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Photoaffinity Labeling of A_1-adenosine Receptors*

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Karl-Norbert Klotz‡, Gloria Cristalli§, Mario Grifantini§, Sauro Vittori§, and Martin J. Lohse‡

From the ‡Pharmakologisches Institut, Universität Heidelberg, Im Neuenheimer Feld 366, 6900 Heidelberg, Federal Republic of Germany and the §Dipartimento di Scienze Chimiche, Università di Camerino, Via S. Agostino 1, 62032 Camerino, Italy

The ligand-binding subunit of the A_1-adenosine receptor has been identified by photoaffinity labeling. A photolabile derivative of *R*-N^6-phenylisopropyladenosine, *R*-2-azido-N^6-*p*-hydroxyphenylisopropyladenosine (*R*-AHPIA), has been synthesized as a covalent specific ligand for A_1-adenosine receptors.

In adenylate cyclase studies with membranes of rat fat cells and human platelets, *R*-AHPIA has adenosine receptor agonist activity with a more than 60-fold selectivity for the A_1-subtype. It competes for [^{3}H]N^6-phenylisopropyladenosine binding to A_1-receptors of rat brain membranes with a K_i value of 1.6 nM. After UV irradiation, *R*-AHPIA binds irreversibly to the receptor, as indicated by a loss of [^{3}H]N^6-phenylisopropyladenosine binding after extensive washing; the K_i value for this photoinactivation is 1.3 nM.

The *p*-hydroxyphenyl substituent of *R*-AHPIA can be directly radioiodinated to give a photoaffinity label of high specific radioactivity (^{125}I-AHPIA). This compound has a K_D value of about 1.5 nM as assessed from saturation and kinetic experiments. Adenosine analogues compete for ^{125}I-AHPIA binding to rat brain membranes with an order of potency characteristic for A_1-adenosine receptors. Dissociation curves following UV irradiation at equilibrium demonstrate 30–40% irreversible specific binding.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates that the probe is photoincorporated into a single peptide of M_r = 35,000. Labeling of this peptide can be blocked specifically and stereoselectively by adenosine receptor agonists and antagonists in a manner which is typical for the A_1-subtype.

The results indicate that ^{125}I-AHPIA identifies the ligand-binding subunit of the A_1-adenosine receptor, which is a peptide with M_r = 35,000.

Adenosine appears to be a modulator of a great variety of biological functions, including the nervous and cardiovascular systems, immune responses, and metabolism (for an overview, see Ref. 1). Most of these effects seem to be mediated via membrane-bound receptors. On the basis of pharmacological and biochemical studies, these receptors have been subdivided into the A_1 (or R_i)- and the A_2 (or R_a)-subtypes (2, 3). The A_1-receptor mediates an inhibition, and the A_2 receptor a stimulation of adenylate cyclase activity.

The A_1-subtype seems to be involved in the inhibition of neuronal firing and neurotransmitter release in the central nervous system. In the periphery, effects of adenosine, such as the negative inotropy in the heart and the inhibition of lipolysis in fat cells, have been attributed to the A_1-receptor (1, 4).

Direct ligand binding studies have been used to characterize the A_1-receptor both in the central nervous system and in peripheral tissues (4, 5). More recently, radioiodinated ligands of high specific radioactivity have been prepared, which allow identification of the A_1-receptor in tissues with low receptor densities (6–9).

Covalent labeling of receptors can be achieved with photolabile probes which, upon UV irradiation, bind irreversibly to the receptor (10). This approach has been successfully applied to several receptors and has considerably advanced their biochemical characterization (11–16).

In order to apply this approach to the A_1-adenosine receptor, we describe here the preparation of a photoaffinity label and its ability to identify the ligand-binding subunit of the receptor in rat brain membranes.

EXPERIMENTAL PROCEDURES

Materials

Carrier-free Na^{125}I was purchased from Amersham-Buchler, Braunschweig, Federal Republic of Germany. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from British Drug House, Poole, United Kingdom. All other materials were from sources described previously (8, 17) and were of the highest quality available.

Methods

Synthesis of R-AHPIA[1]—The synthesis of *R*-AHPIA was achieved starting from 9-(2,3,5-O-acetyl-D-ribofuranosyl)-2,6-dichloropurine and will be described in detail elsewhere.[2] The compound was dissolved at a concentration of 10 mM in dimethylsulfoxide and diluted in water in one step to 100 μM. Photolability of the compound was assessed by taking UV spectra after varying times of UV irradiation.

Preparation of ^{125}I-AHPIA—*R*-AHPIA was radioiodinated according to the method of Hunter and Greenwood (18). All reagents were dissolved in water. For the radioiodination, 10 μl of Na^{125}I (1 mCi) were added to 50 μl of 0.3 M potassium phosphate buffer, pH 7.4. 20 μl of 100 μM AHPIA were added, followed by 10 μl of chloramine T (2 mg/ml). The tube was vortexed for 50 s, and the reaction was stopped by addition of 10 μl of sodium bisulfite (2 mg/ml). The separation of the reaction products was done by chromatography on a Sephadex G-25 superfine column (0.9 × 20 cm) which was eluted with a 0.1 M potassium phosphate buffer, pH 7.4, at 4 °C. Fractions of approximately 2 ml were collected. 10-μl aliquots of the fractions

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[1] The abbreviations used are: *R*-AHPIA, *R*-2-azido-N^6-*p*-hydroxyphenylisopropyladenosine; ^{125}I-AHPIA, *R*-2-azido-N^6-^{125}I-*p*-hydroxyphenylisopropyladenosine; *R*-/*S*-PIA, *R*-/*S*-N^6-phenylisopropyladenosine; NECA, 5'-*N*-ethylcarboxamidoadenosine; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate.

[2] G. Cristalli, M. Grifantini, S. Vittori, K.-N. Klotz, and M. J. Lohse, manuscript in preparation.

were counted for radioactivity, and 2.5-μl aliquots were tested for binding activity as described below. As ^{125}I-AHPIA appears to stick to polypropylene, CHAPS was added to give a final concentration of 0.1%. The same concentration of the detergent was also present in all experiments with the radioiodinated compound; this concentration does not alter the binding characteristics of the A_1-adenosine receptor. ^{125}I-AHPIA was stored at −20 °C.

Preparation of Membranes—Rat brain membranes were prepared as described previously (19), except that after the last centrifugation, the pellets were resuspended in 10 mM EDTA, 50 mM Tris-HCl, pH 7.4, centrifuged as before, and resuspended in 50 mM Tris-HCl, pH 7.4.

Human platelet membranes were prepared as described by Hoffman *et al.* (20), but $MgCl_2$ was omitted from the resuspension buffer.

Rat fat cells were isolated according to the method of Rodbell (21), and fat cell membranes were prepared as described by McKeel and Jarett (22).

The protein concentration of the membrane preparations was determined according to Lowry *et al.* (23).

Adenylate Cyclase Assay—The activity of adenylate cyclase of rat fat cell and human platelet membranes was determined as described by Jakobs *et al.* (24). [α-^{32}P]ATP was synthesized according to Walseth and Johnson (25). The incubation medium contained in a total volume of 100 μl of 50 mM Tris-HCl, pH 7.4, approximately 300,000 cpm of [α-^{32}P]ATP, 100 μM unlabeled ATP, 10 μM GTP, 100 μM cyclic AMP, 1 mM $MgCl_2$, 100 μM EGTA, 150 mM NaCl, 500 μM Ro 20-1724, 0.4 mg/ml creatine kinase, 5 mM creatine phosphate, 2 mg/ml bovine serum albumin, and 0.2 unit/ml adenosine deaminase. For human platelet membranes, the GTP concentration was reduced to 1 μM and NaCl was omitted. Reactions were initiated by addition of membranes (10 μg of protein for fat cells and 20 μg for platelets), and the incubation time was 10 min at 37 °C.

Binding Assays—The binding of [^{3}H]PIA (1 nM) to rat brain membranes (0.4 mg of protein/ml) was measured in a total volume of 250 μl with an incubation time of 2 h at 25 °C as described (17).

The binding of ^{125}I-AHPIA was measured in the same way with a radioligand concentration of approximately 70 pM unless stated otherwise. Nonspecific binding of ^{125}I-AHPIA (as of [^{3}H]PIA) was determined in the presence of 1 mM theophylline and amounted to approximately 30% of total binding at the indicated ligand concentration.

Photoincorporation—Rat brain membranes (0.4 mg of protein/ml) were incubated with *R*-AHPIA or ^{125}I-AHPIA for 2 h at 25 °C and then put on ice. After centrifugation at 5000 × *g* for 10 min, the pellets were resuspended in incubation buffer (0 °C) without ligand in order to remove unbound ligand. Due to the very slow dissociation of the ligand at 0 °C, this procedure does not affect the specific binding, but reduces nonspecific binding. The samples were then irradiated for 3 min with a Mineralight TM 15 UV lamp at a distance of 15 cm.

In experiments investigating the photoinactivation of [^{3}H]PIA binding by *R*-AHPIA, any ligand not covalently bound was removed by extensive washing at 25 °C. This was done by adding 10 ml of Tris-HCl and centrifugation at 5000 × *g* for 10 min. The pellets were resuspended in 10 ml of the same buffer, left to stand for 10 min, and centrifuged as above. This procedure was repeated twice, and the final pellets were resuspended at a protein concentration of 1 mg/ml and tested for [^{3}H]PIA binding as described above.

SDS-Polyacrylamide Gel Electrophoresis—After UV irradiation, samples (300 μg of protein) were centrifuged at 15,000 × *g* for 2 min. The pellets were dissolved in 100 μl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 25 mM dithiothreitol, 1 mM EDTA, 5% SDS, 10% glycerin, and 0.003% bromphenol blue) and kept at 95 °C for 3 min. Subsequently, 80-μl aliquots were electrophoresed on a 10% SDS-polyacrylamide gel (140 × 110 × 1.5 mm) with a 4% stacking gel (acrylamide:*N,N'*-methylenebisacrylamide, 100:2.7) according to Laemmli (26). Gels were then dried for autoradiography, or, in the case of gels with molecular weight markers, stained with Coomassie Blue.

Data Analysis—Equilibrium binding data were analyzed by nonlinear curve fitting with the aid of the program SCTFIT (27). Kinetic binding data were fitted by nonlinear regression using the equations and curve-fitting procedures described (17). All experiments shown were repeated at least three times.

RESULTS

The structure of *R*-AHPIA is given in Fig. 1. The photolability of the compound was seen in UV spectra before and after irradiation by a decrease of an absorption maximum at 277 nm and the appearance of a new maximum at 262 nm.

The biological activity of the compound at adenosine receptors was tested in adenylate cyclase studies using membranes from subtype-selective cells. Rat fat cell membranes were used for the A_1-subtype and human platelets for the A_2-subtype (4). *R*-AHPIA inhibited the adenylate cyclase activity of rat fat cell membranes to the same extent (65%) as do other adenosine analogues; the IC_{50} value was 35 nM (Fig. 2*A*). The presence of 100 μM theophylline shifted the curve to the right to give an IC_{50} value of 140 nM. This competitive antagonism by theophylline indicates that *R*-AHPIA inhibits adenylate cyclase activity via an A_1-adenosine receptor.

The adenylate cyclase of human platelet membranes was stimulated by NECA to a greater extent than by other adenosine analogues (Fig. 2*B*); this has also been reported by Hüttemann *et al.* (28). *R*-AHPIA had the same efficacy as 2-chloroadenosine and *R*-PIA and an EC_{50} value of 2.2 μM. Again, 100 μM theophylline shifted the curve to the right, resulting in an EC_{50} value of 9 μM, indicating an action of *R*-AHPIA via an A_2-receptor. High concentrations of *R*-PIA and 2-chloroadenosine inhibited adenylate cyclase activity in the platelet membranes, most likely via the intracellular P-site (29). Such an inhibition was not seen with *R*-AHPIA, indicating the absence of an interaction with the P-site.

On the basis of the EC_{50} values for the A_2-receptor and the IC_{50} values for the A_1-receptor, selectivity ratios can be estimated. This gives a more than 60-fold selectivity of *R*-AHPIA for the A_1-subtype, compared to a factor of 39 for *R*-PIA and 13 for 2-chloroadenosine.

The affinity of *R*-AHPIA for the A_1-receptor was determined in competition experiments using [^{3}H]PIA as radioligand (Fig. 3). Under nonphotolyzing conditions, *R*-AHPIA competed for [^{3}H]PIA binding to rat brain membranes with a K_i value of 1.6 nM (Fig. 3*A*). The monophasic competition curve suggests a competitive antagonism at a single site. Preincubation of rat brain membranes with *R*-AHPIA, followed by UV irradiation and extensive washing to remove *R*-

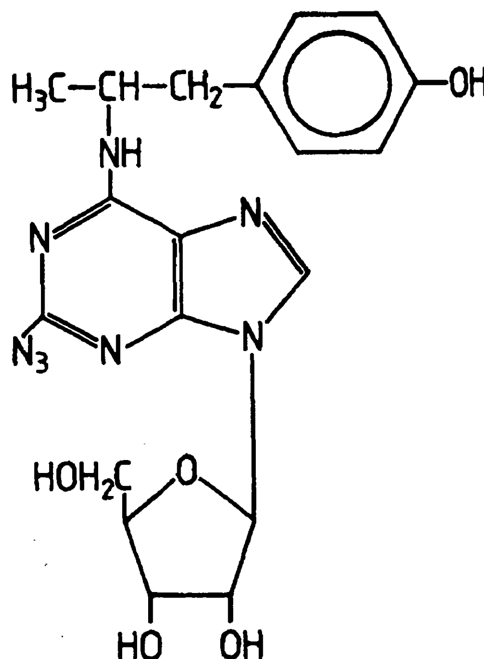

FIG. 1. Structure of *R*-AHPIA.

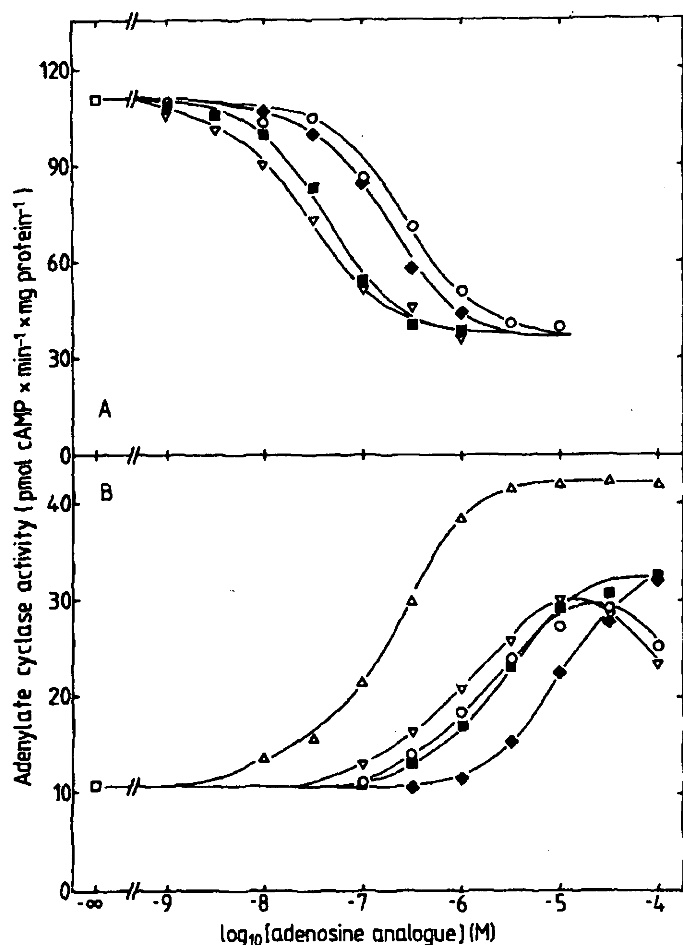

FIG. 2. **Effects of *R*-AHPIA and adenosine analogues on adenylate cyclase.** The activity of the enzyme in the presence of various concentrations of adenosine analogues was determined as described under "Methods." ■, *R*-AHPIA; ◆, *R*-AHPIA in the presence of 100 μM theophylline; ▽, *R*-PIA; ○, 2-chloroadenosine; △, NECA. *A*, inhibition of basal adenylate cyclase activity in rat fat cell membranes. IC_{50} values determined from Hill plots were: 35 nM for *R*-AHPIA, 140 nM for *R*-AHPIA in the presence of 100 μM theophylline, 25 nM for *R*-PIA, and 150 nM for 2-chloroadenosine. *B*, stimulation of basal adenylate cyclase activity in human platelet membranes. EC_{50} values were: 2.2 μM for *R*-AHPIA, 9.0 μM for *R*-AHPIA in the presence of 100 μM theophylline, 190 nM for NECA, 0.96 μM for *R*-PIA, and 1.9 μM for 2-chloroadenosine.

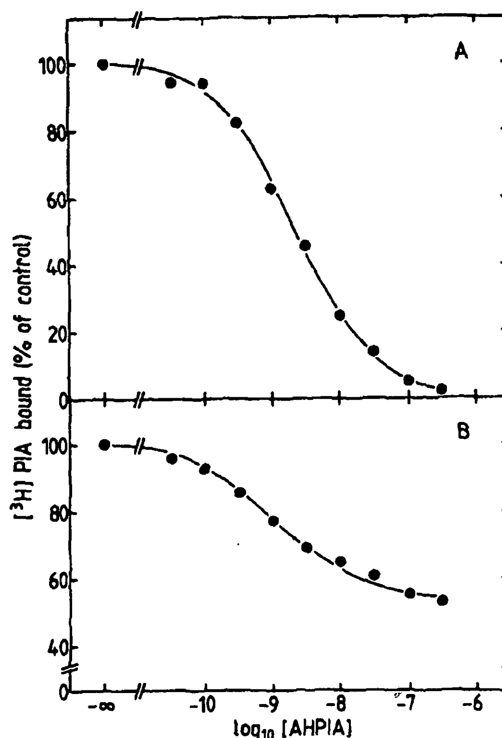

FIG. 3. *A*, competition of *R*-AHPIA for [^{3}H]PIA binding to rat brain membranes. Binding of 1 nM [^{3}H]PIA to rat brain membranes in the presence of various concentrations of *R*-AHPIA was done as described under "Methods." Nonlinear curve fitting gives a K_i value for *R*-AHPIA of 1.6 nM. *B*, photoinactivation of [^{3}H]PIA binding to rat brain membranes by *R*-AHPIA. Rat brain membranes were incubated for 2 h at 25 °C with various concentrations of *R*-AHPIA, UV-irradiated for 3 min, and then extensively washed as described under "Methods." [^{3}H]PIA binding was subsequently determined as described. Without UV irradiation, the binding of [^{3}H]PIA was not affected by preincubation with *R*-AHPIA (*i.e.* all values 100%). The apparent K_i value for the photoinactivation was calculated to be 1.3 nM.

AHPIA not covalently bound, resulted in a concentration-dependent loss of [^{3}H]PIA binding (Fig. 3*B*). This photoinactivation occurred with an apparent K_i value of 1.3 nM, and the maximal inhibition was about 40%. Again, the curve was monophasic, indicating the interaction with a single class of binding sites. These experiments demonstrate that *R*-AHPIA can be used as a photoaffinity label for A_1-adenosine receptors.

In order to obtain a radioactive probe, *R*-AHPIA was radioiodinated at its *p*-hydroxyphenyl substituent according to the method of Hunter and Greenwood (18). Fig. 4 shows the elution profile of the reaction products from a Sephadex G-25 superfine column. The first radioactivity peak, which was without binding activity, represents $^{125}I^-$ eluting with the total volume of the column. Due to interactions of heterocyclic aromatic and iodinated compounds with the column material, ^{125}I-AHPIA followed as a broad peak in fractions 10–40; the binding activity of this peak paralleled the radioactivity, providing evidence for a constant specific radioactivity of the compound. Unlabeled *R*-AHPIA was eluted in fractions 10–16, as assessed by the inhibition of [^{3}H]PIA binding by aliquots of the fractions of a separate run with the unlabeled compound.

Saturation experiments of ^{125}I-AHPIA binding to rat brain membranes using concentrations of 0.1–5 nM radioligand gave a K_D value of 2 nM and a B_{max} value of 220 fmol/mg of protein, representing the high affinity state of the receptor (17). For [^{3}H]PIA, we observed a B_{max} value of 240 fmol/mg of protein for the high affinity state in the same membrane preparation. The binding of ^{125}I-AHPIA was promoted by divalent cations and reduced by guanine nucleotides similarly to the binding of other A_1-receptor agonist radioligands (17, 30).

Adenosine analogues competed for the binding of ^{125}I-AHPIA with an order of potency characteristic for the A_1-receptor (Table I). There was good agreement with the K_i values of the compounds obtained from competition for [^{3}H]PIA binding. In particular, *R*-PIA was considerably more potent than *S*-PIA and also more potent than NECA, which is typical for the A_1-receptor.

Kinetic experiments were done to obtain a kinetic estimate of the affinity of ^{125}I-AHPIA and to measure the photoincorporation of the compound (Fig. 5). The association of the ligand reached equilibrium after about 2 h, and the ligand dissociated almost completely from the binding site after addition of 1 mM theophylline with a half-life of about 25 min. Nonlinear curve fitting gives a kinetic K_D of 1.4 nM, which agrees well with the value obtained from saturation experiments. When the samples were UV-irradiated before the start of the dissociation reaction, about 35% of the specific

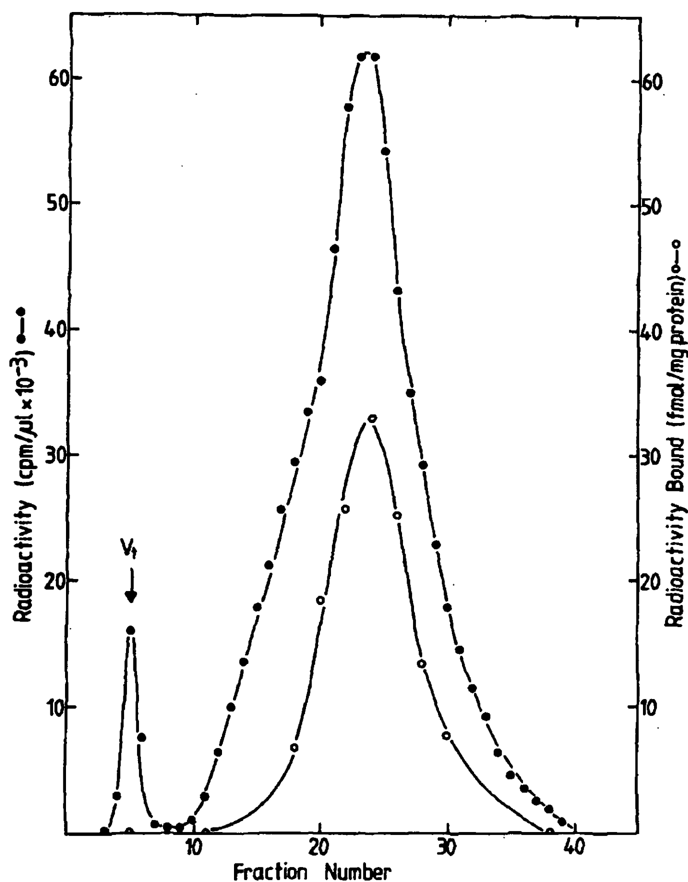

FIG. 4. **Elution profile of the reaction products of the radioiodination of *R*-AHPIA.** The total reaction volume (approximately 100 µl) was loaded onto a Sephadex G-25 superfine column (0.9 × 20 cm) and eluted with 0.1 M potassium phosphate buffer, pH 7.4. Fractions of approximately 2 ml were collected. The first peak of radioactivity, representing $^{125}I^-$, eluted with the total volume of the column (V_t). Binding activity of the eluted radioactive products for A_1-adenosine receptors of rat brain membranes was assessed from the specific binding of a 2.5-µl aliquot determined as described under "Methods."

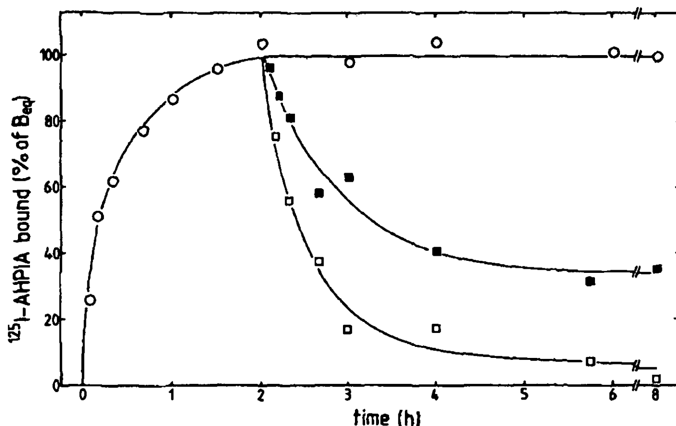

FIG. 5. **Kinetics of ^{125}I-AHPIA binding to rat brain membranes.** Binding of ^{125}I-AHPIA (approximately 55 pM) to rat brain membranes (approximately 70 µg protein/250 µl) at 25 °C was determined as described under "Methods." The dissociation reaction was initiated by addition of 10 µl of theophylline to give a final concentration of 1 mM. *Open squares* represent non-irradiated samples; *closed squares* represent samples that were UV-irradiated for 3 min prior to the initiation of the dissociation reaction as described under "Methods." B_{eq}, binding at equilibrium. Nonlinear curve fitting gives the following values. Control samples: $k_- = 0.0293$ min^{-1}; $k_+ = 0.0212$ nM^{-1} min^{-1}; $K_D = 1.4$ nM. UV-irradiated samples: $k_- = 0.0184$ min^{-1}.

TABLE I

Competition for ^{125}I-AHPIA and [^{3}H]PIA binding to rat brain membranes

Competition curves using 10 different concentrations of the competing ligands were measured as described under "Methods," and the K_i values were calculated by nonlinear curve fitting.

Compound	^{125}I-AHPIA K_i	[^{3}H]PIA K_i
	nM	*nM*
R-PIA	0.9	1.0
NECA	5.4	7.1
S-PIA	31	52
8-Phenyltheophylline	120	78
Theophylline	7500	6200

binding could no longer be dissociated. This indicates photoincorporation of the label. The dissociation of the label which was not covalently bound after UV irradiation followed a time course similar to the non-irradiated control.

After photoincorporation of ^{125}I-AHPIA, samples were subjected to SDS-polyacrylamide gel electrophoresis. The autoradiograms of the gels shown in Fig. 6 indicate that, in rat brain membranes, ^{125}I-AHPIA covalently labels a peptide of $M_r = 35,000$ with the specificity expected of the A_1-adenosine receptor. Labeling of this peptide was prevented in the presence of 1 mM theophylline (Fig. 6*A*). It was also prevented in the presence of both isomers of PIA, with the *R*-isomer being more than 10 times as potent as the *S*-isomer (Fig. 6*A*). Similarly, labeling was abolished in the presence of 10 µM NECA, but was not altered by a number of compounds specific for other membrane-bound receptors (Fig. 6*B*).

^{125}I-AHPIA also labeled a band with $M_r = 40,000$, but labeling of this band was not abolished in the presence of any A_1-receptor ligand. In addition, apparently specific radioactivity was observed near the bromphenol front; this labeling was not completely abolished in samples containing concentrations of A_1-receptor ligands sufficiently high to completely abolish labeling of the $M_r = 35,000$ band. However, it was considerably reduced when GTP was added to the samples after UV irradiation in order to rapidly dissociate ^{125}I-AHPIA not covalently bound, resulting in a reduction of total radioactivity in the samples (Fig. 6*A*).

Membrane preparations and incubations were also done with buffers containing either of two protease inhibitor preparations: (*a*) 0.1 mg/ml α_2-macroglobulin, an inhibitor of most proteases (31); or (*b*) 5 µg/ml leupeptin, 15 µg/ml benzamidine, 5 µg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 20 mM EDTA, a preparation which inhibits, for example, degradation of dopamine receptors from rat striatum (16). Both of these procedures did not alter the autoradiographic pattern.

Under nonreducing conditions, *i.e.* in the absence of dithiothreitol in the sample buffer, the position of the specifically labeled band ($M_r = 35,000$) remained unchanged.

DISCUSSION

R-AHPIA is a photolabile adenosine analogue that binds with high affinity and selectivity to the A_1-adenosine receptors. One of the main advantages of the compound is that it can be directly radioiodinated to give ^{125}I-AHPIA with a specific radioactivity identical to that of carrier-free Na^{125}I (2050 Ci/mmol).

Judging from adenylate cyclase studies, the compound has a more than 60-fold selectivity for the A_1-receptor compared to the A_2-receptor; this compares favorably with the parent compound *R*-PIA, one of the most selective A_1-receptor agonists. The inhibition of adenylate cyclase in rat fat cell membranes shows that *R*-AHPIA is a full agonist at A_1-receptors, whereas (like most adenosine analogues (28)) it has a slightly

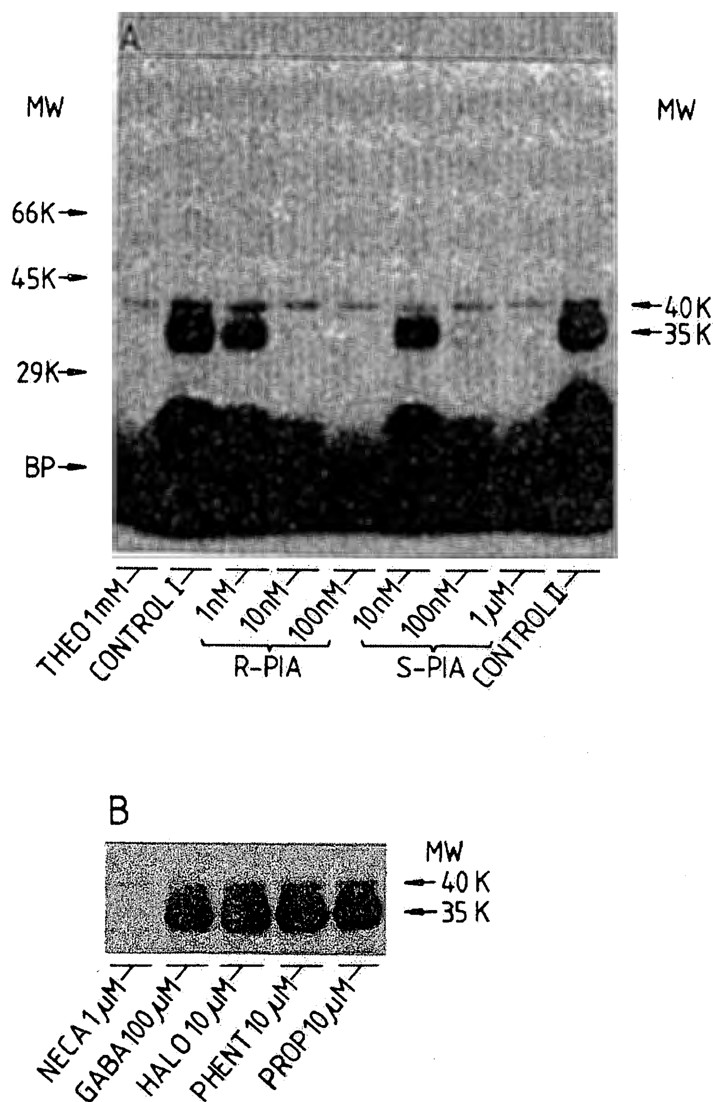

FIG. 6. **SDS-polyacrylamide electrophoresis of rat brain membranes labeled with ^{125}I-AHPIA.** Shown are the autoradiograms of the electrophoresis gels. *A*, stereospecific inhibition of the labeling by *R*- and *S*-PIA. Membranes were incubated with different concentrations of the isomers, and UV irradiation was done as described under "Methods." 100 μM GTP (final concentration) was added after the UV irradiation to all samples except *Control II*. *THEO*, theophylline; *BP*, bromphenol blue. *B*, effects of A_1-receptor ligands and ligands for other receptors on the labeling. The incubation was done in the presence of the indicated concentrations of NECA, γ-aminobutyric acid (*GABA*), haloperidol (*HALO*), phentolamine (*PHENT*), and propranolol (*PROP*).

lower efficacy than NECA at the A_2-receptor of human platelet membranes. Finally, in concentrations up to 100 μM, *R*-AHPIA does not seem to interact with the intracellular P-site. Thus, *R*-AHPIA is a selective probe for the A_1-receptor.

Competition of *R*-AHPIA for [^{3}H]PIA binding to rat brain A_1-receptors and photoinactivation of the binding sites by *R*-AHPIA both occur with a K_i value of about 1.5 nM. Thus, the affinity of the compound is similar to that of *R*-PIA. Furthermore, these results suggest that photoinactivation of and competition for the binding of [^{3}H]PIA occur at the same site.

The radioiodinated compound, ^{125}I-AHPIA, binds to a single class of non-interacting sites. Competition for the binding by adenosine analogues and methylxanthines characterizes this site as an A_1-adenosine receptor. The monophasic kinetic and saturation curves indicate that, at the concentrations used, ^{125}I-AHPIA does not bind to either the low affinity state of the A_1-receptor or the A_2-receptor. The affinity of ^{125}I-AHPIA for the receptor is 1–2 nM, as determined both in equilibrium and kinetic experiments. Thus, the iodination does not markedly alter the affinity of the compound, in contrast to aminobenzyladenosine (9), but in agreement with observations with the parent compound N^6-*p*-hydroxyphenylisopropyladenosine (8).

Dissociation of the radioligand after UV irradiation is incomplete, leaving about 35% specific binding. This indicates that 35% of ^{125}I-AHPIA bound to the receptor is photoincorporated. Unlabeled *R*-AHPIA used in saturating concentrations inactivates, after UV irradiation, about 40% of the binding sites for [^{3}H]PIA. It can be concluded that the photoincorporation of one molecule of *R*-AHPIA inactivates one binding site for [^{3}H]PIA. Consequently, no interactions between the binding sites can be assumed, a conclusion which is in accordance with the linear Scatchard plot seen in saturation studies. This contrasts with results obtained for the benzodiazepine receptor, where the photoincorporation of one molecule of flunitrazepam inactivates four binding sites for [^{3}H]flunitrazepam; from this observation, it has been suggested that the benzodiazepine receptor might form tetramers (11).

After UV irradiation, ^{125}I-AHPIA covalently labels a peptide with M_r = 35,000 seen in SDS-polyacrylamide gel electrophoresis. Photoincorporation of the label into the peptide is inhibited with the appropriate specificity for A_1-adenosine receptors. In particular, *R*-PIA appears to be considerably more potent than *S*-PIA. ^{125}I-AHPIA also labels another peptide with M_r = 40,000, but labeling of this peptide can not be prevented by adenosine analogues or methylxanthines, suggesting that the binding to this peptide is nonspecific. In contrast to observations with other receptors (15, 16), the presence of protease inhibitors does not alter the appearance of the autoradiogram. This indicates that, under the preparation and incubation conditions used, no proteolytic degradation of the binding subunit of the A_1-adenosine receptor occurs.

In addition to this band with M_r = 35,000, apparently specific radioactivity was also seen near the bromphenol front, which might be due to small degradation products of the receptor. However, it was also seen in the presence of various protease inhibitors and also, although to a lesser extent, in nonspecific lanes. Thus, although these and other experiments cannot definitively exclude that this radioactivity is due to degradation products of the receptor, we assume that it does not represent specific labeling.

The presence of dithiothreitol in the sample buffer does not change the pattern of the specifically labeled band. This indicates that, upon reduction, no separation of polypeptide chains occurs and that the specifically labeled band is composed of a single peptide chain.

The investigation of the molecular size of the solubilized A_1-adenosine receptor with gel filtration (32) or sucrose density gradient centrifugation (33) suggested much larger receptor molecules than reported here. However, with these techniques, receptors coupled to the inhibitory guanyl nucleotide-binding regulatory subunit of adenylate cyclase are examined. In addition, the solubilized receptors are present as detergent-protein complexes with an apparent molecular size larger than the protein itself. In our study, we demonstrated A_1-adenosine receptors on SDS-polyacrylamide gel electrophoresis, *i.e.* only the binding subunit.

We conclude that ^{125}I-AHPIA labels a peptide of M_r = 35,000 representing the binding subunit of the A_1-adenosine receptor. ^{125}I-AHPIA can be used to identify the binding site of the receptor in membrane preparations. The compound is easily prepared from *R*-AHPIA, and its high specific radio-

activity along with a relatively high photoincorporation will make it a powerful tool for the characterization of the A_1-adenosine receptor.

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