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Solubilization of [^{3}H]Leukotriene D_4 Receptor Complex from Guinea Pig Lung Membranes

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SUMMARY

Guinea pig lung membrane leukotriene D_4 (LTD_4) receptors were prelabeled with [^{3}H]LTD_4 and solubilized using digitonin, 3-[(3-cholamidopropyl)- dimethylammonio]-1-propane sulfonate, and other non-ionic, zwitterionic, and ionic detergents. [^{3}H]LTD_4 remains tightly associated with the receptor complex in the digitonin solubilized state. The dissociation rate of [^{3}H]LTD_4 from the soluble receptor complex was increased in the presence of guanine nucleotides and sodium ions in a manner similar to that observed for the receptors in the membrane-bound state. The soluble [^{3}H]LTD_4 receptor complex was retained on wheat germ lectin affinity columns and destabilized by heat (40 ± 4°), trypsin, and chymotrypsin treatment, suggesting that the receptor is a glycoprotein. Size exclusion high pressure liquid chromatography of the soluble receptor complex showed that an apparent molecular weight of the soluble receptor complex, in the presence of digitonin, is in the range of 240,000–500,000. An approximately 20-fold enrichment of receptor-radioligand complex was achieved by passing the solubilized LTD_4 receptor preparation successively through size exclusion and wheat germ lectin chromatography columns. These data provide the first step toward the purification and chemical characterization of LTD_4 receptors.

Employing radioligand binding techniques, several laboratories have demonstrated specific, high affinity, and stereoselective binding of LTD_4 and LTC_4 to distinct sites in the membrane fractions of human and guinea pig lung (1–4). The results obtained from radioligand competition binding studies and smooth muscle contraction studies showed that the specificity profile of the LTD_4- but not the LTC_4-binding sites on guinea pig lung membranes linearly correlated with the myotonic activities of the LTD_4 agonists and antagonists (5). We conclude that the LTD_4 specific binding sites on guinea pig lung membranes represent biochemically and pharmacologically important receptors. Sodium ions and guanine nucleotides have been demonstrated to specifically modulate the binding of [^{3}H]LTD_4 to its receptors on guinea pig and human lung membranes (2, 6). Evidence from these receptor regulation studies suggests that membrane-localized receptors for LTD_4 are coupled, via G_i protein, to intracellular effector systems (7).

LTD_4-induced smooth muscle cell contraction (8) and guinea pig lung contraction (9) have been demonstrated to be largely mediated through prostanoid metabolites of arachidonic acid. Arachidonic acid (and its metabolites) have been implicated as an intracellular mediator for many types of membrane receptors (10) such as chemotaxis peptide (11, 12) and possibly LTD_4 (8). The molecular mechanisms of signal transduction for the LTD_4 receptor, however, remain undefined. To characterize further the molecular mechanism(s) of receptor-ligand interaction and to understand the biochemical nature of LTD_4 receptors as well as the mechanisms of signal transduction, solubilization and purification of the receptors from membranes are essential. This is, however, complicated by the fact that the leukotrienes themselves are highly lipophilic and have detergent-like characteristics. In the present study, we have solubilized and partially purified the [^{3}H]LTD_4-bound receptor complex from guinea pig lung membranes. The biochemical mechanisms of receptor-ligand interaction are also characterized.

Materials and Methods

The natural chiral form 5*S*,6*R*-LTD_4, the unnatural chiral form 5*R*,6*S*-LTD_4, and racemic mixtures, LTC_4 and LTE_4, were prepared by chemical synthesis and were supplied by Dr. J. Gleason (Department of Medical Chemistry, SKF Laboratories). Purity, as defined by HPLC analysis (13), was greater than 96%. The isotopically labeled [^{3}H]LTD_4 was obtained from New England Nuclear (Boston, MA). It was reported to have greater than 96% purity with specific activity of 37–40 Ci/mmol. The purity, chirality, and identity of the radioligand were also confirmed by HPLC methods (3). Digitonin was purchased from Gallard-Schlesinger Co. (Carle Place, NY). Chymotrypsin, trypsin, soy-

ABBREVIATIONS: LTC_4, diastereoisomeric mixture of 5(*R*)-hydroxy-6(*S*) and 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; LTD_4, natural chiral form of 5(*S*)-hydroxy-6(*R*)-*S*-1-cysteinylglycyl-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; LTE_4, 5(*S*)-hydroxy-6(*R*)-*S*-*l*-cysteinyl-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; HPLC, high pressure liquid chromatography; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; NP-40, Nonidet P-40; GTPγS, guanosine-5′-*O*-3-thiotriphosphate, Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; PEG, polyethyleneglycol 8000; WGL, wheat germ lectin; NAG, *N*-acetylglucosamine.

bean trypsin inhibitor, bacitracin, benzamidine, phenylmethylsulfonyl fluoride, Gpp(NH)p, GTPγS, phosphoenopyruvate, myokinase, CHAPS, NP-40, Triton X-100, Lubrol PX, Brij 56, Tween-80, polidocanol, lysolecithin, and sodium deoxycholate were purcahsed from Sigma Chemical Co. (St. Louis, MO). Lentil lectin-sepharose 4B, WGL-Sepharose 6MB, and concanavalin A-Sepharose were purchased from Pharmacia Co. (Uppsala, Sweden).

Guinea pig lung membrane preparation. Male albino guinea pigs (Hartley strain, 500–800 g body weight) were sacrificed by decapitation. The lungs were removed, frozen in liquid nitrogen, and stored at −70° until used. The frozen tissue was thawed and placed in 10 volumes of homogenization buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 2 mM ethylenediaminetetraacetate, and the following protease inhibitors: soybean trypsin inhibitor, (10 μg/ml), bacitracin (100 μg/ml), benzamidine (100 μM), and phenylmethylsulfonyl fluoride (100 μM) (buffer A). The tissue was homogenized with a Brinkman PT-20 Polytron at 0° for a total of 1 min with 10-sec pulses, at a setting of 6. The homogenate was centrifuged (1,000 × *g* for 10 min) to remove tissue clumps, unbroken cells, and nuclei. The supernatant was centrifuged at 40,000 × *g* for 20 min. The pellets were washed twice in buffer B (10 mM Tris-HCl, pH 7.5) by resuspension and centrifugation at 40,000 × *g* for 20 min. The pellets were finally resuspended with a Teflon homogenizer in 5 ml of buffer B at a concentration of 10–20 mg/ml of protein. Partial purification of the lung membranes was accomplished using discontinuous sucrose density gradient centrifugation. The 40,000 × *g* crude membrane pellets were resuspended in buffer B containing 10% sucrose at a protein concentration of 2 mg/ml. Twenty ml of the membrane suspension were then gently layered on a 10-ml 40% sucrose cushion, in Beckman SW-28 nitrocellulose centrifuge tube. The discontinuous sucrose gradients were centrifuged in an SW-28 rotor at 25,000 rpm (83,000 × *g*) for 1 hr at 4°. The material at the boundary of the sucrose gradient layers was collected, diluted three times with buffer B, and centrifuged at 45,000 rpm (150,000 × *g*) in a Ti-70 rotor for 30 min to pellet the membranes. These membranes were further washed by resuspension and centrifugation in buffer B once. The concentrations of protein were determined by the method of Bradford (14) using bovine serum albumin as the standard. The pH of all the solutions and buffers was adjusted with potassium hydroxide.

[^{3}H]LTD$_4$ binding to membrane receptors. Results obtained from radioligand binding studies indicated that [^{3}H]LTD$_4$ binding to membrane receptors was affected by monovalent and divalent cations, guanine nucleotides, the temperature of incubation, pH, and the species of the buffer used. The standard conditions employed to study LTD$_4$ membrane receptors have been described in detail previously (3). Briefly, the incubation mixture contained 2 nM [^{3}H]LTD$_4$, with or without 2 μM LTD$_4$, in 10 mM Pipes buffer (pH 6.5) containing 5 mM cysteine, 5 mM glycine, 10 mM $CaCl_2$, 10 mM $MgCl_2$ (binding buffer, buffer C), and guinea pig lung membrane protein (500–800 μg/ml) in a volume of 1.5 ml for 30 min at 20°. This was selected to assure a minimal degree of bioconversion of [^{3}H]LTD$_4$, a maximal increase of receptor density, and high affinity of [^{3}H]LTD$_4$ binding to its receptor. Free ligand was separated from membrane-bound ligand by vacuum filtration through Whatman GF/C filters and by washing with 20 ml of buffer B at 0°. Total and nonspecific binding values were defined as the [^{3}H]LTD$_4$ bound to the membranes in the absence or presence of a 1000-fold excess of unlabeled LTD$_4$, respectively. The specific binding was defined as that amount of [^{3}H]LTD$_4$ bound to the membrane when the nonspecific component was subtracted from the total binding. Specific binding represented 80–85% of the total binding and has been characterized previously to be equivalent to the LTD$_4$ receptor-binding component (4, 5).

Solubilization of [^{3}H]LTD$_4$ receptor complex. [^{3}H]LTD$_4$ (2 nM) was incubated with lung membranes (500–800 μg/ml of protein) in buffer C as described above for 40 min at 20°. After incubation, 18.5 ml of buffer B at 0° were added, and the membranes were pelleted by centrifugation at 40,000 × *g* at 0° for 20 min. All subsequent procedures were performed at 0°–4° unless otherwise noted. The supernatants were removed and the membrane pellets were gently rinsed with buffer B. Detergent solutions (from 0.8 to 2.5 ml) were added to submerge membrane pellets. A small stirring bar was added into each tube to resuspend the membranes in the detergent solutions by gentle stirring for 60 min. The resulting solutions containing residual membranes were centrifuged at 100,000 × *g* for 1 hr in a Ti-70 rotor. The supernatants were then assayed for the [^{3}H]LTD$_4$-labeled receptor activity. To determine nonspecific binding of the radioligand, membranes were incubated with [^{3}H]LTD$_4$ (2 nM) and LTD$_4$ (2 μM) under standard conditions and processed for solubilization as described above.

PEG precipitation of solubilized [^{3}H]LTD$_4$ bound receptor complex. The method of PEG precipitation was employed to measure the solubilized [^{3}H]LTD$_4$ receptor complex as described by Homcy *et al.* (15). Briefly, bovine γ-globulin (100 μg/ml) was incubated with 100–200 μl of solubilized supernatant (100–300 μg of solubilized membrane protein) and PEG (20% w/v) at 0° in a total volume of 1 ml for 10 min in triplicate assay tubes. The contents were poured onto filter paper discs (Whatman GF/C) and the precipitates were retained on the filters. The filters were immediately washed with 5 ml of PEG solution (8%, w/v) at 0°, for four times. The nonspecific binding of [^{3}H]LTD$_4$ to solubilized membrane proteins was determined in triplicate, from the soluble preparations that contained [^{3}H]LTD$_4$ (2 nM), LTD$_4$ (2 μM), and membranes in the original incubation mixture as described above. The [^{3}H]LTD$_4$ specific binding to the soluble protein was defined as the difference of total and nonspecific binding. Ten ml of scintillation fluid (HP/b Ready-Solv) were added into each counting vial, and the radioactivity of the filters was determined by scintillation spectrometry with an efficiency of 30–40%.

Bioconversion of [^{3}H]LTD$_4$ in soluble membrane receptor preparation. Reverse phase HPLC (Beckman Instruments, with model 165 variable wavelength detector) was employed to study the biotransformation of radioligands in the receptor-binding experiments. The lung membranes (400 μg/ml) were incubated with [^{3}H]LTD$_4$ (1 nM) under conditions described above for 30 min at 20°. Following incubation, the reaction mixture was poured into a 24-mm borosilicate glass filter holder; the particulate membranes were retained on a Whatman GF/C filter and washed immediately with 2 ml of ice-cold buffer B. The filtrate was collected for analysis. The radioactivity associated with the receptors on the filter paper was immediately extracted with 2 ml of methanol/H_2O (1:1). The methanol was evaporated under argon and a 200-μl aliquot of the remaining aqueous solution was subjected to HPLC analysis. The radioactivity of the filtrate and methanol/H_2O extract was determined from small (50-μl) aliquots to estimate the total radioactivity of each fraction before HPLC analysis. The time required to extract the filter-bound radioactivity was approximately 20–25 sec. Greater than 98% of the membrane-bound radioactivity was routinely extracted. No endogenous leukotrienes were detected in the membrane preparations employed in this study; thus, the samples to be analyzed were mixed with LTC$_4$, LTD$_4$, and LTE$_4$ standards (1 μg/ml) and injected for HPLC analyses. The recovery of radioactivity for each chromatogram was 100 ± 5% of the total radioactivity injected.

The integrity of soluble receptor-bound and the partially purified soluble receptor-bound radioactive leukotrienes was assayed by direct chromatography on reverse phase HPLC. Alternatively, methanol extracts of PEG precipitates from the Whatman GF/C filter paper discs were also assayed by reverse phase HPLC. The C_{18} reverse phase LiChrosorb column (RP-18, 10 μm, 0.46 × 25 cm) was used with acetonitrile/10 mM phosphate buffer (pH 6.7) as mobile phase, at a flow rate of 2 ml/min for the separation of LTC$_4$, LTD$_4$, and LTE$_4$ standards. A 25–35% acetonitrile gradient was used in the first 10 min, followed by a constant 35% acetonitrile for the last 10 min. The radioactivity of 0.5- or 1-ml fractions was determined by liquid scintillation spectrometry.

Chromatography of solubilized receptor-ligand complex by size exclusion. TSK-3000 and TSK-4000 size-exclusion HPLC col-

umns (7.5 mm i.d. × 30 cm) connected in series were used to fractionate the solubilized radioligand-receptor complex. The columns were pre-equilibrated with elution buffer (buffer D; 10 mM Pipes buffer, pH 6.5, containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, 5 mM cysteine, 5 mM glycine, and 0.5% digitonin) and then calibrated with the following marker protein standards: ferritin (M_r 540,000), catalase (M_r 240,000), aldolase (M_r 158,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000), chymotrypsinogen A (M_r 25,000), and cytochrome *c* (M_r 12,500). Solubilized receptor-radioligand complex (200 μl) or the NaCl, Gpp(NH)p-treated receptor-radioligand complex (containing 100–200 μg of protein) was injected into the column and eluted with buffer D at a flow rate of 0.5 ml/min. Soluble protein-bound radioactivity in 0.5- or 1-ml fraction was determined directly by scintillation spectrometry or by the PEG precipitation method described above.

Chromatography of solubilized receptor-ligand complex by lectin affinity column. Lectin affinity chromatography columns were prepared in Pasteur Pipettes with a bed volume of 2 ml. The lectins employed were WGL-Sepharose 6MB, lentil lectin-Sepharose 4B, or concanavalin A-sepharose 4B. One to 2 ml of solubilized supernatant or the pooled fractions of solubilized receptor-radioligand complex obtained from size exclusion HPLC were loaded onto the columns that were pre-equilibrated with the binding buffer C and incubated for 10 min at room temperature. Columns were then washed eight times with 1 ml of buffer C in a stepwise fashion. The receptor-radioligand complex was then eluted with 1 ml of buffer C containing 0.1 M NAG. Rate of elution was maintained at approximately 0.5 ml/min. Finally, 10 ml of 1 M KCl was added to the columns to elute the tightly bound proteins and to regenerate the columns. Receptor-radioligand complex in 1-ml fraction was assayed by the PEG precipitation method or by direct scintillation spectrometry as described above.

Results

The dissociation constant (K_d) and maximum number of bonding sites (B_{max}), under the standard conditions, were 0.18 ± 0.05 nM and 650 ± 150 fmol/mg of protein, respectively, for [^{3}H]LTD_4 binding to guinea pig lung membrane receptors. In partially purified (sucrose density gradient) guinea pig lung membranes, the K_d and B_{max} values were 0.15 ± 0.03 nM and 2100 ± 250 fmol/mg protein, respectively (fig. 1).

Solubilization of receptor-ligand complex from guinea pig lung membranes. Unless otherwise noted, crude guinea pig lung membranes were employed for all of the following receptor solubilization experiments. Receptor-radioligand complexes were formed by incubating guinea pig lung membranes with 2 nM [^{3}H]LTD_4. Under the conditions employed, the radioligand occupied more than 90% of the receptor sites in the membrane (Fig. 1a). The receptor-radioligand complexes can be solubilized by several types of detergents. However, the efficiency of extraction (yield), the specific activity (enrichment), and the stability of solubilized receptor-radioligand complex varied substantially depending on the concentration and the detergents employed. Several types of zwitterionic and non-ionic detergents were employed to maximize the yield (Fig. 2) of the soluble receptor-radioligand complex. Under the experimental conditions employed, a greater than 20% yield, at optimal concentrations of the detergents, was obtained by using polyethoxy C_9O_{12} (Polidocanol), digitonin, NP-40, CHAPS, and sodium taurocholate. Yields of solubilization less than 5% were obtained using polyethoxy-type non-ionic detergents, Tween-80 (Fig. 2), Brij 56, and Lubrol PX, and zwitterionic detergents, lysolecithin, sodium deoxycholate (results not shown). The nonspecific binding of [^{3}H]LTD_4 to the detergent-solubilized membrane protein was, in general, low (less than 10% of total binding) for all of the detergents and independent of the

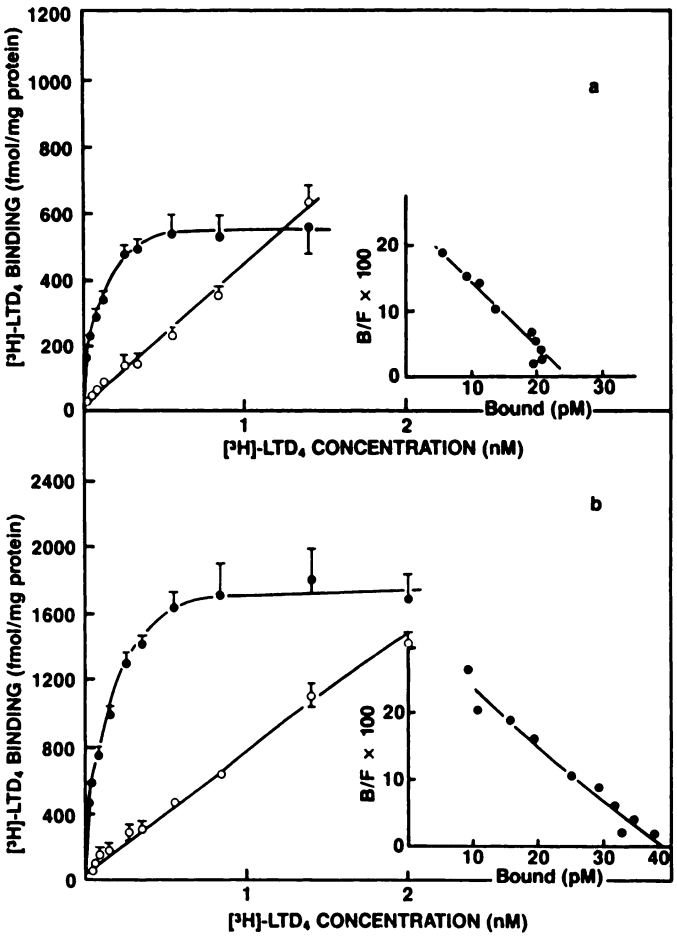

Fig. 1. Saturation binding of [^{3}H]LTD_4 to crude and purified guinea pig lung membranes. a, Crude guinea pig lung membranes (50 μg/ml) were incubated with 0.04–2 nmol of [^{3}H]LTD_4 in 0.5 ml of binding buffer for 40 min at 20°. Binding of [^{3}H]LTD_4 to the membranes was determined by a vacuum filtration method. Nonspecific binding (○) of [^{3}H]LTD_4 to membrane was determined by including 1000-fold LTD_4 in the incubation buffer as described in Materials and Methods. Specific binding (●) was determined by subtracting the nonspecific binding from the total binding of [^{3}H]LTD_4 to the membranes. *Inset*, Scatchard analysis of the saturation binding data. b, Discontinuous sucrose density gradient, partially purified guinea pig lung membranes were employed for saturation binding analyses. The conditions used were identical to those described above except that the protein concentration was reduced to 20 μg/ml.

concentrations of the detergents employed. However, only digitonin and CHAPS solubilized receptor-radioligand complexes that were stable enough for further characterization (see Fig. 3). Data shown in Fig. 2 demonstrated that two detergents, CHAPS (0.6–2%) and digitonin (0.5–2%), can be employed to solubilize the receptor-ligand complex effectively.

Receptor specific activity of the receptor-radioligand complex (as indicated by the PEG-precipitable radioactivity) increased 2.5- and 2-fold after solubilization with digitonin or CHAPS, respectively. At an effective concentration range of detergents employed, digitonin (0.5%, 4.2 mM; at a protein/detergent ratio of 0.5) and CHAPS (0.9%, 15 mM; at a protein/detergent ratio of 0.18) solubilized 44 and 20% of membrane-bound receptors, and 18 and 28% of total membrane protein, respectively (Table 1). The degree of enrichment and yield of soluble receptor complex were equivalent when the centrifugation procedure employed was 100,000 × 60 *g*·min, 200,000 × 60 *g*·min or 100,000 × 120 *g* min.

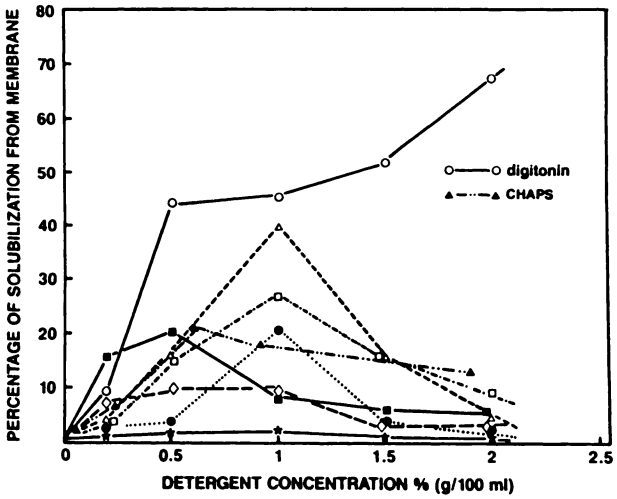

Fig. 2. Solubilization of [^{3}H]LTD$_4$-bound receptors from guinea pig lung membranes. The membranes were incubated with 2 nmol of [^{3}H]LTD$_4$ and pelleted by centrifugation as described in Materials and Methods. Varying concentrations of digitonin (○), CHAPS, (▲), NP-40 (■), sodium taurocholate (△), Triton X-100 (◇), Octyl-β-glucoside (●), Polidocanol (□), and Tween-80 (★) were employed to solubilize [^{3}H]LTD$_4$ receptors. The radioactivity in 100 μl of 100,000 × *g* supernatant (soluble preparation) was determined by the PEG precipitation method as described in Materials and Methods. The standard deviation of each point was 5–10% of the mean (not shown for clarity of the figure). The specific binding of [^{3}H]LTD$_4$ to soluble protein and to lung membrane receptors was calculated as described in Materials and Methods. The membrane LTD$_4$ receptor-binding capacity labeled with 2 nmol of [^{3}H]LTD$_4$ was defined as 100%.

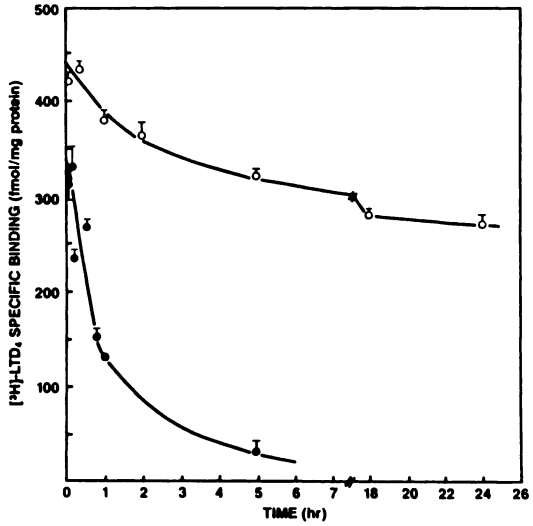

Fig. 3. Dissociation of [^{3}H]LTD$_4$ from digitonin- and CHAPS-solubilized receptor. Membrane receptors were labeled with 2 nM [^{3}H]LTD$_4$ and solubilized with 0.5% digitonin (○) or 10 mM CHAPS (●) as described in Materials and Methods. Two hundred μl of digitonin-soluble preparation (containing 135 μg of protein) or CHAPS-soluble preparation (containing 100 μg of protein) were incubated at 20° from 0 to 24 hr. The [^{3}H]LTD$_4$ specific binding activity, at each indicated point of time, was determined by the PEG precipitation method and expressed as fmol/mg of soluble protein.

Two LTD$_4$ antagonists, FPL 55712 (16) and SKF 102922 (17), were employed to study the specificity of the LTD$_4$ receptor for the solubilization studies. These two receptor antagonists have binding affinities (K_i) to membrane LTD$_4$ receptors of 2 μM and 0.5 μM, respectively. When FPL 55712 (20 μM) and SKF 102922 (5 μM) were included in the binding reaction mixture with [^{3}H]LTD$_4$, they inhibited [^{3}H]LTD$_4$ binding to the membrane receptors by 50 ± 5% and 50 ± 6% (18). When the membrane pellets of these incubation mixtures were solubilized with 0.5% digitonin, the amount of [^{3}H]LTD$_4$-labeled soluble receptor complex was reduced to 60 ± 5 and 55 ± 6%, respectively (results not shown). These results suggest that the radioligand-labeled soluble complexes are LTD$_4$ receptors.

Under the experimental conditions employed, the receptor-radioligand complex appeared to be stable in digitonin solutions. Dissociation of radioligand from the membrane (6) or digitonin-solubilized receptor (Fig. 3) was slow at 20°. Dissociation of [^{3}H]LTD$_4$ was biphasic and relatively more rapid when CHAPS was employed for solubilization. None of the other detergents employed in this study yielded a soluble receptor-radioligand complex that was more stable than that solubilized with CHAPS (results not shown). The dissociation rates of [^{3}H]LTD$_4$ were slower at 0 or 4° than at 20 or 37° (results not shown).

Modulation of the prelabeled [^{3}H]LTD$_4$ receptor complex by nucleotides and monovalent cations. Previous publications have demonstrated that sodium ions and guanine nucleotides specifically modulated [^{3}H]LTD$_4$ binding to membrane receptors by promoting a rapid dissociation of [^{3}H]LTD$_4$ from the membrane receptor complex (4, 6). The effects of nucleotides and monovalent cations on the solubilized receptor-radioligand complex were also investigated for comparison. As shown in Fig. 4, addition of NaCl or Gpp(NH)p to the digitonin-solubilized receptor-radioligand preparation also induced dissociation of radioligand from the soluble complex. KCl (120 mM) was less effective than NaCl. The effects of sodium ions and Gpp(NH)p appeared to be specific since KCl (120 mM), CsCl (120 mM), ATP, and GTP (100 μM each) were much less effective than NaCl or GTPγS or Gpp(NH)p (results not shown). The concentration-response relationships of monovalent cations and guanine nucleotides were further investigated. Fig. 5 shows that monovalent cations induced a concentration-dependent decrease in the amount of radioligand bound to the soluble receptor as determined by the PEG precipitation method. The IC_{50} concentrations of the monovalent cations tested were 120 and 230 mM for NaCl and LiCl, respectively. the IC_{50} concentrations for KCl, RbCl, and CsCl were greater than 300 mM. The counter-anion chloride appeared to have no measurable effects on the solubilized receptor at concentrations as high as 200 mM. The IC_{50} for NaCl was even lower than 120 mM when the concentrations of $MgCl_2$ and $CaCl_2$ were lowered to 1 mM (results not shown). Sodium ions were the most effective monovalent cation species in promoting the dissociation of [^{3}H]LTD$_4$ from the receptor-radioligand complex. The rank order potency of monovalent cations in modulating the [^{3}H]LTD$_4$ binding to the membrane receptors is identical to that of the receptor-radioligand complex in the solubilized state. Thus, these results indicate that the response of the receptors to the monovalent cations and guanine nucleotides after solubilization were comparable to those in the membranes.

The effects of nucleotides on the solubilized receptor-radioligand complex were studied in more detail (Figure 6). The nucleotides induced a concentration-dependent displacement of [^{3}H]LTD$_4$ associated with receptor as determined by PEG precipitation. The rank order of potency of various species of nucleotides tested was GTPγS > Gpp(NH)p ≫ GTP > dGTP. The IC_{50} values for GTPγS, Gpp(NH)p, and GTP were 0.7, 0.9, and 100 μM, respectively. The rank order potency and IC_{50}

TABLE 1

Solubilization of [^{3}H]LTD_4 receptor complex by digitonin and CHAPS

Detergent (concentration)	Specific binding[a]	Specific activity of receptor[b]	Total receptor activity solubilized	Yield membrane receptor[c]	Enrichment[d]
	fmol/200 μl	*fmol/mg protein*	*fmol*	%	*-fold*
CHAPS (0.6%; 10 mM)	49 ± 2.5	630 ± 30	110 ± 5.6	23	2.0
CHAPS (0.9%; 15 mM)	42 ± 2.0	448 ± 22	95 ± 4.5	20.3	1.4
Digitonin (1%; 8.3 mM)	55.4 ± 4.0	720 ± 51	209 ± 15.7	45	2.4
Digitonin (0.5%; 4.2 mM)	56.3 ± 2.0	810 ± 31	217 ± 7.3	44	2.6

[a] The LTD_4 receptor specific binding activity in 0.2 ml of detergent-solubilized supernatant was determined using the PEG precipitation method.

[b] The soluble receptor specific activity was determined by normalizing the [^{3}H]LTD_4 specific binding activity (in 0.2 ml) to the concentration of solubilized membrane protein determined previously (14).

[c] The total LTD_4 receptor binding activity was determined by labeling the crude membrane protein with 2 nM [^{3}H]LTD_4 under standard conditions. The receptor binding capacity, under this condition, was defined as 100%.

[d] Enrichment of receptor activity was defined as the extent of increase of soluble receptor activity determined as specific activity of receptor.

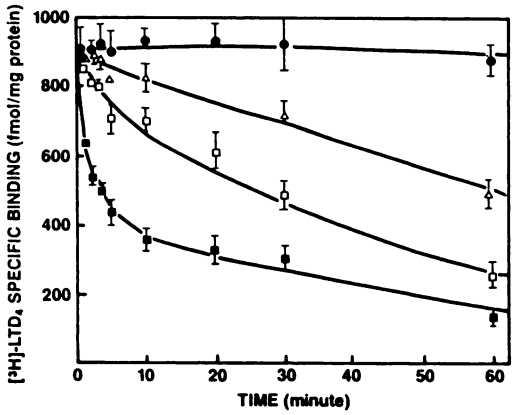

Fig. 4. Effects of K^+, Na^+, and Gpp(NH)p on the dissociation kinetics of [^{3}H]LTD_4 from solubilized receptor complex. Receptors were prelabeled with 2 nM [^{3}H]LTD_4 and solubilized with 0.5% digitonin. The solubilized receptor complex (200 μl containing 73 μg of protein) was incubated alone (control; ●), with 2 μM Gpp(NH)p (■), 120 mM NaCl (□), or 120 mM KCl (△) at 20° from 0 to 60 min. The [^{3}H]LTD_4 specific binding at each indicated point of time was determined by PEG precipitation and calculated as fmol of binding/mg of soluble protein. Standard deviations, when not shown, are smaller than the symbols employed.

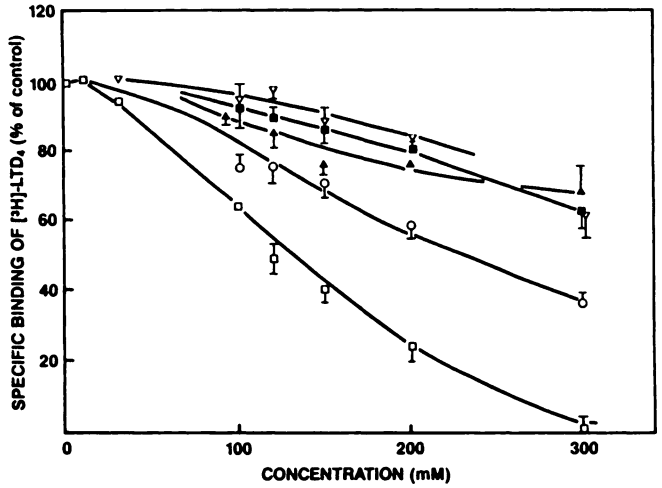

Fig. 5. Specificity of the effects of monovalent cations on the soluble LTD_4 receptor complex. [^{3}H]LTD_4-bound receptor was solubilized with 0.5% digitonin as described in the legend to Fig. 3. Aliquots (200 μl) of the soluble receptor preparations were incubated with 20 μl of varying concentrations of LiCl (○), NaCl (□), CsCl (▲), KCl (▽), or RbCl (■) at 20° for 30 min. [^{3}H]LTD_4 specific binding was determined as described in Materials and Methods. Standard deviations, when not shown, are smaller than the symbols employed.

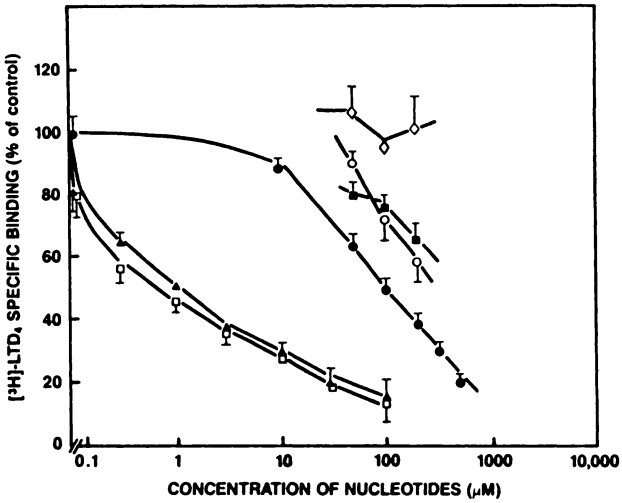

Fig. 6. Specificity of the effects of nucleotides on the soluble LTD_4 receptor complex. [^{3}H]LTD_4-bound receptor was solubilized with 0.5% digitonin as described in the legend to Fig. 3. Twenty μl of varying concentrations of GTPγS (□), Gpp(NH)p (▲), ATP (◇), dGTP (○), CTP (■), and GTP (●) were mixed with 200 μl of soluble receptor preparation and incubated at 20° for 30 min. [^{3}H]LTD_4 specific binding was determined by the PEG precipitation method as described in Materials and Methods.

concentrations were comparable to those observed for LTD_4 receptors in membrane preparations (6). Thus, these results demonstrate that the two major mechanisms of LTD_4 receptor regulation: effects of guanine nucleotides and monovalent cations are retained when the [^{3}H]LTD_4 receptor complexes are solubilized from the membranes.

To characterize the radioligand that was receptor bound or dissociated from the soluble receptors, radioligand was extracted from the PEG-soluble and PEG-precipitable fractions and subjected to HPLC analyses. These studies were performed on soluble radioligand receptor complexes obtained under optimal conditions and after Gpp(NH)p and NaCl treatment. The results (Fig. 7a) demonstrate that the bulk of the radioligand extracted from the PEG precipitate or released by Gpp(NH)p treatment (Fig. 7b) co-eluted with the LTD_4 standard, indicating the [^{3}H]LTD_4 was not degraded or metabolized in the process of solubilization or subsequent treatments. Results similar to those in Fig. 7 were obtained when radioligand released with NaCl treatment (200 mM) was analyzed by HPLC (results not shown).

Chromatography of solubilized receptor-radioligand complex by lectin columns. Digitonin-solubilized receptor-radioligand complex was passed through lentil lectin, WGL,

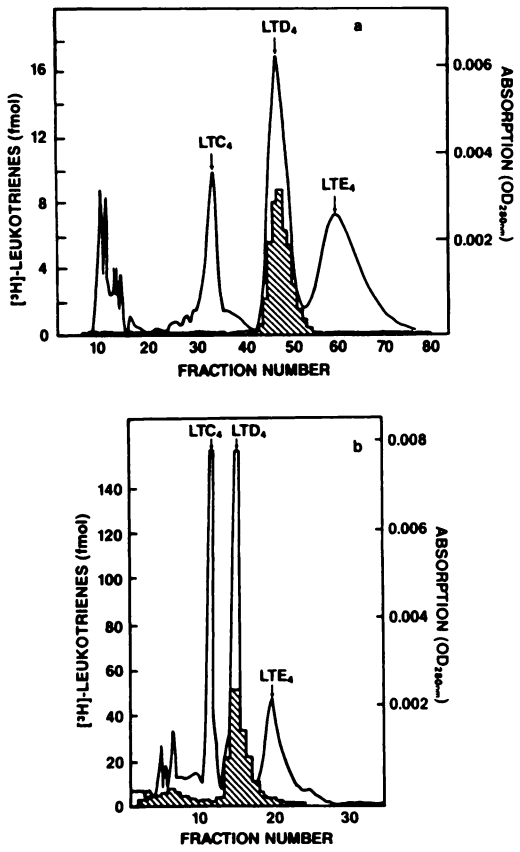

Fig. 7. Reverse phase HPLC of receptor-bound and dissociated radioligand. Soluble [^{3}H]LTD$_4$ receptor complex was prepared as described in the legend to Fig. 3. a, The soluble [^{3}H]LTD$_4$ receptor complex (200 μl, containing 150 μg of soluble protein) was precipitated with PEG on a Whatman GF/C filter paper disc and washed four times with PEG solution as described in Materials and Methods. The radioactive materials on the filter paper disc was extracted with 2 ml of methanol and then analyzed by reverse phase HPLC. The radioactivity in the 0.5-ml fraction was collected and determined. b, The soluble receptor preparation (2.0 ml, containing 3.5 mg of protein) was incubated with 100 μM Gpp(NH)p at 20° for 30 min. The reaction mixture was mixed with LTC$_4$, LTD$_4$, and LTE$_4$ standards and injected for HPLC analysis as described in Materials and Methods. The radioactivity in 1-ml fractions was collected and determined. The recovery of the radioactivity was 98 ± 4% of the total radioactivity injected into the column.

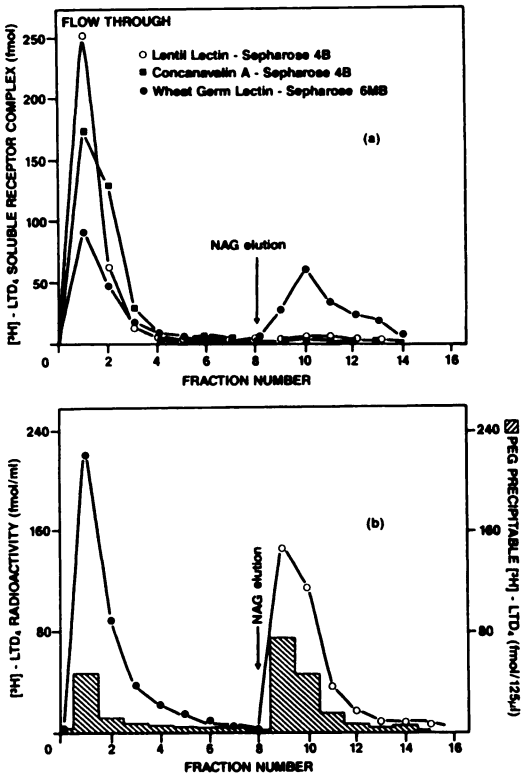

Fig. 8. Lectin affinity chromatography of solubilized receptor complex. a, [^{3}H]LTD$_4$-labeled soluble receptor complex was prepared and loaded on lentil lectin-Sepharose, concanavalin A-Sepharose 4B, or WGL-Sepharose 6MB columns as described in Materials and Methods. The columns were then washed in a stepwise manner with 1 ml of buffer E for eight times as flowthrough. NAG (0.1 M in buffer C) was used to elute the radioactivity bound to the column in a stepwise manner with 1 ml of NAG elution in each fraction. b, The radioactivity of flowthrough and eluted fraction was monitored by the PEG precipitation method or direct scintillation spectrometry of the soluble fractions.

and concanavalin A affinity columns. As shown in Fig. 8a, greater than 96% of soluble receptor-ligand complex was recovered in the flowthrough fractions when lentil lectin and concanavalin A columns were used, indicating that these two columns did not retain significant amounts of the soluble receptor complex. The WGL column, however, retained 47% of total radioactivity loaded, and the radioactive material retained on the WGL column was eluted with NAG. NAG-eluted radioactivity was stable in 0.5% digitonin elution buffer and was precipitated by PEG (Fig. 8b), suggesting that the radioligand was bound to high molecular weight macromolecular complexes. Of the total radioactivity in the flowthrough fraction, 19.4% was PEG precipitable. In the NAG-eluted fractions, 44% of the total radioactivity was PEG precipitable (Fig. 8b). These results suggest that the WGL column preferentially retained and enriched the solubilized LTD$_4$ receptor by 2.3-fold.

Radioligand bound to NAG-eluted material was dissociated when incubated with 100 μM Gpp(NH)p or 200 mM NaCl at 20° for 30 min (results similar to those in Figs. 4–6; not shown). Also, 0.2%, 95% and 0.5% of the Gpp(NH)p-dissociated radioligand from the WGL column retained soluble receptor complex co-eluted with LTC$_4$, LTD$_4$ and LTE$_4$ standards, respectively, on a reverse phase column HPLC (results similar to Fig. 7; not shown). Similar results were observed when the WGL affinity column was employed to fractionate CHAPS-solubilized preparations, suggesting that little or no [^{3}H]LTD$_4$ bound to the solubilized receptors was converted to other [^{3}H]leukotrienes or degraded during the WGL column chromatography. The stability of the CHAPS-soluble preparation, after passing through the WGL columns, was less than that prepared by digitonin (results not shown).

Incubation of receptor-radioligand complex with trypsin or chymotrypsin (Fig. 9a) or in the presence of increasing temperatures resulted in dissociation of the radioligand. DNAse, RNAse, or heat-inactivated proteases had no significant effects on the receptor complex. When the receptor complex was incubated from 0 to 80° for 10 min under the experimental conditions, the temperature that inactivated 50% of receptor was 40 ± 4° (Fig. 9b). These results suggest that the soluble receptor-radioligand complexes are heat-labile proteins or have heat-labile protein components. The results from these experiments suggest that the soluble receptor complex is a membrane-localized glycoprotein containing sialic acid or *N*-acetylglucosamine residues.

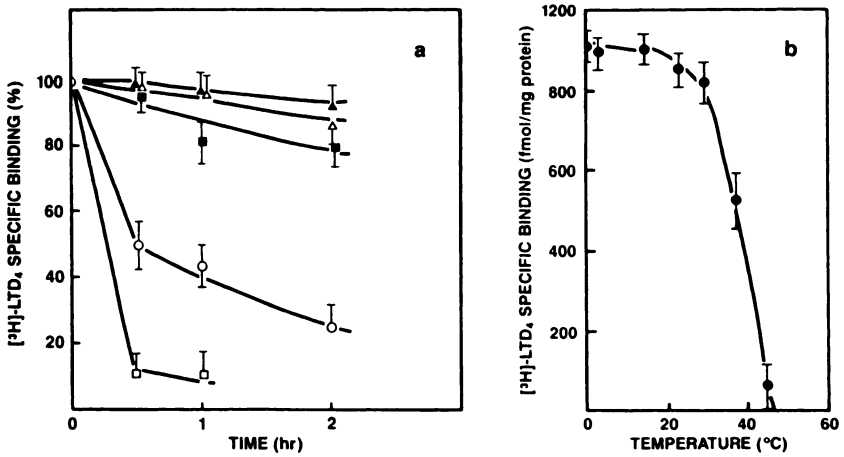

Fig. 9. Protease and heat sensitivity of soluble receptor complex. a, [^{3}H]LTD_4-bound receptor was solubilized using 0.5% digitonin. The soluble receptor complex (150 μl, containing 130 μg of soluble protein) was incubated with 10 μg of heat-inactivated (100° boiled for 10 min) trypsin (▲), 10 μg of heat-inactivated chymotrypsin (△), water (■) as control, or 10 μg of preactivated trypsin (○), and 10 μg of chymotrypsin (□) in a volume of 200 μl of 20° from 0 to 2 hr. Trypsin and chymotrypsin were prepared in 10 mM Tris-HCl buffer (pH 7.5) at a concentration of 1 mg/ml and preincubated at 37° for 1 hr before being used. The [^{3}H]LTD_4 specific binding at each indicated point of time was determined. The PEG-precipitable radioactivity at zero time was determined as 100%. b, The soluble receptor preparation (150 μl, containing 130 μg of protein) was incubated at 0–80° for 10 min and rapidly chilled in an ice-bath. the PEG-precipitable radioactivity was determined as described above.

Size exclusion HPLC and partial purification of receptor-radioligand complex. Size exclusion HPLC was employed to fractionate the digitonin-solubilized receptor preparation. As shown in Fig. 10a, the radioligand that complexed to high molecular weight proteins (peak A) eluted with a retention time between molecular weight standard proteins of ferritin (M_r 540,000) and catalase (M_r 240,000). Approximately 19.5% of the radioligands (peak B) eluted with a retention time identical to that of the free [^{3}H]LTD_4, suggesting either dissociation of the receptor-bound radioligand during chromatography or that free [^{3}H]LTD_4 was not thoroughly washed from the membranes before solubilization. Employing reverse phase HPLC, the radioligand extracted from peaks A and B was identified as [^{3}H]LTD_4 (results not shown; similar to Fig. 7). When the high molecular weight peak (peak A) was rechromatographed on the same column (Fig. 10b), nearly 95% of the radioactivity was recovered with a retention time identical to that in the first chromatography, suggesting that dissociation of the receptor-bound [^{3}H]LTD_4 was insignificant during size exclusion chromatography. The high molecular weight protein-bound radioligand was also released by sodium ions and guanine nucleotides specifically in a manner identical to that described above (Figs. 4–6).

Using discontinuous sucrose density gradients and a combination of size exclusion HPLC and lectin affinity chromatographic methods, we have solubilized and partially purified the receptor-radioligand complex from guinea pig lung membranes. The data shown in Table 2 quantitate the purification results. The procedures employed resulted in a 20-fold purification with a yield of 3%. Sodium ions and guanine nucleotides also modulate the partially purified soluble receptor complex. Incubation of the partially purified soluble receptor preparation with Na^+ (120 mM) and Gpp(NH)p (200 μM) resulted in rapid dissociation of the radioligand. Analysis of the dissociated radioligand by reverse phase HPLC showed that greater than 95% of the dissociated radioligand was [^{3}H]LTD_4 (results not shown).

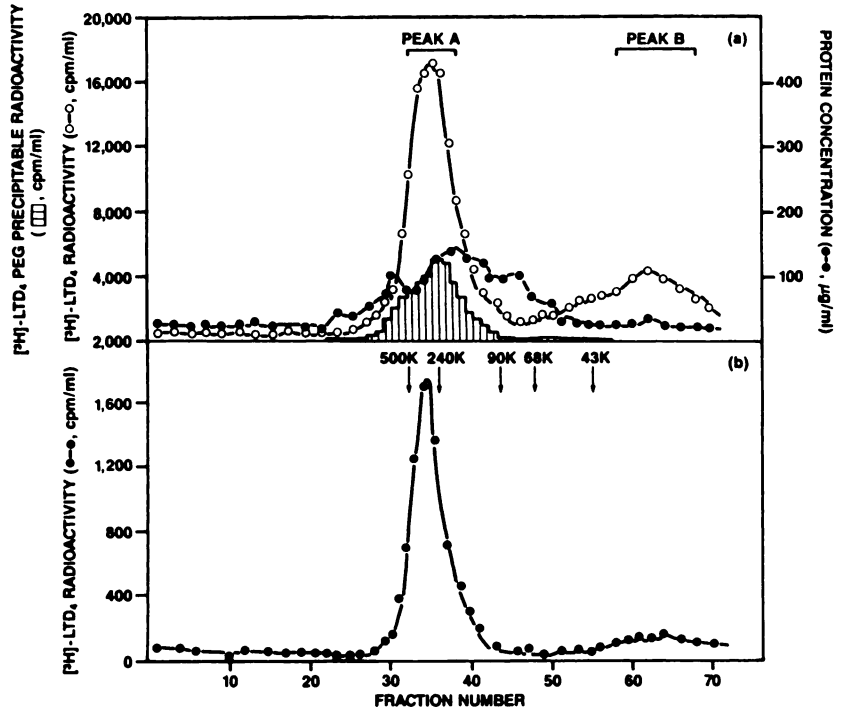

Fig. 10. Size exclusion chromatography of solubilized receptor-ligand complex. A TSK 4000 and TSK 3000 size exclusion chromatography column was connected in series to fraction the [^{3}H]LTD_4-bound soluble receptors. The column was eluted with buffer E containing 0.5% digitonin at a flow rate of 0.5 ml/min. a, The radioactivity of PEG-precipitable material in 200 μl of each fraction was determined (□). Alternatively, the radioactivity in 100 μl of each fraction was directly determined (○). The protein concentration in each fraction was determined by the Bradford method (14) (●) as the presence of digitonin interfered with UV absorption spectra or the method of Lowry *et al.* (18a) for determination of protein concentration. b, The fractions 32–38 of the chromatogram in a were pooled and injected into the same columns together with the molecular weight protein markers under identical conditions described above. The radioactivity in the fraction 58–68 (peak B) area corresponds to free [^{3}H]LTD_4.

TABLE 2

Partial purification of LTD_4 receptor complex from guinea pig lung membranes

Procedures	Protein Concentration	Total protein	Receptor activity	Yield	Specific activity	Enrichment
	μg/ml	μg	fmol	%	fmol/mg	-fold
A. Crude membrane protein[a]	1,750	4,725	2,948	100	594	1
B. Solubilization[b]	450	450	1,500	51	3,330	5.6
C. Size exclusion chromatography[c]	40	120	594	20	4,950	8.3
D. WGL column[d]	7	7	85	3	12,080	20.3

[a] Crude guinea pig lung membranes were employed.
[b] The LTD_4 receptors were labeled with 2 nM [^{3}H]LTD_4 under standard conditions, and were solubilized with 0.5% digitonin as described in Materials and Methods.
[c] TSK 3000 and TSK 4000 size exclusion HPLC columns were connected in series to fractionate the solubilized receptor complex as described in the legend to Fig. 10.
[d] A WGL column was employed to fractionate the solubilized [^{3}H]LTD_4 receptor complex as described in the legend to Fig. 9. The concentration of protein, the specific activity of receptor, the degree of enrichment, and the percentage yield were calculated as described in the legend to Table 1.

Discussion

Experimental evidence provided by several laboratories has demonstrated that, in guinea pig lung, LTD_4 binds to membrane receptors with high affinity and a high degree of specificity. The pharmacological specificity profile of the membrane-localized receptors corresponds to the agonist and antagonist activities of leukotriene D- and leukotriene E-type analogs in smooth muscle contraction assays (5). Sodium ions and guanine nucleotides have been demonstrated to specifically regulate the association and dissociation rates of radioligand to and from the membrane receptor complex. These studies clearly demonstrated that the LTD_4 specific binding sites in guinea pig lung membrane are physiologically and pharmacologically important receptors.

Results obtained in the current study demonstrate that membrane-localized receptors can be prelabeled with [^{3}H]LTD_4 and solubilized from guinea pig lung as a radioligand-receptor complex in digitonin. This is supported by several lines of evidence. First, receptor agonist and antagonists (SKF 102922 and FPL 55712), which specifically inhibited [^{3}H]LTD_4 binding to receptors, resulted in equivalent reduction in radioligand-receptor complex that can be solubilized by digitonin. Second, when bound to the receptor, either in the soluble or membrane-bound state, the radioligand remained tightly associated with the receptor complex with a minimal degree of bioconversion or chemical degradation. These results indicate that [^{3}H]LTD_4-bound proteins or protein complexes are probably not leukotriene-metabolizing enzymes such as γ-glutamyltranspeptidase(s) or dipeptidase(s). Third, sodium ions and guanine nucleotides specifically regulated the dissociation of [^{3}H]LTD_4 from the radioligand-receptor complex in a fashion analogous to that observed in the membranes. Furthermore, the rank order potencies of monovalent cations and purine nucleotides in modulation of the solubilized receptor-radioligand complex were identical to those characterized previously for LTD_4 membrane receptor. These results suggest that the key features of receptor regulation are retained throughout the process of solubilization and, in concert with the other lines of evidence, demonstrate that the solubilized radioligand-receptor complex represents the membrane-bound LTD_4 receptors.

Radioligand receptor binding studies reported from several laboratories have clearly demonstrated that the LTD_4 membrane receptor is regulated by guanine nucleotides, indicating that the G protein(s) is (are) involved in regulation of LTD_4 receptors. The effects of sodium and guanine nucleotides on LTD_4 receptors are analagous to those in the platelet α_2-adrenergic receptor system (19, 20) or the opiate receptors in central nervous system (21), suggesting that the G_i protein may regulate the LTD_4 receptors. A ternary complex model for agonist-receptor-G protein (L-R-G complex) has been proposed (22) to describe the molecular mechanism of G protein regulation of receptor-radioligand interaction. Furthermore, solubilized L-R-G complexes have been demonstrated in α_2-adrenergic (23) and dopamine D_2-receptor systems (24). Because the results obtained from the current study have clearly demonstrated that the solubilized [^{3}H]LTD_4 radioligand-receptor complex was also specifically modulated by guanine nucleotides and sodium ions, it is likely that a G protein is also present in the prelabeled solubilized LTD_4 receptor-radioligand complex.

Digestion of solubilized receptor complex with proteases, but not DNAse or RNAse, resulted in rapid dissociation of [^{3}H]LTD_4 from the radioligand-receptor complex, suggesting that the soluble receptor complexes are proteins. Also, the radioligand-receptor complex bound to WGL affinity column and was eluted with NAG, suggesting that the radioligand-receptor complex may be a glycoprotein complex containing NAG or sialic acid. Since G proteins that have been isolated and purified (25–27) do not contain NAG or sialic acid, it is probable that the receptor moiety is a membrane glycoprotein and that the soluble L-R-G complex may bind to the WGL column through NAG or a sialic acid group(s) on the R subunit of the complex.

Employing size exclusion and HPLC, we have partially purified the soluble LTD_4 receptor complex 20-fold. In the presence of digitonin, the apparent molecular weight of receptor complex micelle was in the range of 240,000–500,000. The true molecular weight, oligomeric structure, stokes radius, and sedimentation coefficient of the receptor and radioligand-receptor-complex, however, are yet to be determined accurately as the detergent digitonin can significantly bias many of the hydrodynamic measurements (28).

Evidence in the literature has documented that many receptors that are functionally coupled to the inhibition of adenylate cyclase are directly regulated by the G_i protein (29). G_i protein can be selectively and specifically ADP-ribosylated in the presence of islet-activating protein isolated from *Bordetella pertussis*. The G_i protein-coupled α_2-adrenergic receptor and opiate receptors share a common feature in that these receptors are also regulated by sodium ions specifically. Thus, it was hypothesized initially that the LTD_4 receptors may be coupled to the G_i protein since the LTD_4 receptor was also specifically regulated by sodium ion (4). In our laboratory, we have not been able to demonstrate that the LTD_4 receptor is coupled to the inhibition of a basal or stimulated state of adenylate cyclase (6). However, recent studies have shown that G_i proteins also

regulate receptors that are coupled to the activation of phospholipase A_2 and phospholipase C (12, 30). These observations are consistent with the effects of LTD_4 observed in smooth muscle cell and endothelial cell systems (8) and in guinea pig lung.[1] In these systems, LTD_4 receptors induce activation of phospholipase A_2, mobilization of arachidonic acid, and subsequent biosynthesis of prostanoids. Mobilization of arachidonic acid and biosynthesis of prostanoids in these systems can be inhibited by islet-activating protein treatment.[2] Thus, these results provide suggestive evidence for the involvement of G_i protein in the regulation of LTD_4 receptors that are coupled to phospholipases and that resulted in the release of arachidonic acid.

In most of the G protein-regulated receptor systems, only agonist binding to the receptors is mediated through G protein via the formation of L-R-G ternary complex. Agonists promote the ternary complex formation, whereas antagonist binding is independent of G protein regulation and does not promote the high affinity complex formation (22). In addition, detergent solubilization of membranes invariably disrupts the coupling between R and G proteins unless the agonist is prebound. Because, in the absence of high affinity agonist, detergent may have uncoupled the receptor-G protein complex, it is expected that binding of [^{3}H]LTD_4 to the solubilized receptor cannot promote L-R-G ternary complex formation and, thus, cannot be detected by the PEG precipitation method.[1] Nevertheless, the approach of solubilization and characterization of the agonist-labeled LTD_4 receptors has yielded important insights into the molecular and biochemical nature of receptor-ligand-G_i protein interaction. Inasmuch as the antagonist binding to LTD_4 receptor may be independent of G protein regulation, it may be possible to solubilize the *antagonist*-occupied or -unoccupied LTD_4 receptors to measure binding of the high affinity radiolabeled antagonist to the soluble receptors. High affinity radiolabeled antagonists are now being synthesized.

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