-198615, directly correlated with the antagonist activities in the functional (smooth muscle contraction) assay (Table 1).

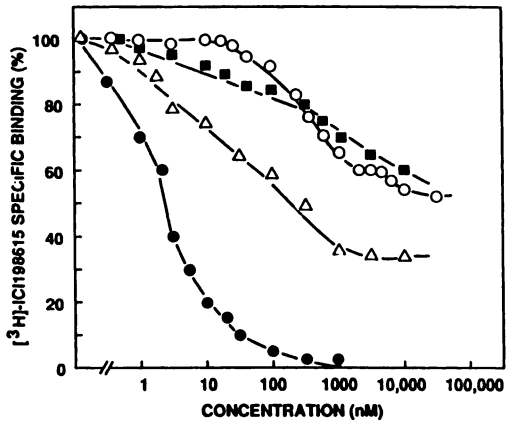

Fig. 5. Determination of pharmacological specificity by ICI-198615 and LTD_4 competition. Guinea pig lung membranes (100 μg/ml) were incubated with 0.2 nM [^{3}H]ICI-198615, under standard conditions, and varying concentrations of LTD_4 (△), LTE_4 (■), (5*R*,6*S*)-LTD_4 (○), or ICI-198615 (●), as described in Materials and Methods.

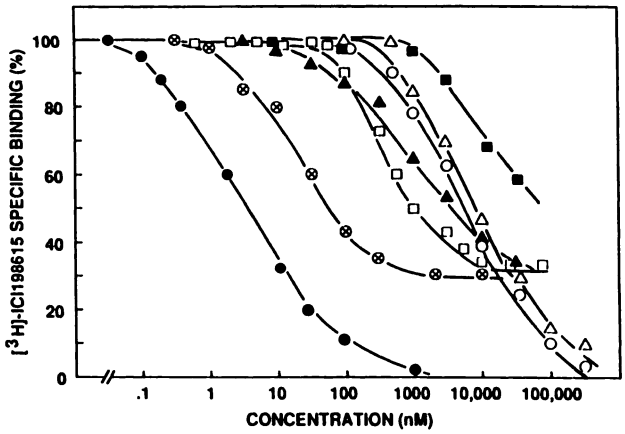

Fig. 6. Determination of pharmacological specificity of [^{3}H]ICI-198615 binding by antagonist competition. Guinea pig lung membranes were incubated with 0.3 nM [^{3}H]ICI-198615, under standard conditions, and varying concentrations of ICI-198615 (●), WY-48252 (□), WY-45911 (■), SKF 104353 (⊗), SKF 104373 (▲), FPL-55712 (○), or LY 171883 (△), as described in Materials and Methods.

These results indicate that these antagonists were binding to or sharing the pharmacologically and physiologically relevant LTD_4 receptor in guinea pig lung membranes. A fraction (~30%) of specific sites labeled by [^{3}H]ICI-198615 was not shared by antagonists SK&F 104353, SK&F 104373, or WY-48252, nor by agonists LTD_4, (5*R*,6*S*)-LTD_4 or LTE_4. This component does not represent the "low affinity state" binding for agonists, because, even in the presence of EDTA or guanine nucleotides (see below), this component was not displaceable at high concentrations of LTD_4 or other agonist analogs.

Shifting of agonist binding affinity state. Previous reports demonstrated that LTD_4 can bind to receptors and promote the formation of a ligand-receptor-G protein high affinity ternary complex (20). Guanine nucleotide and Na^+ facilitate the dissociation of [^{3}H]LTD_4 from the ternary complex, thus favoring the binding of [^{3}H]LTD_4 to the receptor in the low affinity state. With the availability of a potent, high affinity, radiolabeled receptor antagonist, we investigated the effects of Na^+, divalent cations, and guanine nucleotides, singly or in combination, on the affinity states for agonist binding. These results are shown in Fig. 7. In the absence of Na^+, GTPγS, or EDTA, the LTD_4 competition curve was heterogeneous and shallow, as shown in Fig. 5. However, in the presence 3 mM EDTA or 100 μM GTPγS, the LTD_4 competition curve was shifted to the right (Fig. 7). NaCl (100 mM) alone also caused a rightward shift of the LTD_4 competition curve (results not shown). The LTD_4 competition curve was shifted even further when 100 mM NaCl and 100 μM GTPγS were added together in the assay (results not shown). The relative proportions and the receptor binding affinity states for LTD_4 were calculated and are summarized in Table 3. GTPγS induced an increase of 35% of the receptor in the lower affinity state. When EDTA and Na^+ were used together, a preponderance of the receptor (83%) was shifted to the low affinity state, in an additive manner.

When EDTA, Na^+, and GTPγS were added in combination, a maximal 100-fold rightward shift of the LTD_4 displacement curve was observed. In fact, under this condition, and this condition only, the LTD_4 displacement curve was best described by a single-site model, with a binding affinity of 200 ± 30 nM and greater than 98% of the receptor in this state for LTD_4 binding (Table 3). This result indicates that Na^+, GTPγS, and divalent cations individually regulate the binding of agonist to the LTD_4 receptors. GTPγS and, to a lesser extent, Na^+ or EDTA can shift the relative proportion of the LTD_4 receptors in the high or low affinity states. The regulation of the binding affinity states by Na^+, GTPγS, and EDTA appeared to be additive, and the combination of all three was required to have LTD_4 binding completely shift to the low affinity state.

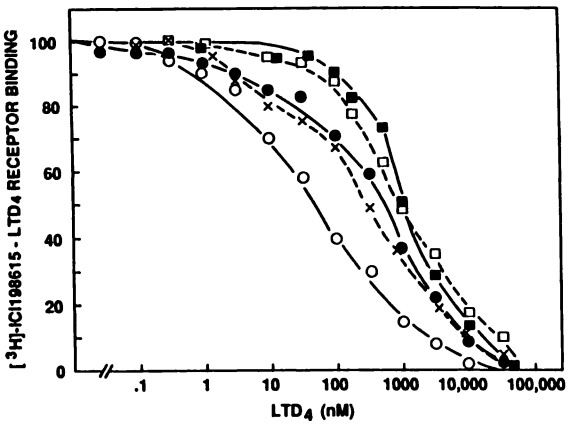

Fig. 7. Shift of the binding affinity states of LTD_4 to receptor by divalent cations Na^+, GTPγS, or a combination. Guinea pig LTD_4 membranes (100 μg/ml) were incubated with 0.3 nM [^{3}H]ICI-198615, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, in 10 mM PIPES buffer that contained varying concentrations of LTD_4 (○). This standard condition was varied by adding 3 mM EDTA (×), 100 μM GTPγS (□), 100 mM NaCl plus 3 mM EDTA (●), or 100 mM NaCl plus 3 mM EDTA plus 100 μM GTPγS (■) in combination. Nonspecific binding of [^{3}H]ICI-198615 was defined in the presence of 20 μM LTD_4.

TABLE 3

Effects of cations and guanine nucleotide on guinea pig lung LTD_4 receptor binding affinity states

Additions of 3 mM EDTA, 100 mM NaCl, or 100 μM GTPγS were used. The results from each experiment were analyzed using a SCATFIT program to determine the $K_{D\cdot H}$, $K_{D\cdot L}$, $\%R_H$, and $\%R_L$. Averaged results from three experiments were pooled and are shown as mean ± standard error.

	High Affinity		Low Affinity	
	K_{DH}	$\%R_H$[a]	K_{DL}	$\%R_L$
	nM		nM	
Standard condition[b]	2.2 ± 1.0	52 ± 3	140 ± 30	48 ± 3[c]
Standard condition + EDTA	0.5 ± 0.2	24 ± 3	60 ± 7	76 ± 3[d]
Standard condition + GTPγS	10 ± 7	17 ± 5	250 ± 32	83 ± 5
Standard condition + NaCl	2.4 ± 1.7	40 ± 9	130 ± 30	60 ± 10
Standard condition + EDTA + NaCl	1.0 ± 0.7	17 ± 3	130 ± 15	83 ± 3[e]
Standard condition + EDTA + NaCl + GTPγS	NA[g]		200 ± 30[h]	98 ± 5[f]

[a] $\%R_H$, percentage of receptors in the high affinity state; $\%R_L$, percentage of receptors in the low affinity state.

[b] Standard condition, 1 mM $CaCl_2$; 1 mM $CaCl_2$; 10 mM PIPES (pH 6.5), 0.3 nM [^{3}H]ICI 198615, and 100 μg/ml guinea pig lung membrane in 1-ml assay mixture.

[c–f] $\%R_L$ was significantly different ($p < 0.01$, Student *t* test) between *c* and *d*, *c* and *e*, *e* and *f*.

[g] NA, not applicable.

[h] The binding affinity K_{DL} (200 ± 30 nM) is statistically different ($p < 0.01$) from the K_{DH} (2.2 ± 1.0 nM).

Discussion

Considerable advancement in the understanding of the molecular pharmacology of peptidoleukotrienes has been noted in recent years. Evidence suggests that there is only one class of receptor in human airway tissue that is shared by LTC_4, LTD_4, and LTE_4 (18, 27). In guinea pig lung, there is evidence to suggest that LTC_4 may interact with a separate population of receptors (18, 25, 27). Many high affinity antagonists for LTD_4 have been synthesized and reported. In addition, studies have also revealed that inositol-1,4,5-trisphosphate and diacylglycerol may serve as intracellular messengers for LTD_4 receptors in the receptor-bearing cells (11, 12). Cyclooxygenase-dependent and -independent effects induced by LTD_4 are the consequences of LTD_4 receptor activation and are secondary to the formation of intracellular messengers (11, 13). Therefore, high affinity receptor antagonists have been used to inhibit most of the peptidoleukotriene-induced pathophysiological effects (11–19, 24, 25, 27). However, there are still many unanswered questions regarding the molecular mechanisms of receptor-ligand binding and signal transduction. We have utilized a high affinity receptor antagonist to address these issues.

The specific binding of antagonist [^{3}H]ICI-198615 to guinea

pig lung membranes was rapid, saturable, reversible, membrane protein dependent, and selective for LTD_4, LTE_4, and three different classes of LTD_4 receptor antagonists. Also, binding of [^{3}H]ICI-198615 to guinea pig membrane specific sites was stereoselective and the rank order of potency for agonists and antagonists in the binding assay was closely correlated with those derived from functional assays (Table 1) and from [^{3}H] LTD_4 receptor competition assays. Thus, these results indicate that [^{3}H]ICI-198615 is binding to the pharmacologically relevant LTD_4 receptor site in guinea pig lung membranes. The profile of [^{3}H]ICI-198615-labeled sites in guinea pig lung membranes, however, was somewhat heterogeneous. Agonists and three classes of functionally defined "receptor antagonists," which bear little structural similarity, were used to define the subtypes of the [^{3}H]ICI-198615-labeled specific sites. These included (a) hydroxyacetophenones, e.g., FPL-55712 and LY-171883; (b) the leukotriene structure-based antagonists, e.g., SK&F 104353 and SK&F 104373; and (c) the novel quinoline analogs of REV-5901, compounds such as WY-45911 and WY-48252 (25). All of these functionally defined LTD_4 receptor antagonists displaced [^{3}H]ICI-198615 binding to specific sites in a dose-dependent manner, with a rank order equivalent to that reported (Table 1) for functional studies. The hydroxyacetophenone-type antagonists (FPL-55712 and LY-171883) competed with [^{3}H]ICI-198615, with affinities in the range of 0.5–2 μM. The ICI-198615-like compounds (WY-48252 and WY-45911) also competed with the radioligand quite effectively (K_i = 35 and 850 nM, respectively) and yet incompletely (≤65%), perhaps because they lack the hydrophobic cyclopentane moiety. Thus, the displacement isotherms suggested that ICI-198615 was binding to two distinct sites in guinea pig lung membranes. The leukotriene structure-type antagonists (SK&F 104353 and SK&F 104373) could also displace [^{3}H]ICI-198615 specific binding in a dose-dependent and stereoselective manner but maximally to 70 ± 10%. Interestingly, LTD_4 also could maximally displace [^{3}H]ICI-198615 specific binding to the same (70 ± 10%) level at pharmacologically relevant concentrations. Even in the presence of Na^+, EDTA, and GTPγS, where binding of LTD_4 to its receptor is shifted to the low affinity state, LTD_4 could maximally displace 70 ± 10% of [^{3}H]ICI-198615 specific binding sites. These results suggest that a large fraction of [^{3}H]ICI-198615 specific binding sites (70 ± 10%) represents the pharmacologically specific LTD_4 receptors in guinea pig lung membranes, and this fraction represents both the high and low affinity state receptor for LTD_4 binding. There are currently no available data to indicate to what the other 30% of the [^{3}H]ICI-198615 binding relates. This fraction of [^{3}H]ICI-198615 specific binding could be related to pharmacological functions other than LTD_4 receptor blockade or could merely be unique for binding to guinea pig lung membranes.

Using radiolabeled agonists ([^{3}H]LTD_4 and [^{3}H]LTE_4), it has been shown (5–7) that binding of agonist to the receptor is specifically regulated by Na^+, divalent cations, and guanine nucleotides. These results suggest that a G protein (non-G_s), tightly coupled to the LTD_4 receptor, is a key regulatory protein that can communicate between the receptor and the catalyst, phosphoinositide-specific phospholipase C (4). Agonist binding to the receptor in membrane preparations induces the formation of a high affinity, ligand-receptor-G protein ternary complex (20). It is also hypothesized that, depending on the functional state of the G protein, binding of the agonist to the receptor can be in either a high affinity or a low affinity state (11). GTP and the nonhydrolyzable analogs (GTPγS and Gpp(NH)p), via binding and then activation of the G protein, can alter the ligand-receptor-G protein ternary complex equilibrium and thus result in a downward shift of the LTD_4 binding affinity. With the availability of [^{3}H]ICI-198615, a radiolabeled receptor antagonist, evidence to support such a model is provided in this study. In the presence of divalent cations, GTPγS induced a shift of binding affinity for LTD_4 and a substantial increase of LTD_4 receptor in the low affinity state. Na^+ also appeared to be effective and facilitated the effects of EDTA or GTPγS on the binding of LTD_4 to the low affinity states. Divalent cations, reported to favor the formation of agonist-receptor-G protein complex (5–7, 9, 10), also shifted the affinity states of the receptor for LTD_4 binding, although opposite to that induced by GTPγS and/or Na^+. These results directly demonstrate, for the first time, that LTD_4 binding to the receptor can exist in high or low affinity states, K_{D_H} = 0.5 ± 0.2 to K_{D_L} = 200 ± 30 nM. A complete transition of the receptors from high affinity to low affinity state requires GTPγS, Na^+, and the absence of high concentrations of divalent cations. These studies demonstrate that there is only a *single class* of LTD_4 receptors, with interconvertible agonist binding affinity states, depending on the presence of Na^+, divalent cations, and guanine nucleotides in guinea pig lung membrane preparation *in vitro*.

For several classes of the G protein-regulated membrane receptors, e.g., the adrenergic, dopaminergic, muscarinic, and serotonergic receptors, nucleotide-regulated interconversion of receptor affinity states is well established (28–33). This paradigm of affinity state transition was useful in interpreting the kinetic and molecular mechanisms of receptor-ligand binding. Also, it was used to extrapolate the possible regulatory mechanisms of these receptors, the corresponding G protein, and signal transduction processes in the receptor-containing cells or tissues.

In analogy with the model proposed for other G protein-coupled receptor systems (28–33), the results and conclusions derived from the LTD_4 receptors provide clues as to how the LTD_4 receptor may function in its target cells or tissues. For example, because the intracellular concentration of GTP is relatively high (at levels that are high enough to regulate the affinity states of LTD_4 receptors) and divalent cation concentrations are low (less than 1 μM), it is conceivable that the majority of the plasma membrane LTD_4 receptors in the intact cells exist in a low affinity state, independent of G protein. Presentation of agonist LTD_4 to the receptors may recruit a G protein to form a LTD_4 receptor-G protein high affinity complex, but only transiently. Intracellular GTP can bind to and activate the G protein and, thus, rapidly facilitate the dissociation of the ternary complex. The transducer G protein can then activate the catalyst phosphoinositide-specific phospholipase C, resulting in the formation of intracellular messengers. Thus, an initial agonist (LTD_4) signal is transduced via the receptor to the G protein. Intracellularly Na^+ and GTP facilitate the signal transduction to phosphoinositide-specific phospholipase C, resulting in the amplification and cascade of the signal to generate the intracellular messengers, diacylglycerol and inositol-1,4,5-trisphosphate.

In conclusion, the radiolabeled [^{3}H]ICI-198615 is specific for

the LTD_4 receptors in guinea pig lung membranes. It is useful for the characterization of the LTD_4 receptor affinity states and helps us to better define the molecular mechanisms of signal transduction for LTD_4 receptors in guinea pig lung.

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