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^{125}I-BW-A844U, an Antagonist Radioligand with High Affinity and Selectivity for Adenosine A_1 Receptors, and ^{125}I-Azido-BW-A844U, a Photoaffinity Label

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SUMMARY

3-(4-Amino)phenethyl-1-propyl-8-cyclopentylxanthine (BW-A844U) has been synthesized and shown to bind with high affinity to adenosine A_1 receptors of bovine brain membranes (K_D = 0.23 nM). This compound is highly selective for A_1 receptors; the K_i for binding to A_2 receptors of human platelet membranes is 2.0 μM (A_2/A_1 ratio = 8700). Radioiodination of the 3-aminophenethyl group resulted in ^{125}I-BW-A844U, a radioligand that retains high affinity for A_1 receptors in bovine brain membranes (K_D = 0.14 nM) and to 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate-solubilized receptors (K_D = 0.34 nM). Specific binding of ^{125}I-BW-A844U represented >90% of the total binding at the K_D. From the association constant ($K_1 = 5.0 \times 10^8$ $M^{-1}min^{-1}$) and the dissociation constant (K_{-1} = 0.064 min^{-1}), the kinetic K_D (K_{-1}/K_1) in membranes was calculated to be 0.13 nM. NaCl (1 M) had little effect on the binding affinity of ^{125}I-BW-A844U, in contrast to the large effect of salt on the binding affinity of acidic antagonist radioligands. 8-Sulfophenyltheophylline inhibited radioligand binding with a Hill coefficient of 1.0, indicative of a single affinity binding state for the antagonist. By comparison, two distinct agonist affinity states of A_1 receptors for the agonist (*R*)-phenylisopropyladenosine could be resolved, a high affinity state (62%, K_H = 74 pM) and a low affinity state (K_L = 3.83 nM). The addition of 0.1 mM guanylylimidodiphosphate converted all receptors to the low affinity state. Addition of NaCl (0.5 M) decreased the fraction of receptors in the high affinity state and increased both K_H and K_L, suggesting that NaCl alters coupling of receptors to G proteins and influences the conformation of the receptor polypeptide, whether or not the receptor is coupled to a G protein. Conversion of the arylamine on the 3-position of ^{125}I-BW-A844U to an aryl azide resulted in a photoaffinity label, ^{125}I-azido-BW-A844U. Upon photoactivation, the photoaffinity label was specifically photoincorporated into the 34,000-dalton polypeptide of the adenosine A_1 receptor.

Adenosine receptors on the extracellular surfaces of cell membranes have been classified into A_1 and A_2 subtypes based on the potency order of adenosine analogs and their effects to either inhibit or stimulate adenylate cyclase activity, respectively (1, 2). Alkylxanthines such as caffeine and theophylline have been found to be adenosine receptor antagonists, and modifications of the structure of these compounds led to the development of 1,3-dipropyl-8-phenylxanthine derivatives, which have increased potency and selectivity for adenosine A_1 receptors (3–10).

The 3-propyl substituent of 1,3-dipropyl-8-phenylxanthine derivatives can be replaced with arylamines, radioiodinated, and converted to aryl azides with little change in A_1 binding affinity. This approach was used to make the first radioiodinated adenosine receptor antagonist radioligands and photoaffinity labels (9–11). Recently, it was found that replacing the 8-phenyl substituent of 1,3-dipropyl-8-phenylxanthine with an 8-cyclopentyl group results in greater affinity and selectivity for A_1 receptors in the resulting antagonist, CPX (12, 13). These observations provided the rationale for this study in which we describe the synthesis and characterization of a new ^{125}I-labeled 3-arylamine-8-cyclopentylxanthine, ^{125}I-BW-A844U, which is superior in its A_1 binding characteristics to 8-phenylxanthines. The corresponding aryl azide derivative, ^{125}I-azido-BW-A844U, is a photoaffinity label that specifically labels the 34-kDa peptide component of the adenosine A_1 receptor.

Materials and Methods

Bovine cortices obtained from Pel-Freez (Rogers, AR) were homogenized in 10 volumes of buffer A (10 mM HEPES, 10 mM EDTA, 10%,

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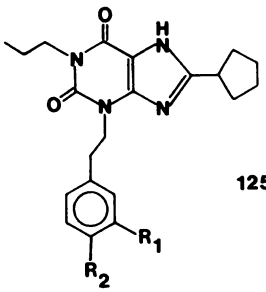
ABBREVIATIONS: CPX, 1,3-dipropyl-8-cyclopentylxanthine; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-proanesulfonate; MeOH, methanol; HEPES, N-2-hydroxyethylpiperazine N′-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography PIA, phenylisopropyladenosine; Gpp(NH)p, guanosine 5′-(β,α-imido)triphosphate; BW-A844U, 3-(4-amino)phenethyl-8-cyclopentyl-1-propyl-9*H*-purine-2,6(1*H*,3*H*)dione; BW-A827U, 2-[4-[3-(4-aminophenethyl)- 1,2,3,6-tetrahydro-2,6-dioxo-1-propyl-9H-purin-8-yl]phenoxy]acetic acid; XAC, 1,3-dipropyl-8-[$H_2N(CH_2)_2NHCOCH_2O$-phenyl]xanthine.

w/v, sucrose, 10 μg/ml PMSF, and 0.1 mM benzamidine, pH 7.4 at 21°). The homogenate was filtered through two layers of gauze and centrifuged at 40,000 × *g* for 30 min. The pellet was resuspended in an equal volume of buffer B (PMSF- and sucrose-free buffer A with EDTA reduced to 1 mM) and recentrifuged. Membranes were resuspended in 5 volumes of buffer B and stored in aliquots (5 mg of protein/ml) at −70°. In some cases an additional step was added in which the membranes were centrifuged at 500 × *g*, the pellet discarded, and the supernatant processed as described above. This resulted in a 60–80% increase in the number of receptors per mg of protein detected but no change in other receptor binding characteristics. CHAPS- solubilized receptors were prepared by adding an equal volume of 1% (w/v) CHAPS in buffer B to cortical membranes. After centrifugation at 100,000 × *g* for 1 hr, solubilized receptors in the supernatant were stored in aliquots at −70°.

Synthesis of BW-A844U. A solution of 5,6-diamino-1-(4-nitrophenethyl)-3-propyl uracil (8) (0.49 g, 1.5 nmol) and cyclopentanecarboxaldehyde (93%, 0.15 g, 1.5 mmol) in nitrobenzene (15 ml) was warmed slowly to reflux. Water formed in the reaction and nitrobenzene (total volume, 10 ml) were distilled via a Dean Stark trap during 20 min at reflux. The cooled black mixture was diluted with ether (20 ml) and filtered to give a black solid, which was dissolved in CH_2Cl_2, decolorized with activated carbon, flash chromatographed on silica gel, and eluted with CH_2CL_2 to give 8-cyclopentyl-3-(4-nitrophenethyl)-1-propyl-9*H*-purine-2,6(1*H*,3*H*) dione, (0.16 g, 26%), compound **1** in Fig. 1. A solution of **1** in glacial acetic acid (25 ml) was shaken under hydrogen (20 psi) with 5% palladium on carbon (0.1 g added in two portions at 2.0 hr). Catalyst was removed by filtration through Celite after 2.5 hr, and after evaporation of solvent the solid was triturated in 2% ethanol/diethyl ether, filtered, washed with ether, and air dried to give BW-A844U as a buff solid (0.11 g, 73%), m.p. 240–243° (dec.). Analysis for $C_{21}H_{27}N_5O_2 \cdot 1/2\ H_2O$: C, 64.59; H, 7.23; N, 17.94. Found: C, 64.82; H, 7.21; N, 17.81. The structure was confirmed by ^1H NMR and mass spectrometry. UV (0.1 N NaOH):λ_{max}, 282 (ϵ = 16,300), 218.5 (ϵ = 38,600). This compound is identical in structure to CPX (13) except that the 3-propyl substituent has been replaced by 3-aminophenethyl.

Melting points are uncorrected and were determined with a Meltemp. ^1H NMR spectra were recorded with a Varian XL-200 in d_6-dimethylsulfoxide solution. Chemical ionization (CH_4) mass spectra were provided by Oneida Research Services (Whitesboro, NY). Elemental analysis was provided by Atlantic Microlabs, Inc. (Atlanta, GA). UV spectra were recorded with a Varian Superscan 3. Products were homogeneous by thin layer chromatography (silica gel, Whatman type MK6F) when developed with 95% $CHCl_3$/MeOH.

Synthesis of ^{125}I-BW-A844U. To 50 μl of BW-A844U (1 mM in MeOH) was added 1–2 mCi of $Na^{125}I$ in 0.3 M phosphate buffer, pH 7.5, followed by 5 μl of chloramine T (1 mg/ml in H_2O). The iodination reaction was terminated after 5 min by the addition of 50 μl of sodium metabisulphite (5 mg/ml in H_2O). Radioiodinated products were purified by HPLC (IBM LC9533) over a C_{18} column (250 × 4.5 mm) and eluted with an isocratic buffer, 75% MeOH/25% 5 mM phosphate buffer, pH 6.0, at a flow rate of 1 ml/min. The eluate was monitored for UV absorption at 280 nm and for radioactivity by an in-line Beckman 170 Radioisotope detector. The fraction containing the ^{125}I-BW-A844U was stored at −20°.



	R_1	R_2
1	H	NO_2
BW-A844U	H	NH_2
^{125}I-BW-A844U	^{125}I	NH_2
^{125}I-Az-BW-A844U	^{125}I	N_3

Fig. 1. Structures of xanthine analogs refered to in the text. *^{125}I-Az-BW-A844U*, ^{125}I-azido-BW-A844U.

Synthesis of ^{125}I-azido-BW-A844U. BW-A844U was radioiodinated as described above and the reaction products were extracted into ethyl acetate, dried under N_2, and taken up in 100 μl of MeOH. Fifty μl of 1 N HCl were added, followed by 50 μl of 0.5 N sodium nitrite. After 10 min, 50 μl of 0.5 N sodium azide were added for an additional 10 min. All reactions were carried out on ice and in the dark. The products were extracted into ethyl acetate, dried, taken up in HPLC buffer (80% MeOH/5 mM phosphate buffer, pH 6), and chromatographed as described above with the UV lamp off. A small aliquot of the reaction product was chromatographed with the UV lamp on to ascertain complete separation of azido-BW-A844U from ^{125}I-azido-BW-A844U. The photolabel was stored in the dark at −20°.

Radioligand binding assays. Binding assays were initiated by adding 50 μl of ^{125}I-BW-A844U (0.01–2 nM) in buffer B plus 2 units/ml adenosine deaminase to 50 μl of either membranes or CHAPS-solubilized receptors (1 mg of protein/ml) in triplicate and incubating them for 2 hr at 21°. Nonspecific binding was determined by adding 1 mM theophylline or 10 μM (*R*)-PIA with equivalent results. In some cases NaCl was added as noted in the text. In a few experiments the agonist radioligand ^{125}I-aminobenzyladenosine, prepared as described (10), was used in companion assays to compare the number of binding sites detected by the two radioligands. Incubates were filtered over Whatman GF/C filters (for membranes) or Whatman GF/B filters pretreated with 0.3% (v/v) polyethyleneimine (14) (for solubilized receptors) using a modified Brandel Cell Harvester. The filters were rinsed with 3 × 4 ml of ice-cold 10 mM Tris. HCl buffer, pH 7.4, with or without 0.25 M NaCl and the filters were counted in a Beckman 5500 gamma counter. The K_D of ligands for A_1 receptors, determined by competition for ^{125}I-N^6-aminobenzyladenosine binding to bovine brain membranes, and the K_I of ligands for A_2 receptors, determined by inhibition of *N*-ethylcarboxamidoadenosine-stimulated adenylate cyclase activity in human platelet membranes, were evaluated as described (10). Proteins were measured by fluorescamine fluorescence against bovine serum albumin standards (15).

Photolabeling experiments. Bovine or rat cortical membranes, 100–200 μg of protein in buffer B with 2 units/ml of adenosine deaminase, were incubated with 0.1–0.2 nM of either ^{125}I-azido-BW-A844U or ^{125}I-azido-BW-A827U (9, 11) in a final volume of 2 ml, with or without 1 mM theophylline at 4° for 2 hr. Photolabeled polypeptides were separated and visualized as described previously (9, 11, 16). In brief, after binding reached equilibrium in the dark, aryl azides were photoactivated on ice by UV light from a Black-Ray lamp, model XX-15, at a distance of 10 cm for 15 min. Membranes were pelleted and washed once by centrifugation at 40,000 × *g* and dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis denaturing buffer containing 6% (v/v) 2-mercaptoethanol. After electrophoresis according to the method of Laemmli (17), autoradiographs were prepared by exposing dried gels to Kodak XK-1 X-ray film for 48–72 hr.

Data analysis. Equilibrium binding parameters (K_D and B_{max}) were calculated by nonlinear least-squares interpolation to an equation for a single site binding model (15). Inhibition of specific radioligand binding (*B*) by a competitive inhibitor was fit to the equation:

$$B/B_0 = 1/f \sum_{i=1}^{n} F_i \cdot L/K_D\ (1 + I/K_{Ii}) + L)$$

in which *f* is the fractional occupancy of the receptor in the absence of inhibitor, *I*; *F* is the fraction of receptor in each state; B_0 is the specific binding of radioligand in the absence of inhibitor; *L* and *I* are the free concentrations of radioligand and inhibitor, respectively; K_D and K_I are dissociation constants for *L* and *I*, respectively; and *n* is the number of different affinity binding states for *I*. Due to the high affinity of bovine brain receptors for ^{125}I-BW-A844U and (*R*)-PIA, it was essential to calculate the free concentrations of radioligand and inhibitor for each concentration of inhibitor used. This was accomplished by an iterative process to simultaneously solve the above equation, a similar equation

describing inhibitor binding, and conservation of mass equations ($L_T = L + B$; $I_T = I$ + bound inhibitor).

Results

Characterization of BW-A844U affinity for A_1 and A_2 receptors. Based on competition for ^{125}I-N^6-aminobenzyladenosine binding to A_1 receptors of bovine brain, we found that the K_D of BW-A844U was 0.23 ± 0.06 nM (three experiments). The K_I for A_2 receptors of human platelets for the same compound was much higher, 2.0 ± 0.4 μM (three experiments). The ratio (A_2/A_1) is 8700.

Synthesis of ^{125}I-BW-A844U and the photoaffinity label ^{125}I-azido-BW-A844U. After radioiodination, ^{125}I-BW-A844U was separated by HPLC from the uniodinated parent compound, BW-A844U, and other minor iodinated products (Fig. 2A). The Yield of the mono-^{125}I-BW-A844U was 60–65% of the added ^{125}I. After storage for 60 days at −20° or incubation with bovine brain membranes for 2 hr at 21°, >98% of the remaining ^{125}I coeluted with the native radioligand when subjected to a second HPLC run.

The photoaffinity label ^{125}I-azido-BW-A844U was separated from the parent uniodinated compound, azido-BW-A844U, and other minor products by HPLC (Fig. 2B). Both of these radioligands were synthesized from carrier-free ^{125}I, resulting in specific activities of 2200 Ci/mmol.

Equilibrium binding studies. ^{125}I-BW-A844U bound to a single site in crude bovine cortical membranes with high affinity, K_D = 0.14 nM (Fig. 3A). In three experiments in which the same batches of membranes were used, the number of binding sites detected with ^{125}I-aminobenzyladenosine (high plus low affinity binding sites) agreed with the number of binding sites for ^{125}I-BW-A844U to within 10%. Reversible specific and nonspecific binding of ^{125}I-azido-BW-A844U, measured in the dark, were nearly identical to that of the parent amine (data not shown). We have shown previously that high concentrations of salts (>0.5 M) selectively cause large increases (>10-fold) in the binding affinities of acidic antagonists. As expected, 1M NaCl only slightly increased the affinity of the neutral antagonist ^{125}I-BW-A844U (Fig. 3). The binding characteristics of the new radioligand to receptors solubilized in CHAPS were similar to its binding to membranes, in the absence or presence of NaCl (Fig. 3B).

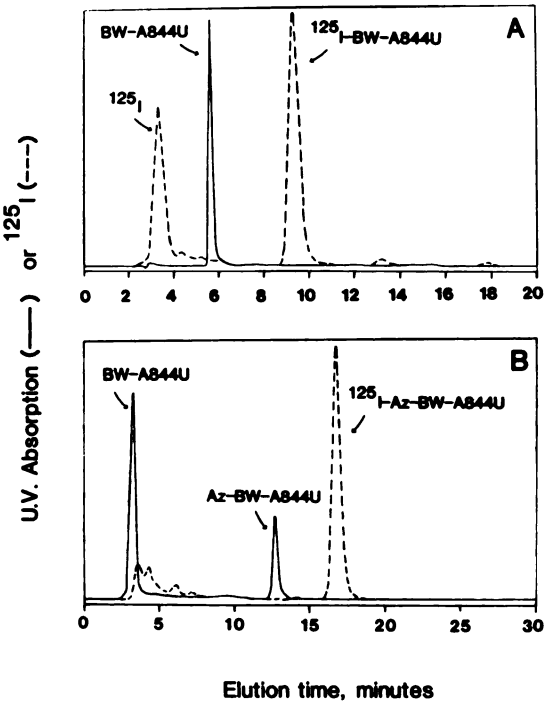


Fig. 2. Purification of ^{125}I-BW-A844U and ^{125}I-azido-BW-A844U. BW-A844U (A) and azido-BW-A844U (*Az-BW-A844U*) (B) iodination reaction products were separated by HPLC as described in Materials and Methods.

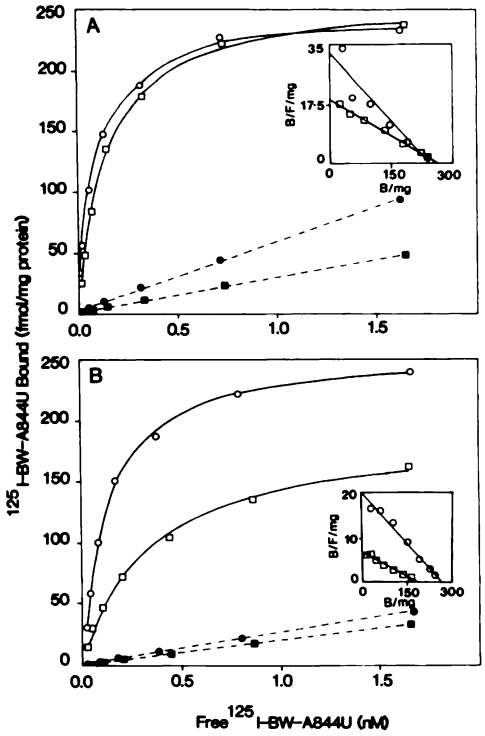


Fig. 3. Equilibrium binding of ^{125}I-BW-A844U to bovine brain membranes and CHAPS-solubilized receptors with or without NaCl. Various concentrations of ^{125}I-BW-A844U were incubated with (A) membranes (45 μg of protein) or (B) CHAPS-solubilized receptors (50 μg of protein) in a final volume of 0.1 ml, with or without 1 M NaCl, for 2 hr. Each *point* represents the mean of three closely agreeing replicates. Binding parameters (estimated by Scatchard plots, which are shown as *insets*) were fit to a single site binding model as described in Materials and Methods. B_{max} (fmol/mg of protein) and K_D (nM) in the presence and absence of NaCl, respectively, were (A) 248, 0.089 and 261, 0.14; and (B) 257, 0.12 and 192, 0.34. The results are typical of three experiments.

In the absence of NaCl we found the following K_D values (nM) for the binding of ^{125}I-BW-A844U to adenosine A_1 receptors of various species: bovine, 0.14; rat, 0.43; porcine, 0.57; and murine, 0.75; in all cases the addition of 1 M NaCl increased the binding affinity by 2–3 fold. In all species the number of binding sites (B_{max}) was increased 60–80% in membranes partially purified as described under Materials and Methods.

Kinetics of ^{125}I-BW-A844U binding. The rates of association and dissociation of ^{125}I-BW-A844U to adenosine A_1 receptors of bovine brain membranes were determined from experiments shown in Fig. 4. The kinetically determined K_D (K_{-1}/K_{+1}) was 0.13 nM, in good agreement with that determined from equilibrium binding studies, K_D = 0.14 nM.

Competition for ^{125}I-BW-A844U binding to brain membranes by other ligands. Bruns *et al.* (13) found that antagonists competed for [^{3}H]CPX binding in rat brain membranes with Hill coefficients near unity, whereas Hill coeffi-

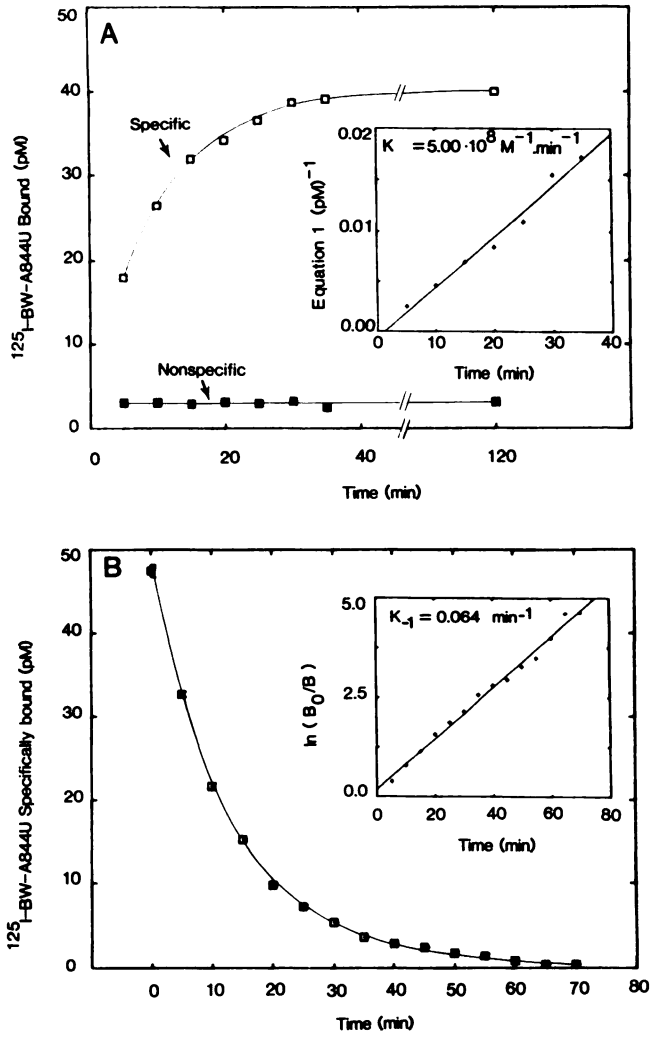


Fig. 4. Kinetics of ^{125}I-BW-A844U binding to bovine brain membranes. A, To determine the rate of association of the radioligand, brain membranes (46 μg of protein) were incubated for various times with 104 pM ^{125}I-BW-A844U in the absence or presence of 1 mM theophylline (nonspecific binding). Specific binding at each point was calculated by subtracting nonspecific binding at the time point indicated. The *inset* shows a plot of incubation time versus the binding data transformed by Eq. 1: $\{\ln [B_e(H-B\cdot B_e/R_T)/(H(B_e-B))]\}/(H\cdot R_T/B_e-B_e)$ in which B_e is the specifically bound radioligand at equilibrium (40 pM) and H, the initial free concentration of radioligand, was approximated by the total radioligand concentration (104 pM). B_{max} was determined from equilibrium binding experiments to the same batch of membranes (210 fmol/mg of protein). The second order rate constant, K_1, given by the slope of the linear regression fit to the line (r = 0.990), was 5.00×10^8 M^{-1} min^{-1}. B, To determine the dissociation rate constant of ^{125}I-BW-A844U, brain membranes (46 μg of protein) were preincubated with radioligand (116 pM) for 2 hr at 21°, in a final volume of 0.1 ml. Dissociation of the radioligand from receptors was monitored at 5-min intervals after addition of 1 mM theophylline. Specific binding at each point was calculated by subtracting nonspecific binding, measured 2 hr after the addition of theophylline. The *inset* shows a plot of ln (B_0/B) as a function of time where B_0 represents specific binding before the addition of theophylline and B represents specific binding at the indicated time points. The rate constant for dissociation, K_{-1}, given by the slope of the linear regression fit (r = 0.996), is 0.064 min^{-1} and from this value the $t_{½}$ for dissociation (ln 2)/K_{-1} was calculated to be 10.8 min. Each *point* represents the mean of three determinations. Standard errors were smaller than the symbols.

cients for agonists ranged between 0.6 and 0.75. Our results, based on competition of ligands for the binding of ^{125}I-BW-A844U to bovine brain membranes were similar (Fig. 5). The Hill coefficient for the antagonist 8-sulfophenyltheophylline was 1.00, whereas optical isomers of the agonist PIA had Hill coefficients <0.65. The difference in potency of the stereoisomers of PIA was 17.5-fold. Hill coefficients <1 for agonists may be indicative of multiple receptor affinity states for agonists. In order to more thoroughly analyze agonist affinity states, and to reexamine the effects of NaCl and guanine nucleotides on agonist radioligand binding, the binding of (*R*)-PIA over a wide concentration range was determined under various conditions of NaCl and Gpp(NH)p (Fig. 6 and Table 1). In the absence of Gpp(NH)p, two agonist affinity states could be demonstrated, which differed in their affinities by 52-fold. The addition of 0.1 mM Gpp(NH)p resulted in conversion of all the receptors into the lower affinity state (Table 1). The addition of 0.5 M NaCl, which had a minimal effect on the affinity of receptors for the neutral antagonist radioligand (Fig. 3), decreased agonist binding affinity of both the high and low affinity states in the absence or presence of Gpp(NH)p. NaCl also reduced the proportion of receptors in the high affinity state from 62 to 44% ($p < 0.01$).

Photoaffinity labeling of brain adenosine A_1 receptors. ^{125}I-Azido-BW-A844U and ^{125}I-azido-BW-A827U were used to photolabel A_1 adenosine receptors in bovine (Fig. 7) and rat (not shown) brain membranes. Both photolabels were specifically incorporated into a 34-kDa peptide. Due to its acidic nature, photolabeling of ^{125}I-BW-A827U is greatly enhanced by the addition of 1 M NaCl, which may mask charge repulsion between the receptor and the ligand (11), whereas NaCl has little effect on the degree of photolabeling that can be achieved with the new neutral antagonist photoaffinity label ^{125}I-azido-BW-A844U (Fig. 7A). A similar pattern of photolabeling was observed with CHAPS-solubilized receptors (not shown). Based on the affinity of ^{125}I-azido-BW-A844U and the data of Fig.

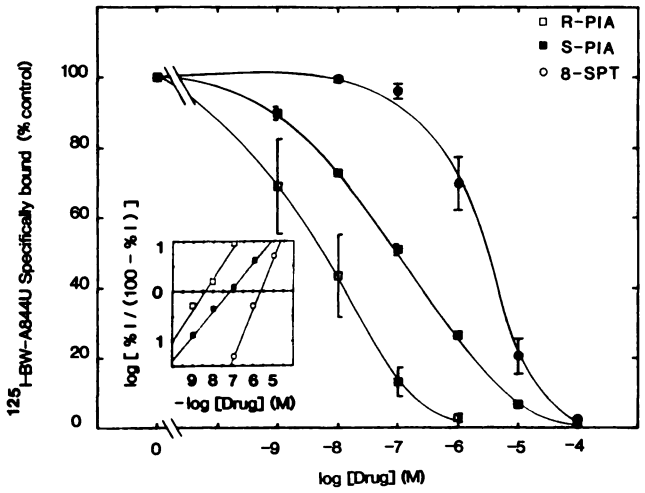


Fig. 5. Competition for ^{125}I-BW-A844U binding to brain membranes by 8-sulfophenyltheophylline (*8-SPT*) and optical isomers of PIA. Brain membranes (46–56 μg of protein) were incubated with 90–100 pM ^{125}I-BW-A844U and various concentrations of drugs in a final volume of 0.1 ml. Each *point* represents the mean ± standard error of three pooled experiments assayed in triplicate on different days. The *inset* shows Hill plots of the same data. IC_{50} values for (*R*)-PIA, (*S*)-PIA, and 8-sulfophenyltheophylline, respectively, were 4, 70, and 2230 nM; Hill coefficients were 0.62, 0.48, and 1.00.

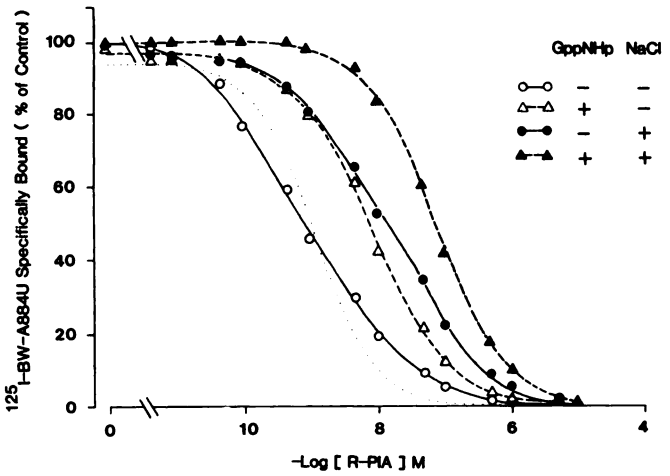


Fig. 6. Inhibition of ^{125}I-BW-A844U binding to bovine brain membranes By (*R*)-PIA. Brain membranes (60–70 μg) were incubated with ^{125}I-BW-A844U (125 pM), 2 units/ml adenosine deaminase, and 5 mM $MgCl_2$ with or without Gpp(NH)p (0.1 mM) and/or NaCl (500 mM) in a final volume of 0.1 ml. Binding assays were terminated and analyzed as described in Materials and Methods. Each experiment was repeated three times in triplicate, and the pooled data points were fit to binding equations (the means of nine determinations are plotted). The calculated binding parameters are shown in Table 1. The *dotted line* shows the fit to a one site model in the absence of Gpp(NH)p and NaCl to illustrate the improvement in the fit obtained by adding a second site.

TABLE 1

Receptor affinity states for (*R*)-PIA in bovine brain membranes

Where indicated Gpp(NH)p (0.1 mM) or NaCl (500 mM) were added. Data modeled for binding to one, two, or three states were derived from composites of three experiments. The appropriate number of states were determined from *F*-tests evaluated at $p < 0.001$. Data are means ± standard errors.

Conditions		No. of states	High[a]		Low, K_D
Gpp(NH)p	NaCl		*f*	K_D	
			%	nM	nM
–	–	2	62.0 ± 2.9	0.074 ± 0.011	3.83 ± 0.80
+	–	1			3.49 ± 0.24
–	+	2	43.7 ± 3.0	0.67 ± 0.10	22.4 ± 2.96
+	+	1			26.01 ± 1.02

[a] Agonist affinity states. *f* = percentage of receptors in the highest affinity state; K_D = dissociation constant(s) for (*R*)-PIA.

7B, we calculated that 3.7% of the reversibly bound antagonist was specifically incorporated into the 34-kDa polypeptide. In the presence of NaCl, the efficiency of photoincorporation of ^{125}I-azido-BW-A844U and ^{125}I-azido-BW-A827U into the receptor polypeptide was 3.5 and 6%, respectively.

Discussion

By comparison with 1,3-dipropyl-8-phenylxanthines, ^{125}I-BW-A844U is well suited for characterizing adenosine A_1 receptors. It combines the advantage of replacing the 8-phenyl substituent with 8-cyclopentyl, which results in higher affinity and A_1 selectivity, and the advantage of replacing the 3-propyl group with a 3-arylamine, which introduces an iodinatable substituent. The resulting radioligand can be readily converted to a photoactive aryl azide. These modifications do not appreciably change the A_1 binding characteristics of the modified compounds.

For binding to adenosine A_1 receptors, ^{125}I-BW-A844U compares favorably with two other high affinity antagonists, [^{3}H]CPX (13) and [^{3}H]XAC (18, 19). It has higher specific activity, 2200 versus 117 and 103 Ci/mmol. It binds with comparable or higher affinity to A_1 receptors (K_D for binding to rat brain membranes assayed in the absence of NaCl, 0.43 nM versus 0.46 and 1.2 nM). It has greater selectivity for A_1 versus A_2 receptors with K_i/K_D ratio (A_2/A_1) of 8700 or 1744 versus 740 and 20.[1] Radioligands suitable for detecting adenosine receptors in brain may be less useful in other tissues in which the density of adenosine receptors is lower. The general utility of a radioligand can be estimated from its ratio of specific to nonspecific binding. By this criterion ^{125}I-BW-A844U also compares favorably with an ^{125}I-labeled derivative of XAC, which binds with a relatively low ratio of specific to nonspecific binding (20).

There has been some disagreement about whether one or two affinity states of adenosine A_1 receptors exist when assayed in the absence of guanine nucleotides. Lohse *et al.* (21), Yeung and Green (22) and Green (23) could detect only a single affinity state of [^{3}H]PIA binding to rat brain membranes, although addition of GTP revealed a lower affinity state. In contrast, Patel *et al.* (24) and Williams and Risley (25) detected two affinity states in rat brain for [^{3}H]N^6-cyclohexyladenosine and [^{3}H]2-chloroadenosine, respectively. It may be difficult to detect specific binding of agonist radioligands to low affinity states due to high levels of nonspecific binding, which arise when agonist concentrations are raised to high enough levels to saturate these sites. Bruns *et al.* (13) were able to resolve two agonist affinity states in rat brain membranes assayed in the absence of guanine nucleotides for several different agonists based on competition for binding of the antagonist [^{3}H]CPX. In bovine brain [^{3}H]N^6-cyclohexyladenosine was reported to bind to two affinity states of A_1 receptors and 63% of the receptors were found in the high affinity state (3). These data are in excellent agreement with the results of this study in which two affinity states for (*R*)-PIA were found and the fraction of high affinity binding sites was found to be 62% based on competition for binding with ^{125}I-BW-A844U (Table 1). Goodman *et al.* (26) also noted that competition for [^{3}H]diethylphenylxanthine binding by agonists in bovine brain was shallow, indicative of multiple agonist affinity states. 8-Cyclopentylxanthine radioligands such as [^{3}H]CPX and ^{125}I-BW-A844U are particularly well suited for examining multiple agonist affinity states of A_1 receptors in brain, because they bind with a high ratio of specific to nonspecific binding (>90% at the K_D), and they bind with high selectivity, thus minimizing a possible interaction with A_2 receptors. The binding of (*R*)-PIA was shifted to a single low affinity state upon the addition of Gpp(NH)p. These data are consistent with the idea that the low affinity sites reflect receptors uncoupled from G proteins. In the presence of NaCl (0.5 M) two affinity states for (*R*)-PIA were also observed, but both bound the agonist with lower affinity than in the absence of NaCl. These data suggest that NaCl has an effect on receptor conformation even when the receptor is uncoupled from G proteins.

High affinity agonist binding is dependent on the presence of magnesium or other divalent cations (26) and NaCl counters the effect of added magnesium (9). Increasing the concentration of magnesium also has been shown to reduce the ability of

[1] K_i values for A_2 receptors were determined from inhibition of NECA-stimulated adenylate cyclase activity in human platelet membranes (XAC) or by competition for radioligand binding to rat striatal membranes (CPX). BW-A844U was evaluated by both methods, resulting in K_i determinations of 2.0 and 0.75 μM, respectively. The latter assay was performed by R. Ferris and F. Tang of the Burroughs Wellcome Co.

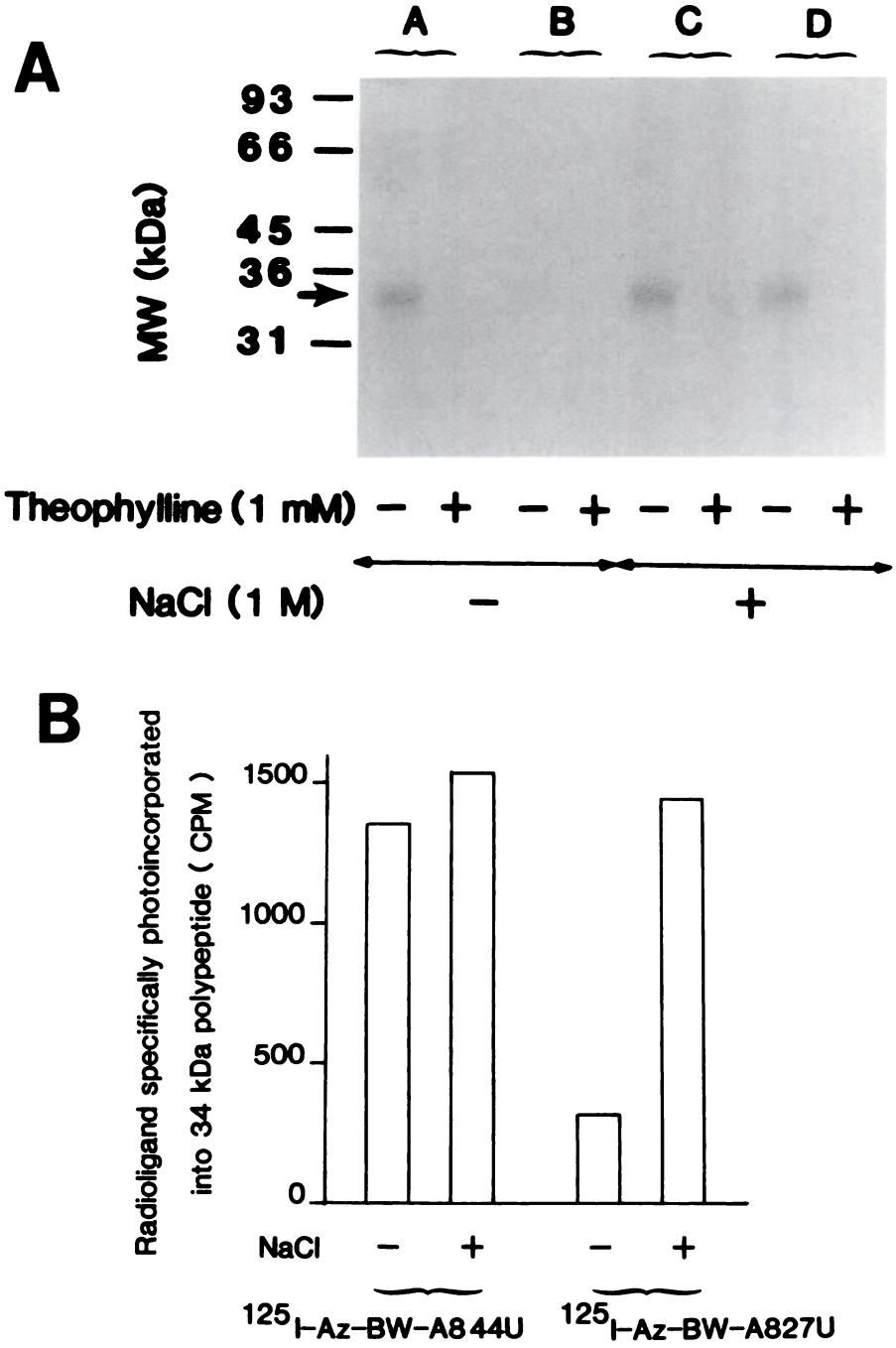


Fig. 7. Photoaffinity labeling of brain membranes with ^{125}I-azido-BW-844U and ^{125}I-azido-BW-827U. A, Bovine brain membranes (108 μg of protein) were incubated with ^{125}I-azido-BW-844U (0.15 nM; *lanes A* and *C*) or ^{125}I-azido-BW-827U (0.17 nM; *lanes B* and *D*) with or without NaCl (1 M). Photolabeled peptides were detected after photoactivation by electrophoresis and autoradiography as described under Materials and Methods. B, Histogram showing the amount of ^{125}I incorporated into the 34-kDa receptor polypeptide in the absence and presence of NaCl.

guanine nucleotides to shift the adenosine A_1 receptor out of the high affinity agonist binding state (26). Sodium, on the other hand, appears to promote conversion of receptors to the low affinity state, decreasing the fraction of receptors in the highest affinity state even in the absence of Gpp(NH)p (Table 1). Thus, magnesium and sodium may have reciprocal actions to either attenuate or enhance the action of GTP on agonist binding. Also, receptors that activate or inhibit adenylate cyclase activity modulate the sensitivity of the enzyme to sodium (27, 28) and magnesium (29, 30). Thus, the effects of NaCl on agonist binding affinity may be of physiological relevance, in addition to complicating the comparison of ligand affinities *in vivo* and *in vitro*. It should be appreciated that the use of membranes to measure agonist binding will result in overestimation of agonist binding affinities compared with intact tissues because these affinities are generally measured in the absence of sodium or guanine nucleotides.

Agonists such as (*R*)-PIA bind more tightly to the high affinity state of A_1 receptors of bovine brain ($K_D < 0.1$ nM; Table 1) than of rat brain (K_D = 3–3.8 nM; Refs. 13 and 23). This variation could reflect a difference in the properties of receptors or G proteins. Agonist binding to the low affinity receptor state may be a truer reflection of the receptor per se than binding to receptor-G protein complexes. It is therefore notable that there is a substantial difference in the K_D of (*R*)-PIA for binding to low affinity states in bovine brain (3.5–4 nM; Table 1) versus rat brain (>1 μM; Ref. 12) suggesting that the receptors themselves vary among species. Species differ-

ences in the binding affinity of antagonists noted in this and other (3, 31, 32) studies support this conclusion.

The effects of high concentrations (>0.5 M) of NaCl on the binding affinity of antagonists should not be confused with the effect of NaCl on agonist binding. The binding affinity of bovine brain A_1 receptors for ^{125}I-BW-A844U is increased 2-fold by the addition of 1 M NaCl. In contrast, addition of 1 M NaCl increased the binding affinity of the acidic antagonist ^{125}I-BW-A827U by 43-fold (10). This differential effect of NaCl on acidic versus neutral antagonist binding is a general phenomenon, which we have attributed to the ability of salt to mask a negatively charged region on or near the receptor. The effect is graded and nonsaturable. In contrast, the effect of sodium on agonist binding occurs at lower concentrations and probably is the result of conformational changes in the receptor polypeptide, as discussed above.

^{125}I-Azido-BW-A844U specifically photoincorporates with an efficiency of 3.5–3.7% into the same 34–35-kDa A_1 receptor peptide in bovine and rat brain membranes as has been extensively characterized by agonist photolabels (9, 16, 33, 34) and acidic antagonist photolabels (9, 11). The highest efficiency of photolabeling was achieved with the acidic 8-phenylxanthine derivative ^{125}I-azido-BW-A827U (6%), but this compound requires high NaCl for high affinity binding. Cross-linking of an amine-containing antagonist radioligand to receptors with a heterobifunctional agent resulted in low efficiency (2%) cross-linking of a larger (40-kDa) polypeptide (20). A possible explanation for this size discrepancy is that alkylation of the amines on the receptor polypeptide with the cross-linking agents results as an anomalously high M_r determination.

The new compounds described in this report may be particularly useful for characterizing adenosine A_1 receptors in tissues with a low density of receptors, where tissue mass is limiting, such as cells in culture. The combination of high specific activity, high A_1 selectivity, and low nonspecific binding make them attractive for use in tissues with low receptor density or low mass or those that contain mixed populations of adenosine receptor subtypes.

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