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Post Soluble Binding to the Leukotriene D_4 Receptor from Guinea Pig Lung Membranes

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SUMMARY

Guinea pig lung membranes were extracted with 1% digitonin and yielded a preparation that contained soluble leukotriene D_4 (LTD_4) receptor. Specific binding of the high affinity radiolabeled receptor antagonist [^{3}H]ICI-198615 to the soluble LTD_4 receptor was time dependent and reversible. The dissociation constant (K_d) and the density (B_{max}) of [^{3}H]ICI-198615 binding to the soluble LTD_4 receptor was 0.2 ± 0.08 nM and 380 ± 40 fmol/mg of protein, respectively. Radioligand competition studies showed several classes of structurally diverse, functionally defined, receptor antagonists competed with [^{3}H]ICI-198615 binding to the soluble receptor. The rank order of potency and specificity of these antagonists in binding to the soluble receptor were equivalent to those determined from the membrane-bound receptor binding assay and from the smooth muscle contraction assay. Binding of LTD_4 to the soluble receptor was observed, in the competition assay, only in the low affinity state (K_i = 2 μM). Size-exclusion chromatography of the soluble LTD_4 receptor showed that the apparent molecular weight of the LTD_4 receptor in digitonin micelle was approximately 300,000.

Peptidoleukotrienes LTC_4, LTD_4, and LTE_4 are potent mediators of immediate-type hypersensitivity responses and may be involved in a spectrum of lung diseases (1, 2). The membrane receptor for LTD_4 has been demonstrated and well characterized (3, 4). The results indicated that the LTD_4 receptor is a heat-sensitive glycoprotein (5), preferentially localized in the plasma membrane of smooth muscle cells, and is coupled to a G protein(s) (3–6).

The first step in studying the molecular architecture of the receptor is to construct method and assay systems to solubilize the membrane-bound receptor protein for purification. Attempts to use radiolabeled agonists ([^{3}H]LTD_4 or [^{3}H]LTE_4) to bind the detergent-solubilized receptor protein were unsuccessful (5). The results of this study led to the realization that agonist binding promoted and stabilized the agonist-receptor-G protein ternary complex (5). In addition, detergent solubilization of membranes invariably disrupted the coupling of receptor and G protein, unless the agonist ternary complex was preformed. Therefore, it was apparent that a radiolabeled high affinity receptor antagonist was necessary to bind to the detergent-solubilized LTD_4 receptor, because LTD_4 receptor antagonist binding to the receptor is a bimolecular event and independent of G protein regulation. The result presented in this study is the first to demonstrate the feasibility of using a radiolabeled high affinity receptor antagonist to label detergent-solubilized LTD_4 receptors from guinea pig lung membranes.

Materials and Methods

Chemicals and reagents. The natural chiral form of LTD_4, (5*S*,6*R*)-LTD_4, the unnatural chiral form 5*R*,6*S*-LTD_4, SKF 104353, SKF 104373, FPL 55712, and LY-171883 were prepared by total chemical synthesis (greater than 98% purity) and supplied by the Department of Medicinal Chemistry, SK&F Laboratories. Isotopically labeled [^{3}H]ICI-198615 (60 Ci/mmol) was obtained from New England Nuclear (Boston, MA). The purities were greater than 98% by HPLC analyses. Digitonin (90% pure) was purchased from Gallard-Schlesinger Co. (Carle Place, NY). Protease inhibitors, nucleotides, detergents, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The TSK 4000 and TSK 3000 HPLC columns were obtained from Beckman Instruments (Irvine, CA). WY-48252 and WY-49511 were gifts from Dr. J. Chang, Ayerst-Wyeth Laboratories (Princeton, NJ). ICI-198615 was obtained from Dr. R. Krell at Stuart/ICI Pharmaceutical Inc. R-12525 was obtained from Dr. H. Campbell at Rorer Central Research.

GPL membrane preparation. GPL membranes were prepared from cryopreserved lung by homogenization, differential centrifugation, and discontinuous density gradient centrifugation, as described previously (5).

Solubilization of LTD_4 receptors. GPL membranes (30 mg of protein) were resuspended in 16 ml of solubilization buffer (20 mM

ABBREVIATIONS: LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4 leukotriene E_4; GPL, guinea pig lung; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; GTPγS, guanosine-5′-*O*-(γ-thio)triphosphate; HPLC, high pressure liquid chromatography; G protein, guanine nucleotide-binding protein; PEG, polyethylene glycol.

PIPES, pH 6.5, containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1% digitonin) (buffer A) and incubated at 4° for 40 min. The protein to detergent ratio was set at 1:5 to 1:8. The suspension was then centrifuged at 150,000 × *g* for 90 min. The supernatant, containing the solubilized receptor, was used for binding and biochemical analyses. Protein concentration was determined by a modified method of Lowry *et al.* (5, 7) using bovine serum albumin as the standard.

Binding of [^{3}H]ICI-198615 to soluble receptor was quantitated by incubation at 22° for 90 min in 0.5 ml of 20 mM PIPES (pH 6.5) containing (unless otherwise indicated) 0.5 nM [^{3}H]ICI-198615, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 250 μl of solubilized preparation or column fraction in 0.5% digitonin. Proteins were precipitated by addition of 1 ml of bovine γ-globulin (50 μg) and 1 ml of PEG (20%, w/v) at 0°, as described previously (5, 8). The precipitates were retained on GF/C filter paper and washed immediately with 20 ml of 8% PEG (in 10 mM Tris·HCl buffer, pH 7.4) at 0°. Nonspecific binding of [^{3}H]ICI-198615 was determined in the presence of 500 nM unlabeled ICI-198615 (1000-fold). Five milliliters of scintillation fluid (HP/b; Ready-Solv) were added to the counting vial containing the filter. Radioactivity on the filter was determined by scintillation counting with an efficiency of 35–40%. The results were expressed as mean ± standard error, averaged from duplicate determinations from three experiments.

HPLC of soluble receptor. A TSK-4000 and a TSK-3000 size-exclusion HPLC column were connected in tandem to fractionate the solubilized GPL preparation. The column was preequilibrated with 15 mM PIPES buffer, pH 6.5, containing 0.5% digitonin, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 20 mM KCl (buffer B) and was then calibrated with the following molecular weight marker protein standards: dextran blue (M_r 2,000,000), thyroglobulin (M_r 660,000), β-amylase (M_r 200,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 29,000), and aprotinin (M_r 6,500). One milliliter of solubilized GPL preparation was injected and eluted with buffer B at 0.5 ml/min. One-minute fractions were collected and fractions from five chromatographic runs were pooled. The [^{3}H] ICI-198615 specific binding activity was determined as described above.

Results

Kinetics of [^{3}H]ICI-198615 binding to digitonin-solubilized receptor. Several types of nonionic and zwitterionic detergents were employed to solubilize and extract GPL membranes for [^{3}H]ICI-198615 specific binding protein. The degergents used (1%) were digitonin, Nonidet P-40, Triton X-100, Tween-80, CHAPS, deoxycholate, octylglucoside, and polyethoxy C_9O_{12} (Polidocanol). Under the conditions employed, digitonin, CHAPS, and octylglucoside yielded soluble preparations that have [^{3}H]ICI-198615 specific binding activities. Digitonin (1%, protein to detergent ratio, 1:5) yielded [^{3}H]ICI-198615 specific binding activity 8 to 10 times higher than those with octylglucoside or CHAPS (results not shown). Digitonin was selected to solubilize GPL LTD_4 receptor for the following studies.

[^{3}H]ICI-198615 bound to the soluble membrane protein in the solubilized GPL preparation in a time-dependent manner and gradually reached a steady state level after 70 min (Fig. 1). Nonspecific binding of 0.5 nM [^{3}H]ICI-198615 was also time dependent and accounted for 40% of total binding at the steady state condition. The specific binding was time dependent and reached a steady state level after 70 min. Addition of excess unlabeled ICI-198615 (2 μM) to the incubation mixture at the steady state (Fig. 1, *arrow*) resulted in a gradual reduction of PEG-precipitable binding of [^{3}H]ICI-198615, indicating the specific binding of [^{3}H]ICI-198615 was reversible. The K_d could be calculated based on the association and dissociation rate constants. However, the K_d thus determined was quite variable, when compared with that determined from saturation binding experiments (see Fig. 2). Specific binding of [^{3}H]ICI-198615 increased in proportion to the amount of solubilized membrane protein from 20 to 200 μg of soluble protein and was not affected by NaCl (100 mM), KCl (100 mM), GppNHp (100 μM), GTPγS (100 μM), or GTP (1 mM) (results not shown). The results indicate that [^{3}H]ICI-198615 specific binding to solubilized GPL membrane was time dependent, reversible, and protein concentration dependent.

Saturation binding of [^{3}H]ICI-198615 to solubilized GPL membrane protein. Varying concentrations of [^{3}H]ICI-198615 were incubated with soluble membrane protein. The specific binding of [^{3}H]ICI-198615 increased, dependent on the concentration of radioligand from 0 to 0.6 nM, and gradually reached a plateau level of 0.8 nM. The nonspecific binding of [^{3}H]ICI-198615 was linearly dependent upon the concentration of the radioligand (Fig. 2). These results indicate that binding of [^{3}H]ICI-198615 was saturable. The saturation binding data were converted by the method of Scatchard (9) and yielded a linear plot (Fig. 2, *inset*). The K_d and the B_{max} were determined with a least squares best-fit computer program to be 0.2 ± 0.08 nM and 380 ± 40 fmol/mg, respectively (mean ± standard error of three experiments). These results show that, in the concentration range of the radioligand employed, [^{3}H]ICI-198615 binds to a single class of specific sites in the soluble GPL membrane preparation with high affinity and saturable density.

Competition and specificity of the [^{3}H]ICI-198615 binding. The specificity of [^{3}H]ICI-198615 binding to the GPL soluble receptor was characterized by using several classes of pharmacologically active agents. The following compounds, at a concentration of 10 μM or less, did not compete with more than 10% of [^{3}H]ICI-198615 specific binding: platelet-activating factor, platelet-activating factor antagonist CV-3988, adenosine, ADP, ATP, epinephrine, norepinephrine, prazosin, propranolol, histamine, vasopressin, prostaglandin E, prostaglandin D_2, thromboxane antagonists SQ29548 and BM104505, dopamine, serotonin, hydrocortisone, dexamethasone, ibuprofen, indomethacin, neomycin, streptomycin, verapamil, amiloride, NADPH, and yohimbine (results not shown). Thus, binding of [^{3}H]ICI-198615 to the GPL soluble protein was selective and was not affected by these agents.

The specificity of [^{3}H]ICI-198615 was also evaluated by using LTD_4, LTE_4, and three classes of structurally distinct receptor antagonists. ICI-198615 displaced 100% of [^{3}H]ICI-198615 (K_i = 0.4 ± 0.1 nM), under standard conditions (Fig. 3). Another class of LTD_4 receptor antagonists, SK&F 104353 and its stereoisomer SK&F 104373, are structurally unrelated to ICI-198615 or acetophenone-type (e.g., FPL 55712) antagonists. SK&F 104353 and SK&F 104373 displaced [^{3}H]ICI-198615 specific binding to solubilized GPL receptor in a concentration-dependent and a stereoselective manner (Fig. 3). The maximal level of displacement for SK&F 104353 and SK&F 104373, (20–30 μM), however, was 80 ± 6%. The maximum [^{3}H]ICI-198615 specific binding displaceable by other structurally different receptor antagonists (e.g., WY-48252) (Fig. 3) also approached 80 ± 5%. It appeared that a majority of the [^{3}H]ICI-198615 specific binding in the soluble GPL preparation was to the LTD_4 receptor component. The binding affinities of SKF 104353 and SKF 104373 were calculated from this "LTD_4 receptor component" of [^{3}H]ICI-198615 specific binding as 4 ± 1.5 and 150 ± 36 nM, respectively. Currently, there is no evidence to indicate that the 20% of the [^{3}H]ICI-198615 specific

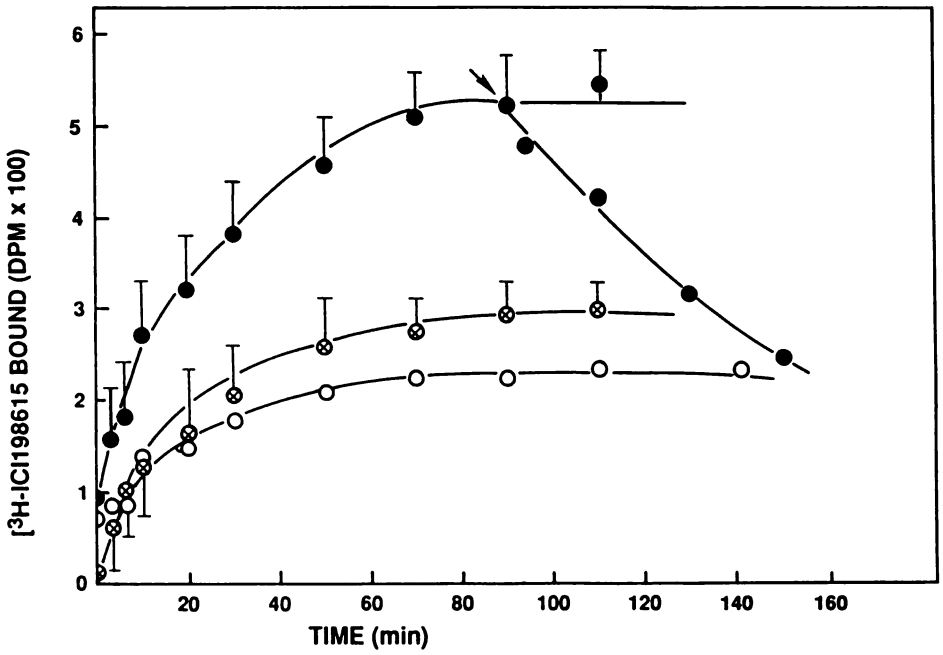

Fig. 1. [^{3}H]ICI-198615 binding to digitonin-solubilized GPL membrane protein. [^{3}H]ICI-198615 (0.5 nM) was incubated with 200 μl of digitonin-solubilized protein for the indicated length of time to determine the total (●) and the nonspecific (○) binding. The specific binding (⊗) was calculated by subtraction. At the indicated point (*arrow*), 2 μl of 100 μM ICI-198615 was added to the incubation mixture.

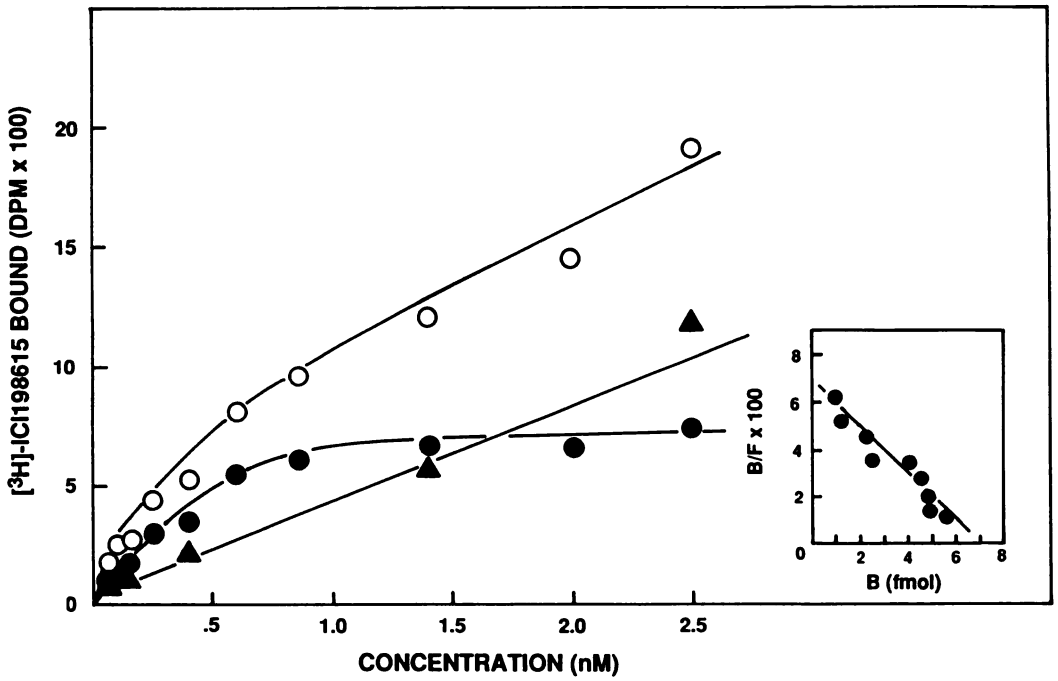

Fig. 2. Saturation binding of [^{3}H] ICI-198615 to solubilized GPL preparation. Varying concentrations of [^{3}H]ICI-198615 (0.05 to 2.5 nM) were incubated with 200 μl of digitonin-solubilized GPL preparation in the presence or absence of 1000-fold ICI-198615, for 90 min. The specific binding (●) was calculated from total (○) and nonspecific (▲) binding under standard conditions, as described in Materials and Methods. *Inset*, conversion of the specific binding data by the Scatchard (9) method.

binding not displaced by these antagonists was related to the soluble LTD_4 receptor. Further saturation or competition experiments to characterize this non-receptor-related component were unsuccessful and discontinued. Two quinoline-type receptor antagonists, WY-48252 and WY-49511 (10), structurally related to ICI-198615, displaced binding of [^{3}H]ICI-198615 to the LTD_4 receptor component in soluble GPL, with K_i values of 60 ± 15 and 750 ± 120 nM, respectively. Another recently reported quinoline type LTD_4 receptor antagonist, R-12525, competed with the LTD_4 receptor component of [^{3}H]ICI-198615 binding with a K_i of 30 ± 12 nM. The agonist LTD_4 competed dose dependently with [^{3}H]ICI-198615 binding; the K_i was 2 ± 0.45 μM. LTE_4 also competed with [^{3}H]ICI-198615 binding to the soluble receptor preparation.

The binding affinities of these three classes of antagonists differ slightly from those determined by using [^{3}H]LTD_4 in the GPL membrane receptor binding assay (Table 1). However, the general rank order of binding affinities directly correlated with the antagonist activities in the functional (smooth muscle contraction) assay and membrane receptor binding assay (Table 1). The results show that these antagonists were binding to the pharmacologically and physiologically relevant LTD_4 receptors solubilized from GPL membranes.

Size-exclusion chromatography of solubilized LTD_4 receptor. Size-exclusion HPLC was employed to further characterize the soluble LTD_4 receptor. Digitonin-solubilized LTD_4 receptor was fractionated through TSK-4000 and TSK-3000 columns connected in tandem. A major peak of [^{3}H]ICI-198615

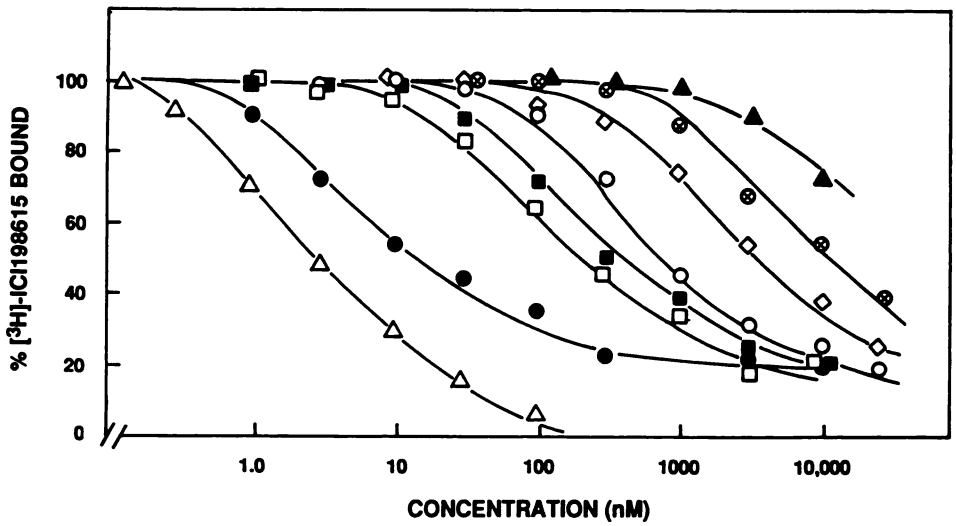

Fig. 3. Competition of [^{3}H]ICI-198615 specific binding by LTD$_4$ and receptor antagonists. The incubation mixture contained 0.5 nM [^{3}H]ICI-198615, 200 μl of soluble protein, and varying concentrations of ICI-198615 (△), SK&F 104353 (●), R-12525 (□), WY-48252 (■), SK&F 104373 (○), WY-49511 (◇), LTD$_4$ (⊗), and LTE$_4$ (▲). The [^{3}H]ICI-198615 bound in the presence of 0.5 μM ICI-198615 in each competition experiment was defined as 100% inhibition. Pooled results from four experiments were averaged and shown. The standard errors, usually 5–8% of the mean for each point, is not shown for clarity.

TABLE 1

Comparison of the binding affinities of LTD$_4$ receptor ligands

Compounds	Binding affinity		Functional activity	
	Membrane LTD$_4$ receptor K_i[a]	Soluble LTD$_4$ receptor K_i[b]	Agonist EC_{50}[c]	Antagonist $[K_B]$[d]
	nM		nM	nM
LTD$_4$	0.15 ± 0.05	2000 ± 450	5 ± 3	
WY-49511	820 ± 120	750 ± 120		350
SKF-104373	370 ± 35	150 ± 36		160
WY-48252	35 ± 12	60 ± 15		25
R-12525	12 ± 5	30 ± 12		10
SKF-104353	3 ± 1.5	4 ± 1.5		2.5
ICI-198615	0.06 ± 0.03	0.4 ± 0.1		0.5

[a] Binding affinity determined in GPL membrane receptor binding assay (11) using 0.3 nM [^{3}H]ICI-198615, taken from previous reports (10–13) and unpublished results.

[b] Binding activity determined using digitonin-solubilized GPL membrane receptor preparation as described in Fig. 3.

[c] Agonist activity taken from guinea pig tracheal smooth muscle contraction assay reported by Lewis *et al.* (2).

[d] Antagonist activity taken from previous (10–13) publications.

specific binding activity was detected (Fig. 4) with a retention time of 29 min, between the molecular weight marker proteins thyroglobulin (M_r 660,000) and β-amylase (M_r 200,000). To ensure that the [^{3}H]ICI-198615 binding to the protein fractions represented the soluble LTD$_4$ receptor, instead of the minor [^{3}H]ICI-198615 binding component not related to the soluble receptor, we utilized 0.5 μM ICI-198615 and 10 μM WY-48252 to define the soluble LTD$_4$ receptor component. The results showed that the binding of [^{3}H]ICI-198615 to the protein in the peak fractions was displaceable by ICI-198615 and WY-48252, indicating that the peak contained soluble LTD$_4$ receptors. The apparent molecular weight of the GPL membrane receptor, in digitonin micelle, was approximately 300,000 (Fig. 4, *inset*).

Discussion

A prerequisite step of LTD$_4$ receptor isolation and purification is to establish methods that can reliably and efficiently solubilize the membrane receptor protein while maintaining the ligand-binding function. Previous studies with [^{3}H]LTD$_4$ or [^{3}H]LTE$_4$ suggested that a high affinity radiolabeled receptor antagonist was necessary for this purpose. The present results demonstrated that [^{3}H]ICI-198615 is such an agent, which can be used to label the solubilized LTD$_4$ receptor with high affinity and the expected pharmacological specificity. [^{3}H]ICI-198615 binding to the soluble GPL receptor was time and protein concentration dependent, reversible, saturable, and stereoselective. Most importantly, several structural classes of functionally defined LTD$_4$ receptor antagonists competed with [^{3}H]ICI-

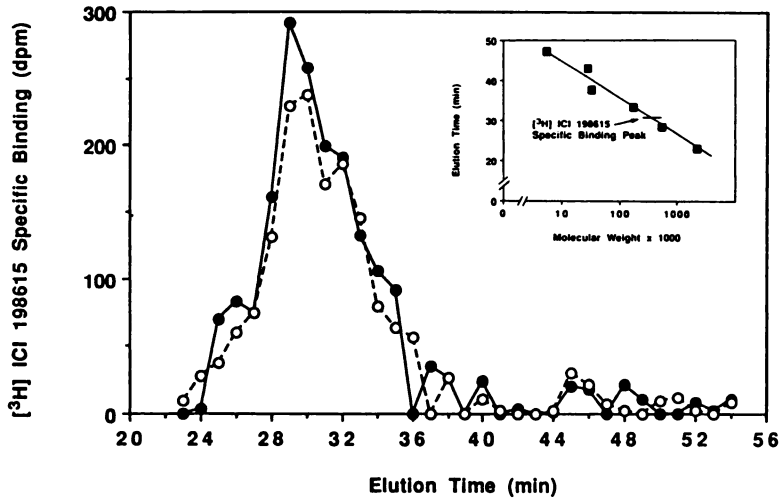

Fig. 4. Size-exclusion chromatography of digitonin-solubilized receptor preparation. A, One milliliter of digitonin-solubilized preparation was injected and eluted with buffer B. The chromatography was repeated five times to accumulate enough soluble material for binding experiments. The [^{3}H]ICI-198615 binding activity in each pooled fraction was determined by incubating 0.5 nM [^{3}H]ICI-198615 with 400 μl of each fraction in the presence (or absence) of 0.5 μM ICI-198615 (●), or 10 μM WY-48252 (○) to determine the LTD$_4$ receptor specific binding. Results shown are representative data of a single experiment repeated three times.

198615 binding to a major portion (80%) of the specific binding sites labeled by [^{3}H]ICI-198615. In addition, the receptor binding affinity of these antagonists, for either the soluble or the membrane LTD_4 receptor, correlated well with the pharmacological activities defined by the functional assay. These results indicate that the major portion of [^{3}H]ICI-198615-labeled soluble specific binding sites fully reflect the functional and biochemical specificity of the previously characterized LTD_4 receptor in GPL.

A corollary from previous membrane receptor [^{3}H]LTD_4 binding studies (4, 5) and the receptor-ligand-G protein ternary complex solubilization studies was that, depending on the functional state of the G protein, LTD_4 binding to the receptor could exist in a high affinity (K_d = 0.15 nM) or a low affinity (K_d > 100 nM) state. Such a G protein-regulated affinity state transition model in the α_2-adrenergic receptor (14), β-adrenergic receptor (15), and dopaminergic receptor (16) systems has been well established and, thus, lends strong support to the LTD_4-receptor-G protein ternary complex model. By analogy to this molecular model, the high affinity state receptor for LTD_4 binding (K_d = 0.15 ± 0.05 nM) was stabilized by the G protein and the agonist. Solubilization of the membranes, in the absence of the agonist, disrupted the coupling between the receptor and the G protein and yielded the receptor only in the low affinity state. Binding of LTD_4 to the G protein-uncoupled (i.e., in the soluble) state represented the low affinity state binding. Using the radiolabeled antagonist [^{3}H]ICI-198615, the binding affinity of LTD_4 to the digitonin-solubilized receptor was determined as 2 ± 0.45 μM. Thus, these experimental observations further supported the previously predicted LTD_4 receptor affinity state transition model.

Using size-exclusion HPLC, results in this study suggested that the apparent molecular weight of a digitonin-solubilized GPL LTD_4 receptor preparation was approximately 300,000. This is not widely different from the estimated apparent molecular weight of the prelabeled [^{3}H]LTD_4-receptor-G protein ternary complex (5). It is apparent that the hydrodynamic properties of the receptor-detergent complex could be profoundly affected by the hydrophobic interaction with the HPLC column matrix. The actual molecular weight and the oligomeric structure of the receptor protein or protein subunits cannot be deduced from the current studies. Attempts to obtain better resolution of the proteins in the M_r 100,000–300,000 range have not been successful; therefore, the difference in the molecular weight of prelabeled and postlabeled receptor was not apparent in this study. Nevertheless, it is clear that the molecular mechanism of [^{3}H]ICI-198615 binding to the soluble LTD_4 receptor is different from that for [^{3}H]LTD_4. The current studies have established a method of LTD_4 receptor solubilization and initial fractionation from GPL membranes. These studies have also established a radiolabeled receptor antagonist binding assay for the soluble LTD_4 receptor and, thus, open the door for receptor purification, structural determination, and molecular cloning in the future.

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