Inflammation, Vol. 14, No. 4, 1990

IDENTIFICATION OF β_2-ADRENOCEPTORS ON GUINEA PIG ALVEOLAR MACROPHAGES USING (−)-3-[^{125}I]IODOCYANOPINDOLOL

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Abstract—The β-adrenoceptor antagonist (−)-3-[^{125}I]iodocyanopindolol ([^{125}I]ICYP) binds with high affinity and in a saturable way to membranes of guinea pig alveolar macrophages. The equilibrium dissociation constant for [^{125}I]ICYP is 24.3 ± 1.2 pM, and the number of binding sites is 166.3 ± 13.7 fmol/mg protein ($N = 4$, ± *SEM*). Displacement studies with selective antagonists showed that [^{125}I]ICYP labels β_2-adrenoceptors on guinea pig alveolar macrophages.

INTRODUCTION

Inflammatory processes are currently considered to contribute to the development of bronchial hyperresponsiveness, which is often encountered in allergic conditions. Recently it has been shown that activated pulmonary macrophages can cause an impairment of guinea pig tracheal β-adrenoceptor function through the release of reactive oxygen radicals (1). Moreover, via the release of thromboxane A_2 canine lung macrophages have been reported to facilitate cholinergic neurotransmission in airway smooth muscle (2). These data indicate that pulmonary macrophages might play an important role in the development of airway obstruction.

Macrophage activity is closely correlated to intracellular cAMP concentrations. β-Adrenergic agents have been shown to increase cellular cAMP levels (3–5) and concomitantly to reduce cellular activity (6). Since the cAMP-pro-

0360-3997/90/0800-0421$06.00/0 © 1990 Plenum Publishing Corporation

ducing receptors on alveolar macrophages might be important in the regulation of airway inflammation, study of these receptors is needed. Recently we observed that ovalbumin sensitization results in an augmented β-adrenoceptor-stimulated cAMP production of guinea pig alveolar macrophages (unpublished results). There are indications that this elevation of the cAMP production is regulated at the level of the β-adrenoceptor. Since previous attempts to label the β-adrenoceptor of guinea pig alveolar macrophages were rather unsuccessful (3), we evaluated the binding of the nonselective β-adrenoceptor antagonist (−)-3-[^{125}I]iodocyanopindolol ([^{125}I]ICYP) to membranes of guinea pig alveolar macrophages in more detail.

MATERIALS AND METHODS

Materials. (−)-[^{125}I]ICYP (free base, specific activity: 1950 Ci/mmol) was obtained from Amersham International plc (Amersham, U.K.). Timolol maleate was purchased from Sigma Chemical Co. (St. Louis, Missouri). l-Propanolol hydrochloride, *d*-propranolol hydrochloride, ICI 118.551 and ICI 89.406 were kind gifts from Imperial Chemical Industries (Macclesfield, U.K.).

Membrane Preparation of Guinea Pig Alveolar Macrophages. Male Hartley guinea pigs (300–500 g) were anesthetized by injecting sodium pentobarbitone (70 mg/kg intraperitoneally), the trachea was cannulated and bronchoalveolar cells were collected by repeated lavages of 8-ml aliquots of 0.9% saline. The cells were recovered from the lavage fluid by centrifugation (400*g*, 10 min, 4°C), resuspended in Gay balanced salt solution (pH 7.4), and a Ficoll-Isopaque gradient centrifugation procedure was carried out (400*g*, 30 min., 4°C). Enriched macrophage populations consisted of >95% alveolar macrophages as differentiated by May-Grünwald-Giemsa staining. Viabilities ranged from 90 to 95% as tested by trypan blue exclusion. Macrophages were homogenized in 50 mM Tris HCl, containing 0.25 M sucrose, 25 mM KCl, and 5 mM $MgCl_2$ (pH 7.4) using a Heidolph pestle. The homogenate was centrifuged at 50,000*g* for 120 min at 4°C, and the pellet was resuspended in 50 mM Tris HCl containing 10 mM $MgCl_2$ (pH 7.4).

[^{125}I]ICYP Binding Assay. In the saturation experiments, membrane suspension (10 μg) was incubated with increasing concentrations of [^{125}I]ICYP (5–80 pM) in the absence or presence of 0.1 μM timolol to define total and nonspecific binding. The incubation was performed in triplicate in a total volume of 200 μl in 50 mM Tris HCl, containing 10 mM $MgCl_2$ (pH 7.4 at 37°C). The incubation was started with the addition of 50 μl membrane suspension and was performed for 60 min at 37°C. After 60 min, the glass tubes were quickly transferred to 4°C, rinsed with 3 ml cold 50 mM Tris HCl containing 10 mM $MgCl_2$ (pH 7.4 at 4°C), and rapidly filtered through Whatman GF/C glass fiber filters (Whatman International Ltd., Maidenstone, England). This procedure was repeated once, whereafter the filters were washed twice with 3 ml cold buffer. Radioactivity was counted in a gamma-counter (Packard) with an efficiency of 68%.

The characterization of the [^{125}I]ICYP binding was performed by displacement studies with several selective β_1- and β_2-adrenergic antagonists. In these experiments membrane suspension was incubated with a single concentration [^{125}I]ICYP (60 pM) and increasing amounts of unlabeled agents. Binding data were evaluated with the nonlinear regression program LIGAND (7).

Protein Determination. Protein was assayed as previously described (8), using bovine serum albumin as standard.

RESULTS

Specific [^{125}I]ICYP binding to membranes of guinea pig alveolar macrophages was defined as the difference of [^{125}I]ICYP binding in the absence (total binding) or presence of 0.1 μM timolol (nonspecific binding). Under these conditions [^{125}I]ICYP bound with a high affinity and in a saturable way to the membrane fraction. Analysis of the binding data by nonlinear regression revealed the presence of a single binding site for [^{125}I]ICYP with an equilibrium dissociation constant (K_d) of 24.3 $\pm$ 1.2 pM and a maximal binding capacity (B_{max}) of 166.3 $\pm$ 13.7 fmol/mg protein (mean $\pm$ *SEM*, N = 4). Fits including more binding sites did not reach higher significance. The presence of a single binding site was confirmed by the transformation of the specific binding into a Scatchard plot. This transformation resulted in linear relationships, indicating the interaction of the radioligand with a single binding site (data not shown).

Displacement of the [^{125}I]ICYP binding by some β-adrenoceptor antagonists occurred in a biphasic manner (Figure 1); all displacements curves were significantly best fitted according to a two-binding-site model. Timolol and l-propranolol both bound with high affinity to the [^{125}I]ICYP binding site (K_i = 0.2–0.5 nM), but also showed an interaction with the [^{125}I]ICYP binding sites at 1000-fold lower concentrations. The stereoisomers of propranolol only showed a stereospecificity in favor of the l-isomer for the high-affinity binding site (Table 1).

To discriminate between β_1- and β_2-adrenoceptors, the two selective agents ICI 118.551 and ICI 89.406 were used. The β_2-receptor selective ICI 118.551

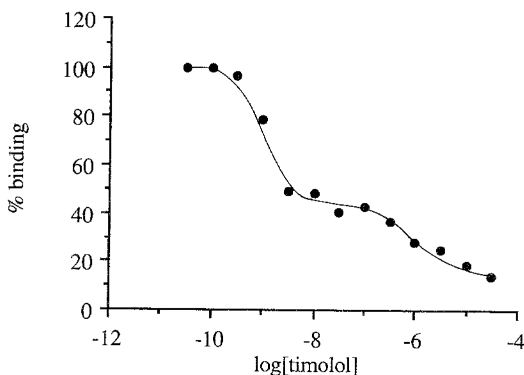

Fig. 1. Displacement of [^{125}I]ICYP binding to membranes of guinea pig alveolar macrophages by the nonselective β-adrenoceptor antagonist timolol. A typical experiment out of three is shown. The binding data could be best fitted according to a two binding site model.

Table 1. Inhibition Constants of Some β-Adrenoceptor Antagonists for Displacement of [^{125}I]ICYP Binding from Membranes of Guinea Pig Alveolar Macrophages[a]

Drug	K_{i1} (nM ± *SEM*)	K_{i2} (μM ± *SEM*)
Timolol	0.18 ± 0.06	3.1 ± 1.4
l-Propranolol	0.55 ± 0.21	13.0 ± 5.3
d-Propranolol	42.9 ± 5.0	19.1 ± 1.8
ICI 118.551	1.7 ± 0.4	12.6 ± 2.4
ICI 89.406	205.2 ± 50.2	19.2 ± 3.8

[a] Data shown are the results from simultaneous fitting procedures of three independent experiments.

proved to be 100-fold more effective in displacing [^{125}I]ICYP binding than ICI 89.406 (Table 1). These two agents also showed a low-affinity binding component in the micromolar range.

DISCUSSION

Alveolar macrophages form a major defense mechanism in the lung towards inhaled substances. Elevation of the intracellular cAMP levels has been shown to be reflected by marked changes in macrophage activity (6). Previously it has been shown that the β-adrenoceptor system of guinea pig and human alveolar macrophages is functionally coupled to the cAMP-producing adenylate cyclase (3–5). Experiments with selective agents suggested that the β_2-adrenoceptor subtype was involved in this response (3–5).

However, radioligand binding studies on membranes of guinea pig alveolar macrophages with [^{125}I]ICYP could not substantiate these findings. Whereas a K_d value of approximately 42 pM for [^{125}I]ICYP is expected for binding to β-adrenoceptors (9), Henricks et al. observed a 20-fold lower affinity (850 pM) for the binding of [^{125}I]ICYP to guinea pig alveolar macrophages (3). Recently we observed that ovalbumin sensitization results in an enhanced β-adrenoceptor-stimulated cAMP production of guinea-pig alveolar macrophages (unpublished results). This augmentation of the cAMP generation is probably regulated at the level of the β-adrenoceptor. Therefore we examined the binding of [^{125}I]ICYP to guinea pig alveolar macrophages in more detail. In contrast to previous findings (3), [^{125}I]ICYP binds with high affinity (K_d = 24 pM) to membrane fractions of the macrophages in this study. This value corresponds well with the initially reported K_d value of 42 pM on guinea pig left ventricle membranes (9). The discrepancy with the previous study, which reports a value

of 850 pM (3), can be explained by some methodological differences. For example, a Ficoll-Isopaque centrifugation step was included to purify the cells after bronchoalveolar lavage in our study. Moreover, based on displacement studies, the determination of the nonspecific binding in the saturation experiments was performed with a 100-fold lower concentration of timolol (0.1 μM vs. 10 μM) in the present study. Initial experiments, using 10 μM timolol, also yielded rather low affinities for [^{125}I]ICYP and were usually best fitted according to a two-binding-site model. The secondary binding site for [^{125}I]ICYP also is shown in the displacement experiments. This site showed low affinities for all tested agents. Moreover, the secondary binding site did not show any stereospecificity for the two enantiomers of propranolol, suggesting that it is not related to the β-adrenoceptor.

The high-affinity binding site displayed several characteristics of the β_2-adrenoceptor subtype. The 80-fold difference in displacing activity of the two isomers of propranolol is indicative of the presence of β-adrenoceptors. Using the β_1- and β_2-selective agents ICI 89.406 and ICI 118.551, it was possible to characterize the [^{125}I]ICYP binding site as a β_2-adrenoceptor. The K_i values of these compounds, found in this study, correspond well with previous reported findings on β_2-adrenoceptors of guinea pig lung and rat colon (9, 10). Therefore this study shows the presence of β_2-adrenoceptors on guinea pig alveolar macrophages, which resemble β_2-adrenoceptors in other tissues. Moreover, based on these findings, it is clear that radioligand binding studies should be handled with great caution. Stereoisomers appear to be nice tools in this respect. Displacement studies with such ligands (if available) should be performed for defining radioligand binding sites as receptor sites.

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