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IDENTIFICATION OF LEUKOTRIENE D_4 RECEPTOR BINDING SITES IN GUINEA PIG LUNG HOMOGENATES USING [^{3}H]LEUKOTRIENE D_4

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We have characterized [^{3}H]leukotriene D_4 binding to guinea pig lung homogenates. Both biphasic dissociation kinetics and curvilinear Scatchard plots indicated the presence of [^{3}H]leukotriene high and low affinity states of the binding sites. The rank order of potency for the competition study was leukotriene C_4 = leukotriene D_4 > leukotriene E_4 >> arachidonic acid, and for their contractile effect on lung strips was leukotriene C_4 = leukotriene D_4 = leukotriene E_4 >> arachidonic acid. FPL-55712 was the only other agent tested that inhibited binding. These results suggest that binding of [^{3}H]leukotriene D4 to the homogenate is consistent with its binding to specific leukotriene D_4 receptor sites.

While the mechanism for leukotriene (LT) C_4, D_4 and E_4-induced airway contraction is unclear, the extremely potent effect and the selective antagonism by FPL-55712 suggest involvement of a specific receptor site in the action at the end organ level (1-4). By analyzing LTC_4 and LTD_4 contraction following FPL-55712 treatment, several investigators further propose the presence of two LT receptor sites in the lung parenchyma of guinea pigs (3) and that the airway LTD_4 receptor is qualitatively different from the receptor in ileum (5) and uterus (6). However, the interpretation of the results from the contraction experiments (1-6) is often complicated by the indirect participation of released prostaglandins and thromboxanes in the LT-induced response (7-11). To explore the biochemical aspects of the airway LT receptor, we set out to use [^{3}H]LTD_4 to quantitate its binding to guinea pig lung homogenates and to directly assess interactions of LTs and many other compounds with the binding sites.

Abbreviation: LT, leukotriene; DMPP, dimethyl-4-phenylpiperazinium; TMB-8, 8-(N,N-diethylamino)octyl-2,4,5-trimethoxybenzoate; AA, arachidonic acid.

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METHODS

LTB_4, LTC_4, LTD_4 and LTE_4 were gifts from Dr. J. Rokach (Merck Frosst Canada, Inc.). FPL-55712 was from Dr. R.C. Murphy (Univ. of Colorado at Denver). U-60257 was from Dr. M.K. Back (Upjohn Co.). Prostaglandins, thromboxane B_2 and DMPP, a nicotinic receptor stimulant, were purchased from Sigma Co. (St. Louis, Mo.). TMB-8, a Ca^{2+} blocking agent (12), was purchased from Aldrich Chemical Co. (Ann Arbor, Mich.). Sources of other compounds were indicated elsewhere (13). [14,15-^{3}(H)]LTD_4 (specific activity = 40.3 Ci/mmol) was purchased from New England Nuclear Co. (Boston, Mass.).

The stock solutions of LTs were prepared according to the procedures provided by Merck Frosst Canada, Inc. The actual concentrations of their stock solutions were determined by UV spectrophotometery at 280 nm or 270 nm (for LTB_4) before each experiment began and were corrected accordingly. LTs in the stock solution remained 75-100% active after completion of all binding and contraction experiments. [^{3}H]LTD_4 was stored under argon at -20°C.

We prepared crude lung membrane homogenates of Hartley guinea pigs (200-350 g) and performed [^{3}H]LTD_4 binding assays according to the methods reported previously (14), except for a pH of 7.0 for the 50 mM Tris HCl buffer (25°C), 30 min incubation period and a protein concentration of 1.0 mg/ml in this study. Specific [^{3}H]LTD_4 binding, defined as binding in the absence of LTD_4 minus binding in the presence of 2.7×10^{-7} M LTD_4, was about 70-85% of total [^{3}H]LTD_4 binding (0.6 nM). Assays were performed in duplicate in each experiment. The dissociation constants and concentrations of lung [^{3}H]LTD_4 binding sites were determined from a Scatchard plot (Fig. 2) using a nonlinear least-squares curve fitting program for 2 independent sites (SCATFIT-59, contributed by Dr. P.J. Munson, N.I.H.). The IC_{50} value was the concentration of a compound that reduced [^{3}H]LTD_4 binding by 50%.

For contraction experiments, guinea pig lung parenchymal strips were prepared according to the method of Drazen and Schneider (15) and suspended in a modified Krebs-Ringer medium (27°C) (13) at 0.2-0.4 g tension. After equilibration for approximately 2-hours (no spontaneous airway relaxation), the response to cumulative concentrations of LTC_4, D_4 and E_4 (10^{-11}-3×10^{-8} M), AA (10^{-8}-3×10^{-5} M) and histamine (10^{-8}-10^{-4} M) was determined. We used the same solutions of these compounds for both binding and contraction studies. The concentration of the compound which produced 50% of the maximal contraction, a ED_{50} value, was then estimated from each dose-response curve. In this study, values are generally expressed as mean ± SE.

RESULTS AND DISCUSSION

In the guinea pig lung homogenates, specific [^{3}H]LTD_4 binding was linear with protein concentration from 0.25 to 1.0 mg/ml, and binding to the preparation at 25°C was at moderate speed with half-maximal binding of 7.6 ± 1.2 min (n=3) (Fig. 1A). Equilibrium was reached in 20 min and was constant for 70 min. [^{3}H]LTD_4 dissociated from the binding sites with the addition of 2.7×10^{-7} M LTD_4. Kinetic analysis of the [^{3}H]LTD_4 dissociation curve revealed the presence of both fast ($t_{1/2}$ = 15.9 ± 1.5 min, n=4) and slow ($t_{1/2}$ = 85.2 ± 14.4 min) components (Fig. 1B). The biphasic dissociation kinetics suggest dissociation of [^{3}H]LTD_4 from two

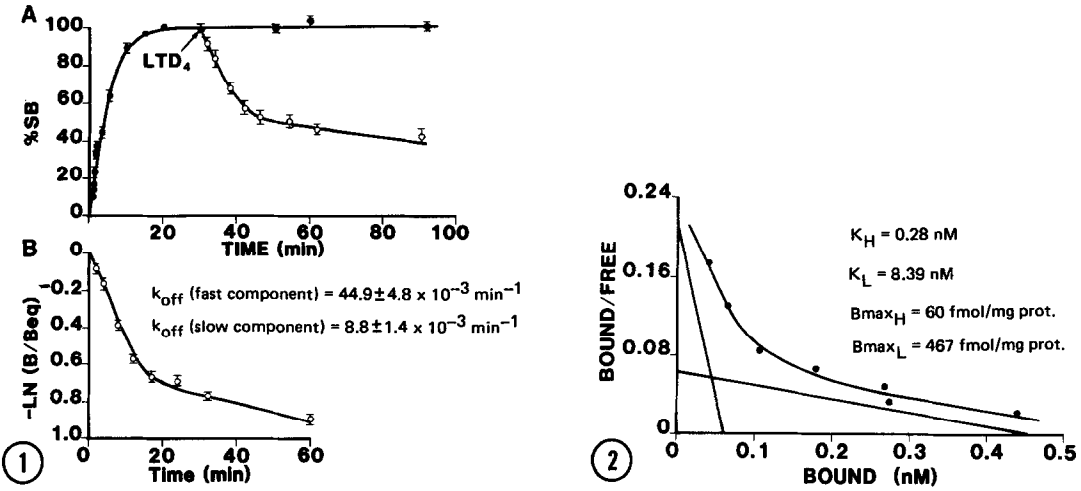

Fig. 1. Kinetic analysis of [^{3}H]LTD$_4$ binding to the guinea pig peripheral lung preparation. A. Association and dissociation of [^{3}H]LTD$_4$ binding as a function of time. Data are expressed as a percentage of specific [^{3}H]LTD$_4$ binding (SB) at equilibrium (B_{eq}) (maximal SB = 1,200-1,500 cpm). B. First-order dissociation rate plot based on the data shown in Fig. 1A. The slope of the fast and slow component of the curve determined by linear regression was regarded as the rate constant for dissociation (k_{off}). Each point represents mean ± SE of 3-4 experiments.

Fig. 2. Scatchard plot of [^{3}H]LTD$_4$ binding to the guinea pig peripheral lung preparation. Lines are best-fit plots determined from the curve fitting program. The inset shows the dissociation constants and concentrations of low and high affinity binding sites. K_H and $Bmax_H$ are the dissociation constant and concentration of high affinity binding sites and K_L and $Bmax_L$ are for low affinity binding sites.

affinity states of the binding sites, which is further evident from the result of subsequent equilibrium binding experiments.

A Scatchard analysis of the data of the experiment showed a curvilinear plot (Fig. 2) consistent with the presence of a heterogeneous population of [^{3}H]LTD$_4$ binding sites. Analysis of the data by the curve fitting program for two sites resulted in the dissociation constants and concentrations of high and low affinity binding sites shown in Fig. 2. In three separate experiments, K_H and K_L were 0.85 ± 0.29 and 5.44 ± 1.87 nM, and $Bmax_H$ and $Bmax_L$ were 131 ± 52 and 378 ± 55 fmol/mg prot., respectively.

The IC_{50} values of LTs and other compounds from competition experiments are listed in Table 1. LTC$_4$, LTD$_4$, LTE$_4$, AA, and FPL-55712 at the highest concentration tested were capable of maximally inhibiting [^{3}H]LTD$_4$ binding

Table 1. IC_{50} values of compounds tested in the guinea pig peripheral lung homogenate.

Compound	IC_{50}(M)*
Arachidonic Acid (AA)	$4.99\pm1.04\times10^{-5}$(6)
AA + 0.1 mM U-60275	$6.48\pm1.45\times10^{-5}$(2)
LTB_4	$> 2.47\times10^{-5}$ (4)
LTC_4	$2.20\pm0.15\times10^{-9}$(5)**
LTD_4	$4.75\pm0.90\times10^{-9}$(11)**
LTD_4 + 0.1 mM cysteine	$4.67\pm0.72\times10^{-9}$(3)
LTE_4	$2.63\pm0.19\times10^{-8}$(4)**
FPL-55712	$5.45\pm0.36\times10^{-6}$(5)
Others	
Prastaglandin A_1, A_2, B_1, D_2, E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$,and I_2; thromboxane B_2, Glutamic acid, Cysteine, Glycine, Glutathione, Histamine, DMPP, Serotonin, Methacholine, Diphenhydramine, Propranolol, Methysergide, Atropine, Verapamil, Nefedipine, Diltiazem, Cromolyn, TMB-8, U-60257, Indomethacin	No effect at 10^{-5} and 10^{-4}M***

*Mean ± SE of numbers of experiments indicated in parenthesis.
**There is a significant difference between IC_{50} values of LTC_4 or LTD_4 and LTE_4 ($p < 0.05$), whereas the difference between the values of LTC_4 and LTD_4 is not significant (analysis of variance and Duncan's multiple range test).
***Each concentration of compounds was performed at least 2 times.

(data not shown), resembling a competitive inhibition of binding. LTC_4 and LTD_4 were the most potent competitors among the compounds tested, and there was no statistical difference between the ID_{50} values of both competitors in competing with $[^3H]LTD_4$ for the binding sites. LTE_4 was about 6-fold less potent in competition that LTD_4. AA could also inhibit binding but it competed 10,000-fold less effectively that LTD_4 when 50% of the sites were occupied. LTB_4 at 2.47×10^{-5} M had little effect.

Except for LTC_4, LTD_4, LTE_4 and AA, we found that FPL-55712 was the only agent tested that inhibited $[^3H]LTD_4$ binding. The listed prostaglandins, thromboxane B_2, amino acids, glutathione, agonists, antagonists and enzyme inhibitors did not inhibit $[^3H]LTD_4$ binding (Table 1). Moreover, we found that U-60257, a glutathione-S-transferase inhibitor (16), and cysteine,

an aminopeptidase inhibitor (17), did not significantly alter the competition curve or IC_{50} value of AA and LTD_4 in this preparation (Table 1).

Using the same solutions prepared for the binding studies, we also demonstrated that LTC_4, LTD_4, LTE_4 and AA produced a graded contraction of the isolated guinea pig lung strip. The contractile response to these compounds was slower in onset and longer in duration when compared with the contraction due to histamine (data not shown). The ED_{50} values for LTC_4, LTD_4, LTE_4, AA and histamine were $2.05 \pm 0.41 \times 10^{-9}$ (n=13), $3.33 \pm 0.74 \times 10^{-9}$ (n=13), $1.04 \pm 0.12 \times 10^{-9}$ (n=8), $2.46 \pm 0.40 \times 10^{-6}$ (n=3) and $1.02 \pm 0.56 \times 10^{-6}$ M (n=12), respectively. The difference among the ED_{50} values of LTC_4, LTD_4 and LTE_4 was not significant ($p > 0.05$, analysis of variance). This is consistent with the recent result of Dahlen et al. (18).

In this study, the high potency of LTC_4, LTD_4 and LTE_4 interacting at the binding sites apparently reflects the specificity of the receptor sites, and fits well with the potent contractile effect on guinea pig lung strips. The specificity of the receptor binding sites is also supported by the effective inhibition of $[^3H]LTD_4$ binding by FPL-55712 but not by LTB_4 and other compounds tested (see Table 1). Although nifedipine, verapamil, and TMB-8 have recently been shown to be effective in antagonizing the contraction of the lung strip to LTD_4 (9, 11, 19), our binding study proves that this is not a direct effect. Lack of the direct inhibition of $[^3H]LTD_4$ binding by prostaglandins and thromoboxane B_2 would exclude the possible mole of in vitro release of these substances in competing with $[^3H]LTD_4$ for binding sites. This fact and the failure of U-60275 to alter the inhibition of $[^3H]LTD_4$ binding by AA would demonstrate a direct effect of AA at the binding sites.

Both present and previous studies (20) have shown that the rank order of potency of LTs in competing with $[^3H]LTD_4$ for the binding sites appears to be $LTC_4 = LTD_4 > LTE_4$. However, we have found their potency order in producing airway contraction to be $LTC_4 = LTD_4 = LTE_4$. The reason for such discrepancy is unclear, but could be related to many factors previously

reported (10, 11, 18). It is also possible that [^{3}H]LTD_4 in the homogenate may bind to non-airway muscle cell types which contain a subclass of LTD_4 receptors and in such receptor, LTE_4 is less effective in terms of competition (21). The rank order of their potency in inhibiting [^{3}H]LTD_4 binding apparently differs from that of their ability to compete with [^{3}H]LTC_4 for LTC_4 receptor sites (22).

Our biphasic dissociation kinetics and curvilinear Scatchard plot. provide direct evidence for the first time for the existence of two affinity states of airway LTD_4 receptor sites in guinea pigs. We have quantitated the dissociation constants and concentrations of the low and high affinity binding sites. Each dissociation constant of the [^{3}H]LTD_4 binding sites is about 100-fold less than the corresponding K_L or K_H value for an acetylcholine interaction with airway muscarinic receptors (23). This may account for the much greater potency of LTD_4 in producing the airway contraction compared to muscarinic agonists. Further studies concerning the functional significance of the [^{3}H]LTD_4 low and high affinity binding sites are needed.

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