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SPECIFIC BINDING OF LEUKOTRIENE B_4 TO GUINEA PIG LUNG MEMBRANES

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Summary: We have demonstrated binding sites for LTB_4 in guinea pig lung membranes. Binding of [^{3}H]-LTB_4 was of high affinity (Kd = 0.76 nM), saturable and linear with protein concentration (0.2 - 1.2 mg/ml). Scatchard and Hill's plot analysis indicated a single class of binding site with a Hill's cofficient of 0.99 ± 0.08 (n=4). [^{3}H]-LTB_4 was unmetabolized during incubation with membrane preparations, as indicated by high performance liquid chromatography. Divalent cations such as Mg^{2+} and Ca^{2+} enhanced binding capacity without changing the Kd. Na^+ ions decreased binding in a concentration-dependent manner. Guanine nucleotides, GTP, GTPγS and Gpp(NH)p also decreased the number of binding sites. Finally, competition experiments demonstrated the following order of potency for displacement of [^{3}H]-LTB_4 from its receptor site: LTB_4 > 20-OH-LTB_4 >> 20-COOH-LTB_4 = 6-trans-12-epi-LTB_4 > LTC_4 = LTD_4 = 5-HETE. These data indicate that a specific LTB_4 receptor, in addition to the previously documented LTC_4 and LTD_4 receptors, exists in guinea pig lung.

Leukotriene (LT) B_4 [5(S), 12(R)-dihyroxy-eicosa-6,14-cis-8,10-trans-tetraenoic acid], is a product of the lipoxygenase pathway of arachidonic acid (AA) metabolism(1-3). LTB_4 production has been shown to occur in rabbit neutrophils (2), human neutrophils (4), human lung (5), and guinea pig lung (6). On a molar basis, LTB_4 is the most potent chemotaxin factor for human, rabbit and rat neutrophils (7-10). LTB_4 also induces neutrophil aggregation, generation of superoxide and degranulation in the presence of cytochalasin B (7-10). Another important action of LTB_4 is its ability to contract guinea pig

ABBREVIATIONS: LT - Leukotriene, 5-HETE - 5-Hydroxy Eicosatetraenoic Acid, AA - Arachidonic Acid, HPLC - High Performance Liquid Chromatography, Kd - Dissociation Constant.

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lung parenchymal strips and human isolated airways (11). In this regard, LTB_4 is 100-1000 times more potent than histamine on a molar basis; however, it is less potent than the peptidoleukotrienes LTC_4 and LTD_4. Binding studies have demonstrated that receptor sites for LTB_4 occur on the extracellular side of human neutrophil membranes (12-14) and that receptors for LTC_4 and LTD_4 exist on human and guinea pig lung membranes (15-17). A noticeable absence of LTB_4 binding data in lung tissue is apparent. In this communication we have documented receptor sites for LTB_4 on guinea pig lung tissue and have studied some of the biochemical mechanisms regulating this specific receptor site.

MATERIALS AND METHODS

Materials: [14,15-^{3}H(N)]-leukotriene B_4 (32 Ci/mmole) was purchased from New England Nuclear. LTC_4, LTD_4, LTB_4, 20-OH-LTB_4, 20-COOH-LTB_4, 5-HETE, and 6-trans-12-epi-LTB_4 were generous gifts from Dr. J. Rokach, Merck-Frosst, Inc., Point Claire-Dorval, Quebec. Unlabelled drugs were stored in aliquots at -70°C until day of use. Dilutions were made with water and used within 2 days. Purity was regularly checked by HPLC. Guanosine-5′-(3-O-thio) triphosphate ($GTP\gamma S$), guanosine triphosphate (GTP) and guanyl-5′-(β,γ,imino) triphosphate (Gpp(NH)p) were purchased from Calbiochem-Behring (San Diego, CA). All other chemicals and reagents used were of analytical grade.

Preparation of Lung Membranes: Lungs from freshly stunned and exsanguinated guinea pigs (Male, 300 g, English short-hair, Connaught Laboratories, Toronto, ON) were chopped into cubes 2 x 2 x 2 mm, in 10 volumes of homogenization buffer of the following composition: Tris-HCl and Tris base 10 mM (pH 7.0 at 4°C), Ethylenediaminetetracetic acid (EDTA) (10 mM), phenylmethylsulphonyl fluoride (PMSF) (200 μM), Dithiothreitol, (1mM) and Sucrose (0.25M). The lung fragments were then disrupted using a polytron homogenizer at a setting of 6 for 30 seconds. The homogenate was centrifuged at 1000 g for 10 minutes to pellet unbroken chunks of tissue, nuclei and undisrupted cells. After filtration through a layer of gauze, the supernatant was centrifuged at 110,000 g for 30 minutes. The pellet was re-suspended in the above buffer prepared without EDTA and PMSF. The suspended pellet was re-centrifuged two more times at 110,000 g for 30 minutes each. The final pellet was then suspended to a protein concentration of 3 - 4 mg/ml and stored in aliquots under liquid nitrogen until the day of use. Protein was determined by the method of Lowry (18) using bovine serum albumin as standard.

[^{3}H]-LTB_4 Binding Assay: All incubations were carried out in duplicate in micro-centrifuge tubes (capacity 1.5 ml). Total

assay volume was 250 µl except in time course assays where the volume was scaled up appropriately (10-20 fold). In this case, clean scintillation vials were used. Drugs, Tris buffer 50 mM (pH 7.0), and [^{3}H]-LTB_4 were added and protein was used to start the reaction. Non specific binding was measured in duplicate tubes containing unlabelled LTB_4 (1.0 µM). Specific binding was defined as the difference between total and nonspecific binding. The latter component was approximately 10-25% in all experiments. Tubes were vortexed to ensure the protein was suspended adequately. Binding was carried out at 25°C unless otherwise stated. Separation of the bound from free ligand was carried out by vaccum filtration through 25 mm Whatman GF/B filters and rapid washing with 3, 5 ml ice cold aliquots of Tris buffer 10 mM (pH 7.0). Dried filters were added to scintillation vials with 10 ml of liquid scintillant and the radioactivity counted in a scintillation counter at approximately 45% efficiency with quench correction. Data was analyzed by a computer program. Saturation analysis was aided by computer fitting of curves by the method of least squares. All data presented are the mean of duplicates and is representative of at least two other experiments performed on different membrane preparations.

RESULTS AND DISCUSSION

[^{3}H]-LTB_4 binding to lung membrane was found to be saturable and of high affinity (figure 1). Initial studies showed that at 25° binding was optimal at pH 7.0, dependent on protein concentration and attained equilibrium after 10 min. Scatchard plots (figure 1, inset A) indicate a single class of binding sites with a Kd of 0.76 ± 0.04 nM (n=3), and a binding capacity (B_{max}) of 254 ± 21 fmoles/mg protein (n=3) for incubations carried out in the presence of 10 mM Ca^{2+}. Hill's analysis of the data (inset B) yielded a Hill's coefficient of 0.99 + 0.1 (n=4) thus reinforcing the absence of cooperative interactions between receptors. The observed Kd value for LTB_4 binding in lung is similar to observed Kd values for LTB_4 binding to neutrophils (Kd = 0.46 nM, ref. 13) and for LTC_4 and LTD_4 binding to specific receptors in lung tissue (15-17).

HPLC analysis of [^{3}H]-LTB_4 recovered and isolated after incubation with membrane protein indicated that previously bound [^{3}H]-LTB_4 was chemically unchanged; in followup experiments the recycled ligand was able to bind lung membranes in a fashion

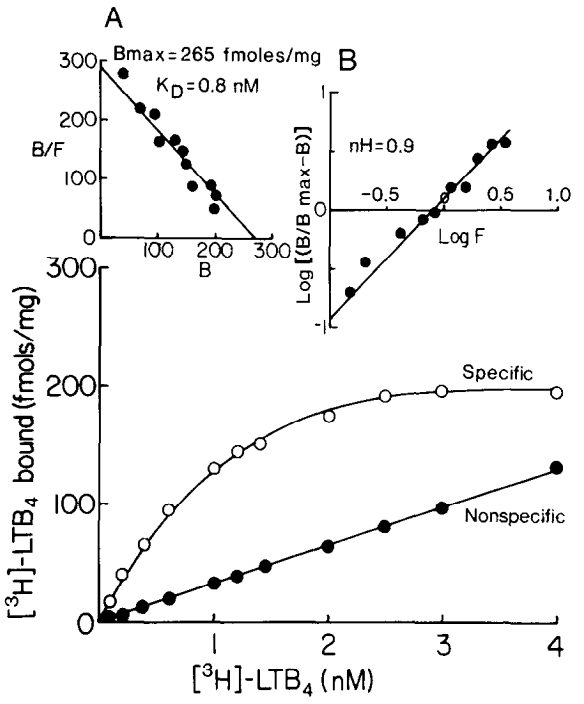


Figure 1. Saturation analysis of [^{3}H]-LTB$_4$ binding to lung membranes, lung membranes (0.36 mg/ml) were incubated with increasing concentrations of [^{3}H]-LTB$_4$ in the presence of Ca^{2+} (10 mM) at 25°C for 30 minutes. Similar tubes contained LTB$_4$ (1 μM) to determine the nonspecific binding component. Curves were fitted by the method of least squares. Scatchard analysis of the data is shown in inset A. Hill plot analysis is shown in inset B.

identical to pure [^{3}H]-LTB$_4$ (data not shown). It appears that during the binding process, [^{3}H]-LTB$_4$ is not metabolized to a significant extent by lung tissue. These findings agree with other studies (19,20) which show that rat, human adult and human fetal lung do not metabolize LTB$_4$ to its 20-hydroxy or 20-carboxy derivatives.

Figure 2 shows that in incubations carried out with membranes prepared in the presence of 10 mM EDTA, divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}) increased the level of specific binding. Ca^{2+} was the most effective and at 10 mM increased the binding capacity by 37% (n = 3) but did not alter the Kd. Monovalent cations, Na^+, Li^+, and K^+ reduced specific binding of ([^{3}H]-LTB$_4$ (figure 2B). The most effective cation, 1 M Na^+,

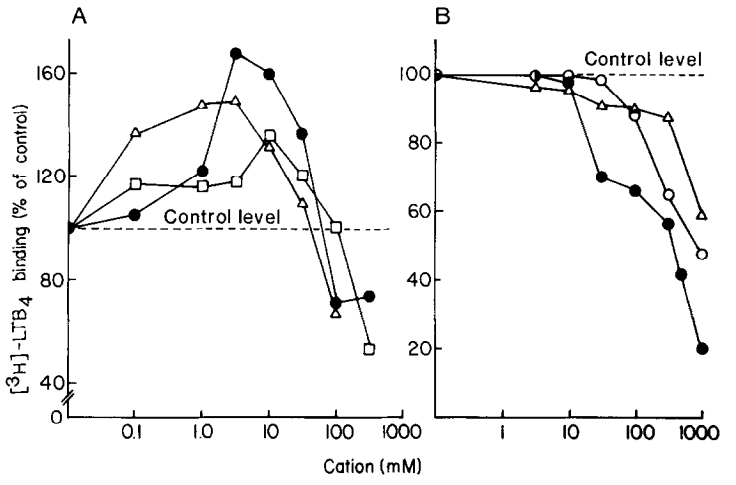
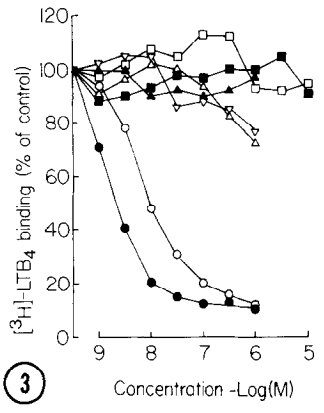


Figure 2. The effect of cations on [^{3}H]-LTB_4 binding. Lung membranes were incubated with [^{3}H]-LTB_4 (1nM), at 25°C for 30 minutes. The divalent cations Mn^{2+} (△), Mg^{2+} (□), and Ca^{2+} (●) were used in part A. The monovalent cations Na^+ (●), K^+ (○), and Li^+ (△) were used in part B. Results are expressed as a percentage of control specific binding (153.0 ± 4.7 fmoles/mg). Points are the means of duplicate assays and are representative of at least 2 experiments.

reduced binding by 80%. These results are reminiscent of previous studies demonstrating similar effects of divalent and monovalent cations on the binding of LTD_4 to guinea pig lung (15-17).

In competition assays (figure 3), we found that LTB_4 and its hydroxy derivative 20-OH-LTB_4 were the most potent inhibitors of the binding of [^{3}H]-LTB_4 to lung membranes. The rank order of potency for inhibition of binding was LTB_4 > 20-OH-LTB_4 >> 20-COOH-LTB_4 = 6-trans-12-epi-LTB_4 > LTC_4 = LTD_4 = 5-HETE. Thus, the receptor we studied seems to be specific for LTB_4 as LTC_4 and LTD_4 failed to compete for this binding site. The receptor also seems to have stereoselectivity since the stereoisomer of LTB_4, 6-trans-12-epi-LTB_4 was significantly less potent than LTB_4 in displacing [^{3}H]-LTB_4 from its binding sites. The relative potency of LTB_4 > 20-OH-LTB_4 >> 20-COOH-LTB_4 in competing for the lung LTB_4 binding site is the same as that observed in



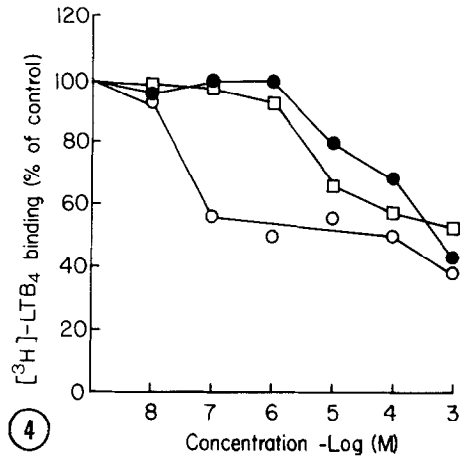


Figure 3. Competition for [^{3}H]-LTB_4 binding by lipoxygenase products. Lung membranes (0.4 mg/ml) were incubated with [^{3}H]-LTB_4 (0.76 nM) in the presence of Ca^{2+} (10 mM) at 25°C for 30 minutes. Unlabelled competing ligands were LTB_4 (●), 20-OH-LTB_4 (○), 20-COOH-LTB_4 (△), LTC_4 (□), LTD_4 (■), 5-HETE (▲), and 6-trans-12-epi-LTB_4 (▽). Points are means of duplicates expressed as a percentage of control binding (158.5 ± 3.2 fmoles/mg) and representative of 3 other experiments.

Figure 4. The effect of guanine nucleotides on [^{3}H]-LTB_4 binding. Lung membranes (0.3 mg/ml) were incubated with [^{3}H]-LTB_4 (0.76 nM) in the absence of any added cations. GTP (●), GTPγS (○), and Gpp(NH)p (□) were present in increasing concentrations in duplicate tubes. Binding was carried out at 25°C for 30 minutes. Volumes are expressed as a percentage of control total binding (166.3 ± 16.1 fmoles/mg).

competition experiments in human neutrophils (13) and in contractility studies on guinea pig lung parenchymal strips (27).

Guanine nucleotides GTP, GTPγS and Gpp(NH)p regulate the LTD_4 receptor (15) and in our experiments the binding capacity of [^{3}H]-LTB_4 to lung membranes was depressed in their presence (figure 4). The nonhydrolysable analog GTPγS was the most potent with GTP being the least potent. The regulation of LTB_4 binding by guanine nucleotides and cations suggests that this receptor may be linked to guanine nucleotide binding (N) proteins (15-17, 21-22). Originally it was thought that the N protein might be Ni, the inhibitory regulatory protein of adenylate cyclase, since LTB_4 stimulated responses were blocked by pertussis toxin. However in light of recent findings that other

pertussis toxin substrate proteins exist in stimulated cells (23, 24) the identity of the LTB_4 regulating N proteins remains an open question. There is also evidence LTB_4 may generate second messengers via different pathways. For example, Holian showed that pertussis toxin blocks LTB_4 stimulated superoxide production and phosphatidyl turnover in guinea pig alveolar macrophages (25). However, Volpi et al (26) demonstrated that in rabbit neutrophils, LTB_4 mobilizes Ca^{2+} without influencing polyphosphoinositide production. Further work is needed to clarify these aspects.

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