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Subcellular Localization of Leukotriene D_4 Receptors in Sheep Tracheal Smooth Muscle

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SUMMARY

The distribution of [^{3}H]leukotriene D_4 ([^{3}H]LTD_4) receptors in subcellular membrane fractions obtained from sheep tracheal smooth muscle was studied. Using differential centrifugation and discontinuous sucrose density gradient centrifugation, the subcellular membranes were separated into six fractions. The [^{3}H]LTD_4 receptor distribution profile in these fractions correlated with markers for the plasma membrane (5′-nucleotidase and alkaline phosphodiesterase) and did not correlate with markers for the mitochondria (cytochrome *c* oxidase and succinate-dependent cytochrome *c* reductase). The dissociation constant (K_d) and maximum number of binding sites (B_{max}) for [^{3}H]LTD_4 binding to the receptors in the crude mixture of membranes (PII) were 0.38 ± 0.2 nM and 77 ± 14 fmol/mg of protein, respectively. The K_d and B_{max} of [^{3}H]LTD_4 binding to the receptors in the plasma membrane-enriched fraction (FII) were 0.40 ± 0.2 nM and 268 ± 46 fmol/mg of protein, respectively. The specificity profile of the [^{3}H]LTD_4 receptors in the plasma membrane-enriched fraction was equivalent to that observed in the crude membrane and correlated with the agonist myotonic activities in the smooth muscle contraction assay system. Furthermore, the binding of [^{3}H]LTD_4 to the plasma membrane receptors was modulated by guanine nucleotides in a manner analogous to that observed in crude membranes, suggesting that agonist interaction with the receptors was regulated by guanine nucleotide binding protein. These results suggest that, in sheep tracheal smooth muscle, the plasma membrane is the primary location of specific LTD_4 receptors.

The peptidoleukotrienes LTC_4, LTD_4, LTE_4 are major components of slow reacting substance of anaphylaxis (1, 2) and are potent smooth muscle spasmogens *in vitro* and *in vivo* (1, 3–5). Studies in our laboratory and others have demonstrated that specific binding sites for LTC_4 and LTD_4 are present in crude membrane fractions derived from a variety of tissues and cells in culture (6–15). It has been shown that LTC_4 binding sites differ from those for LTD_4 and that LTE_4 binds to LTD_4-specific sites (6–8, 10–16). Moreover, LTD_4-specific binding sites in guinea pig and human lung are coupled to a guanine nucleotide-binding protein and are physiologically coupled to contraction, i.e., they are receptors (12–14), and our data suggest that LTD_4 receptors in the guinea pig heart may differ from those in the guinea pig lung (7, 8). In contrast, it is likely that only a fraction of the specific LTC_4 binding sites identified are receptors coupled to smooth muscle contraction (12, 16); in rat liver cytosol, e.g., the bulk of the LTC_4-specific binding sites could be glutathione transferase (17).

Although LTC_4- and LTD_4-specific binding sites have been identified in crude membrane preparations, because of the lipophilicity of the leukotrienes it is conceivable that the receptors for the leukotrienes are located intracellularly rather than in the plasma membrane. In fact, recent reports have shown that specific LTC_4 binding sites are in mitochondrial, microsomal, and plasma membrane preparations (15–18).

LTD_4 receptors have been identified and characterized in guinea pig lung, trachea, and heart, and in human lung (11–14). They have also been identified in sheep tracheal smooth muscle and these studies have confirmed that, in this tissue, LTD_4 binding sites represent receptors physiologically coupled to contraction (current study). Thus, because it is a tissue composed of a relatively homogeneous population of smooth muscle cells that have LTD_4 receptors clearly coupled to a physiologic response, we have studied the subcellular localization of the receptors in sheep tracheal smooth muscle. We have fractionated the tissue into various components, characterized the fractions by using marker enzymes, and determined that LTD_4 receptors are located primarily in the plasma membrane-enriched fraction. We discuss these results in contrast to the recently reported subcellular localization studies on LTC_4 binding sites.

ABBREVIATIONS: LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; LTD_1, (5*S*)-hydroxy-(6*R*)-*S*-1-cysteinylglycyl-(7*Z*)-eicosenoic acid; homo-LTD_1, (6*S*)-hydroxy-(7*R*)-*S*-1-cysteinylglycyl-(8*Z*)-heneicosenoic acid; GppNHp, guanyl-5′-yl-imido-diphosphate.

Materials and Methods

Preparation of sheep tracheal smooth muscle membrane fractions. A simplified scheme of differential centrifugation and discontinuous sucrose gradient fractionation is shown in Fig. 1 (17). The smooth muscle was dissected from the C-opening of the trachea. The luminal epithelium and serosal layers of connective tissue were removed. The remaining tissue contained primarily smooth muscle (greater than 95%) and a small portion (less than 5%) of glandular cells. The smooth muscle (30 to 40 g) was minced with a McIlwain tissue chopper into 1- to 2-mm^3 blocks and the following procedures were performed at 0 to 4°. The minced smooth muscle was homogenized with a Polytron PT-20 tissue grinder for 20 sec, at a setting of 6, in 10 volumes of homogenization buffer [0.25 M sucrose, 10 mM Tris·HCl (pH 7.5), containing EDTA (5 mM), soybean trypsin inhibitor (10 μg/ml), bacitracin (100 μg/ml), benzamidine (100 μM), and phenylmethylsulfonyl fluoride (500 μM)]. Nuclei and unbroken cells were removed by a low speed (1000 × *g* for 10 min) centrifugation. A large fraction of mitochondria in the supernatant was sedimented by centrifugation (8000 × *g* for 10 min) and called PI. The supernatant was centrifuged at 100,000 × *g* for 60 min and the pellet was defined at PII. The PII pellets were resuspended with a Teflon homogenizer in a 65% sucrose solution at a concentration of 20 mg/ml. The PII components were fractionated by discontinuous density sucrose gradients using the buoyant density floatation method. The PII membrane suspension in 65% sucrose solution (1 ml) was overlayed with 3 ml of 50, 40, 30, and 15% sucrose solution in a nitrocellulose SW-41 ultracentrifuge tube. The discontinuous sucrose gradients were centrifuged in a SW-41 rotor for 100,000 × *g* for 16 hr. The membrane fractions F(I), F(II), F(III), and F(IV) at the boundary layers of 15/30, 30/40, 40/50, and 50/65 were collected and labeled. These fractions were diluted with 10 mM Tris (pH 7.5) to 5 times the original volume and centrifuged at 150,000 × *g* for 60 min.

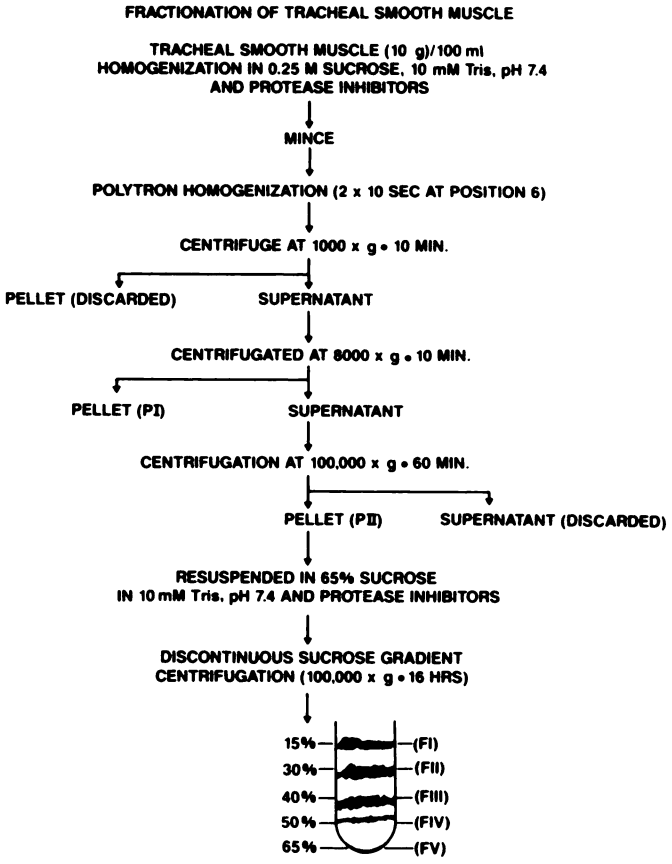

Fig. 1. Fractionation of subcellular membranes from sheep tracheal smooth muscle.

The pellets were resuspended in 10 mM Tris·HCl at a protein concentration of 1 to 2 mg/ml and used for the determination of marker enzyme activity or [^{3}H]LTD_4 receptor binding activity within 24 hr.

Marker enzyme activity determination. The assay procedures for marker enzyme activities have been described previously (17–20). Briefly, (a) as plasma membrane marker enzymes, 5′-nucleotidase and alkaline phosphodiesterase activities were determined at 37°, under optimal conditions, as μmol of phosphate released from 5′-AMP per mg of protein per 20 min and μmol of *p*-nitrophenol released from *p*-nitrophenol-5′-thymidylate per mg of protein per 20 min, respectively; (b) as microsomal marker enzymes, nucleoside diphosphatase and glucose-6-phosphatase activities were determined at 37°, under optimal conditions, as μmol of phosphate released from inosine diphosphate per mg protein per 20 min and μmol of phosphate released from glucose-6-phosphate per mg of protein per 20 min, respectively; and, (c) as mitochondria marker enzymes, succinate dependent cytochrome *c* reductase and cytochrome *c* oxidase activities were measured as the rate of formation of the reduced form of cytochrome *c* (OD_{550}) from succinate per mg of protein per min and the rate of formation of the oxidized form of cytochrome *c* per mg of protein per min, respectively. Under the experimental condition used for each of the marker enzymes, the enzymatic activity was linear with respect to the time of incubation and the concentration of membrane protein. The extent of utilization of the substrate for each enzyme was limited to up to 5%.

Protein concentration determination. The concentrations of protein in each fraction of membranes and PI and PII fractions were determined by the method of Bradford (21). The results obtained were similar to those determined by the method of Lowry *et al.* (22).

LTD_4 receptor binding assay. Binding of [^{3}H]LTD_4 to membrane fractions was performed at 23° in triplicate, in 20 mM Tris·HCl buffer (pH 7.5), in a volume of 0.5 ml, containing 10 mM cysteine, 10 mM glycine, 10 mM $MgCl_2$, 10 mM $CaCl_2$ (standard condition), membrane protein (50 to 200 μg/ml), [^{3}H]LTD_4 (0.04 to 5 nM), and unlabeled LTD_4 (40 nM to 5 μM) or other compounds as noted in the figure legends. Membrane-bound [^{3}H]LTD_4 was separated from unbound [^{3}H]LTD_4 by filtration and washing with 20 ml of 10 mM Tris·HCl (pH 7.5, at 0°) under reduced pressure. The filter paper discs were transferred to a scintillation vial containing 5 ml of Ready-Solv. The radioactivity was determined in a Beckman LS-7800 liquid scintillation counter, with an efficiency of 30–40%. Total and nonspecific binding of [^{3}H]LTD_4 to the membranes were defined as the amount of [^{3}H]LTD_4 bound to the membrane in the absence or presence of 1000-fold unlabeled LTD_4, respectively. The percentage of specific binding and nonspecific binding was variable, depending upon the membrane fractions used for binding studies. At the K_d concentration of [^{3}H]LTD_4 (0.4 nM), the nonspecific bindings were 8 and 30% of total binding for F(II) and PII membranes, respectively. The specific bindings were 90 and 70% for F(II) and PII membranes, respectively. The specific binding of [^{3}H]LTD_4 in every fraction of membranes was linearly dependent upon the concentration of protein, from 10 μg/ml up to 300 μg/ml. All the values were expressed as mean ± standard error, determined from triplicate samples. Averaged results from three experiments are shown, with the exception of those shown in Fig. 2 and Table II, in which the representative experimental result from a single experiment is shown.

Sheep tracheal smooth muscle contraction. Sheep tracheae were carefully trimmed to remove fatty and connective tissues. Tracheae were cut into rings and placed in a 10-ml water-jacketed bath and connected via silk sutures to a Grass model FT03C force-displacement transducer (Grass Instrument Co., Quincy, MA) for recording isometric tension. The tracheal rings were bathed in modified Krebs-Henseleit (KRH) solution containing the following composition (mM): NaCl, 118; KCl, 4.6; $MgSO_4$, 0.5; $CaCl_2$, 1.8; $NaHCO_3$, 24.9; KH_2PO_4, 1.0; and glucose, 11.1. The tracheal rings were maintained at 37° continually aerated with 95% O_2/5% CO_2, placed under 2 g of passive tension, and equilibrated for 60 min. Before the tissues were challenged with leukotrienes, they were washed with 15 ml of KRH solution and treated

with 1 μM meclofenamic acid for 45 min to inhibit the release of prostanoids. Leukotriene was added to the tissue bath in a cumulative close-response manner when the contraction response reached a plateau. At the end of the experiment, a maximally effective concentration of carbachol (100 μM) was added to the tissue to record the maximal contraction. This response was used as the 100% reference point.

Reagents. The [^{3}H]LTD$_4$ (40 Ci/mmol) was purchased from New England Nuclear Co (Boston, MA). All the synthetic leukotrienes (23) and analogs were supplied by Dr. J. Gleason (Medicinal Chemistry Departments, Smith Kline & French Laboratories). All the buffers, salts, and substrates for marker enzymes were obtained from Sigma Chemical Co (St. Louis, MO).

Results

In sheep tracheal smooth muscle tissue bath preparations, LTD$_4$ induced potent and prolonged contractile effect. The 50% concentration (ED$_{50}$) inducing maximal contractile effect (compared with that induced by 100 μM of carbachol) was 14 ± 5 nM. The maximal contraction force was 37 ± 5% of that induced by carbachol (Fig. 2, Table 1). (5*R*,6*S*)-LTD$_4$ also induced smooth muscle contraction; the ED$_{50}$ was 560 ± 80 nM and the maximal contraction was approximately 23 ± 5% of that induced by carbachol. LTE$_4$, on the other hand, appeared to function as a partial agonist that has, maximally, 7 ± 4% contractile activity when compared with that induced by carbachol (Fig. 2).

Fig. 3 shows the distribution of marker enzymes of [^{3}H]LTD$_4$ receptors in subcellular fractions derived from sheep tracheal smooth muscle. Fraction PI was composed primarily of mitochondria (cytrochrome *c* oxidase and succinate-dependent cytochrome *c* reductase). PII, the pellet obtained after centrifugation at 100,000 × *g* for 60 min, displayed a number of marker enzyme activities and was further fractionated into fractions F(I) to F(IV). F(I) was enriched in microsomal marker enzymes (nucleoside diphosphatase and glucose-6-phosphatase) and to a lesser extent in plasma membrane marker enzymes (5′-nucleotidase and alkaline phosphodiesterase). F(II) was highly enriched in plasma membrane marker enzymes and slightly enriched in microsomal enzymes. F(III) was enriched in mitochondrial marker enzymes. F(IV) was also enriched in mitochondrial marker enzymes but the total activity was low.

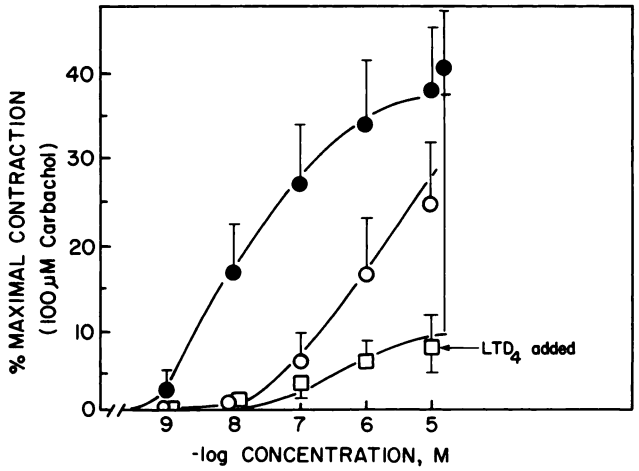

Fig. 2. Contraction of sheep tracheal smooth muscle induced by leukotrienes. Sheep tracheal smooth muscle rings (n = 6) were prepared as described in Materials and Methods. Increasing concentrations of LTD$_4$ (●), LTE$_4$ (□), and (5*R*,6*S*)-LTD$_4$ (○) were added in the tissue bath. Contractile force was recorded and shown. In the experiments for LTE$_4$ a large dose (10 μM) of LTD$_4$ was added to the rings that were previously exposed to 100 μM LTE$_4$, to elicit further contraction.

TABLE 1

Smooth muscle contractile activity of peptide-leukotrienes

Compound	ED$_{50}$[a]	Maximal contraction[b]
	nM	%
LTD$_4$	14 ± 5	37 ± 5
LTE$_4$	ND[c]	7 ± 4
(5*R*,6*S*)-LTD$_4$	560 ± 80	23 ± 5

[a] ED$_{50}$ concentration required to elicit 50% contractile response induced by 100 μM methacoline.
[b] Maximal extent of contractile response of the agonist (100 μM) relative to that induced by 100 μM carbachol.
[c] Not determined.

Table 2 summarizes the recoveries of protein and [^{3}H]LTD$_4$-specific binding sites in each fraction and ratios of [^{3}H]LTD$_4$-specific binding to mg of protein recovered in each fraction. The recoveries of marker enzymes ranged from 45 to 95% depending on the enzyme (see legend to Fig. 3). PI and PII contained 39 ± 9% and 61 ± 8%, respectively, (three experiments) of total membrane protein in the post-nuclear supernatant that can be sedimented by ultracentrifugation (100,000 × *g* for 60 min). Of the total protein in PII, 56 ± 9% was recovered in the discontinuous sucrose density gradient [fractions F(I)–F(IV)]. Greater than 90 ± 5% of the total [^{3}H]LTD$_4$ receptor binding was present in PII and, of this, 81 ± 10% (three experiments) was recovered in F(I)–F(IV) after discontinuous sucrose density centrifugation. F(II) contained 42 ± 7% of the total [^{3}H]LTD$_4$ specific binding activity and the receptor-specific activity (fmol of [^{3}H]LTD$_4$ binding/mg of protein) was enriched 5-fold in this fraction when compared with starting (PII) membrane protein.

Table 3 further demonstrates that the [^{3}H]LTD$_4$ specific binding sites are preferentially located in the plasma membranes, inasmuch as the coefficient of correlation of [^{3}H]LTD$_4$ specific binding activity with the two plasma membrane marker enzyme activities was 0.98 and 0.99. In contrast, coefficients of correlation with markers for microsomes and mitochondria were significantly less (Fig. 4).

To further characterize the [^{3}H]LTD$_4$ specific binding in the crude membrane fraction (PII) and plasma membrane-enriched fraction F(II), we have performed saturation experiments, using F(II) or PII membranes and [^{3}H]LTD$_4$ to determine the maximal binding sites (B_{max}) and the dissociation constant (K_d) for [^{3}H]LTD$_4$ binding to the specific sites. The results showed (Fig. 5) that [^{3}H]LTD$_4$ bound to the specific sites in PII and F(II) membrane in a concentration-dependent manner and reached a plateau level of 80 and 270 fmol/mg of protein, respectively. The Scatchard transformation of these data (Fig. 5, *insets*) yielded linear plots, indicating a single class of saturable specific binding sites for [^{3}H]LTD$_4$ in these two fractions of membrane protein. The K_d and B_{max} values for the [^{3}H]LTD$_4$ specific binding in F(II) and PII membrane fractions were also determined using a computer-based best fit analysis (24). The K_d and B_{max} for [^{3}H]LTD$_4$ specific binding in PII were 0.38 ± 0.2 nM and 77 ± 14 fmol/mg, respectively. The K_d and B_{max} for the F(II) membrane were 0.40 ± 0.2 nM and 268 ± 46 fmol/mg, respectively. These results indicated that the B_{max} of [^{3}H]LTD$_4$ binding sites in F(II) was 3.5-fold that in PII membrane whereas the affinity of binding of the radioligand was equal in these two fractions. The binding of [^{3}H]LTD$_4$ to the nonspecific

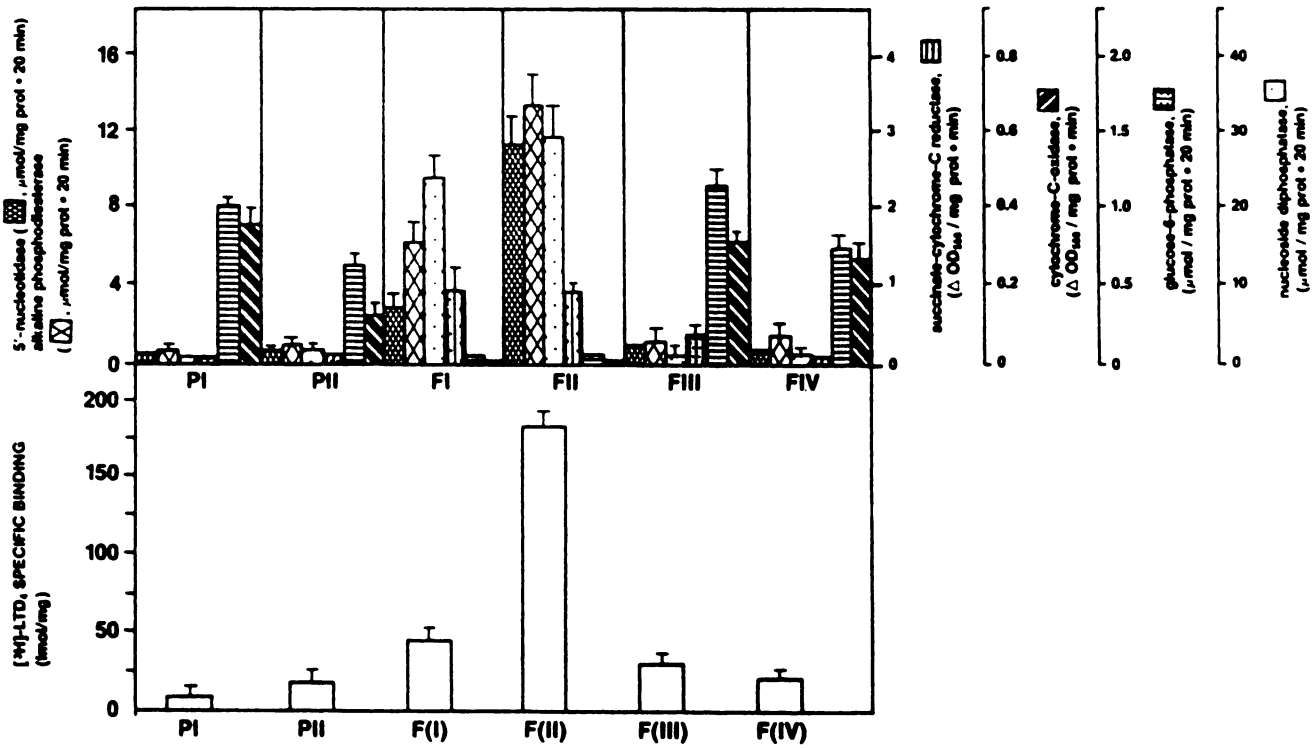

Fig. 3. Distribution of marker enzymes and [^{3}H]LTD_4 specific binding sites in sheep smooth muscle subcellular membrane fractions. The values shown are mean ± standard error obtained from a representative experiment of three. The PI and PII fractions were obtained by differential centrifugation of Polytron-homogenized smooth muscle. The F(I), F(II), F(III), and F(IV) fractions were obtained by discontinuous sucrose density gradients as shown in Fig. 1. The specific activity of the marker enzymes and [^{3}H]LTD_4 receptor binding were define in Materials and Methods. The average recoveries (three experiments) of various marker enzymes were as follows: 5′-nucleotidase, 95%; alkaline phosphodiesterase, 72%; glucose-6-phosphatase, 86%; nucleoside diphosphatase, 80%; cytochrome *c* oxidase, 93%; and succinate-dependent cytochrome *c* reductase, 71%.

TABLE 2

Recovery of Membrane Protein and [^{3}H]-LTD_4 Receptors in Sheep Tracheal Smooth Muscle Membrane Fractionation

Results from a representative experiment (using 15 g of sheep tracheal smooth muscle) are shown. The recovery of protein and receptor binding activity of FI to FIV fractions are based on P(II) membrane protein as starting material.

Membrane Fraction	Protein recovery		[^{3}H]LTD_4 receptor recovery		[^{3}H]LTD_4 Receptor
	mg	%	*fmol*	%	*fmol/mg of protein*
P(I)	11.2	32	91 ± 10	8	8.1
P(II)	24	68	1014 ± 52	92	42.3
F(I)	0.7	3	65 ± 8	6.4	92.9
F(II)	1.8	7.5	392 ± 29	39	217.8
F(III)	7.0	29.2	320 ± 19	32	45.8
F(IV)	4.7	20	135 ± 7	13	28.7
Total	14.2	59.7	912	90.4	

TABLE 3

Correlation between LTD_4 specific binding activity and marker enzyme activities in subcellular fractions

The [^{3}H]LTD_4 specific binding activity in each fraction of membranes was determined using 0.8 nM [^{3}H]LTD_4, under the optimal conditions for binding. The marker enzyme activities for each of the six membrane fractions from each experiment was determined as described in Fig. 2, and averaged results from three experiments are shown.

	Linear correlation coefficient, γ
Plasma membrane marker enzymes	
5′-Nucleotidase	0.98 ± 0.02
Alkaline phosphodiesterase	0.97 ± 0.02
Microsomal marker enzymes	
Nucleoside diphosphatase	0.82 ± 0.03
Glucose-6-phosphatase	0.68 ± 0.18
Mitochondrial marker enzymes	
Succinate cytochrome *c* reductase	0.51 ± 0.12
Cytochrome *c* oxidase	0.52 ± 0.14

component in F(II) was considerably lower than that in the PII fraction.

To characterize the specificity of the [^{3}H]LTD_4 binding in F(II) and PII membranes, radioligand competition studies were performed in the presence or absence of several LTD analogs and the antagonist FPL 55712 (7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroperoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid). The competition curves of each of these compounds, in the F(II) membrane [^{3}H]LTD_4 specific binding sites, were parallel (Fig. 6), indicating that these agents bound to the LTD_4 specific sites with different affinities. The binding affinities for LTD_4, (5*R*,6*S*)-LTD_4, (5*S*,6*R*)-LTD_1, (5*R*,6*S*)-LTD_1, (6*S*,7*R*)-homo-LTD_1, (6*R*,7*S*)-homo-LTD_1, and FPL 55712 were 0.6, 90, 20, 720, 180, 2050, and 800 nM, respectively. The rank order potency of binding for these compounds is equivalent to that reported in guinea pig lung membranes (12) and that derived from sheep tracheal smooth muscle contraction studies (Fig. 2 and Table 1). In addition, the rank order potency of binding for these compounds in F(II) membrane LTD_4-specific sites was the same as those in PII membranes (results not shown). The coefficient of correlation for the binding affinities of these compounds in the two fractions was 0.98. Thus, these results demonstrate that the LTD_4 spe-

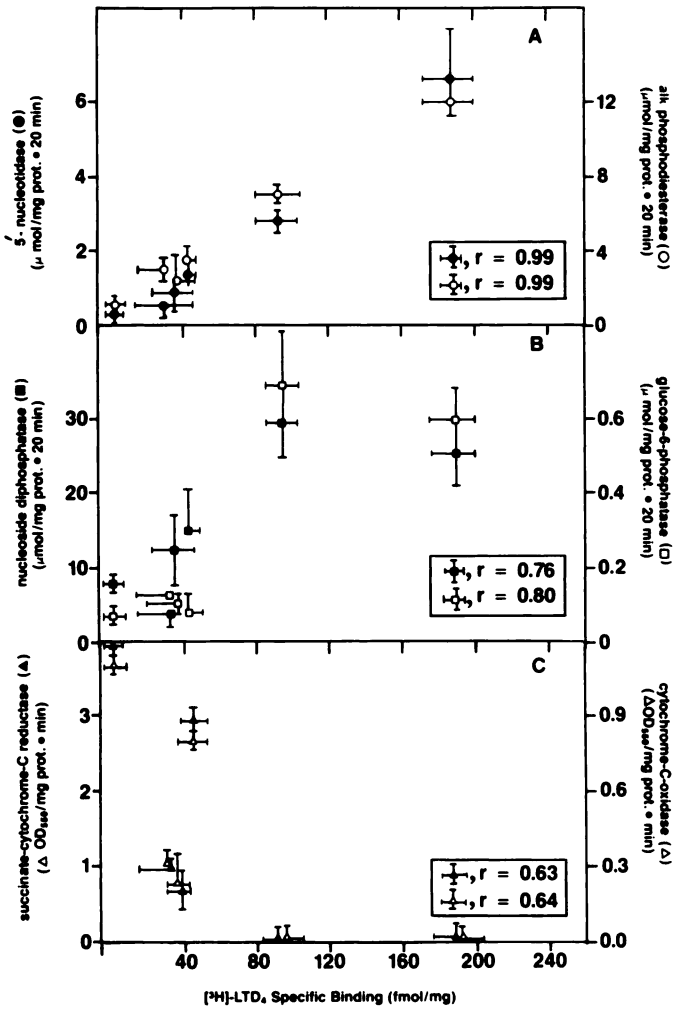

Fig. 4. Correlation between [^{3}H]LTD$_4$ specific binding and membrane marker enzymes. The LTD$_4$ specific binding activity in each of the subcellular membrane fractions was determined using 0.8 nM [^{3}H]LTD$_4$ under standard conditions. The marker enzyme activities were taken from the average of three experiments. A, Plasma membrane marker enzyme 5′-nucleotidase (●) and alkaline phosphodiesterase (○); B, microsomal marker enzymes glucose-6-phosphatase (□) and nucleoside diphosphatase (■); and C, mitochondrial marker enzyme succinate-dependent cytochrome *c* reductase (▲) and cytochrome *c* oxidase (△).

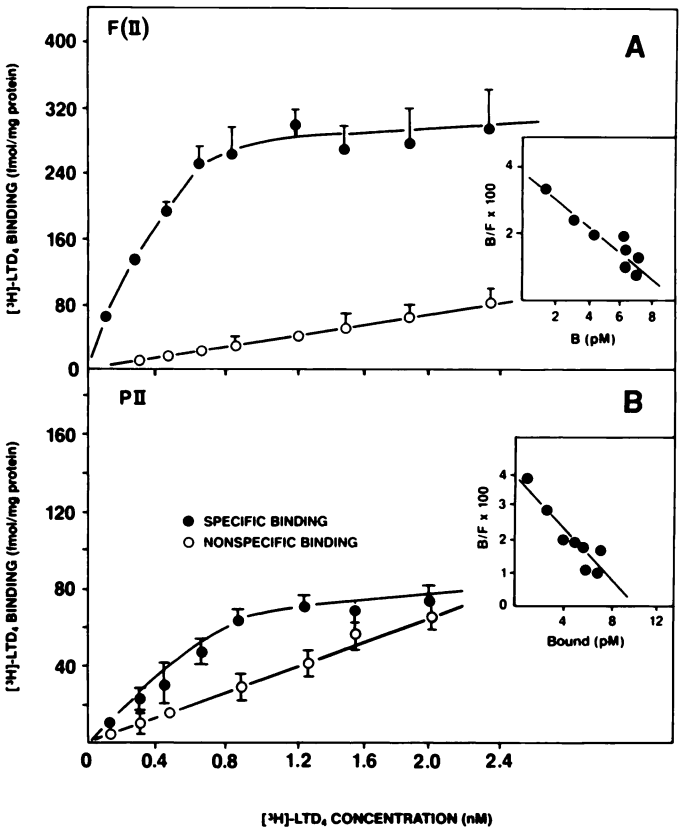

Fig. 5. Saturation binding of [^{3}H]LTD$_4$ to receptors in PII and F(II) membrane fractions. [^{3}H]LTD$_4$ (from 0.05 to 2.5 nM) was incubated in 20 mM Tris·HCl buffer containing 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 50 μg/ml of F(II) membrane protein (A) or 150 μg/ml of PII membrane protein (B) at 23° for 40 min to determine total binding. Nonspecific binding of [^{3}H]LTD$_4$ (○) was determined similarly except that the LTD$_4$ was included at concentrations that were 1000-fold that of [^{3}H]LTD$_4$ under identical conditions. Specific binding (●) was determined by subtracting the nonspecific binding from the total binding. *Inset*, Scatchard plot of the saturation binding data.

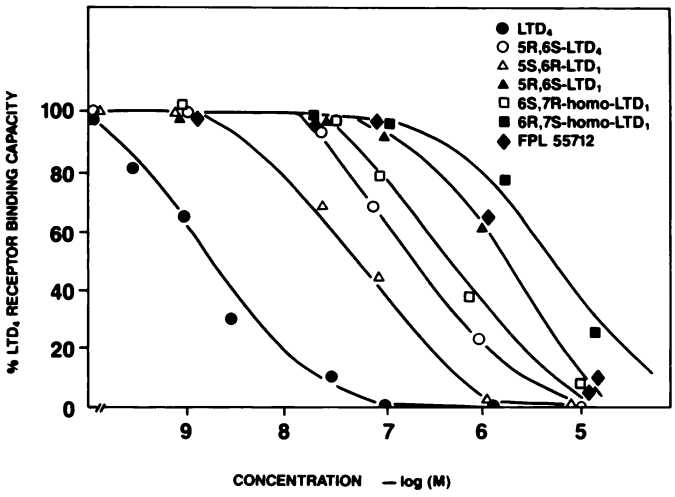

Fig. 6. Competition of [^{3}H]LTD$_4$ receptor binding by leukotriene analogs. The F(II) membrane protein (50 μg/ml) was incubated with varying concentrations of leukotriene agonists and the antagonist FPL 55712, [^{3}H]LTD$_4$ (0.8 nM) in a final volume of 0.5 ml under the standard conditions described in Materials and Methods. Complete inhibition (0% of specific binding) was defined as the amount of [^{3}H]LTD$_4$ specific binding in the presence of 0.8 μM LTD$_4$.

cific binding sites in F(II) and PII preparations are nearly identical. Specific binding of [^{3}H]LTD$_4$ to the F(II) membrane was inhibited when GTP and GppNHp were presented in the incubation mixtures (Table 4), indicating the binding of [^{3}H]LTD$_4$ to the specific sites was regulated by guanine nucleotide binding protein as shown previously in crude membrane preparations (11–13, 15). In preliminary experiments, pertussis toxins (islet-activating protein) inhibited the calcium mobilization and phosphoinositide metabolism in primary culture smooth muscle cells derived from sheep trachea, indicating that a pertussis toxin-sensitive guanine nucleotide binding protein is critically involved in regulation of LTD$_4$ receptors (results not shown).

Discussion

Several subcellular membrane fractionation studies (17–19) have provided evidence that membrane-bound 5′-nucleotidase,

TABLE 4

Characteristics of LTD_4 receptors in subcellular membrane fractions of sheep smooth muscle

The numbers indicate the pooled results from three experiments.

	PII	F(II)
K_d (nM)	0.38 ± 0.2	0.40 ± 0.2
B_{max} (fmol/mg)	77 ± 14	268 ± 40
Guanine nucleotide regulation		
GTP, IC_{50} concentration (μM)	400 ± 50	450 ± 30
GppNHp, IC_{50} concentration (μM)	13 ± 4	20 ± 8
Rank order specificity of agonists binding	$LTD_4 > LTE_4 > LTD_1 >$ homo-LTD_1	$LTD_4 > LTE_4 > LTD_1 >$ homo-LTD_1

alkaline phosphodiesterase, and Mg-ATPase activities co-purify during differential centrifugation and isopycnic sucrose centrifugation. In uterine smooth muscle, these marker enzymes have been shown to be preferentially localized in plasma membranes and to co-purify with the oxytocin receptors (18). In addition, studies using detergent (digitonin) to extract the lipids of the subcellular membranes demonstrated that the marker enzymes 5′-nucleotidase, Mg-ATPase, and alkaline phosphodiesterase can be shifted to higher density due to the higher cholesterol content of the smooth muscle plasma membrane (28, 29). Thus, these enzymes, used in this study, are reliable markers for plasma membranes in smooth muscle cells. Likewise, the two enzymes, cytochrome *c* oxidase and succinate dependent-cytochrome *c* reductase, used for markers of mitochondria, have been well established as extremely reliable markers in dog tracheal and other types of smooth muscle (18–20, 26). Microsomal membranes are a heterogenous mixture of membrane fragments and vesicles that usually contain rough and smooth endoplasmic reticula and possibly membranes from the Golgi apparatus and lysosomal membranes. Due to the extreme variability of the buoyant density of each of these membrane particles, we did not attempt to further fractionate the microsomal membranes. Glucose-6-phosphatase and inosine diphosphatase have been shown to be reliable marker enzymes for the endoplasmic reticula (18–20, 27). Currently, marker enzymes for microsomal membranes other than endoplasmic reticula have not been well defined, particularly for ovine tracheal smooth muscle. Therefore, the possibility of the presence of LTD_4 receptors in the microsomal membranes still exists. Alternatively, the presence of LTD_4 receptors in microsomes could simply be due to incomplete separation of plasma membranes in the microsomal membrane fraction. Data provided in the paper indicate that the preponderance of LTD_4 receptors co-purify or co-enrich with the plasma membrane marker enzymes, thus suggesting that the most likely location for the bulk of the LTD_4 receptors in ovine tracheal smooth muscle is in the plasma membrane.

In previous studies, we have shown that [^{3}H]LTD_4 binding sites are physiologically coupled to contraction in guinea pig lung and are receptors (12). In the present study, we have demonstrated that greater than 39% of the [^{3}H]LTD_4 binding sites are located in the plasma membrane fraction of sheep tracheal smooth muscle cells. Moreover, the results shown in this study also demonstrate that these binding sites are receptors by several criteria; the binding sites in F(II) are highly specific and saturable and the K_d values for [^{3}H]LTD_4 binding in F(II) and in crude membrane fractions are nearly identical (K_d = 0.4 ± 0.2 nM and K_d = 0.38 ± 0.2 nM, respectively). Furthermore, the binding of [^{3}H]LTD_4 to specific sites in F(II) was highly stereoselective and the binding was modulated by guanine nucleotides in a fashion analogous to that observed in crude lung membranes (11–13, 15). Finally, the rank order of potencies of various agonists and antagonist binding to the specific sites in F(II) was equal to that in crude membranes and correlated with smooth muscle contractile activities (12). Thus, we conclude that the [^{3}H]LTD_4 specific binding sites on F(II) membranes represent the biochemically and pharmacologically important receptors. The specific LTD_4 receptors are most likely localized primarily in the plasma membrane of smooth muscle cells.

The observations reported here for LTD_4 receptor subcellular localization differ from those recently reported for LTC_4 binding sites (15–18). However, we have previously shown that LTD_4 receptors are biochemically and pharmacologically different from LTC_4 specific binding sites, and only a fraction of the LTC_4 binding sites in guinea pig lung are likely to be receptors coupled to smooth muscle contraction (12, 17). Thus, many of the specific binding sites identified in the LTC_4 binding study may not be receptors coupled to the contraction of smooth muscle. More detailed studies are required to determine whether LTC_4 receptors are localized to plasma membranes or are in other subcellular membranes.

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