Volume 244, number 2, 263–267 FEB 06813 February 1989

[^{3}H]AHN 086 acylates peripheral benzodiazepine receptors in the rat pineal gland

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Received 21 December 1988

AHN 086, an isothiocyanato derivative of Ro 5-4864 (4'-chlorodiazepam), inhibits radioligand binding to peripheral benzodiazepine receptors with characteristics of an irreversible (acylating) ligand. We now report that [^{3}H]AHN 086 labels a ≈30 kDa protein in the rat pineal gland determined by both SDS-polyacrylamide gel electrophoresis and gel filtration high-performance liquid chromatography of digitonin-solubilized membranes. Specific incorporation of [^{3}H]AHN 086 into this protein was inhibited by preincubating membranes with excess AHN 086. Moreover, significant specific binding of [^{3}H]AHN 086 was not observed in either bovine pineal gland (which does not possess high-affinity binding sites for Ro 5-4864) or ovalbumin. These findings suggest that the ≈30 kDa protein labeled by [^{3}H]AHN 086 in rat pineal gland is associated with peripheral benzodiazepine receptors in this tissue.

Pineal gland; Peripheral benzodiazepine receptor; Radioligand-receptor binding inhibitor; AHN 086, [^{3}H]-

1. INTRODUCTION

There is unequivocal evidence that the principal psychopharmacological effects of benzodiazepine (BZ) agonists are mediated through specific receptors in the central nervous system [1,2]. During the past decade, the biochemical and molecular properties of BZ receptors located in tissues derived from the neural crest have been well described [3–5]. In comparison, much less is known about the function and molecular organization of recognition sites for BZ receptors found outside the central nervous system (termed peripheral benzodiazepine receptors; PBR). While PBR was initially characterized in peripheral tissues [6] and transformed cell lines of neural origin [7] using [^{3}H]diazepam (which also binds to BZ receptors), it is now evident that BZ receptors and PBR have distinct subcellular distributions [8,9], apparent molecular masses [10–12] and pharmacological specificities [6,7,13–16].

Recently, we reported [17,18] that AHN 086 (1-(2-isothiocyanatoethyl-7-chloro-1,3-dihydro-5-(4-chlorophenyl)-2H-1,4-benzodiazepine-2-one hydrochloride), an isothiocyanato derivative of Ro 5-4864 (4'-chlorodiazepam, the prototype PBR ligand) inhibited radioligand binding to PBR in rat renal membranes with the characteristics of an irreversible ligand. In contrast, AHN 086 did not affect radioligand binding to central BZ receptors [18]. Here, we used a radioactive form of AHN 086 to label irreversibly PBR in rat pineal gland, which has been reported to possess a high density of these sites [13,19]. We now report that [^{3}H]AHN 086 specifically labels a ≈30 kDa protein with properties characteristic of PBR.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)

Table 1

Incorporation of [^{3}H]AHN 086 (pmol/mg protein)

Tissue	Total	Nonspecific	Specific
Rat pineal	15.8	9.5	6.3
Bovine pineal	11.6	10.1	1.5
Ovalbumin	0.17	0.17	–

Specificity of [^{3}H]AHN 086: Samples were prepared as described in section 2. Nonspecific incorporation was measured by preincubating samples with AHN 086 (10 μM) for 30 min prior to the addition of 20 nM [^{3}H]AHN 086. The amount of radioligand specifically incorporated into PBR was defined as the difference observed in the presence and absence of AHN 086 (10 μM). Protein quantities for rat and bovine pineal and ovalbumin were 153 μg protein/ml, 440 μg protein/mg and 2 mg protein/ml, respectively. Rat and bovine pineal data are mean pmol/mg protein of two separate experiments. Analysis using ovalbumin was based on one determination as a control

2. MATERIALS AND METHODS

2.1. *Tissue preparation*

Adult, male Sprague-Dawley rats (Zivic-Miller, Allison Park, PA) were housed in a 12 h light/dark cycle with free access to laboratory chow and water. Animals were killed by decapitation, the pineal glands were removed, frozen rapidly on solid CO_2 and then stored at −70°C. Tissues were homogenized in 100 vols ice-cold 0.32 M sucrose using a motor-driven Teflon pestle and glass homogenizer (10–12 passes). The homogenate was centrifuged at 1000 × g for 10 min (4°C). The resulting pellet (P_1) was discarded and the supernatant centrifuged at 23000 × g for 20 min at 4°C. The enriched mitochondrial pellet (P_2) was resuspended with a Brinkman Polytron (setting 5, 5 s) in 100 vols of 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.0). Frozen bovine pineal glands were processed in an identical manner.

2.2. *Tissue labeling with [^{3}H]AHN 086*

Homogenates were incubated in buffer with 20 nM [^{3}H]AHN 086 for 30 min at 4°C. In some experiments, tissues were preincubated for 30 min with 10 μM AHN 086 prior to addition of the radiolabeled compounds. The radioactivity incorporated in an aliquot of this tissue was determined by liquid scintillation counting (Beckman LS 5801). Protein content was routinely determined according to Bradford [20].

2.3. *Gel-filtration chromatography*

Pineal membranes prelabeled with [^{3}H]AHN 086 were centrifuged at 23000 × g (4°C) and the pellet resuspended (Brinkman Polytron) in 100 vols of 50 mM Tris-HCl (pH 7.4) buffer containing 1% digitonin. The membrane suspensions

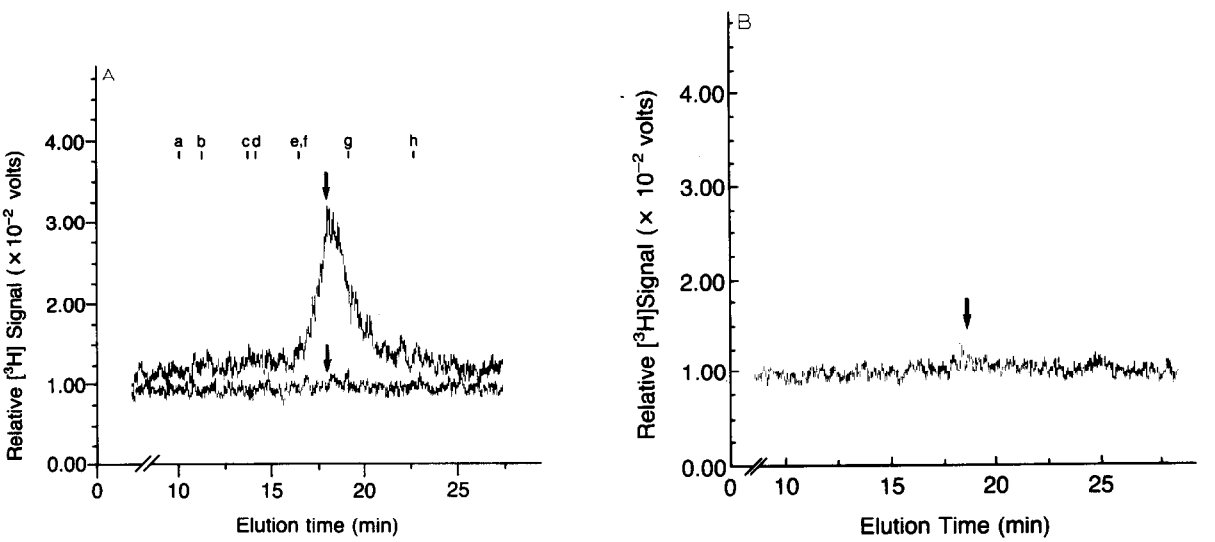

Fig.1. (A) BioSil TSK-250 chromatography of [^{3}H]AHN-labeled, digitonin-solubilized membranes. Disrupted P_2 membrane fractions from rat pineal were incubated with [^{3}H]AHN 086 (20 nM) or preincubated with AHN 086 (10 μM) prior to incubation with radiolabel as described in section 2. Aliquots (200 μl) were applied to a Bio-Rad BioSil TSK-250 column at a flow rate of 0.5 ml/min and radioactivity monitored directly from the column using a Flo-One Beta Radioactive flow detector. Abscissa: analog tritium signal. Molecular mass markers (kDa) monitored at A_{280}: (a) void volume (blue dextran) and thyroglobulin, 690; (b) ferritin, 440; (c) IgG, 160; (d) aldolase, 158; (e) ovalbumin (Bio-Rad), 44; (f) ovalbumin (Pharmacia), 43; (g) myoglobin, 17.5; (h) cyanocobalamin, 1.4. The center of the peak of radioactivity (top line) corresponds to 27 kDa (arrow). Preincubating tissue with AHN 086 eliminated the 18.2 min peak (lower trace). (B) Chromatogram representing [^{3}H]AHN 086 incorporation into bovine pineal gland. Tissues were prepared and analyzed in the same fashion as in (A). No analog tritium signal was observed in the same 27 kDa region as in the rat pineal. Data are representative of at least three separate determinations. Equal amounts of radioactivity were analyzed in both rat and bovine pineal gland.

were maintained at 4°C for 20 min. Samples were then centrifuged at 100000 × g in a Beckman Airfuge. Aliquots (200 μl) of supernatant were applied to a BioSil TSK-250 column and eluted at a flow rate of 0.5 ml/min with 20 mM Na_2HPO_4 buffer (pH 6.8) containing 0.5% octyl-β-D-glucopyranoside and 50 mM Na_2SO_4. Radioactivity was monitored as an analog signal using a Radiomatic Instruments Flo-One Beta Radioactive flow detector. In some experiments, 1 ml aliquots were collected and the radioactivity monitored by liquid scintillation counting. The column was calibrated with molecular mass standards (Bio-Rad, Pharmacia and Sigma; range, 1–690 kDa).

2.4. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

Frozen membrane pellets labeled with [^{3}H]AHN 086 were thawed and solubilized in 10% (w/v) SDS with 100 mM Tris-HCl (ph 7.4), 5 mM β-mercaptoethanol, 0.1 g/ml sucrose and 0.02 mg/ml bromophenol blue tracking dye [21–23]. Samples were placed in boiling water for 5 min and 40-μl aliquots ($\leq$100 μg protein/well) were loaded on 1.4 mm thick 10% polyacrylamide slab gels [24]. The gels were electrophoresed using a running buffer (0.1% SDS, 25 mM Tris and 190 mM glycine) with a potential of 80 V applied for the first 1.5 h and a 180 V for the remaining 2.5 h [22,23]. The gels were vacuum dried for 2 h and exposed to Kodak X-Omat R film [25] for 3 weeks. The apparent molecular masses of the radioactive bands were determined by comparing their migration with standard protein markers (Rainbow Markers, Amersham, Arlington Heights, IL) of 14–200 kDa.

2.5. *Materials*

AHN 086 was prepared as described [17]. [^{3}H]AHN 086 (spec. act. 45 Ci/mmol) was synthesized from *N*-desmethyl-4′-chlorodiazepam by Dupont-NEN, Boston, MA. All other materials were obtained from standard commercial sources.

3. RESULTS

While [^{3}H]AHN 086 was incorporated into rat or bovine pineal proteins as well as ovalbumin, significant specific incorporation was detected only in rat pineal (table 1). Gel-filtration chromatography of digitonin-solubilized membranes from rat pineal revealed a single major peak of radioactivity with a retention time of 18.2 ± 0.1 min corresponding to 27 ± 2.1 kDa (fig.1A). This peak was reduced by preincubation with AHN 086 (10 μM). A corresponding peak was not observed when an equal amount of radioactivity from bovine pineal was applied to this column (fig.1B). Collection and scintillation counting of 1 ml fractions of [^{3}H]AHN 086 labeled rat pineal membranes with and without preincubation in the presence of AHN 086 revealed a 47% reduction in radioactivity corresponding to the same retention time as the 27 kDa peak. In contrast, preincubation of bovine pineal with AHN 086 did not result in a decrease in radioactivity at the corresponding retention time (not shown). A major band corresponding to a 30.6 kDa protein by [^{3}H]AHN 086 was also observed using SDS-PAGE (fig.2). Preincubation of rat pineal membranes with 10 μM AHN 086 reduced incorporation of [^{3}H]AHN 086 into the primary protein band (not shown). Minor protein bands were present slightly above (31.1 kDa) and below (30.1 kDa) the primary 30.6 kDa protein band (fig.1).

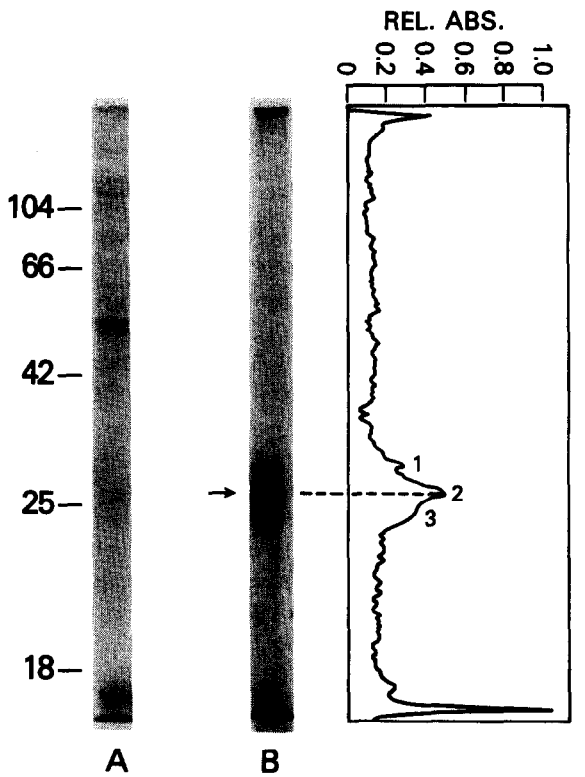

Fig.2. SDS-PAGE of [^{3}H]AHN 086 (20 nM) binding to rat pineal. P_2 fractions of rat pineal were incubated with [^{3}H]AHN 086, solubilized, and SDS-PAGE performed as described in section 2. (A) Coomassie blue stain of proteins of rat pineal membranes. (B) Fluorography of irreversible [^{3}H]AHN 086 binding to rat pineal membranes identifies a ≈30 kDa protein band. Rainbow markers (14–200 kDa) were included to calibrate the gel with known molecular masses. Densitometric scan of the fluorogram indicates relative densities of the bands found in the ≈30 kDa range. The results are representative of at least three separate determinations using identical procedures.

4. DISCUSSION

AHN 086 is an isothiocyanato derivative of Ro 5-4864, the prototype ligand used to characterize PBR [14,26–28]. This compound was shown to inhibit potently and specifically radioligand binding to PBR with characteristics of an irreversible (acylating) ligand [17,18]. The synthesis of [^{3}H]AHN 086 afforded a high-affinity, selective radioligand for characterization of PBR.

Rat pineal gland was selected for study, since it has been shown to possess a high density of PBR [13,19]. In preliminary experiments, preincubation of rat pineal membranes with Ro 5-4864 (10 μM) or PK 11195 (10 μM) did not significantly reduce incorporation of [^{3}H]AHN 086 (not shown). These observations are consistent with previous findings [18] that under identical incubation conditions, neither PK 11195 nor Ro 5-4864 could block the irreversible inhibition of radioligand binding to PBR produced by AHN 086 [18]. However, prelabeling pineal membranes with AHN 086 (10 μM) reduced the binding of [^{3}H]AHN 086 by 37–49% (table 1). Several lines of evidence suggest that this reduction in radioligand binding represents specific binding to PBR. A similar effect of AHN 086 was observed in neither [^{3}H]AHN 086-labeled bovine pineal (which lacks high-affinity binding sites for Ro 5-4864) [29] nor ovalbumin (table 1). Moreover, this concentration of AHN 086 has been shown to block reversible radioligand binding to PBR [18].

Gel-filtration chromatography of rat pineal extracts prelabeled with [^{3}H]AHN 086 revealed a major peak of radioactivity with a retention time of 18.2 min, corresponding to 27 kDa. Preincubation of membranes with AHN 086 reduced specific binding which suggests that this peak corresponds to material specifically labeled by [^{3}H]AHN 086. In addition, application of equal amounts of radioactivity from bovine pineal did not yield a peak of radioactivity with a parallel retention time (fig.1B). SDS-PAGE also demonstrated a major band of radioactive material of ≈30 kDa (fig.2) which was reduced in membranes prelabeled with cold AHN 086 (not shown). This finding further supports the suggestion that the protein labeled by [^{3}H]AHN 086 is associated with PBR in rat pineal.

The apparent molecular mass of PBR in rat pineal obtained with [^{3}H]AHN 086 is in good agreement with both the target size of PBR (34 ± 4 kDa) in rat estimated by radiation inactivation using [^{3}H]diazepam [10] and by photoaffinity labeling with [^{3}H]flunitrazepam [30]. In this latter study, proteins of ≈30 and ≈35 kDa were photoaffinity labeled, with the larger protein exhibiting characteristics of a voltage-dependent anion channel (VDAC) protein [30]. In contrast, several studies using [^{3}H]PK 14105 (a nitrophenyl derivative of PK 11195) to photoaffinity label PBR from a number of tissues observed 17–18.5 kDa proteins [31–33] with SDS-PAGE.

These differences in apparent molecular mass could be attributed to the methodologies used to identify and label PBR. Alternatively, isoquinolinecarboxamides (such as PK 11195) and 1,4-BZs may label distinct PBR proteins. Thus, while B_{max} estimates of PBR using [^{3}H]PK 11195 and [^{3}H]Ro 5-4864 are in good agreement in many rodent tissues [9,18,29], there does not appear to be a high-affinity binding site for PBR ligands with a 1,4-BZ structure in other mammalian species including cow and man [29,34]. This hypothesis is further supported by gel-filtration chromatography of digitonin-solubilized rat or bovine adrenal glands, where [^{3}H]PK 14105 labeled a protein of 215 kDa [11], a value in good agreement with that found in rat renal membranes solubilized with Triton X-100 and detected with [^{3}H]Ro 5-4864 [35]. While it is possible that this apparent molecular mass reflects detergent molecules bound to protein [11,31], these findings may indicate a multimeric arrangement of 18 and 30 kDa proteins in rodent tissues. Future studies with molecules capable of covalent attachment to PBR should prove valuable in determining their molecular arrangement, structure and function.

Acknowledgements: R.T.M. is a recipient of a Pharmacology Research Associate Training (PRAT) Fellowship (1988-90) from the National Institute of General Medical Sciences. J.A.S. is a Fellow in the Cystic Fibrosis Training Program, NIDDK.

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