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Characterization of Guinea Pig Myocardial Leukotriene C_4 Binding Sites

Regulation by Cations and Sulfhydryl-Directed Reagents

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SUMMARY

Using [^{3}H]leukotriene C_4 (LTC_4) and radioligand-binding techniques, specific leukotriene C_4 binding sites have been identified in membranes derived from guinea pig ventricular myocardium. High performance liquid chromatography analyses indicated that, in the presence of the γ-glutamyl transpeptidase inhibitor L-serine-borate (80 mM), less than 2% of membrane-bound [^{3}H]LTC_4 was converted at 20° to [^{3}H]leukotriene D_4 or [^{3}H] leukotriene E_4. The specific binding of 4 nM [^{3}H]LTC_4, in the presence of 80 mM L-serine-borate, reached a stable steady state within 15 min at 20° (pH 7.5). A monophasic Scatchard plot of saturation binding data yielded a dissociation constant (K_d) of 27.5 ± 6.0 nM and a maximum number of binding sites (B_{max}) of 19.9 ± 5.2 pmol/mg of membrane protein. Competition binding studies of [^{3}H]LTC_4 with synthetic leukotriene C_4, leukotriene D_4, and leukotriene E_4 and the putative peptidoleukotriene antagonists FPL 55712, SKF 88046, and 4*R*-hydroxy-5*S*-1-cysteinylglycine-6*Z*-nonadecanoic acid revealed an order of potency of leukotriene C_4 >> 4*R*-hydroxy-5*S*-1-cysteinylglycine-6*Z*-nonadecanoic acid > SKF 88046 > LTE_4 > LTD_4 > FPL 55712. The specific [^{3}H]LTC_4 binding was stimulated by the divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+} and to a lesser degree by the monovalent cations Na^+, K^+, Li^+, and NH_4^+. $CaCl_2$ (3 mM) and NaCl (150 mM) stimulated the LTC_4 binding by increasing the B_{max} to 42.6 ± 5.9 and 35.0 ± 2.0 pmol/mg, respectively, but had minimal effects on K_d. Pretreatment of the heart membranes with the sulfhydryl reagent *N*-ethylmaleimide decreased the specific [^{3}H]LTC_4 binding in a concentration-dependent manner. The *N*-ethylmaleimide-induced inactivation of [^{3}H]LTC_4 binding sites was protected by occupation of the binding site with the agonist leukotriene C_4, but no protection was observed with the antagonist SKF 88046. Scatchard analyses of saturation isotherms indicated that 30 μM *N*-ethylmaleimide pretreatment reduced the B_{max} of the [^{3}H]LTC_4 binding to 8.2 ± 3.1 pmol/mg with minimal effects on K_d. The data provide direct biochemical evidence for specific [^{3}H]LTC_4 binding sites in the guinea pig heart membranes. The [^{3}H]LTC_4 binding sites appear to be modulated by divalent and monovalent cations and free sulfhydryl group(s) may be associated with the agonist-binding site. The results suggest that the physiological effects of the leukotrienes on the guinea pig heart may be mediated through membrane-bound receptors.

INTRODUCTION

The major active constituents of the slow reacting substance of anaphylaxis have been identified (1) as the peptidoleukotrienes LTC_4,[1] LTD_4, and LTE_4. The peptidoleukotrienes are derivatives of arachidonic acid which contain a conjugated triene chromophore and hydroxyl and peptide substituents (2). LTC_4 and LTD_4 have been shown to induce contractions of guinea pig trachea and lung parenchymal strips (3), guinea pig uterus (4), and pulmonary artery (5) at low concentrations *in vitro*. These agents demonstrate significant bronchoconstrictive activity *in vivo* in guinea pig (3) and *Macaca irus* (6). Consequently, the peptidoleukotrienes are considered to be one of the major mediators of allergic asthma and immediate-type hypersensitivity reactions in the pulmonary system.

Recent pharmacological evidence has shown that LTC_4 and LTD_4 induce a profound depressant effect on myocardial contractility (7). Additional evidence has suggested that the peptidoleukotrienes may be involved in

[1] The abbreviations used are: LTC_4, leukotriene C_4 (other forms are analogous); Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; HPLC, high performance liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NEM, *N*-ethylmaleimide; PHMB, *p*-hydroxymercuribenzoate; 4*R*,5*S*,6*Z*-*nor*-LTD_1, 4*R*-hydroxy-5*S*-1-cysteinylglycine-6*Z*-nonadecanoic acid; SRS-A, slow reacting substance of anaphylaxis.

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immediate-type hypersensitivity reactions in the heart (8). The clinical manifestations of these interactions include cardiac anaphylactic shock (9), sudden cardiac death (10), and adverse drug reactions (11).

In vitro studies in the guinea pig (12) and *in situ* evidence from the dog (13) and pig (14) have suggested that the negative inotropic effects of the peptidoleukotrienes may be mediated in part by the synthesis of cyclooxygenase products (12) or a result of a reduction in coronary flow (12–14). The latter possibility is important when considering that negative inotropic effects in the heart are associated with small decreases in coronary blood flow (15, 16). In addition, studies in several species (7, 13, 14, 17, 18) have indicated that the peptidoleukotrienes decreased myocardial contractility via other receptor-mediated processes that were independent of prostanoid synthesis or alterations in coronary blood flow. A recent study in the guinea pig heart by Burke *et al.* (19) has suggested that LTC_4-induced effects in the coronary vasculature versus the ventricular myocardium may be mediated by separate receptors.

The intent of the present study was to identify specific [^{3}H]LTC_4 binding sites in guinea pig ventricular myocardial membranes. Thus, the interactions of [^{3}H]LTC_4 with its membrane-bound binding sites were studied. Furthermore, the dynamic interactions of the [^{3}H]LTC_4 binding site with regulatory factors such as sulfhydryl-directed reagents and cations were characterized.

MATERIALS AND METHODS

Materials. LTD_4 diastereoisomers (greater than 95% 5*S*,6*R*-LTD_4), LTC_4, and LTE_4 (1:1 diastereoisomeric mixtures of 5*S*,6*R*- and 5*R*,6*S*-[14,15-^{3}H]LTC_4 and LTE_4) were synthesized as described (20) and generously provided by Dr. John Gleason (Department of Medicinal Chemistry, Smith Kline and French). FPL 55712 was kindly provided by Fisons Pharmaceuticals. [14,15-^{3}H]LTC_4 (40–41 Ci/mmol; 98% 5*S*,6*R*-[^{3}H]LTC_4 diastereoisomer) was from New England Nuclear. Other chemicals were of the highest grades available from commercial sources.

Membrane preparations. Male albino guinea pigs were sacrificed by cervical dislocation and exsanguination. The whole hearts were quickly removed, frozen in liquid nitrogen, and stored at −70°. Whole hearts were thawed in buffer A containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.5, 20°), 5 mM EDTA, 10 μM phenylmethylsulfonyl fluoride, 15 μg/ml soybean trypsin inhibitor, 100 μg/ml bacitracin, and 10 μg/ml benzamidine. The right and left ventricles were isolated from the thawed hearts.

The ventricles were blotted dry, weighed, minced well with scissors, and Polytron-homogenized (twice for 10 sec on a setting of 6) in 4 volumes of buffer A. The homogenate was centrifuged at 1,300 × *g* for 10 min. The supernatant was saved and the pellet was homogenized and centrifuged as above. The supernatants were combined and filtered through eight layers of cotton gauze. The filtrate was centrifuged at 155,000 × *g* for 40 min and the supernatant was discarded. The pellet was washed with and resuspended in 10 volumes of buffer B containing 20 mM Tris-HCl (pH 7.5, 20°), 100 mM NaCl, 5 mM EDTA, 5 μg/ml benzamidine, 50 μg/ml bacitracin, 5 μg/ml soybean trypsin inhibitor, and 10 μM phenylmethylsulfonyl fluoride. The resuspended pellet was centrifuged at 155,000 × *g* for 40 min. The supernatant was discarded and the pellet was resuspended in 1 volume of buffer B and used within 12 hr. For binding studies involving the effects of cations (see below), the pellet was resuspended in 1 volume of 10 mM Tris-HCl (pH 7.5, 20°), 50 μg/ml bacitracin, 5 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor, and 10 μM phenylmethylsulfonyl fluoride and then diluted with the appropriate buffer for the binding assay. When necessary, buffers were titrated to pH 7.5 with tetramethylammonium hydroxide.

Binding assay. Unless otherwise stated, assays were performed in a total volume of 1 ml at 20° in buffer C containing 20 mM Tris (pH 7.5, 20°), 80 mM L-serine-borate, 1 mM dithiothreitol, 10 mM cysteine, 10 mM glycine, [^{3}H]LTC_4, and unlabeled LTC_4 as indicated. The free acid form of borate was used in the assay buffer and the pH was adjusted to 7.5 with tetramethylammonium hydroxide. Assays were conducted in siliconized glass tubes under an argon-enriched atmosphere and terminated by filtration at 0° under reduced pressure on Whatman GF/C filters. The filters were rinsed four times under reduced pressure with 4 ml of ice-cold 20 mM Tris-HCl (pH 7.5, 0°) and 100 mM NaCl. The filters were dried and radioactivity remaining on the filters was determined by scintillation spectrometry. Nonspecific binding was determined by the addition of a 1000-fold excess of unlabeled LTC_4 to a separate set of tubes. Nonspecific binding was subtracted from total binding to membranes and filters to determine specific binding. Specific binding represented 85 to 90% of the total binding.

Membrane pretreatment. Guinea pig heart membranes were prepared as described above. Membranes (5 mg/ml) were incubated with the indicated sulfhydryl reagent in buffer D containing 20 mM Tris (pH 7.5), 80 mM L-serine-borate, and 10 mM glycine in a total volume of 0.5 ml for 30 min at 20° and then diluted to 10.5 ml with ice-cold (4°) buffer D. The reaction mixture was centrifuged at 155,000 × *g* for 40 min at 4° and the pellet was resuspended in 1 ml of 10 mM Tris (pH 7.5). Protection experiments with agonists or antagonists included a 45-min preincubation with the indicated agonist or antagonist under identical conditions described above. The indicated sulfhydryl reagent was added subsequently and was incubated, centrifuged, and resuspended as described for treated membranes.

HPLC analyses. Membranes from guinea pig heart were prepared as described above. The membrane preparations and [^{3}H]LTC_4 were incubated in a final volume of 2 ml under identical conditions described above for binding assays. The reaction mixture was incubated at 20° for 45 min and terminated by vacuum filtration at 0° through a Whatman GF/C filter held in a single 24-mm borosilicate glass filter holder. The filter was washed immediately with 2 ml of ice-cold wash buffer. The filtrate was collected and filtered, and 200 μl were analyzed directly by HPLC. The GF/C filter was immediately extracted with 2 ml of methanol:H_2O (1:1). The methanol was evaporated under argon, the remaining aqueous solution was filtered, and a 200-μl aliquot was subjected to HPLC analysis. Aliquots of each fraction were taken before HPLC analysis to determine total radioactivity of the samples. Unlabeled LTC_4 (400 pmol), LTD_4 (400 pmol), and LTE_4 (600 pmol) were added to each aliquot to determine the retention times of the individual leukotrienes. In the absence of added leukotriene standards, extracts of membrane protein (1.0 to 2.0 mg/ml) did not contain endogenous material with retention times predetermined for the leukotrienes which suggests that the heart membranes did not contain levels of endogenous LTC_4, LTD_4, and LTE_4 that might interfere with determinations of K_d or K_i values. Recovery of total radioactivity from the filter was determined to be greater than 95%.

A reverse phase C_{18} Ultrasphere ODS column (0.46 × 25 cm, 5 μm) was used at a flow rate of 1.0 ml/min with a mobile phase of acetonitrile/10 mM phosphate (pH 7.0) for the separation of LTC_4, LTD_4, and LTE_4. The radioactivity of 0.50-ml fractions was determined by scintillation spectrometry.

RESULTS

HPLC analyses. To characterize the [^{3}H]LTC_4 binding sites, assay conditions were developed to minimize the bioconversion of [^{3}H]LTC_4 to other active peptidoleukotrienes. [^{3}H]LTC_4 was incubated with myocardial membrane protein under a variety of conditions to determine the effects of temperature and buffer composition and then analyzed by reverse phase HPLC (Table 1). Preliminary experiments using HPLC indicated that

TABLE 1

Modification of [^{3}H]LTC$_4$ bioconversion in guinea pig heart membrane and soluble fractions by cysteine and serine-borate

Heart membranes were prepared as described under Materials and Methods. The membranes (100 μg/ml) were incubated in a total volume of 2 ml for 45 min under the conditions described below. Assay conditions, sample preparations, and HPLC protocols are described in Fig. 1. Control experiments indicated that 89 to 91% of recovered radioactivity co-eluted with unlabeled LTC$_4$ when [^{3}H]LTC$_4$ *alone* was placed on the column. The data are expressed as percentages of the radioactivity injected into the column and are a representative example of three separate experiments. The amount of ^{3}H-material injected onto the HPLC (100%) was 3000 to 5000 cpm and 15,000 to 20,000 cpm for membrane and soluble fractions, respectively.

Condition	Soluble			Membrane		
	LTC$_4$	LTD$_4$	LTE$_4$	LTC$_4$	LTD$_4$	LTE$_4$
		%			%	
20 mM Tris, 0°, 1 mM DTT[a]	65.4	28.5	0.7	70.1	22.7	0.3
20 mM Tris, 20°, 1 mM DTT	51.4	38.5	0.9	58.5	33.6	0.4
20 mM Tris, 20°, 1 mM DTT, 10 mM glycine	52.2	37.2	0.2	59.1	31.7	0.2
20 mM Tris, 20°, 1 mM DTT, 10 mM glycine, 10 mM cysteine	55.4	36.6	0.1	57.2	30.9	0.1
20 mM Tris, 20°, 1 mM DTT, 10 mM glycine, 10 mM cysteine, 20 mM L-serine-borate	69.8	21.2	0.1	81.3	10.1	<0.1
20 mM Tris, 20°, 1 mM DTT, 10 mM glycine, 10 mM cysteine, 40 mM L-serine-borate	71.7	10.4	<0.1	88.2	2.2	<0.1
20 mM Tris, 20°, 1 mM DTT, 10 mM glycine, 10 mM cysteine, 80 mM L-serine-borate	85.7	3.3	<0.1	90.1	1.1	<0.1

[a] DTT, dithiothreitol.

20 to 30% of the total radioactivity in the filtrate fraction was a decomposition product that eluted at the solvent front (results not shown). The chemical nature of the eluate is presently unknown but its formation was time-dependent. Its formation was minimized if the samples were injected onto the HPLC soon after extraction. The eluate was formed in the absence of membrane protein and was sometimes detected in stock [^{3}H]LTC$_4$ samples.

In the presence of 20 mM Tris (pH 7.5) for 45-min incubation at 0°, greater than 20% of recovered radioactivity was converted to [^{3}H]LTD$_4$ both in the supernatant (filtrate) and membrane-bound fractions. This result is in contrast to the findings of Pong *et al.* (21) who found minimal bioconversion of [^{3}H]LTC$_4$ to [^{3}H]LTD$_4$ under similar conditions in rat lung membranes. The [^{3}H]LTD$_4$ formation was increased if the membranes were incubated with [^{3}H]LTC$_4$ at 20° in the presence of 20 mM Tris alone, Tris with 10 mM glycine, or Tris, glycine, and 10 mM cysteine. The lack of effect of cysteine on [^{3}H]LTC$_4$ bioconversion is similar to previous reports (22, 23). It is interesting to note the minimal formation of [^{3}H]LTE$_4$ even in the absence of cysteine. Additional HPLC analyses of [^{3}H]LTD$_4$ incubated with heart membranes have demonstrated a similar lack of conversion of [^{3}H]LTD$_4$ to [^{3}H]LTE$_4$ in the absence of cysteine.[2]

A recent communication by Hogaboom *et al.* (24) using guinea pig lung membranes indicated that millimolar concentrations of serine-borate were required to inhibit by greater than 95% the bioconversion of [^{3}H]LTC$_4$ to [^{3}H]LTD$_4$. These results were similar to previous reports (25, 26) using purified preparations of γ-glutamyl transpeptidase from porcine kidney. The effects of serine-borate on [^{3}H]LTC$_4$ bioconversion in heart membranes were determined (Table 1). As the concentration of serine-borate was increased to 80 mM, a concentration-dependent decrease in [^{3}H]LTC$_4$ bioconversion in membrane and supernatant fractions was observed. In the presence of 80 mM serine-borate, less than 2% of membrane-bound and less than 4% of the radioactivity in the filtrate co-eluted with unlabeled LTD$_4$. The majority of the remaining radioactivity, 5 to 9 and 3 to 7% in the supernatant and membrane fractions, respectively, eluted at or shortly after the solvent front. The chemical nature of the eluate is presently unknown. An example of the HPLC profile for the membrane-bound radioactivity is shown in Fig. 1. The data suggested that, under the assay conditions employed in Fig. 1, the results of the binding assay are a reflection of the interactions of [^{3}H]LTC$_4$ with its specific binding site and not a metabolic enzyme such as γ-glutamyl transpeptidase. Under these conditions, [^{3}H]LTC$_4$ binding was examined and characterized.

Characteristics of [^{3}H]LTC$_4$ binding. In the presence of 80 mM serine-borate, [^{3}H]LTC$_4$ binding reached a stable steady state within 15 min and remained constant, at equilibrium, for up to 45 min at 20° (Fig. 2). Additional experiments indicated that bound [^{3}H]LTC$_4$ could be dissociated in the presence of 3 μM unlabeled LTC$_4$. Greater than 95% of bound reactivity dissociated in less than 2 min (Fig. 2, inset). The [^{3}H]LTC$_4$ specific binding was linear, between 10 and 100 μg/ml, with respect to added protein (results not shown). The binding assays were conducted at 20 to 80 μg/ml of protein which was within the linear protein range and in which less than 10% of total radioactivity was bound.

The time course for the binding at 0° was very similar whether the binding was measured in the presence of 20 mM Tris alone or in the presence of 20 mM Tris and 80 mM L-serine-borate (results not shown). In each instance, the binding at 0° was rapid and reached a stable steady state within 10 min although at a lower level than

[2] G. K. Hogaboom, S. Mong, and S. T. Crooke, manuscript in preparation.

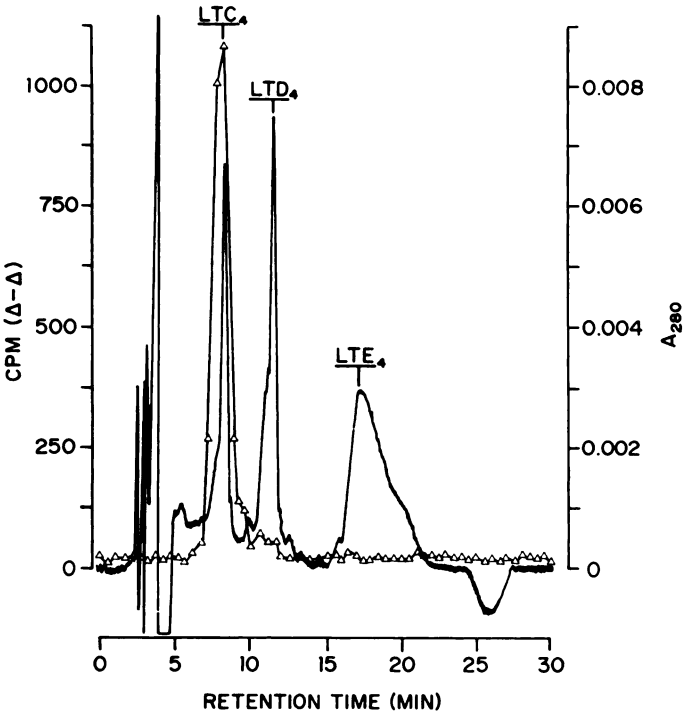

FIG. 1. *HPLC analysis of membrane-bound ^{3}H-metabolites of LTC$_4$*
Guinea pig heart membranes (100 μg/ml) were incubated with 5 nM [^{3}H]LTC$_4$ in 20 mM Tris (pH 7.5), 80 mM serine-borate, 10 mM glycine, 1 mM dithiothreitol, and 10 mM cysteine in a total volume of 2 ml for 45 min at 20°. The membrane-bound fraction was analyzed as described in Materials and Methods. Acetonitrile/10 mM phosphate (pH 7.0) was used as the mobile phase; a 25 to 35% acetonitrile gradient was used the first 10 min, 35% acetonitrile from 10 to 20 min, and 65% acetonitrile from 20 to 30 min.

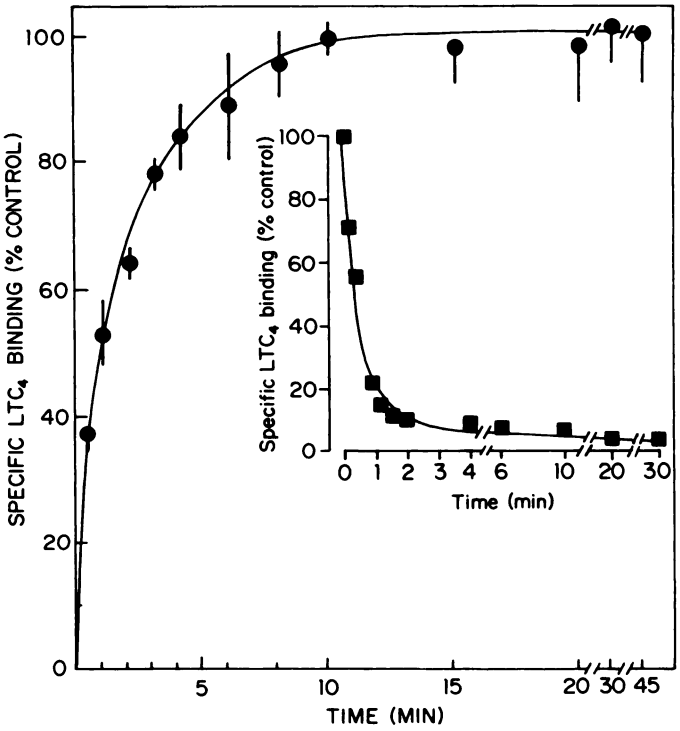

FIG. 2. *Association and dissociation of specific [^{3}H]LTC$_4$ binding*
Membranes (80 μg/ml) from guinea pig hearts were incubated with 4 nM [^{3}H]LTC$_4$ in a final volume of 0.1 ml and binding was measured as described in Materials and Methods. Control binding (100%) was 5727 fmol/mg. Inset, membranes (80 μg/ml) were incubated with 4 nM [^{3}H]LTC$_4$ for 20 min and dissociation was initiated by adding 3 μM unlabeled LTC$_4$ (*0 min*). Control binding (100%) was 5223 fmol/mg. The values are a representative example of three experiments performed in duplicate. The variation was less than 10% between each experiment.

for the binding measured at 20°. The results suggest that, at least for the guinea pig heart membranes, L-serine-borate does not affect the specific [^{3}H]LTC$_4$ binding but is necessary to prevent the bioconversion of [^{3}H]LTC$_4$ to [^{3}H]LTD$_4$ in the binding assay.

Saturability of [^{3}H]LTC$_4$ binding. Specific [^{3}H]LTC$_4$ binding was saturable at approximately 60 nM (Fig. 3). An assay time of 45 min was used to ensure that complete equilibrium was obtained at all concentrations of [^{3}H]LTC$_4$. Less than 10% of total ligand was bound when 20 to 60 μg/ml of membrane protein was used. Scatchard analyses (27) of the saturation binding data yielded a monophasic plot with a K_d of 27.5 ± 6.0 nM and a B_{max} of 19.9 ± 5.2 pmol/mg (n = 3). Analysis of the saturation binding data by the Hill equation (28) yielded a linear plot with a slope of 1.01. These data indicate that, in a concentration range of 2 to 120 nM, a single class of binding sites exists for LTC$_4$ in the guinea pig heart membranes.

Competition of [^{3}H]LTC$_4$ specific binding. The effects of the peptidoleukotrienes LTC$_4$, LTD$_4$, and LTE$_4$ and the end-organ peptidoleukotriene antagonists FPL 55712 (29), SKF 88046, and 4*R*,5*S*,6*Z*-2-nor-LTD$_1$ on specific [^{3}H]LTC$_4$ binding were determined (Fig. 4). Unlabeled LTC$_4$ displaced [^{3}H]LTC$_4$ in a concentration-dependent manner with a K_i (30)[3] of 39.7 nM. As the unlabeled LTC$_4$ was a 1:1 mixture of (5*R*,6*S*)- and (5*S*,6*R*)-LTC$_4$ diastereoisomers, the K_i of the pure (5*S*,6*R*)-LTC$_4$ (i.e., the natural diastereoisomer) would be expected to be less than the (5*S*,6*R*)-(5*R*,6*S*)-LTC$_4$ mixture assuming the binding sites display stereospecificity. This value is near the K_d value for [^{3}H]LTC$_4$ (5*S*,6*R*-LTC$_4$ diastereoisomer) binding as determined by Scatchard analyses (Fig. 3).

In contrast, LTE$_4$ (a 1:1 mixture of 5*R*,6*S* and 5*S*,6*R* diastereoisomers) and LTD$_4$ (the 5*S*,6*R* diastereoisomer) were relatively weak competitors with K_i values of 7.9 and 14.1 μM for LTE$_4$ and LTD$_4$, respectively. These data indicate the [^{3}H]LTC$_4$ binding sites are relatively specific for LTC$_4$ and may not interact with LTD$_4$ and LTE$_4$ at physiologically relevant concentrations (12). FPL 55712, a putative peptidoleukotriene antagonist in perfused heart and isolated ventricle strips (7), displaced [^{3}H]LTC$_4$ binding with a K_i of 25.5 μM. These results support, to some degree, the idea that FPL 55712 is a peptidoleukotriene antagonist (albeit weak) in the guinea pig myocardium. However, FPL 55712 may not be specific, as recent studies have demonstrated a variety of biochemical and pharmacological properties for this agent, especially at concentrations above 50 μM (14, 31–34).

Additional studies with 4*R*,5*S*,6*Z*-2-nor-LTD$_1$, a recently described specific peptidoleukotriene antagonist

[3] K_i is defined by the relationship of $IC_{50}/(1 + [L]/K_d)$ where L is the concentration of the radioligand ([^{3}H]LTC$_4$) and K_d is the dissociation constant for [^{3}H]LTC$_4$ binding.

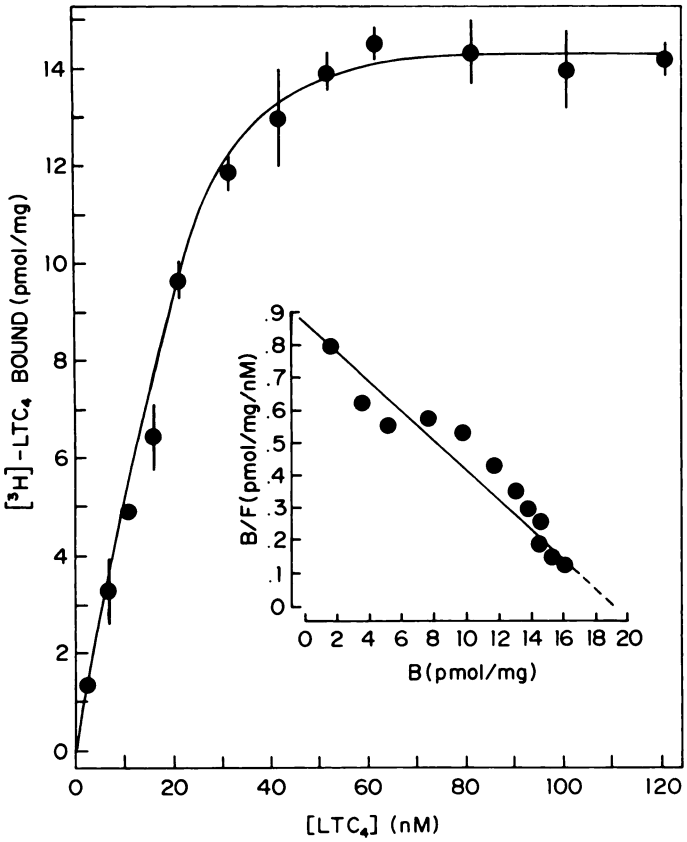

FIG. 3. *Saturation isotherm of [³H]LTC₄ binding*

Heart membrane preparation and general assay conditions are described in Materials and Methods. Protein concentration was 20 μg/ml, assay time was 45 min at 20°, assay volume was 100 μl, and less than 10% of total ligand was bound at each [³H]LTC₄ concentration. Specific binding is presented as a function of free ligand concentration (●). Inset, Scatchard plot of the specific binding data. The data are a representative example of four separate experiments.

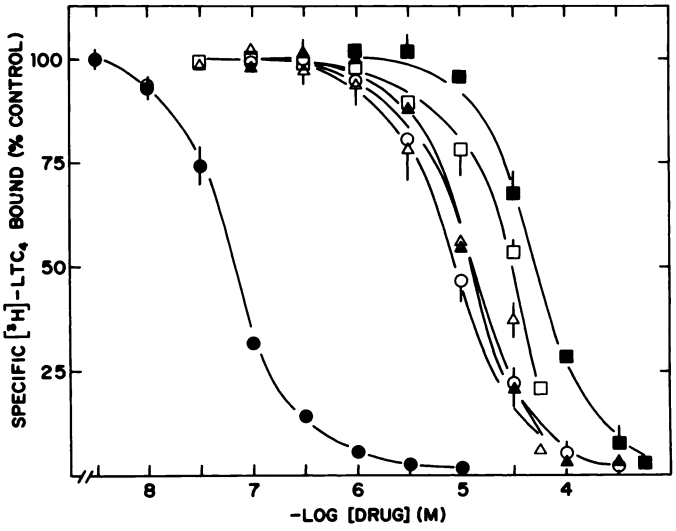

FIG. 4. *Concentration-dependent displacement of specific [³H]LTC₄ binding by unlabeled peptidoleukotrienes and antagonists*

Heart membrane preparation and general assay conditions are described in Materials and Methods. [³H]LTC₄ (27.5 nM) and membrane protein (50 μg/ml) were incubated for 45 min at 20° with LTC₄ (●), LTD₄ (□), LTE₄ (△), FPL 55712 (■), SKF 88046 (▲) or 4*R*,5*S*,6*Z*-2-nor-LTD₄ (○) in a final volume of 0.2 ml. Control (100% specific binding was 1814.6 ± 324.3 fmol/mg and the data are expressed as the mean ± standard error of three separate experiments performed in duplicate.

in the guinea pig pulmonary system (34), demonstrated a concentration-dependent competition of [³H]LTC₄ binding with a K_i of 4.2 μM. SKF 88046, an agent shown to be eight times more potent than FPL 55712 in antagonizing the negative inotropic effects of LTC₄ in the guinea pig perfused heart preparation (19), competed with specific [³H]LTC₄ binding with a K_i of 5.6 μM (4.6 times more potent than FPL 55712). The potency series of FPL 55712 and SKF 88046 provides initial evidence that the [³H]LTC₄ binding may represent a physiologically relevant receptor for LTC₄ in the guinea pig myocardium.

Regulation of specific [³H]LTC₄ binding by monovalent and divalent cations. Recent studies have indicated that divalent cations, primarily Mg^{2+}, can regulate high affinity agonist binding in the β-adrenergic (35) and D_2 dopaminergic (36) receptor systems. In addition, monovalent cations have been shown to modulate α_2-adrenergic agonist and antagonist binding in human platelets (37), and Na^+ has been implicated as having an important role in the hormonal inhibition of adenylate cyclase (38). With these studies in mind, the effects of monovalent and divalent cations on specific [³H]LTC₄ binding were determined.

Preliminary studies indicated that monovalent and divalent cations stimulated specific [³H]LTC₄ binding without affecting bioconversion (results not shown). A concentration-dependent stimulation of specific binding was observed with both monovalent and divalent cations. The concentration-dependent effects of the monovalent cations Na^+, K^+, Li^+, and NH_4^+ are shown in Fig. 5A. Na^+, K^+, and Li^+ increased the specific binding by 2.6- to 3.1-fold with a half-maximal stimulation occurring at 30 to 45 mM. The cation NH_4^+ stimulated the specific binding 3.6-fold at 100 mM with a half-maximal effect at 30 mM. The monovalent cation-dependent stimulation does not appear to be exclusively dependent on cations with small atomic radii (i.e., 0.66 to 1.47 Å) as *N*-methyl-*d*-glucamine, a derivative of deoxysorbitol which has been used as a sodium substitute in receptor studies (37), also stimulated specific [³H]LTC₄ binding in a manner similar to the monovalent cations (Fig. 5A). As control binding is determined in a high ionic strength buffer (i.e., 80 mM L-serine-borate, 10 mM cysteine, 10 mM glycine, and 20 mM Tris), the effects of the monovalent cations are not thought to be a result of *large* changes in ionic strength.

The divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+} also stimulated specific [³H]LTC₄ binding of the heart membranes (Fig. 5B). Ca^{2+} was the most effective in stimulating the LTC₄ binding with a maximal stimulation of 4.6-fold and a half-maximal effect at 0.7 mM. The maximal effect of Mg^{2+} was equivalent to that of Ca^{2+}, but half-maximal stimulation occurred at 1.7 mM. Mn^{2+} stimulated the LTC₄ binding 2.9-fold at 30 mM and half-maximal stimulation was achieved at 1.8 mM. The results suggest that

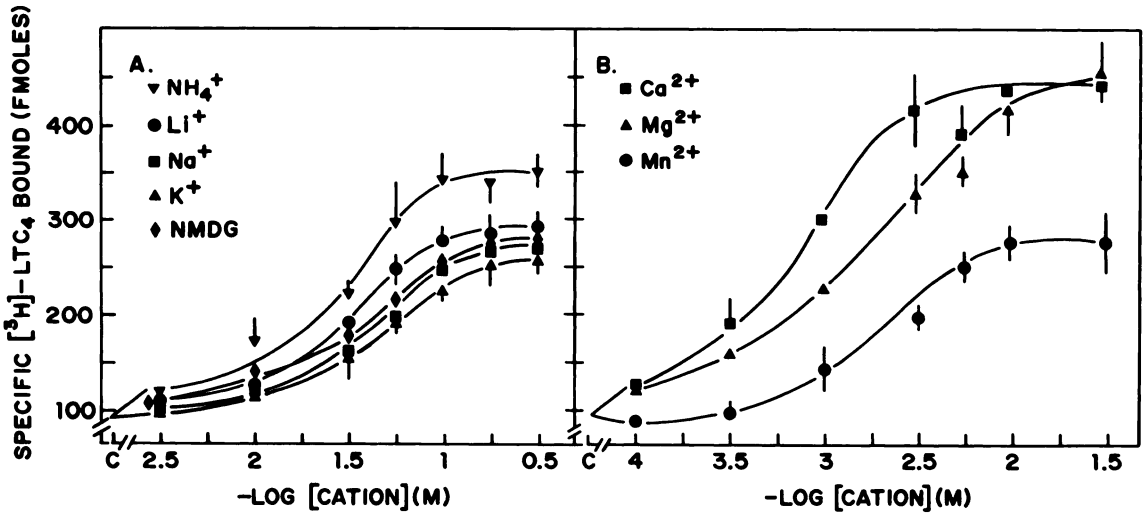

FIG. 5. *Concentration-dependent stimulation by cations of specific [^{3}H]LTC_4 binding*

Membrane preparation and general assay conditions are described in Materials and Methods. Membranes (50 μg/ml) were incubated with 1 nM [^{3}H]LTC_4 in a final volume of 1 ml at 20° for 30 min. A, effect of $(NH_4)_2SO_4$ (▼), KCl (▲), NaCl (■), LiCl (●), and *N*-methyl-*d*-glucamine (♦) (*NMDG*) on specific [^{3}H]LTC_4 binding. B, concentration-dependent effect of $CaCl_2$ (■), $MgCl_2$ (▲), and $MnCl_2$ (●) on specific [^{3}H]LTC_4 binding. The values are a representative example of three experiments performed in duplicate and the variation was less than 10% between each experiment.

TABLE 2

Effect of Ca^{2+} and Na^+ on [^{3}H]LTC_4 binding characteristics in guinea pig heart membranes

Heart membrane preparation and general assay conditions are described in Materials and Methods. Membranes (20 μg/ml) were incubated with 5 to 100 nM [^{3}H]LTC_4 for 45 min at 20° in a final volume of 0.1 ml either in the absence (control; data from Fig. 3) or in the presence of 3 mM $CaCl_2$ or 150 mM NaCl. The B_{max} and K_d values were calculated by the method of Scatchard (27) from saturation isotherms. The data are expressed as means ± of three separate experiments.

Condition	B_{max}	K_d
	pmol/mg	*nM*
Control	19.9 ± 5.2	27.5 ± 6.0
3 mM $CaCl_2$	42.6 ± 5.9	22.9 ± 7.8
150 mM NaCl	35.0 ± 2.0	27.2 ± 2.6

the order of specificity for the stimulation of LTC_4 binding by the divalent cations is $Ca^{2+} > Mg^{2+} > Mn^{2+}$.

Additional studies were conducted to define more clearly the mechanism(s) by which the cations might modulate the [^{3}H]LTC_4 binding in the heart membranes. Saturation experiments in the presence of physiological concentrations of two representative cations, either 3 mM $CaCl_2$ or 150 mM NaCl, were conducted to determine the effects of the cations on affinity and binding site number. These concentrations of $CaCl_2$ and NaCl also had maximal stimulatory effects on the [^{3}H]LTC_4 binding at 1 nM [^{3}H]LTC_4. $CaCl_2$ increased the B_{max} 2.2-fold to 42.6 ± 5.9 pmol/mg and NaCl increased the B_{max} 1.8-fold to 35.0 ± 2.0 pmol/mg (Table 2). Neither $CaCl_2$ nor NaCl had substantial effects on the K_d values when compared to control data. EDTA and EGTA, in concentrations up to 10 mM, and the guanine nucleotides GTP and Gpp(NH)p, in concentrations as high as 1 mM, had minimal effects on [^{3}H]LTC_4 specific binding (data not shown). An effect of EDTA or EGTA would not be expected as the membranes are prepared in the presence of 5 mM EDTA to chelate endogenous cations such as Ca^{2+} and Mg^{2+}. These data are in contrast to specific [^{3}H]LTD_4 binding in guinea pig lung membranes which has been reported to be inhibited by Na^+, stimulated by Ca^{2+} and Mg^{2+}, inhibited by EGTA and EDTA, and reduced by the guanine nucleotides (39).

Regulation of specific [^{3}H]LTC_4 binding by sulfhydryl group modification. Recent studies of β-adrenergic (35, 40) and D_2 dopaminergic (36) receptors have indicated that low concentrations of the sulfhydryl-alkylating reagent NEM affect ligand-receptor interactions. Studies were conducted to determine the effects of sulfhydryl-alkylating reagents on [^{3}H]LTC_4 binding with particular emphasis on the mechanism(s) by which sulfhydryl group modification might regulate the ability of LTC_4 to interact with its binding site.

Initial studies with reducing reagents such as dithiothreitol indicated that these agents had minimal effects on specific [^{3}H]LTC_4 binding (data not shown). This is consistent with the observation that 10 mM L-cysteine, a weak reducing reagent, had little or no effect on binding and is routinely used to inhibit the bioconversion of LTD_4 to LTE_4 (22) in the heart membranes. In contrast, the preincubation of heart membranes with sulfhydryl-alkylating reagents, such as 100 μM NEM, decreased the [^{3}H]LTC_4 binding by more than 95% (Table 3). A similar degree of inhibition of [^{3}H]LTC_4 binding was seen with 1 mM PHMB or 1 mM iodoacetamide (Table 3). HPLC analyses with membranes pretreated with 100 μM NEM indicated that the decrease in membrane-bound [^{3}H]LTC_4 was not a reflection of an alteration of [^{3}H]LTC_4 metabolism or degradation (data not shown). Preincubation of the membranes with 1 mM dithiothreitol before the addition of NEM prevented the inhibition of LTC_4 binding. However, incubation of the membranes with 1 mM dithiothreitol after preincubation with 100 μM NEM or 1 mM reversed the inhibitory effects of PHMB but

TABLE 3

Modification of [^{3}H]LTC$_4$ binding by sulfhydryl reagents

Heart membranes preparation and general assay conditions are described in Materials and Methods. Heart membranes (5 mg/ml) were incubated in 10 mM Tris (pH 7.5) in the presence of the indicated reagents (first incubation) for 30 min at 20° in a final volume of 0.5 ml. Additional reagents were subsequently added (second incubation) and were incubated for 30 min at 20°. The reaction mixture was diluted to 10.5 ml with 10 mM Tris (pH 7.5) and centrifuged at 155,000 × *g* for 40 min at 4° and the pellet was resuspended in 1 ml of 10 mM Tris (pH 7.5). Control binding [100%; none indicates incubation in 10 mM Tris (pH 7.5) alone] was 1571.0 ± 123.2 fmol/mg and the data are expressed as means ± standard error of three separate experiments.

Membrane pretreatment		Specific [^{3}H]LTC$_4$
First incubation	Second incubation	
		% control
None	None	100
None	0.1 mM NEM	9.4 ± 2.2
None	1 mM PHMB	11.3 ± 5.4
None	1 mM iodoacetamide	27.8 ± 7.1
1 mM dithiothreitol	0.1 mM NEM	105.3 ± 9.2
0.1 mM NEM	1 mM dithiothreitol	15.7 ± 4.4
1 mM PHMB	1 mM dithiothreitol	80.1 ± 14.3

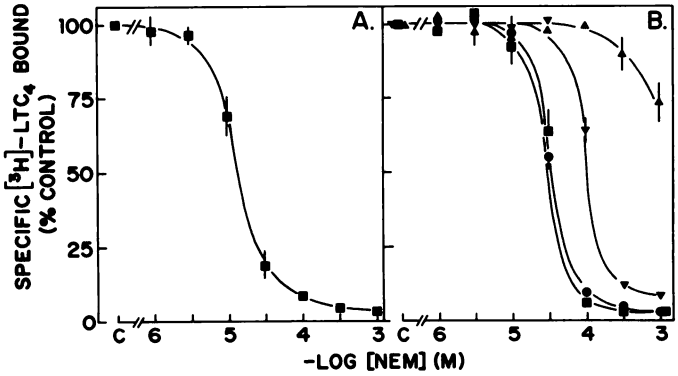

FIG. 6. *Concentration-dependent inhibition of [^{3}H]LTC$_4$ binding by NEM*

Heart membrane preparation and general assay conditions are described in Materials and Methods. A, heart membranes were incubated with the indicated concentrations of NEM, centrifuged, and assayed for specific [^{3}H]LTC$_4$ binding as described in Materials and Methods. Control binding (100%) was 1311.3 ± 197.4 fmol/mg and the values are a representative example of three experiments performed in duplicate. B, heart membranes were incubated with either 10 μM LTC$_4$ (▲), 0.3 mM 4*R*,5*S*,6*Z*-2-nor-LTD$_1$ (▼), or 0.5 mM SKF 88046 (●) for 45 min at 20°. The indicated concentration of NEM was subsequently added and the membranes were incubated an additional 30 min at 20°, centrifuged, and assayed as described in Materials and Methods. Control binding (100%) was 1401 ± 177.8 fmol/mg and the values are a representative example of three separate experiments performed in duplicate.

not those of NEM. The data indicate that sulfhydryl-alkylating reagents such as NEM, PHMB, or iodoacetamide inactivate specific [^{3}H]LTC$_4$ binding sites and preincubation with dithiothreitol can prevent the NEM-dependent inactivation. The inactivation by PHMB, but not NEM, is reversed by a postincubation with dithiothreitol.

Additional studies were undertaken to determine the mechanism(s) by which free sulfhydryl group(s) are associated with or involved in the interactions of LTC$_4$ with its binding site. Preincubation of heart membranes with NEM resulted in a concentration-dependent decrease in specific [^{3}H]LTC$_4$ binding (Fig. 6A) with a half-maximal inhibition at 15 μM NEM. Using a concentration of NEM (30 μM) that inactivates approximately 80% of binding sites at 1 nM [^{3}H]LTC$_4$, the effect of NEM pretreatment of heart membranes on saturation binding data was determined. Scatchard analyses of saturation isotherms indicated that pretreatment of heart membranes with 30 μM NEM decreased the maximum number of binding sites from 20.1 ± 6.7 to 8.2 ± 3.1 pmol/mg with minimal effects on the dissociation constant (Table 4). These data were substantiated by competition binding studies with 1 nM [^{3}H]LTC$_4$, unlabeled LTC$_4$, and SKF 88046 in which the K_i values for [^{3}H]LTC$_4$ binding were equivalent in both treated and control membranes (data not shown).

TABLE 4

Effect of NEM pretreatment of heart membranes on characteristics of [^{3}H]LTC$_4$ binding

General assay conditions, membrane preparation, and pretreatment of membranes with NEM are described in Materials and Methods. Control and pretreated membranes (20 μg/ml) were incubated with 5 to 100 nM [^{3}H]LTC$_4$ for 45 min at 20° in a final volume of 0.1 ml. B_{max} and K_d values were derived from Scatchard analyses (27) of saturation binding data. The data are expressed as means ± standard error of three separate experiments.

Pretreatment	B_{max}	K_d
	pmol/mg	*nM*
Control	20.1 ± 6.7	26.2 ± 5.2
30 μM NEM	8.2 ± 3.1	23.9 ± 7.1

The mechanism of NEM-induced inactivation was further examined by determining the effect of preincubation of heart membranes with LTC$_4$, SKF 88046, and 4*R*,5*S*,6*Z*-2-nor-LTD$_1$. Concentrations of each compound for the preincubation were chosen that displaced [^{3}H]LTC$_4$ by greater than 95% as determined by competition binding studies (see Fig. 4). Preincubation of the heart membranes with 10 μM LTC$_4$ before the addition and subsequent incubation with NEM resulted in a substantial shift to the right of control NEM concentration-response curve (Fig. 6B) with only 27% inhibition occurring at 1 mM NEM. Preincubation of the membranes with 0.3 mM 4*R*,5*S*,6*Z*-2-nor-LTD$_1$ resulted in a partial protection of the NEM-induced inactivation with half-maximal inhibition of [^{3}H]LTC$_4$ binding at 100 μM NEM. Preincubation of the membranes with 0.5 mM SKF 88046 (Fig. 6B) or 0.5 mM FPL 55712 (data not shown) had minimal effects on the NEM-induced inactivation of [^{3}H]LTC$_4$ binding sites.

These data indicate that NEM-dependent inhibition of [^{3}H]LTC$_4$ binding results from a decrease in binding capacity with minimal alterations in binding affinity. The occupation of the binding sites with the agonist LTC$_4$ protects the NEM-induced inactivation of the [^{3}H]LTC$_4$ binding sites. The occupation of the binding sites with antagonists was either less effective (e.g., 4*R*,5*S*,6*Z*-2-nor-LTD$_1$) or ineffective (e.g., FPL 55712 and SKF

88046) in protecting against the inactivation of the [^{3}H]LTC_4 binding sites by NEM. These data suggest that free sulfhydryl group(s) are required for the interactions of [^{3}H]LTC_4 with its binding site and that occupation of the binding site with an agonist such as [^{3}H]LTC_4, but not antagonists, decreases the interactions of the free sulfhydryl groups with NEM.

DISCUSSION

Previously described *in vitro* studies in the guinea pig using isolated ventricular papillary muscle, electrically paced nonperfused atria, or perfused hearts (7) have clearly demonstrated direct myocardial-depressant effects of LTC_4 that were antagonized by FPL 55712. The data in this report substantiate previous pharmacological findings and provide direct, biochemical evidence for the existence of specific [^{3}H]LTC_4 binding sites in the guinea pig ventricular myocardium. The [^{3}H]LTC_4 binding is probably *not* the result of the interactions of LTC_4 with a metabolic enzyme, as the binding, at 0°, was unaffected by L-serine borate, a competitive inhibitor of γ-glutamyl transpeptidase (25, 26). In support of this contention, the bioconversion of [^{3}H]LTC_4 (measured at 20° either in the presence of 20 mM Tris alone or in the presence of 20 mM Tris and 80 mM L-serine-borate) was equivalent in control membranes and membranes pretreated with high concentrations of NEM (1 mM) in which the specific [^{3}H]LTC_4 binding was reduced by greater than 95%.[4] The specific [^{3}H]LTC_4 binding was saturable with a K_d value within the range of LTC_4 concentrations required to induce a negative inotropic effect in the guinea pig (7, 19). The pharmacological profile (Fig. 4) of [^{3}H]LTC_4 showed a high degree of specificity for LTC_4 but not LTD_4 or LTE_4. The putative peptidoleukotriene antagonists FPL 55712 and SKF 88046 competed for specific [^{3}H]LTC_4 binding sites with a potency order similar to that observed in studies with the guinea pig heart (19), suggesting that the binding sites are physiologically linked to the LTC_4-induced negative inotropic effect. Additional studies, however, either in physiological studies with ventricular myocardium or with the determination of a biochemical response (e.g., ion flux, phosphatidylinositol turnover, prostanoid biosynthesis) that is coupled to the LTC_4 binding, are required to functionally define the LTC_4 binding site as a physiological receptor.

Specific [^{3}H]LTC_4 binding sites have been reported for several tissues including guinea pig lung (24), rat lung (21), human lung (41), and cultured smooth muscle cells (42). These binding sites have similarities with respect to their stimulation by cations, rank orders of potency and number of maximum binding sites. The reported B_{max} for the specific [^{3}H]LTC_4 binding sites in membrane preparations is much higher than the B_{max} reported for other receptors such as the catecholamines (36, 37). A complete explanation for this general observation is not known. However, the maximum number of [^{3}H]LTC_4 binding sites reported for intact cultured smooth muscle cells (43) was near 200,000 sites/cell, a value only slightly higher than the reported maximum sites/cell for other lipid- or peptide-containing ligands such as fMet-Leu-Phe (44) or leukotriene B_4 (45). The cultured smooth muscle cells, which have been reported to contract in response to LTC_4 (42), may prove to be a useful model for determining important relationships between receptor number and cellular response.

The specific [^{3}H]LTC_4 binding is stimulated several-fold by both monovalent and divalent cations. Previous agonist binding studies in the D_2 dopamine receptor (36) and β-adrenergic receptor (35) systems have indicated that divalent cations stimulate binding through an increase in B_{max}. However, additional data in these studies using high affinity antagonist radioligands showed the increase in B_{max} for the agonist ligands reflected an increase in the formation of the number of "high affinity" binding sites rather than an uncovering or unmasking of existing sites. Calcium channel binding studies (43) have indicated that divalent cations can increase the B_{max} for [^{3}H]nitrendipine binding in rat cortical membranes. This study related the stimulatory effects on binding to physiological functions of the cations. The present study indicates the maximal number of [^{3}H]LTC_4 binding sites is increased by the divalent cations with a potency order of $Ca^{2+} > Mg^{2+} > Mn^{2+}$. The potency order of the divalent cations in increasing the [^{3}H]LTC_4 binding capacity suggests that the divalent cations may interact with a specific site in or near the LTC_4 binding site and may play a physiological role in regulating cellular levels of LTC_4 receptors. However, the possibility of a specific regulatory site is diminished because of similar stimulatory effects on LTC_4 binding exhibited by a variety of monovalent cations (including *N*-methyl-*d*-glucamine, a charged molecule used as a sodium substitute). These findings suggest that divalent cations may increase the B_{max} for [^{3}H]LTC_4 binding sites through a membrane stabilization phenomenon and that the monovalent cations can mimic this effect but at 50- to 100-fold higher concentrations.

Studies with sulfhydryl-alkylating reagents such as NEM indicate that, for the guinea pig heart membranes, the [^{3}H]LTC_4 binding sites contain free sulfhydryl group(s) that are intimately involved in regulating the interactions of LTC_4 with its binding site. Scatchard analyses of saturation binding data indicate that one mechanism of NEM-induced inactivation of [^{3}H]LTC_4 binding sites is through a decrease in B_{max} without alterations in K_d. Protection experiments with agonists and antagonists provided additional information on the molecular location of the free sulfhydryl group(s) associated with the [^{3}H]LTC_4 binding site. The protection studies suggest that occupation of the binding site with an agonist such as LTC_4 may induce a conformational change in the receptor molecule such that the sulfhydryl groups of interest are hidden or "shielded" from reacting with NEM. The occupation of the LTC_4 binding sites with SKF 88046 and FPL 55712, leukotriene antagonists in the heart (19) with little or no structure similarity to the leukotrienes, provided no measurable effect on the inactivation of [^{3}H]LTC_4 binding sites by NEM. 4*R*,5*S*,6*Z*-2-nor-LTD_1, a leukotriene antagonist in the guinea pig trachea (34) with structural similarity to LTD_4, provided a partial protection of NEM-induced inactivation of

[4] G. K. Hogaboom, unpublished observations.

LTC_4 binding sites which suggests that this compound may induce some degree of conformational change in the binding site to protect the interactions of NEM with the free sulfhydryl groups. 4*R*,5*S*,6*Z*-nor-LTD_1 has partial agonist activity in the guinea pig trachea at high concentrations (34), but its ability either to antagonize LTC_4-induced effects or to mimic LTC_4 actions in the guinea pig heart is presently unknown.

The results of this report and previous pharmacological studies (7, 17–19) strongly suggest the peptidoleukotrienes such as LTC_4 may produce a negative inotropic effect on the heart through a receptor-mediated process. The specific LTC_4 binding sites identified in this report may mediate the physiological effects of LTC_4 in the heart either directly through alterations in intracellular events such as Ca^{2+} translocation or cyclic nucleotide metabolism or indirectly by initiating the synthesis of cyclooxygenase products. The peptidoleukotrienes have been shown to initiate the synthesis of cyclooxygenase products in the heart which not only reduce coronary flow (12) but also decrease myocardial contractility (47). LTC_4 has also been reported to induce thromboxane synthesis in smooth muscle cells in culture (48). As the peptidoleukotrienes have been shown to be released as a result of immediate-type hypersensitivity reactions as well as other immunological and inflammatory events (49, 50), the study of the interactions of LTC_4 with its specific binding sites in the myocardial membranes should provide new insights as to the physiological role of the peptidoleukotrienes in modulating cardiac function.

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