Inhibition of Radiolabeled Leukotriene-Binding by AS-35 in Guinea Pig Lung Membrane Fraction

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ABSTRACT — The inhibitory effects of 9-[(4-acetyl-3-hydroxy-2-n-propylphenoxy)methyl]-3-(1H-tetrazol-5-yl)-4H-pyrido[1,2-a]pyrimidin-4-one (AS-35), a peptide leukotriene (LT) antagonist, on specific bindings of radiolabeled LTC4 and LTD4 in guinea pig lung membrane were investigated to clarify the mechanism by which this agent inhibited LT-induced physiological responses. Binding assays were performed at 20°C in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM CaCl2, 10 mM MgCl2 and 10 mM cysteine in the absence (LTD4 binding assay) or presence (LTC4 binding assay) of 80 mM L-serine borate. Scatchard analysis of each LT specific binding indicated a single and high affinity binding site with a Kd of 0.21 ± 0.05 nM and Bmax of 808 ± 71 fmol/mg protein for [3H]-LTD4, and with a Kd of 21.6 ± 3.8 nM and Bmax of 74.9 ± 2.6 pmol/mg protein for [3H]-LTC4. Competition binding studies showed that AS-35 antagonized [3H]-LTD4 specific binding with a Kd value of 92.7 nM. In contrast, AS-35 was 100 times less effective in inhibiting [3H]-LTC4 specific binding, compared with [3H]-LTD4 specific binding. These results indicate that AS-35 interacts directly with peptide LTs receptors, especially the LTD4 specific binding site to produce its pharmacological effects.

In the preceding paper (1), 9-[(4-acetyl-3-hydroxy-2-n-propylphenoxy)methyl]-3-(1H-tetrazol-5-yl)-4H-pyrido[1,2-a]pyrimidin-4-one (AS-35) has been reported to antagonize the contractions induced by peptide leukotrienes (LTs) such as LTC4, LTD4 and LTE4 in isolated guinea pig ileum without showing any inhibition of ileal contractions induced by histamine, acetylcholine, serotonin and bradykinin. Tracheal contractions induced by LTD4 and LTE4 were also inhibited by AS-35. In addition, i.v.- and p.o.-administration of AS-35 inhibited LTC4-, LTD4- and antigen-induced LT-mediated bronchoconstrictions in guinea pigs.

The potent and selective antagonism by AS-35 suggests involvement of peptide LTs specific binding sites in its action at the end organ level. The present study was undertaken to assess the binding effect of AS-35 on peptide LTs receptors using specific binding assays with [3H]-LTD4 and [3H]-LTC4. The results obtained from the competition studies indicate that AS-35 interacts directly with peptide LTs specific binding sites to produce its pharmacological effects.

MATERIALS AND METHODS

Chemicals

[3H]-LTC4 and [3H]-LTD4 were obtained from New England Nuclear Co. Isotopically labeled compounds were reported to have specific activities of 1420.8 GBq to 1487.4 GBq/mmol and radiochemical purity greater than 98.5%. All of the materials used in this
study were exactly as described in the preceding paper (1).

**Crude membrane preparation from guinea pigs**

The membrane fraction was prepared according to the method of Pong and Dehaven (2). Briefly, male Hartley strain guinea pigs weighing 300 to 500 g were sacrificed, and the lungs were removed and minced into small segments. The tissue was homogenized with a Polytron for total of 1 min with 10-sec pulses at a setting of 6 at 0°C in 10 volumes (w/v) of buffer (50 mM Tris-HCl buffer, pH 7.4) containing soybean trypsin inhibitor (15 μg/ml), bacitracin (100 μg/ml), benzamidine (10 μg/ml) and phenylmethylsulfonyl fluoride (10 μM). The homogenate was centrifuged at 1,000 × g for 10 min to remove tissue clumps, unbroken cells and nuclei; and the supernatant was then obtained by centrifugation at 50,000 × g for 10 min at 4°C. The pellets were resuspended in buffer, homogenized with the Polytron for 10 sec at a setting of 4.5 and then centrifuged at 50,000 × g for 10 min. The final pellets were stored at −80°C. The concentrations of membrane protein were determined by the method of Lowry et al. (3) using bovine serum albumin as a standard.

**Radioligand binding assays**

Binding assays were performed according to the method of Pong and Dehaven (2). Briefly, [3H]-LTD₄ binding assays were carried out in buffer containing 50 mM Tris-HCl buffer (pH 7.4), 10 mM CaCl₂, 10 mM MgCl₂ and 10 mM cysteine at 20°C. Incubation mixtures in a total volume of 400 μl contained lung membrane, [3H]-LTD₄, LTD₄ and drug. For the time course experiments, 250 μg/ml of membrane protein and 2.15 nM [3H]-LTD₄ were used, and the mixtures were incubated for 0 to 90 min. In the experiments for protein dependency, 0.063 to 1.00 mg/ml of membrane protein were incubated with 1.72 nM [3H]-LTD₄ for 90 min. The [3H]-LTD₄ competition studies were conducted under conditions identical to the saturation studies with the exception of the use of [3H]-LTD₄ (1.46 to 2.03 nM) and competitor drugs at various concentrations. The condition for the [3H]-LTC₄ binding assay was similar to that described for the [3H]-LTD₄ binding assays in the presence of 80 mM L-serine borate. For the time course experiments, the mixtures containing 50 μg/ml of membrane protein and 1.66 nM [3H]-LTC₄ were incubated for 0 to 90 min. In the experiments for protein dependency, 0.02 to 0.3 mg/ml of membrane protein were incubated with 2 nM [3H]-LTC₄. The [3H]-LTC₄ saturation experiments were performed using 50 μg/ml of membrane protein and 0.71 to 71.1 nM of [3H]-LTC₄, and the mixtures were incubated for 30 min. The [3H]-LTC₄ competition studies were conducted under conditions identical to the saturation studies with the exception of the use of [3H]-LTC₄ (1.54 to 1.98 nM) and competitor drugs at various concentrations. At the end of incubation, bound radioactivity was separated from free by vacuum filtration through Whatman GF/C filters. Filters were rapidly washed 3 times with 4 ml of ice-cold 50 mM Tris-HCl buffer, placed in scintillation vials and then counted. Each assay was carried out in duplicate. Total and nonspecific bindings were defined as the [3H]-LT bound to the membranes in the absence or presence of a 1000-fold excess of unlabeled LT, respectively. Specific binding was defined as the total binding minus the nonspecific binding. The radioligand competition activities of the analogues were expressed as Kᵢ, defined as Kᵢ = IC₅₀ / [1 + [3H]-LT/Kₐᵢ], where [3H]-LT was the concentration of the radioligand used (4). The dissociation constant (K_d), the density of [3H]-LTD₄ and [3H]-LTC₄ binding (B_max) were determined by Scatchard analysis of saturation binding data (5).

**Analysis of LTs by high-performance liquid chromatography (HPLC)**

Identification of the bound ligand in the membrane preparation was determined by HPLC using Novapac C₁₈ reverse phase col-
umns (Waters). The radioactivity on the filter paper was immediately extracted with 2.5 ml of methanol. Extraction was repeated 2 times, and then the methanol was evaporated. Acetic acid (1 N) containing 10% methanol was added to about 0.5 ml of the remaining aqueous solution and this solution was passed through a Sep-pak cartridge. The cartridge was washed 2 times with 10 ml of distilled water, followed by 10 ml of petroleum ether, and further LTs were eluted with 20 ml of methanol. The LT fraction was evaporated, resuspended with about 50 μl of the eluting system and then subjected to reverse phase-HPLC. Peaks of radioactivity were identified by coelution with LTC4, LTD4 and LTE4 standards in the following solvent system: CH3CN : CH3OH : H2O : CH3COOH = 336:54:610:10 (pH 5.6). Columns were eluted at 1 ml/min, and standards were identified by UV absorbance at 280 nm.

RESULTS

Affinity and binding capacity for [3H]-LTD4 and [3H]-LTC4 specific bindings

Prior to saturation analysis, time and protein concentration dependence of these bindings were examined. Both [3H]-LTD4 and [3H]-LTC4 specific bindings increased by extending the incubation time, reached steady state within 20 min, and remained stable for up to 90 min. Based on these results, [3H]-LTs were incubated with lung membranes for 20 min in the following experiments. In these experiments, specific binding of [3H]-LTD4 and [3H]-LTC4 represented 77% and 83% of the total binding, respectively. For dependence of protein concentration, [3H]-LTD4 and [3H]-LTC4 specific bindings increased linearly with protein concentration in a range from 63 to 750 μg/ml of protein and from 2 to 75 μg/ml of protein, respectively. From these results, 250 μg/ml of protein for the [3H]-LTD4 specific binding assay and 50 μg/ml of protein for the [3H]-LTC4 specific binding assay were used in the following experiments. Scatchard analysis for these two specific bindings showed a single binding site with dissociation constants (Kd) of 0.21 ± 0.05 nM and maximum number of specific binding sites (Bmax) of 808 ± 71 fmol/mg protein for [3H]-LTD4 specific binding, and with Kd of 21.6 ± 3.8 nM and Bmax of 74.9 ± 2.6 pmol/mg protein for [3H]-LTC4 specific binding (Table 1).

Inhibition of [3H]-LTD4 specific binding by LT agonists and antagonists

The ability of LT agonists and antagonists to compete with [3H]-LTD4 specific binding were examined. The competition curve illustrated in Fig. 1 shows that the natural ligand LTD4 competed most effectively with the [3H]-LTD4 binding site (IC50 value of 2.3 nM), indicating that [3H]-LTD4 binding is stereoselective. LTE4 and LTC4 also bound to the [3H]-LTD4 specific binding site, but their efficacies were 10 (LTE4) and 100 (LTC4) times less than that of LTD4, respectively. However, LTB4 at a concentration of 30 μM did not inhibit [3H]-LTD4 specific binding. AS-35 more effectively inhibited [3H]-LTD4 specific binding with an IC50 value of 0.9 μM as compared to FPL-55712 whose IC50 value was 5.2 μM. The order of potency of LT agonists and antagonists for [3H]-LTD4 binding was LTD4 > LTE4 > AS-35 ≈ LTD4 > FPL-55712. Inhibition of [3H]-LTC4 specific binding by LT agonists and antagonists

As summarized in Fig. 2, LTC4 most effectively competed with [3H]-LTC4 for the binding sites (IC50 value of 32 nM), indicating that [3H]-LTC4 binding is stereoselective. LTD4 also bound to the [3H]-LTC4 specific binding site, but its efficacy was 100 times less than that of LTC4. LTE4 slightly inhibited [3H]-LTC4 specific binding at a concentration of 10 μM, but LTB4 at 30 μM did not show any inhibition. AS-35 and FPL-55712 also inhibited [3H]-LTC4 specific binding, each with an IC50 value of 10 nM. FPL-55712 at 100 μM completely inhibited [3H]-LTC4 specific binding, while the same concentration of AS-35 did not produce complete inhibition. The order of potency for the inhibition of [3H]-LTC4 bind-
Table 1. Comparative properties of $^{3}$H-LTD$_{4}$ and $^{3}$H-LTC$_{4}$ specific binding in guinea pig lung membrane

| Specific binding | Affinity ($K_d$) | Density ($B_{max}$) |
|------------------|-----------------|----------------------|
| $^{3}$H-LTD$_{4}$ | 0.21 ± 0.05 nM  | 808 ± 71 fmol/mg protein |
| $^{3}$H-LTC$_{4}$ | 21.6 ± 3.8 nM   | 74.9 ± 2.6 pmol/mg protein |

The affinities and density of $^{3}$H-LTD$_{4}$ and $^{3}$H-LTC$_{4}$ specific bindings were determined by Scatchard analysis. Each value represents the mean ± S.E. of 3 to 5 experiments.

Fig. 1. Inhibition of $^{3}$H-LTD$_{4}$ specific binding by LT agonists and antagonists. Crude lung membranes (250 μg protein/ml) were incubated with $^{3}$H-LTD$_{4}$ (1.46 – 2.03 nM) and LT agonists or antagonists for 30 min at 20°C. Each point represents the mean of 3 experiments. ■: LTD$_{4}$, △: LTE$_{4}$, ▽: LTB$_{4}$, ○: AS-35, △: FPL-55712, ●: LTC$_{4}$ (+ serine borate).

Fig. 2. Inhibition of $^{3}$H-LTC$_{4}$ specific binding by LT agonists and antagonists. Crude lung membranes (50 μg protein/ml) were incubated with $^{3}$H-LTC$_{4}$ (1.54 – 1.98 nM) and LT agonists or antagonists for 30 min at 20°C. Each point represents the mean of 3 experiments. ●: LTC$_{4}$, ■: LTD$_{4}$, △: LTE$_{4}$, ▽: LTB$_{4}$, ○: AS-35, △: FPL-55712.
ing was LTC₄ > LTD₄ ≈ FPL-55712 ≈ AS-35 > LTE₄.

**Kᵢ for LT agonists and antagonists**

As shown in Table 2, Kᵢ values of LTD₄, LTE₄ and LTC₄ for [³H]-LTD₄ specific binding were 0.24 nM, 2.27 nM and 97.0 nM, respectively. These results indicate that whereas the binding site occupied by [³H]-LTD₄ has high affinity and specificity for LTD₄ and LTE₄, this site shows relatively low affinity for LTC₄. Concomitantly, the [³H]-LTC₄ specific binding site has high affinity and specificity for LTC₄, while it displays low affinity for LTD₄ and LTE₄. Kᵢ values of AS-35 for [³H]-LTD₄ and [³H]-LTC₄ binding were 92.7 nM and 10.1 μM, respectively. Therefore, it is clear that AS-35 has approximately 100 times higher affinity for [³H]-LTD₄ specific binding than for [³H]-LTC₄ specific binding.

**HPLC analysis of membrane bound [³H]-metabolite of [³H]-LTD₄ and [³H]-LTC₄**

To ascertain whether the specific binding reflects [³H]-LTD₄ or [³H]-LTC₄ binding, HPLC was employed to analyze the radioactive materials bound to lung membranes. As indicated in Fig. 3, under the standard assay condition for [³H]-LTD₄ binding, greater than 93% of the total membrane radioactivity was co-eluted with unlabeled LTD₄ standard, and approximately 0.4% and 2.5% were co-eluted with a retention time close to that of LTC₄ and LTE₄, respectively. For [³H]-LTC₄ binding, approximately 90% of the total membrane radioactivity co-eluted with the unlabeled LTC₄ standard, less than 5% co-eluted with LTD₄, and no radioactive material was detected in the fractions co-eluted with LTE₄. These findings indicate that, under the assay condition employed, the binding assay reflects the interaction of [³H]-LTD₄ and [³H]-LTC₄ with each specific binding site.

**DISCUSSION**

In the preceding paper (1), we reported that AS-35 antagonized both the contractions of isolated preparations and the bronchoconstrictions induced by peptide LTs in guinea pigs. The present study was undertaken to investigate the inhibitory effect of AS-35 on specific bindings of [³H]-LTD₄ and [³H]-LTC₄ in guinea pig lung membrane. Scatchard analysis of the saturation experiments showed that these two specific bindings consist of a single and high affinity binding site with a Kᵦ of 0.21 ± 0.05 nM and Bₘₐₓ of 808 ± 71 fmol/mg protein for [³H]-LTD₄ specific binding as well as with a Kᵦ of 21.6 ± 3.8 nM and Bₘₐₓ of

| Table 2. Inhibition constant (Kᵢ) for LT agonists and antagonists |
|---------------------|------------------|------------------|
| Ligand              | Compound         | Kᵢ (M)           |
| [³H]-LTD₄           | LTC₄             | 9.70 × 10⁻⁸      |
|                     | LTD₄             | 2.37 × 10⁻¹⁰     |
|                     | LTE₄             | 2.27 × 10⁻⁹      |
|                     | LTB₄             | no inhibition at 3 × 10⁻⁶ M |
|                     | AS-35            | 9.27 × 10⁻⁸      |
|                     | FPL-55712        | 5.35 × 10⁻⁷      |
| [³H]-LTC₄           | LTC₄             | 2.95 × 10⁻⁸      |
|                     | LTD₄             | 8.86 × 10⁻⁶      |
|                     | LTE₄             | slight inhibition at 10⁻⁵ M |
|                     | LTB₄             | no inhibition at 3 × 10⁻⁶ M |
|                     | AS-35            | 1.01 × 10⁻⁵      |
|                     | FPL-55712        | 8.86 × 10⁻⁶      |

Kᵢ values were calculated using the equation Kᵢ = IC₅₀ / (1 + [LT]/K₀).
74.9 ± 2.6 pmol/mg protein for [3H]-LTC₄ specific binding. Data are consistent with the previous results obtained in the lungs of guinea pig (2, 6-10) and humans (11, 12).

The competition studies indicated that AS-35 antagonized the [3H]-LTD₄ specific binding with a Kᵢ value of 92.7 nM, and its potency was approximately 5 times more than that of FPL-55712. The results of binding assays are in keeping with the pharmacological actions of AS-35 that competitively antagonized the contractions of isolated guinea pig trachea induced by LTD₄ (1), and further indicate that AS-35 interacts directly with the LTD₄ specific binding site to produce its pharmacological effects. Mong et al. (9) have also demonstrated that the [3H]-LTD₄ specific binding site in guinea pig lung reflects its pharmacologically and physiologically important receptors, since the radioligand competition activity of LTD₄ was well correlated with the agonist and antagonist smooth muscle contractile responses.

In the competition studies for the [3H]-LTC₄ specific binding in the presence of l-serine borate, known to be an α-glutamyl-transpeptidase inhibitor (13), AS-35 also inhibited the [3H]-LTC₄ specific binding; however, its potency was approximately 100 times less effective in inhibiting the specific binding of [3H]-LTC₄ than that of [3H]-LTD₄, compared with the Kᵢ values of AS-35 for [3H]-LTD₄ and [3H]-LTC₄ specific bindings. The low potency of AS-35 for [3H]-LTC₄ specific binding does not result from its interaction with l-serine borate, because the inhibition of [3H]-LTD₄ specific binding by AS-35 was not affected by l-serine borate in the preliminary experiment (data not shown). This finding supports our previous results that AS-35 had little effect on the contractions of isolated guinea pig trachea induced by LTC₄ in the presence of l-serine borate (1). Unlike the [3H]-LTD₄ specific binding site, the physiological and pharmacological significance of the [3H]-LTC₄ specific binding site, should be made more clear, because the smooth muscle contraction activity of the LTC₄ analogue is not directly correlated with the radioligand competition activity (9). In addition, only a
small part of the binding site detected by the radioligand binding assays may reflect the LTC₄ receptors responsible for its biological response. Thus, there is no definite evidence that the [³H]-LTC₄ specific binding site can be defined as the true LTC₄ receptors.

As to the LTE₄ specific binding, a high affinity, stereoselective binding site for [³H]-LTE₄ has been identified and characterized in guinea pig lung membranes (14–16). From the results that Kᵅ and Bₘₐₓ for [³H]-LTE₄ specific binding are close to those for [³H]-LTD₄ specific binding, it is suggested that LTE₄ binds to the LTD₄ specific binding site and that smooth muscle contraction induced by LTD₄ and LTE₄ may be mediated by identical mechanisms. Indeed, our data show that although the [³H]-LTC₄ specific binding site was slightly antagonized by LTE₄, LTE₄ effectively competed with the [³H]-LTD₄ specific binding site. AS-35 has also been reported to antagonize LTE₄-induced contractions of isolated guinea pig ileum (1). These results indicate that AS-35 is a peptide LT antagonist highly specific for LTD₄ and possibly for LTE₄.

In conclusion, the specific binding sites occupied by [³H]-LTD₄ and [³H]-LTC₄ in guinea pig lung membranes are chemically and physiologically distinct. In addition, competition studies indicate that AS-35 is a selective and competitive inhibitor of [³H]-LTD₄ binding and to a lesser extent [³H]-LTC₄ binding. Thus, it is suggested that AS-35 interacts directly with peptide LT receptors, especially the LTD₄ specific binding site, to produce its pharmacological effects.

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