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CHARACTERIZATION OF [^{3}H]Ro5-4864 BINDING TO "PERIPHERAL" BENZODIAZEPINE RECEPTORS IN GUINEA PIG ALVEOLAR TYPE II CELLS*

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Abstract—The binding of [^{3}H]Ro5-4864 to peripheral benzodiazepine receptors has been demonstrated in a highly purified preparation of alveolar type II cells. [^{3}H]Ro5-4864 binding to guinea pig alveolar type II cells indicated one population of binding sites with high affinity (K_D = 5.7 nM) and saturability (B_{max} = 4582 fmol/mg membrane protein).

Benzodiazepines (BZ) have been used therapeutically as tranquilizers, anticonvulsants, muscle relaxants and sedative or hypnotic drugs [1]. These effects are thought to be mediated through high affinity, stereospecific receptors localized in the central nervous system (CNS) [2–5]. These "central" BZ receptors (CBR) are coupled to both $GABA_A$ receptors and an associated chloride ionophore, and this "supramolecular complex" may be physiologically important in the regulation of neuronal excitability. Another class of recognition sites for BZ has been characterized in many non-neural tissues, including heart [6], mast cells [7], adrenals [8], platelets [9], liver, kidney, testis, and lung [10, 11], as well as several cultured cell lines [12–14]. Several lines of evidence have demonstrated that these "peripheral" benzodiazepine receptors (PBR) are physically [8, 15] and pharmacologically [11, 13, 14, 16–18] distinct from CBR.

Although the physiological function of PBR is not known, recent reports suggest that these receptors may regulate calcium channels in heart [19, 20] and some electrolytes in kidney [21–24] and thus could play a physiological role in ion transport. PBR have been characterized in lung homogenates [10, 11]. However, neither the physiological function nor the cellular origin of these receptors in lung is known. Granular type II cells located in the alveolar epithelium [25, 26] synthesize and secrete surfactant, and have specialized ion transport systems [27–29].

The present study reports the first characterization of [^{3}H]Ro5-4864 binding to peripheral BZ receptors in guinea pig alveolar type II cells. Guinea pigs were selected as a model since their lungs appear more similar in surfactant development to human lungs than to other species [30–33].

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MATERIALS AND METHODS

Isolation of alveolar type II cells. Male Hartley strain guinea pigs (250–300 g) were purchased from Camm Research Laboratories, Wayne, NJ. Type II cells were isolated by digestion of lung with elastase and purified by centrifugal elutriation as previously described [33]. In brief, guinea pigs were anesthetized by intraperitoneal injection of pentobarbital containing heparin. The chest cavity was opened and lungs were perfused by gravity with 2.6 mM phosphate-buffered solution containing 5.6 mM glucose (pH 7.4). Following perfusion, the intact lungs were removed from the thorax and lavaged five times with the perfusion solution. Lungs were instilled with a solution of elastase via a cannula inserted into the trachea, and the perfused lungs were immersed in a solution of 145 mM NaCl for 20 min at 37°. Lungs were then minced with scissors, mixed with fetal calf serum, and filtered sequentially through cotton gauze, 100 μm and 20 μm nylon mesh. The cell suspension was then centrifuged at 200 *g* for 10 min. The cells were washed and then suspended in Hanks' balanced salt solution, pH 7.4, containing 1 mg/ml bovine serum albumin. With the rotor spinning at 2000 rpm, the cell suspension was injected into the mixing chamber and then pumped into the separation chamber of the rotor at a flow rate of 10 ml/min. After all the samples were in the separation chamber, they were eluted by stepwise increase in flow rate (10–12–14–18–22–26–40 ml per min). The fractions collected under 14 and above 26 ml per min were discarded. The fraction collected between 14 and 18 ml per min represented mostly lymphocytes (80% lymphocytes and 20% type II cells), whereas the fraction collected between 22 and 26 ml per min represented homogenous type II cells [33]. For cellular identification, the sample from each fraction was stained with a modified Papanicolou stain, and percentages of type II cells were estimated with the fluorescent compound phosphoine 3R in a Carl Zeiss Standard 16 microscope [33]. The viability of type II

cells was judged by the Trypan blue exclusion test [34].

Receptor binding assay. For receptor binding studies, the cell pellet was washed, suspended in 50 mM Tris–HCl (pH 7.4) (100 μl/10^6 cells), and homogenized by using a Brinkman Polytron (setting 6–7, 15 sec). The homogenate was then centrifuged at 20,000 *g* for 20 min (4°), and the pellet was washed three times and resuspended in 50 mM Tris buffer, pH 7.4 (2–4 mg membrane protein/ml). The binding of [^{3}H]Ro5-4864 to the crude cell membrane fraction was determined in a volume of 1 ml containing: 0.1 ml (30–60 μg) membrane protein, 0.1 ml radioligand (0.5 to 32 nM), 0.1 ml Ro5-4864 (5 μM) (or buffer), and 0.7 ml assay buffer (Tris–HCl, 50 mM, pH 7.4). The reaction was initiated by addition of tissue and terminated after 60 min at 0° by rapid filtration over Whatman GF/B strips using a Brandel M-24R filtering manifold (Brandel Instruments, Gaithersburg, MD), with two 5-ml washes of ice-cold buffer. The specific binding of [^{3}H]Ro5-4864 was defined as the difference in binding obtained in the presence and absence of Ro5-4864 (5 μM). The radioactivity retained by the filters was measured in a Packard B-2450 liquid scintillation spectrometer, using hydrofluor (8 ml) (National Diagnostics, Somerville, NJ) as a scintillant.

Scatchard analysis, Hill plot, and determination of binding constants were performed according to Bennett [35]. Protein concentrations were determined according to Lowry *et al.* [36] using bovine serum albumin as standard.

[^{3}H]Ro5-4864 (86.4 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Nonradioactive Ro5-4864 was donated by Hoffmann-LaRoche, Nutley, NJ.

RESULTS AND DISCUSSION

[^{3}H]Ro5-4864 bound to alveolar type II cells in a saturable fashion (Fig. 1, A and B) with a K_D of 5.7 ± 1.6 nM and a B_{max} of 4582 ± 253 fmol/mg protein. By comparison, the lymphocyte-enriched fraction had a markedly lower maximum binding capacity (B_{max} = 852 ± 99 fmol/mg), although the apparent binding affinity (K_D = 4.4 ± 1.2 nM) of [^{3}H]Ro5-4864 was not significantly different from that of type II cells.

The Scatchard plot (Fig. 1B) indicates an apparently homogenous population of binding sites. The saturation experiments resulted in a Hill coefficient of ~1 (Fig. 1C), which is also indicative of binding site homogeneity and noncooperativity.

The apparent K_D and B_{max} values of [^{3}H]Ro5-

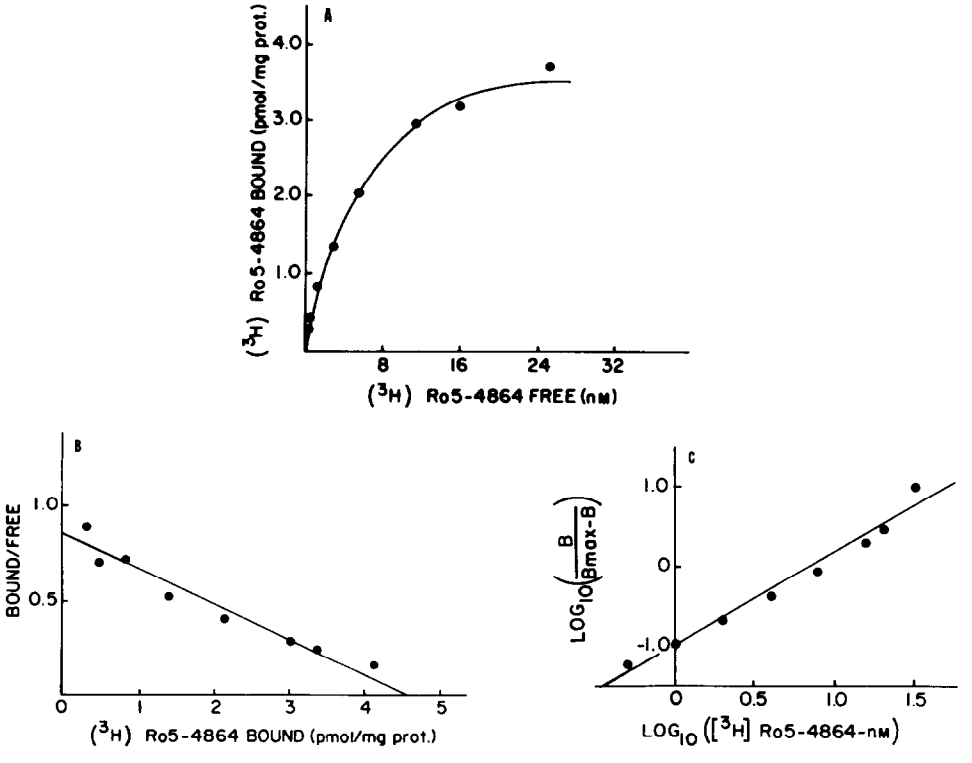

Fig. 1. Saturation isotherm (A), Scatchard analysis (B), and Hill plot (C) of [^{3}H]Ro5-4864 binding to guinea pig alveolar type II cell membranes. For A and B, data shown are representative of three independent experiments. Bound = pmol of specifically bound [^{3}H]Ro5-4864 per mg protein. Free = total concentration of [^{3}H]Ro5-4864 added in the incubation medium — specifically bound [^{3}H]Ro5-4864 per mg protein. The regression line (γ = 0.97) indicates a K_D of 5.54 nM and a B_{max} of 4606 fmol/mg protein. The Hill plot (C) represents mean data of three experiments. [^{3}H]Ro5-4864-nM = concentration of [^{3}H]Ro5-4864, 0.5 to 32.0 nM. B = fmol of specifically bound [^{3}H]Ro5-4864 per mg protein for each concentration of [^{3}H]Ro5-4864. B_{max} = 4582 ± 253 fmol/mg. The regression line (γ = 0.98) gives a Hill slope of 1.14.

4864 binding to guinea pig alveolar type II cells are comparable to values reported in whole lung membranes [21]. Alveolar type II cells constitute approximately 10% of total cell population in guinea pig lung [33]. Since alveolar type II cells and lung homogenates have comparable B_{max} values and the lymphocyte-enriched fraction has a low density of BZ receptors, it is evident that there must be a subset of cells that have even a higher density of peripheral-type sites than the alveolar cells. On the other hand, it could indicate that the alveolar type II cells which we worked with were not pure. But this possibility was ruled out because the cell preparations were highly purified ($> 96 \pm 2\%$) as almost all cells fluoresced following the quick staining with phosphine 3R. As phosphine 3R is specifically taken up by the lamellar bodies, they emitted an initial flare of fluorescence, which rapidly faded into areas of lesser luminosity and ultimately disappeared due to the bleaching effect of ultraviolet light from the fluorescent microscope. Viability, as judged by the Trypan blue dye exclusion technique, indicated that they were predominantly viable (more than 98%). The lymphocyte-enriched fraction contained 20% alveolar type II cells. It is possible that the BZ binding observed in this fraction may be due to alveolar type II cells and that lymphocytes have no BZ binding. In fact, it has been shown recently that human lymphocyte membrane has little or no [^{3}H]diazepam binding [37].

Currently, we are studying the subcellular localization of PBR in alveolar type II cells. A preliminary study has revealed the presence of PBR in the mitochondria of these cells ($B_{max} = 3900$ fmol/mg protein). It is important that we establish the homogeneity of the mitochondria by marker enzyme studies. It is possible that the mitochondria is contaminated with a small percentage of plasma membrane. Basil and Skolnick [38] have recently reported the presence of benzodiazepine receptor in brain mitochondria. Also, Anholt *et al.* [39] have reported the localization of PBR to the outer membrane of mitochondria in adrenal cells.

The high density of binding sites for [^{3}H]Ro5-4864 in alveolar type II cells, coupled with the potential role of this compound in alveolar ventilation [40, 41], ion transport [27–29] and cell differentiation, proliferation, and chemotaxis [42–46], suggest that alveolar type II cells will be a valuable model for elucidating putative pharmacologic and physiological functions of peripheral benzodiazepine receptors. In this regard, it is important to study the binding characteristics of other drugs, such as PK 11195, in mitochondrial membrane of alveolar type II cells.

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