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HETEROGENEITY OF BINDING SITES FOR ICI 198,615 IN HUMAN LUNG PARENCHYMA

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Abstract—We have identified and characterized two different subclasses of binding site for the novel peptido-leukotriene (LT) antagonist, [^{3}H]ICI 198,615, in membranes from human lung parenchyma using a receptor–ligand assay. This novel compound is representative of a new class of LT receptor antagonists and it has been demonstrated to be several orders of magnitude more potent and selective than most other LT antagonists described to date. The binding of [^{3}H]ICI 198,615 is rapid, specific and saturable. Equilibrium was reached within 5–10 min. Non linear fitting of dissociation time courses has revealed the presence of two different components ($K_{off1} = 8.3 \pm 6.8 \times 10^{-4}$ sec^{-1} and $K_{off2} = 0.79 \pm 1.66 \times 10^{-3}$ sec^{-1}) of the kinetic curves, suggesting heterogeneity of the binding sites. Computer analysis of equilibrium binding data obtained at 25° results in a model with two classes of binding sites, a high affinity–low capacity class with $K_{d1} = 0.024 \pm 0.014$ nM and $B_{max1} = 0.015 \pm 0.004$ pmol/mg protein and a low affinity–high capacity class with $K_{d2} = 6326 \pm 3859$ nM and $B_{max2} = 473 \pm 383$ pmol/mg protein. In competition studies, LTD_4 was also found to interact with two classes of binding site ($K_{d1} = 0.016 \pm 0.008$ nM and $K_{d2} = 15195 \pm 8965$ nM). On the contrary, LTE_4 and LTC_4 were found to interact with a homogeneous class of sites only with $K_d = 7466 \pm 4629$ nM and $K_d = 428 \pm 73$ nM, respectively. Furthermore, we have evaluated the effect of a number of LT antagonists on the binding of [^{3}H]ICI 198,615. Ro 24-5913 ($K_d = 3.0 \pm 2.1$ nM), FPL55712 ($K_d = 4945 \pm 2868$ nM), LY171883 ($K_d = 19628 \pm 12365$ nM), SKF 104353 ($K_d = 74.2 \pm 46$ nM) and its enantiomer SKF 104373 ($K_d = 13627 \pm 6813$ nM) were found to interact with a single class of binding sites. The present studies indicate a heterogeneity of binding sites for ICI 198,615 in membranes from human lung parenchyma and that ICI 198,615 is a very potent and selective antagonist of LTD_4 receptors in this tissue.

Peptido-leukotrienes (LTs), known previously as slow reacting substances of anaphylaxis (SRS-A), are potent contractile agents of airways and vascular smooth muscles in different species [1, 2], including humans [3, 4]. They have been demonstrated to induce bronchoconstriction as well as secretion of bronchial mucus and mucosal edema. These properties have suggested that LTs might be important mediators of immediate hypersensitivity reactions, such as asthma [5].

It is now generally accepted that, because of their specificity and stereoselectivity, the pharmacological effects induced by cysteine-containing LTs are receptor-mediated. According to this hypothesis, several laboratories, including ours, have identified and characterized high affinity, specific and saturable binding sites for LTC_4 [6–9] and LTD_4 [10–13] in different tissues and species. Recently, a significant effort has been devoted to the development of selective and specific antagonists of LTs, which might represent potential novel drugs for the therapy of asthma. Since the discovery of the first LT antagonist, FPL55712 [14], several other compounds have been synthesized which antagonize the effects of LTs, such as the structural analog LY171883 [15], the structurally related SKF 104353 and SKF 104373 [16] and, more recently, Ro 24-5913 [17] and ICI 198,615 [18, 19]. The latter compound is representative of a new class of LT receptor antagonists and has been demonstrated to be among the most potent and selective antagonists of LTD_4 receptors yet described.

The object of the present investigation was to identify and characterize specific binding sites for the leukotriene antagonist [^{3}H]ICI 198,615 in human lung parenchyma and its putative interaction with LTD_4 receptors, using a radioligand binding assay.

MATERIALS AND METHODS

Materials. [^{3}H]ICI 198,615 (60 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). ICI 198,615 was kindly provided by the Department of Pharmacology and Medicinal Chemistry, Stuart Pharmaceuticals, Division of ICI America, Inc. (Wilmington, DE, U.S.A.). LTD_4, LTC_4, LTE_4 FPL55712 and Ro 24-5913 were a gift from Hoffman-La Roche, Inc. (Nutley, NJ, U.S.A.). SKF 104353 and SKF 104373 were kindly provided by Smith Kline and French Laboratories (King of Prussia, PA, U.S.A.). LY171883 was kindly provided by Eli Lilly and Co. (Indianapolis, IN, U.S.A.). Serine-borate was prepared as an equimolar solution of serine and boric acid (both from the Sigma Chemical Co., St Louis, MO, U.S.A.) in 50 mM Tris–HCl buffer, pH 7.1. Glycine and cysteine were both from Sigma.

Membrane preparation. Membranes from human lung parenchyma were prepared as described previously [8]. Briefly, macroscopically normal

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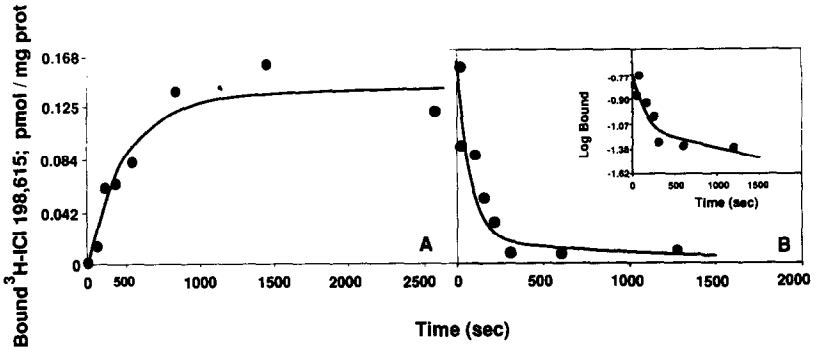

Fig. 1. Panel A. Association time course of specific [³H]ICI 198,615 binding. Panel B. Dissociation time course of specific [³H]ICI 198,615 binding. The inset represents the logarithmic transformation of the data of panel B. [³H]ICI 198,615 was 0.5 nM in both experiments. The interpolated curves were obtained by computerized weighted nonlinear fitting of two experiments, each performed in duplicates. Standard errors (SE) are not shown, but were always less than 15%.

specimens of human lung parenchyma removed during thoracotomy for lung cancer from about 50 different individuals were minced and homogenized at 4° in 50 mM Tris–HCl buffer, pH 7.4 (1:24, w/v) with a Polytron homogenizer. The homogenate was centrifuged at 770 *g* for 10 min (4°) and the supernatant centrifuged at 27,000 *g* for 20 min. The pellet was resuspended in the same buffer and centrifuged again under the same conditions. The final pellet was resuspended in 1/20 of the homogenization volume and stored at −80° for up to 3 months. For the binding assay, the pellet was diluted immediately before use in 50 mM Tris–HCl containing serine-borate (40 mM, final concentration in the sample), glycine (10 mM, final concentration) and cysteine (10 mM, final concentration).

Binding assay. The standard assay was performed in polypropylene tubes in a final volume of 250 μL containing 50 mM Tris–HCl buffer, pH 7.1, 0.2% ethanol, 1 mM $CaCl_2$ and 0.03–0.5 nM [³H]ICI 198,615, and ICI 198,615, LTC_4, LTD_4, LTE_4, FPL55712, LY171883, SKF 104353, SKF 104373 and Ro 24-5913 at the indicated concentrations; serine-borate, glycine and cysteine carried with the membranes (0.15 mg protein/sample). Labeled and unlabeled ICI 198,615 were always freshly prepared with a large excess in Tris–HCl buffer containing 0.5% ethanol and immediately added to the samples. The incubation was started by addition of the membranes and, unless otherwise stated, carried out at 25° for 30 min. After the incubation, bound [³H]-ICI 198,615 was separated from free by filtration through Whatman GF/C fiberglass filters. The filters were washed three times with 5 mL of 50 mM Tris–HCl buffer, pH 7.1. The samples were then counted with 9 mL of Filtercount (Packard). For kinetics experiments, nonspecific binding was defined as that which occurred in the presence of 1×10^{-5} M unlabeled ICI 198,615 and represented up to 50% of total binding. In all the equilibrium experiments, nonspecific binding was estimated as an unknown parameter, as well as K_d and B_{max}, by the computer program used for the analysis.

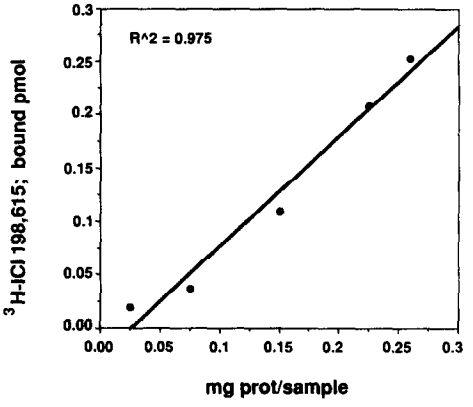

Fig. 2. Relationship between the total [³H]ICI 198,615 (0.5 nM) binding and the amount of proteins present in each sample.

Computer analysis. Analysis of dissociation time courses was performed using a weighted nonlinear least squares (WNLLS) program, EXPFIT [20], for simultaneous analysis of families of exponential decay curves. Analysis of equilibrium ligand binding data were performed by using a different computer program, LIGAND [21]. A series of models of increasing complexity involving from one to three binding sites were considered. Selection of the best fitting model was based on the F test for the extra sum of squares principle [22]. A statistical level of significance of $P < 0.01$ was selected. Whenever possible, data obtained from multiple experiments or multiple membrane preparations were analysed simultaneously. Data are expressed as means ± standard error.

HPLC analysis. Both LTs and ICI 198,615 were checked for purity, and purified if the impurities were above 10% by reversed phase HPLC using a 110B Solvent Delivery Module equipped with an

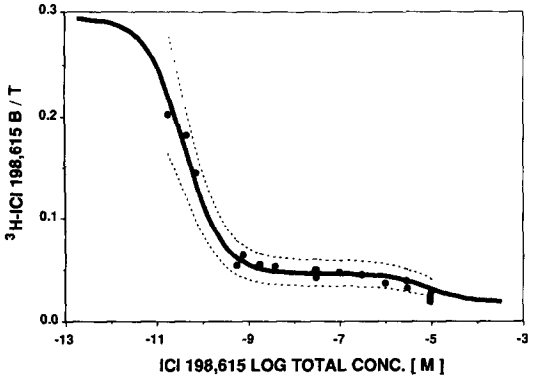

Fig. 3. [^{3}H]ICI 198,615 binding curve expressed as the ratio of total bound concentration over total concentration added (B/T). The first three concentrations were obtained with increasing amounts of [^{3}H]ICI 198,615 (saturation type experiment), while the last nine were obtained by adding increasing concentrations of unlabeled ICI 198,615 to a fixed concentration of labeled ligand (displacement type experiment). The solid line represents the curve calculated from the parameters obtained by the computerized analysis of three different experiments. Each point is the mean of triplicate determinations. The dotted lines represent the 95% confidence limits.

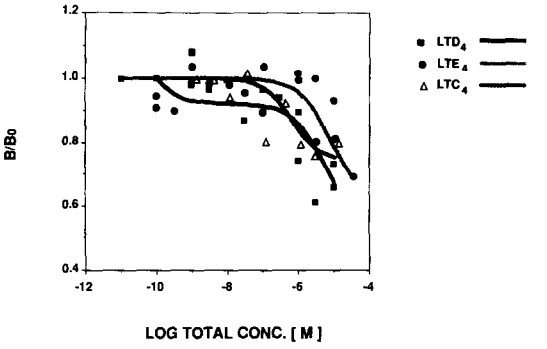

Fig. 4. Heterologous displacement curves of LTD_4 (black squares), LTE_4 (grey circles) and LTC_4 (open triangles) against [^{3}H]ICI 198,615 expressed as the ratio of total bound over total initial binding (B/B_0). The interpolated curves were calculated from the parameters obtained by computerized weighted nonlinear fitting of two experiments for each curve. Each point is the mean of triplicate determinations; 95% confidence limits are not shown for the sake of clarity.

ODS Ultrasphere C18 column (5 μm, 4.6 mm × 25 cm) and a Programmable Detector Module 166 (Beckman). The LTs were eluted isocratically with CH_3OH–H_2O–CH_3COOH (65:35:0.02), pH 5.8 with NH_4OH at 17, 20, 22 ± 0.1 min for LTC_4, LTD_4 and LTE_4, respectively. The flow rate was 1 mL/min. UV detector was set at 280 nm. ICI 198,615 was analysed using CH_3CN–H_2O–CH_3COOH (50:50:0.01), pH 4.4 at a flow rate of 1 mL/min and the compound showed a retention time of 25 min; the UV detector was set at 256 nm. In order to check the purity of [^{3}H]ICI 198,615, fractions were collected every 30 sec with a fraction collector (GILSON mod 201) and the radioactivity of each fraction was assessed by liquid scintillation counting.

RESULTS

The binding of [^{3}H]ICI 198,615 was rapid, specific and saturable. Equilibrium was reached within 5 to 10 min (Fig. 1 panel A). The specific binding was also rapidly reversible (Fig. 1 panel B) after addition of an excess (2×10^{-5} M) of unlabeled ICI 198,615. The logarithmic transformation of the bound data of the kinetic dissociation curve (Fig. 1 panel B insert) was found to be nonlinear, suggesting multiple components for the dissociation phase. Nonlinear fitting and modelling of dissociation time courses have revealed the presence of a biphasic exponential decay curve, in agreement with the hypothesis of the heterogeneity of the binding sites. The two dissociation rate constants, calculated by means of the computer program EXPFIT [20], were identified as being $K_{off1} = 8.3 \pm 6.8 \times 10^{-4}$ and $K_{off2} = 7.9 \pm 1.66 \times 10^{-3}$ sec^{-1}.

In preliminary experiments, the dependence of [^{3}H]ICI 198,615 binding on the protein concentration of the human lung membranes was investigated. As is clear from Fig. 2, the specific binding increased linearly with increasing protein content in the range of 0.01–0.3 mg/sample. For further experiments a concentration of 0.15 mg protein/sample was used.

In order to determine the equilibrium binding curves of ICI 198,615 at the broadest possible concentration range, we performed a particular type of homologous curve called "mixed type experiment" [23], where the first three concentrations were obtained with increasing amounts of [^{3}H]ICI 198,615 (saturation type experiment) and all the others were obtained by adding increasing concentrations of unlabeled ICI 198,615 to a fixed concentration of labeled ligand (displacement type experiment) (Fig. 3). Visual inspection of Fig. 3 suggested the presence of at least two classes of binding site as evidenced by the fact that the curve spanned over two orders of magnitude and by the presence of a plateau clearly separating the two components. Computerized analysis of these data by means of LIGAND [21] revealed the presence of two different classes of binding sites for [^{3}H]ICI 198,615, thus substantiating the data obtained previously with the kinetic experiments. The ICI compound was found to recognize a high affinity–low capacity class of sites with $K_{d1} = 0.024 \pm 0.014$ nM and $B_{max1} = 0.015 \pm 0.004$ pmol/mg protein, and a low affinity–high capacity class with $K_{d2} = 6327 \pm 3859$ nM and $B_{max2} = 473 \pm 383$ pmol/mg protein.

The pharmacological characterization of these binding sites was performed by means of a series of heterologous displacement curves using [^{3}H]ICI 198,615 as the labeled ligand and a number of unlabeled agonists and antagonists. Each experiment consisted of one or more heterologous curves run together with a ICI 198,615 homologous curve in order to reproduce each time the homologous curve

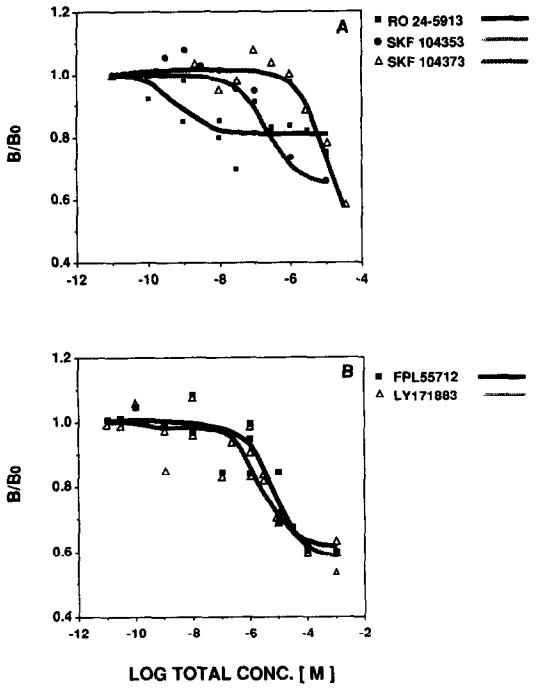

Fig. 5. Heterologous displacement curves of Ro 24-5913 (black squares), SKF 104353 (grey circles) and SKF 104373 (open triangles) (panel A) and of FPL55712 (black squares) and LY171883 (open triangles) (panel B) against [^{3}H]ICI 198,615. All the curves are expressed as the ratio of total bound over total initial binding (B/B_0). The interpolated curves were calculated from the parameters obtained by computerized weighted nonlinear fitting of three different experiments for Ro 24-5913, FPL55712 and LY171883, and two experiments for the SK&F compounds. Each point is the mean of triplicate determinations; 95% confidence limits are not shown for the sake of clarity.

necessary for the subsequent analysis. Figure 4 shows the heterologous displacement curves of three different LT agonists, LTD_4, LTC_4 and LTE_4. The heterologous curves were then analysed simultaneously with the ICI 198,615 homologous curve obtained within the same experiment [21], and it was found that LTD_4, as well as its antagonist ICI 198,615, recognized two different classes of binding site with $K_{d1} = 0.016 \pm 0.008$ nM and $K_{d2} = 15195 \pm 8965$ nM. On the contrary, LTE_4 and LTC_4 were found to recognize a homogeneous class of binding sites with $K_d = 7466 \pm 4629$ nM and $K_d = 428 \pm 73$ nM, respectively.

Figure 5 (panel A) shows that the specific antagonists, Ro 24-5913, SKF 104353 and its enantiomer SKF 104373, dose-dependently inhibited [^{3}H]ICI 198,615 binding. These compounds were found to interact with a single class of binding sites with $K_d = 3.0 \pm 2.1$ nM, $K_d = 74.2 \pm 46$ nM and $K_d = 13627 \pm 6813$ nM, respectively. In panel B are presented the heterologous displacement curves of FPL55712 and LY171883. Both these compounds were found, by computer analysis, to recognize a homogeneous class of binding sites with $K_d = 4945 \pm 2868$ nM and a $K_d = 19628 \pm 12365$ nM, respectively.

DISCUSSION

We have identified specific, saturable and reversible binding sites for [^{3}H]ICI 198,615 in membranes from human lung parenchyma. This compound was characterized previously as interacting with LTD_4 receptors in guinea pig lung membranes by means of binding studies [18] and as being a potent and selective antagonist of LT-induced contraction in both guinea pig trachea and lung strips [19]. High affinity binding sites for [^{3}H]ICI 198,615 had been identified by others in guinea pig lung membranes [24, 25], as well. However, it is well known that there are differences between the pulmonary pharmacology of LTs in rodents and primates [26] and, therefore, it is inadvisable to extrapolate data from lower species to humans.

By computer analysis of both the kinetic and equilibrium ligand binding data we have characterized a heterogeneity of binding sites for [^{3}H]ICI 198,615 in human lung membranes, at variance with the data reported by others [27]. We identified a high affinity–low capacity class of sites with a K_d in the picomolar range and a low affinity–high capacity class of sites with a K_d in the micromolar range (Fig. 3). The identification of this second class of sites depends upon spanning a large range of ligand concentrations, from the very low used in the "saturation" part of the curve, to the very high in the displacement curve. Indeed, by effectively combining both saturation and displacement curves in a single experiment we succeeded in obtaining adequate radioactivity in the lower concentration range and yet reaching high concentrations of the ligand without consuming an excessive amount of labeled compound. In addition, in order to be able to discriminate between the high and low affinity classes, it was important to perform the simultaneous analysis of saturation and displacement curves by means of a computer program. None of these conditions were satisfied previously.

The sites labeled by [^{3}H]ICI 198,615 bind the LT as well (Fig. 4). LTD_4 was found to recognize two different classes of binding site, whereas LTC_4 and LTE_4 recognized a single class of sites, consistent with the data published previously by us [8].

The different antagonists tested, i.e. Ro 24-5913, FPL55712, LY171883, SKF 104353 and its enantiomer SKF 104373, seemed to interact with a homogeneous class of sites, albeit with very different affinities. SKF 104353 could interact also with the low affinity–high capacity class of sites, but this model was not always reproducible. Therefore, further studies using other labeled ligands, e.g. LTC_4, LTD_4 and LTE_4, are necessary in order to establish a definitive model for LT receptors in human lung parenchyma. The biological importance and significance of the two classes of site identified by [^{3}H]ICI 198,615 remain to be established. Whether both classes represent functional LT receptors is an issue that needs further investigation.

In conclusion, the identification and characterization of specific binding sites for a novel and

very potent LT antagonist, such as ICI 198,615, in membranes from human lung parenchyma might help to clarify the role of the LTs in the pathophysiology of asthma. Moreover, the study of different LT agonists and antagonists, with the help of computer analysis and modelling, will serve as a guide for the identification of the underlying model for LT receptors in the human lung. This, in turn, will help in the development of new, more potent and selective drugs for the treatment of asthma.

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