Photoaffinity Labeling Adenosine A_1 Receptors with an Antagonist ^{125}I-Labeled Aryl Azide Derivative of 8-Phenylxanthine†

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We have derivatized a series of ^{125}I-labeled 8-phenylxanthines with photoactive aryl azide groups on the 1- or 3-position of the xanthine ring. A 3-azidophenethyl derivative was found to be optimal for use as an antagonist photoaffinity label for adenosine A_1 receptors. Following photoactivation, radioactivity was covalently and specifically incorporated into a 34000-dalton and, to a lesser extent, into a 24000-dalton polypeptide of rat brain membranes. Photoincorporation into both polypeptides was competitively inhibited by adenosine analogues with a potency order typical of adenosine A_1 receptors, but the 24000-dalton polypeptide bound both agonists and antagonists with lower affinity than the 34000-dalton polypeptide. Specific photolabeling of receptors in brain membranes of rat, guinea pig, dog, and cow did not show any variation in the 34000-dalton adenosine receptor binding subunit. The adenosine agonist photoaffinity label [^{125}I]N^6-(4-azido-3-iodobenzyl)adenosine also specifically photolabeled the 34000-dalton polypeptide, but photoincorporation of the agonist was less efficient than the antagonist and, unlike the antagonist, was greatly reduced by guanosine 5′-(β,γ-imidotriphosphate). The results indicate that the antagonist photoaffinity label may be more useful than agonists particularly for labeling uncoupled receptors.

Adenosine is a regulator of biological functions in the cardiovascular, nervous, endocrine, and immune systems.[1] Many of these effects are mediated via membrane-bound receptors. These receptors have been subdivided into the A_1 (or R_i) and the A_2 (or R_a) subtypes.[2,3] Specific incorporation of adenosine agonist photolabels into A_1 receptor polypeptides (34–38 kDa) has recently been reported. Choca et al.[4] and Patel and Linden[5] photoaffinity labeled peptides from rat and chick brains with [^{125}I]N^6-(4-azido-3-iodobenzyl)adenosine ([^{125}I]AzBA). Photoaffinity labeling was also demonstrated in rat cerebral cortex with [^{125}I]-2-azido-N^6-[(4-hydroxy-3-iodophenyl)isopropyl]-adenosine.[6] [^{125}I]N^6-[2-(4-amino-3-iodophenyl)ethyl]-adenosine was coupled to adenosine receptors in rat brain and adipocyte membranes with use of a photochemical cross-linking agent.[7]

There are drawbacks to the use of agonist compounds for photoaffinity labeling experiments that stem from the fact that agonists do not bind with high affinity to all conformational states of the receptor.[8,9] Also, it is possible that photoincorporation will occur not on the receptor polypeptide but on an adjacent protein such as a G protein. Therefore, we sought to develop an antagonist photoaffinity label for the purposes of (1) improving the efficiency of photoincorporation, (2) comparing the photolabeling pattern with results obtained with agonists, and (3) comparing the photolabeling pattern of antagonists in the absence and presence of agents that uncouple receptors from G proteins.

We have developed high affinity ^{125}I-labeled adenosine antagonists (Figure 1) containing arylamine substituents suitable for conversion into photoactive aryl azides.[10] In this paper, we describe specific photoaffinity labeling and characterization of adenosine receptor binding subunits with IIc ([^{125}I]BWA-947U) in brain membranes from various animal species. The antagonist photoincorporates into adenosine receptors with greater specificity and higher efficiency than the agonist [^{125}I]AzBA. The antagonist labels the same 34-kDa polypeptide as the agonist and to a lesser extent in rat brain, a second 24-kDa polypeptide.

Biological Evaluation

Binding of ^{125}I-Labeled Arylamine and Aryl Azide Antagonists to Rat Brain Membranes. Radioligand binding to brain membranes was examined to determine specific binding, maximum binding, and binding affinity. When photoactive aryl azides were used, binding assays were conducted in dim light to avoid photoactivation. Xanthines with arylamine substituents on the 3-position, Ib and IIb (Figure 1), bound to rat brain membranes with a K_D of 0.8 ± 0.2 nM and a B_{max} of 485 ± 11 fmol/mg of protein (N = 3). The binding affinities of the azide derivatives of these compounds, Ic and IIc, were only slightly lower than the precursor amines with K_D values ranging between 1 and 2 nM (N = 3). Specific binding of xanthines with arylamines or aryl azide substituents on the 1-position, IIIb,c and IVb,c, could not be detected. These radioligand binding data are consistent with the results of experiments in which nonradioactive forms of these ligands were evaluated by competetion assays with other radioligands which bind to adenosine A1 receptors.[10] On the basis of these data, we anticipated that 3-(aryl azide)-substituted 8-phenylxanthine derivatives would prove to be more useful than 1-substituted derivatives as photoaffinity labels for adenosine A_1 receptors.

Antagonist Photoaffinity Labeling of the Adenosine A_1 Receptor Binding Subunit. Figure 2 shows photoaffinity labeling of adenosine A_1 receptors in rat brain membranes by an antagonist and an agonist photoaffinity

† Supported by NIH Grant HL31432 and the Burroughs Wellcome Co. J.L. is the recipient of an Established Investigator Award from the American Heart Association.

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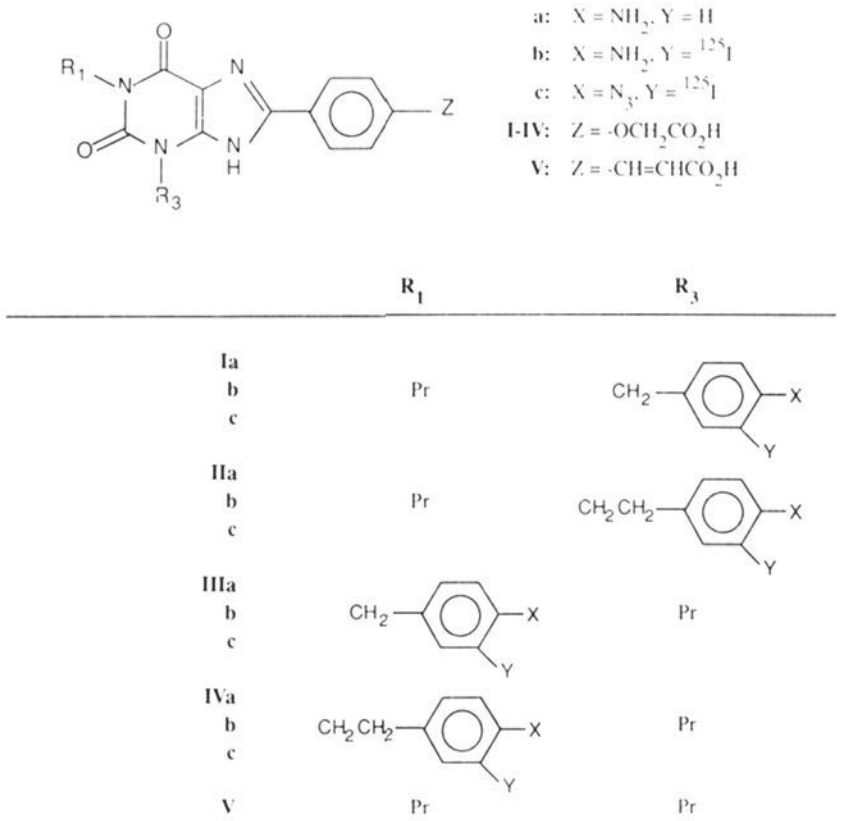

Figure 1. Structures of acidic 8-phenylxanthine derivatives. Iodinatable compounds were prepared by addition of arylamine substituents at the 1- or 3-position of the xanthine ring. Photosensitive compounds were synthesized by converting arylamines to the corresponding aryl azides.

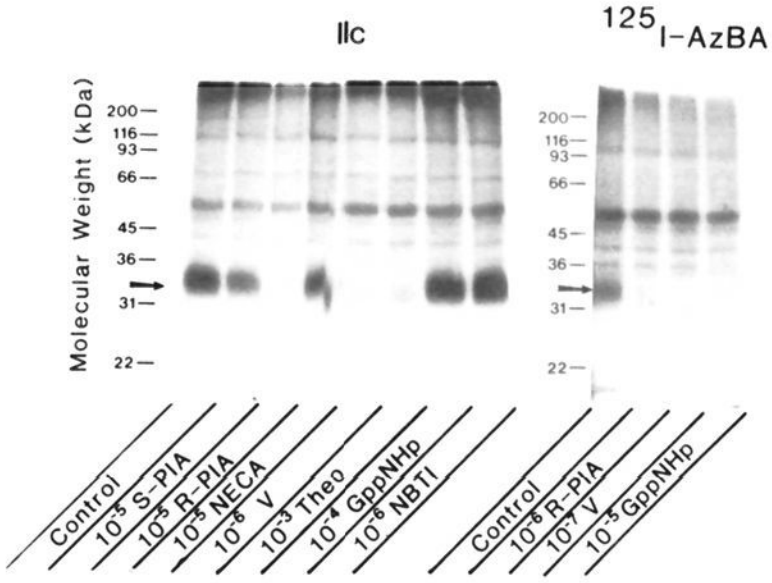

Figure 2. Photoaffinity labeling of rat brain adenosine A_1 receptors by IIc and [^{125}I]AzBA. Aliquots of rat brain membranes (140–168 μg of protein in 100 μL) were incubated with either IIc (1 nM) or [^{125}I]AzBA (1 nM) in the presence of the indicated molar concentrations of competing ligands, washed, and photoactivated as described in the Experimental Section. The samples were subjected to SDS-PAGE and autoradiography. The gels containing IIc were developed for 18 h and those containing [^{125}I]-AzBA for 72 h. Theo = theophylline, GppNHp = Gpp(NH)p. The arrows indicate the position of a prominantly specifically photolabeled 34-kDa polypeptide. The results shown are representative of six similar experiments.

label. Membranes that were incubated with IIc or [^{125}I]-AzBA, irradiated with UV light, and subjected to SDS-PAGE incorporated radioactivity into 10–12 bands. Specific binding was consistently demonstrable in only a 34-kDa and to a lesser extent a 24-kDa polypeptide. Photolabeling of the 34-kDa polypeptide with either the antagonist or the agonist photoaffinity label was inhibited stereospecifically by N^6-(phenylisopropyl)adenosine (PIA) and was inhibited by other adenosine analogues with a potency order typical of A_1 adenosine receptors, i.e., N^6-cyclopentyladenosine (CPA) > (*R*)-PIA > 1-(6-amino-9*H*-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide (NECA) > (*S*)-PIA. Theophylline and V (BWA-1433U), but not the adenosine uptake inhibitor nitrobenzylthio-

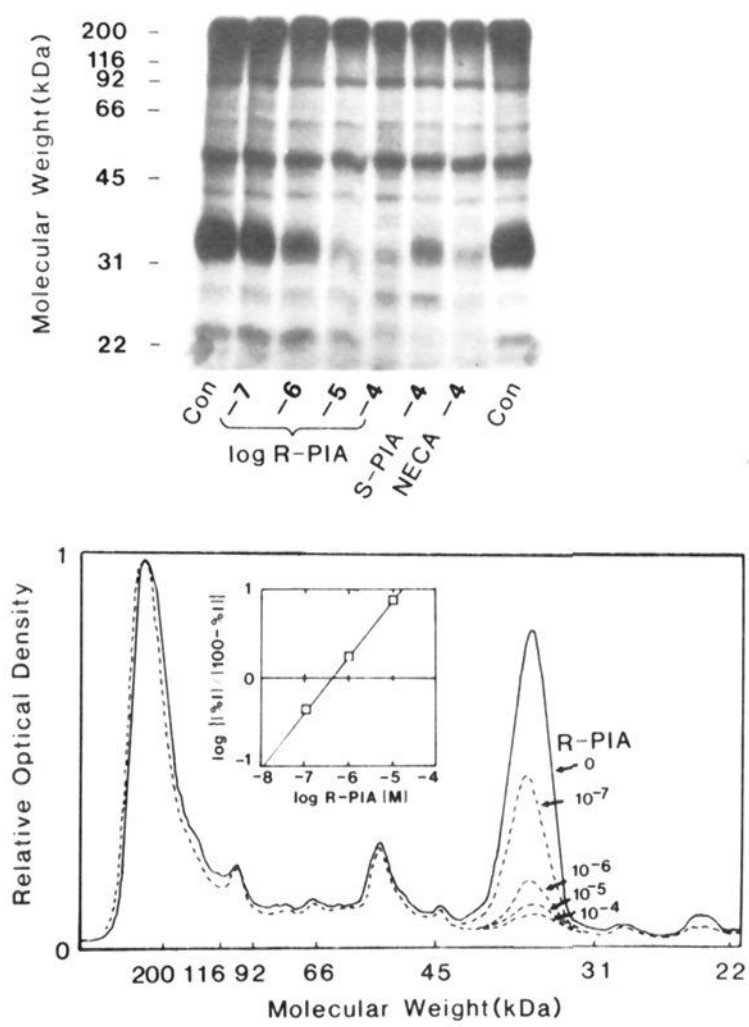

Figure 3. Inhibition of the photoincorporation of IIc into a 34-kDa polypeptide by adenosine agonists. Aliquots of rat brain membranes (140 μg of protein in 100 μL) were incubated with IIc (1 nM) in the absence (con) or presence of the indicated molar concentrations of adenosine agonists (*R*)-PIA, (*S*)-PIA, and NECA. An autoradiograph (top) was preared as described in the Experimental Section and developed for 18 h. Optical scans of the autoradiograph are shown (bottom) for a control lane (solid line) and for 10^{-4} M (*R*)-PIA (dashed line). Traces of lanes treated with other concentrations of (*R*)-PIA are shown as dashed lines under the 34-kDa peak only. The IC_{50} for (*R*)-PIA was calculated from a Hill plot (insert) to be 430 nM for (*R*)-PIA. The results shown are representative of two similar experiments.

inosine (NBTI) (1 μM), inhibited adenosine antagonist and agonist photoincorporation into the 34-kDa polypeptide. Guanosine 5′-(β,γ-imidotriphosphate) (Gpp(NH)p) (100 μM) prevented agonist but not antagonist photoincorporation. The same amount of membrane protein and radioligand were used to photolabel the receptors shown in Figure 2, and the K_D values for binding of these radioligands measured in the dark were similar. However, more antagonist photolabeling was observed despite the fact that the agonist autoradiograph was developed 4 times longer. From this and four other similar experiments, we calculated that photolabeling by the antagonist into the receptor polypeptide occurred with 5–8 times higher efficiency (5.5% of specifically bound ligand) than photolabeling by the agonist. Two other antagonist photolabels, Ic and IIIc, both failed to specifically photolabel any polypeptides.

The dose–response curve for inhibition of photoincorporation of IIc into the 34-kDa polypeptide by the adenosine agonist (*R*)-PIA is shown in Figure 3. The IC_{50} of (*R*)-PIA was calculated to be 430 nM from Hill plots of inhibition of the photolabeling of the 34-kDa receptor polypeptide (Figure 3 insert). This low affinity can be attributed to the presence of 1.0 M NaCl in the incubation medium, which was added to increase the binding affinity of the acidic antagonist photoaffinity label.[10]

The dose–response curves for inhibition of photoincorporation of IIc into the 24- and 34-kDa polypeptides by the adenoine antagonists 1,3-diethyl-8-phenylxanthine

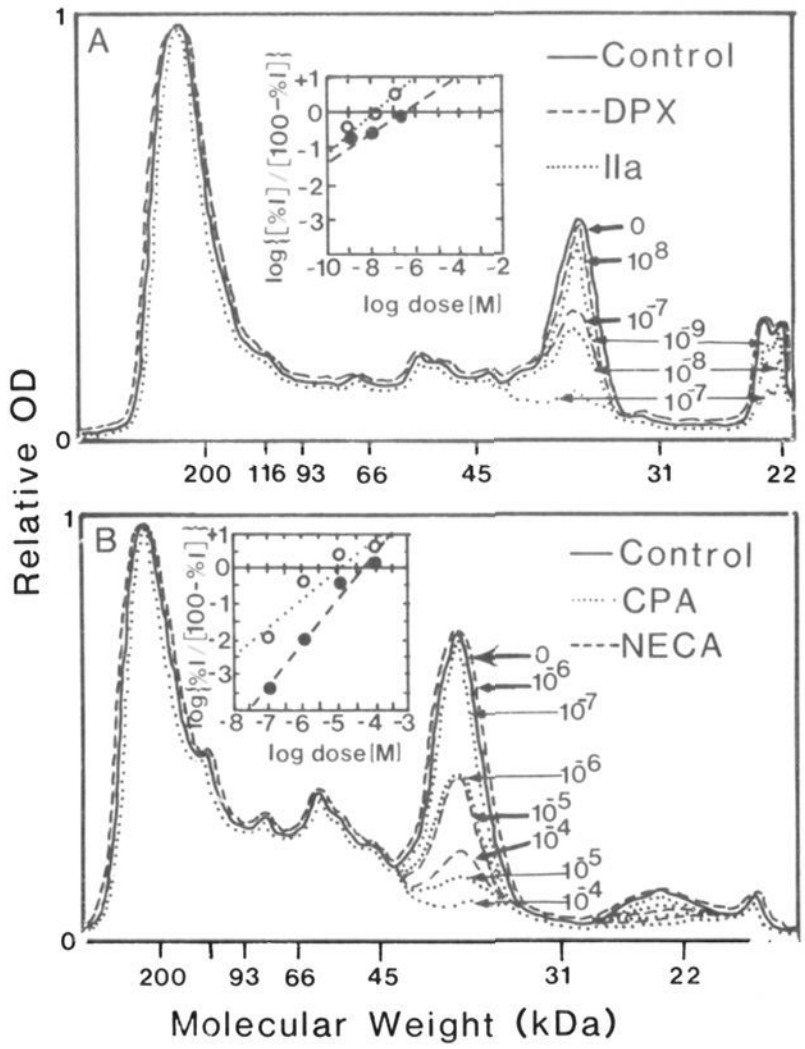

Figure 4. Competetive inhibition of the photoincorporation of IIc into 34- and 24-kDa polypeptides. Aliquots of rat brain membranes (145 μg of protein in 100 μL) were incubated with IIc (912 pM) in the presence of the indicated molar concentrations of other compounds. Following photoactivation, autoradiographs were prepared similar to those illustrated in Figures 2 and 3. (A) Optical scans of radioautiographs showning the effects of DPX (thick arrows) and IIa (thin arrows). IC_{50} values for DPX (closed circles) and IIa (open circles) to inhibit photolabeling of the 34-kDa polypeptide were calculated from Hill plots (insert) to be 366 and 16 nM, respectively. (B) Optical scans of autoradiographs showing the effects of NECA (thick arrows) and CPA (thin arrows). IC_{50} values for NECA (closed circles) and CPA (open circles) to inhibit labeling of the 34-kDa polypeptide were calculated from Hill plots (insert) to be 52 and 11 μM, respectively. IC_{50} values of NECA and CPA for inhibition of photolabeling of the 24-kDa polypeptide were 90 and 14 μM, respectively. The results shown are typical of three experiments.

(DPX) and IIa are illustrated in Figure 4a. IC_{50} values of IIa and DPX for inhibiting photolabeling of the 34-kDa receptor polypeptide were 16 and 366 nM, respectively. The relative order of potency for the adenosine antagonists tested was IIa > DPX > theophylline > caffeine. IIa was also a weak inhibitor of photolabeling of the 24-kDa polypeptide, but DPX was ineffective (Figure 4a).

To determine if the 24-kDa polypeptide is an A_2 adenosine receptor polypeptide, the potency order of adenosine analogues to inhibit the labeling of the 24-kDa polypeptide were examined. The dose–response curves for inhibition of photoincorporation of IIc into the 34- and 24-kDa polypeptide by the A_1-selective adenosine agonist N^6-cyclopentyladenosine (CPA) and by the nonselective agonist NECA[11] were determined. IC_{50} values for inhibiting photoincorporation into the 34-kDa polypeptide by CPA and NECA were 11 and 52 μM, respectively (Figure 4b). CPA was also more potent than NECA for inhibiting the photoincorporation of IIc into the 24-kDa polypeptide, suggesting that it is not an A_2 adenosine receptor binding

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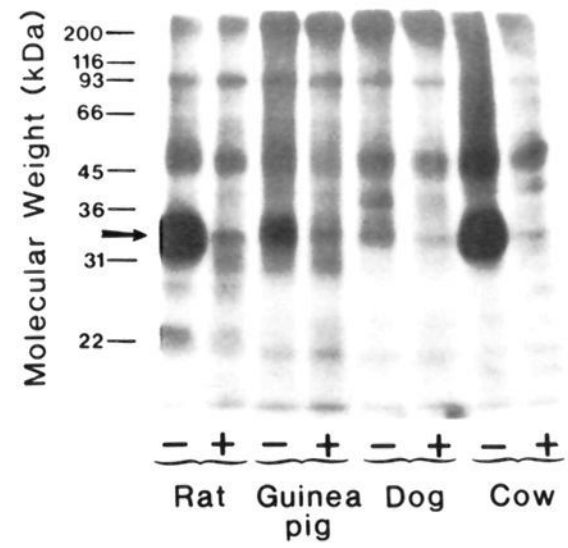

Figure 5. Photoaffinity labeling of A_1 adenosine receptors in the brains of various species with IIc. Aliquots of brain membranes from rat (186 μg of protein), guinea pig (238 μg of protein), dog (274 μg of protein), and cow (204 μg of protein) were incubated with IIc (680 pM) in the presence and absence (+ or −) of V (1 μM). Autoradiographs prepared as described in Experimental Section were developed for 16 h. The results shown are representative of two experiments.

subunit, but rather another component of the A_1 receptor or a degradation product of the receptor.

The results of antagonist radioligand binding studies indicate that there are species differences in the binding affinity of adenosine A_1 receptors for several ligands.[12,13] Figure 5 shows that the same 34-kDa polypeptide is specifically photolabeled in brain membranes of dog, rat, guinea pig, and cow. In the dog brain only, an additional 37 kDa was also labeled. Experiments are ongoing to further characterize this polypeptide. The 34-kDa polypeptide was also labeled in all of these species by the adenosine agonist photolabel, [^{125}I]AzBA.[5] These results indicate that differences in the affinities of radioligands between species are not associated with differences in the molecular weight of the 34-kDa receptor polypeptide.

Discussion

The adenosine antagonist radioligand IIb binds in a saturable and reversible manner to a high-affinity site (K_D = 0.8 nM) in rat brain membranes. The azide derivative of this compound, IIc, binds to the same receptor in the dark, i.e., without photoactivation, with an affinity only slightly lower than the parent amine (K_D = 1.2 nM). The binding affinities of the acidic antagonist radioligands (i.e., Ib and IIb) were greatly increased in the presence of 1 M NaCl.[10] The effect of high salt on acidic antagonist binding provides a useful means for increasing the specificity of antagonist radioligand binding and photolabeling.

1-Substituted arylamine and aryl azide 8-phenyxanthine derivatives bind to adenosine A_1 receptors with lower affinity than 3-substituted derivatives.[10] Thus, IIIb and IVb were not useful radioligands, and not surprisingly, IIIc and IVc were not useful as photoaffinity labels. On the other hand, Ib and its azide derivative Ic were similar in potency to IIb for binding of A_1 receptors in rat brain membranes, but surprisingly, Ic did not photoincorporate as well as IIc into adenosine receptors. The data suggest that the photoactivated azidophenethyl compound, IIc, is more likely to covalently incorporate into the receptor than the photoactivated azidobenzyl compound (Ic) despite the fact that both compounds bind to adenosine receptors with

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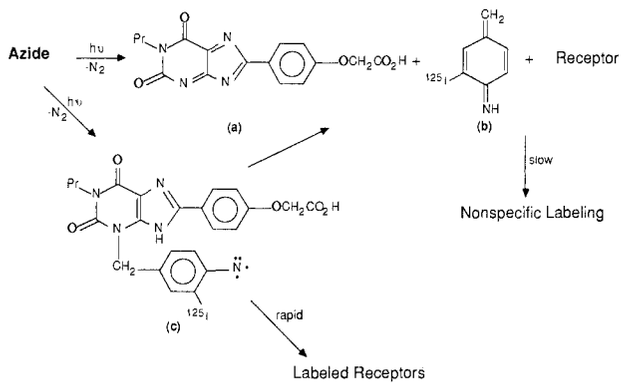

Figure 6. Theoretical pathways for the formation of reactive decay products of Ic following photoactivation. Upon exposure to ultraviolet light, the azidobenzyl group may fragment to a didehydroxanthine (a) and a relatively stable quinoid species (b) and/or to the highly reactive nitrene radical (c). For the purposes of photolabeling receptors, (c) would be preferable to (b) since it would be less prone to cause nonspecific labeling.

similar affinities. Failure of Ic to be effectively incorporated into the receptor on photolysis suggests that benzylxanthines may decompose on photolysis in a manner that results in cleavage of the ^{125}I-labeled benzyl group (Figure 6). The resulting ^{125}I-labeled quinoid fragment (b) would be expected to result in nonspecific labeling since it is likely to be stable enough to diffuse away from the region of the receptor before being attacked by nucleophiles. The nitrene intermediate (c), if formed, could rearrange to give (a) and (b) instead of inserting into a group at the receptor site.[14] In contrast, azidophenethyl compounds such as IIc, which was a useful photoaffinity label, would not be expected to fragment into quinoid species such as (b).

Photoincorporation of the adenosine antagonist radioligand displayed stereospecificity, since (*R*)-PIA was a more potent inhibitor of photoincorporation than (*S*)-PIA. Inhibition of covalent labeling by adenosine analogues exhibited a potency order typical of adenosine A_1 receptors. Gpp(NH)p and NaCl did not prevent antagonist photolabeling, but abolished photolabeling by the adenosine agonist radioligand [^{125}I]AzBA. The fact that the antagonist photolabel incorporates into the same receptor polypeptide under conditions where the agonist no longer binds with high affinity due to the addition of NaCl or guanine nucleotide suggests that the labeled polypeptide is a component of the receptor and not an adjacent polypeptide in close proximity to the receptor, such as the β subunit of a G protein.

In addition to the 34-kDa polypeptide, which was the primary site of specific labeling by both the agonist and antagonist photolabels, a second 24-kDa polypeptide was weakly and inconsistently labeled by the antagonist photoaffinity label in rat brain. Faint and inconsistent labeling of a similar-sized rat receptor polypeptide by an agonist photoaffinity label was noted in another study in which it was found that photolabeling of this polypeptide is sensitive to GTP and has a low affinity for agonists.[6] It is unclear if this low molecular weight poorly photolabeled polypeptide is a degradation product of the adenosine A_1 receptor or a low-affinity binding site unrelated to the receptor. In this study the 24-kDa peptide was found to bind both agonists and antagonists with lower affinity than the 34-kDa polypeptide. Since it bound CPA with higher affinity than NECA, it is unlikely to be a component of the adenosine A_2 receptor. The 34-kDa peptide labeled by IIc and [^{125}I]AzBA appears to be distinct from the purine nucleoside transporter protein, which in erythrocytes has an estimated M_r between 47 and 66 kDa.[15] In this study, NBTI did not inhibit photoincorporation of the antagonist IIc or the agonist [^{125}I]AzBA into the 34-kDa protein. Moreover, this is no evidence that the nucleoside transporter can bind xanthine derivatives with high affinity. The difference in size and pharmacological specificity indicate that the 34-kDa protein labeled by IIc and [^{125}I]AzBA is not a nucleoside transport protein.

The A_1 adenosine receptor photoaffinity label IIc was used to determine if reported differences in the affinities of adenosine A_1 receptors for various ligands among species was associated with alterations in the size of the photolabeled receptor binding subunit. Specific photoincorporation of IIc into brain membranes from rat, guinea pig, dog, and cow did not show any variation in the 34-kDa receptor size. This suggests that (1) the differences in radioligand binding affinities may be due to differences in the receptor coupling proteins and not differences in receptors, (2) subtle differences in the 34-kDa binding subunit in the various species can not be detected by one-dimensional SDS-PAGE, or (3) other membrane components, such as lipids or sugar residues, are responsible for the difference in receptor radioligand binding affinties. The affinity of solubilized β-adrenergic receptors for radioligand binding can be modulated by specific lipids in the membrane.[16]

Compound IIc is the first antagonist photoaffinity label for adenosine A_1 receptors. The property of antagonists to bind to uncoupled receptors as well as to receptor G-protein complexes makes them more useful in the investigation of adenosine receptors than existing agonist radioligands that bind primarily to receptor G-protein complexes.

Experimental Section

8-Phenylxanthine derivatives shown in Figure 1 were synthesized and analyzed as described.[10] Radioactive and nonradioactive compounds were found to cochromatograph by HPLC.

[^{125}I]-2-[4-[3-(4-Amino-3-iodophenethyl)-1,2,3,6-tetrahydro-2,6-dioxo-1-propyl-9*H*-purin-8-yl]phenoxy]acetic Acid (IIb). Two milliCuries of Na^{125}I in 100 μL of 0.3 M KH_2PO_4 (pH 7.5) was added to 10 μL of 10 mM IIa dissolved in 20 mM NaOH, followed by 10 μL of chloramine T (1 mg/mL in H_2O). After the solution was mixed for 1 min, the iodination reaction was quenched with 100 μL of sodium metabisulfite (5 mg/mL in H_2O). The products were extracted five times into 200 μL of ethyl acetate, and 75–85% of the added ^{125}I was detected in the pooled extracts, which were evaporated to dryness under N_2 and dissolved in chromatography buffer (MeOH/5 mM KH_2PO_4 (pH 6.0), 45:55). IIb was purified by isocratic HPLC (Figure 7a). [^{125}I]-IIc was prepared from IIb via intermediate diazonium salts. All procedures described below were carried out in dim light. Compound IIb was dissolved in 300 μL of 350 mM HCl and 100 μL of 10 mM sodium nitrite and then cooled on ice for 10 min. One hundred microliters of 10 mM sodium azide was added for an additional 10 min. The product was extracted into ethyl acetate, evaporated to dryness under N_2, and dissolved in chromatography buffer (MeOH/5 mM KH_2PO_4 (pH 6.0), 60:40). The extracted products were separated by HPLC isocratically at a flow rate of 1 mL/min with the UV detector switched off. Small aliquots of the aryl azide reaction products were monitored by UV absorption at 310 nm to verify separation of IIc from UV-absorbing reaction products (Figure 7b). Ib, IIIb, IVb, and their corresponding azides (Ic, IIIc,

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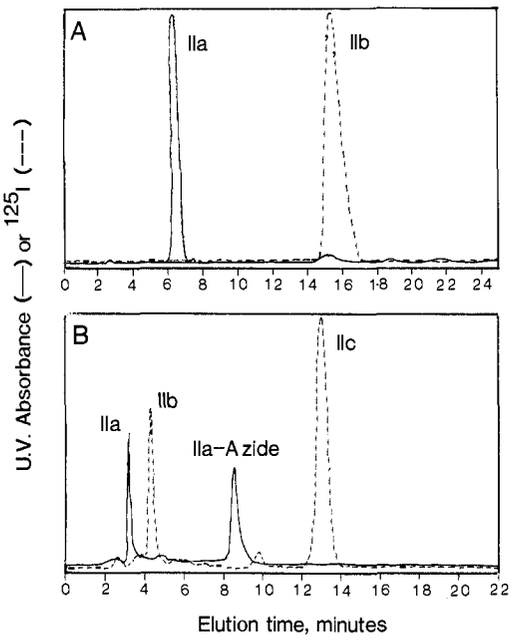

Figure 7. Purification of IIb and IIc. Compound IIa was iodinated with carrier-free ^{125}I as described in the text. The iodinated products were extracted into ethyl acetate and chromatographed isocratically by HPLC (A). Amines were converted to aryl azides, extracted into ethyl acetate, and chromatographed (B). UV absorption was monitored at 310 nm and ^{125}I was monitored by an in-line Beckman Model 170 radioisotope detector. The bulk of the azide products were chromatographed with the UV monitor switched off to avoid photoactivation. Fractions containing purified IIb or IIc were stored at −20 °C in HPLC buffer in the dark.

and IVc) were synthesized and purified by procedures similar to those described above.

[^{125}I]-6-[(4-Azido-3-iodobenzyl)amino]-9-β-D-ribofuranosyl-9*H*-purine. [^{125}I]AzBA, prepared as described,[10] was dissolved in 100 μL of 50 mM HCl. Following the addition of 50 μL of 10 mM sodium nitrite, the solution was cooled for 10 min on ice. Fifty microliters of 10 mM sodium azide was added for an additional 10 min in the dark. The product was extracted into ethyl acetate and evaporated to dryness under N_2. [^{125}I]AzBA was dissolved in HPLC buffer (MeOH/5 mM KH_2PO_4 (pH 6.0), 60:40) and eluted isocratically.

Radioligand Binding Assays. Crude membranes from cerebral cortices of rat, guinea pig, dog, and cow were prepared as described previously.[5] Equilibrium binding assays were conducted for 2 h at 21 °C and terminated by filtration over Whatman GF/C glass fiber filters with use of a modified cell harvester (Brandel, Gaithersburg, MD). Generally, 1 M NaCl was added to the membranes used for antagonist radioligand binding assays because the addition of high salt increased acidic antagonist binding affinity by 10–20-fold.[10] Filters were washed three times with 4 mL of ice-cold 10 mM Tris·HCl, pH 7.4, 2.5 mM $MgCl_2$, and 500 mM NaCl and then counted in a Beckman γ 5500 counter. Nonspecific binding was defined as binding in the presence of 100 μM (*R*)-PIA or 1 μM V^{17} with equivalent results. Fifty microliters of membranes containing 5 units/mL adenosine deaminase were added to 50 μL of radioligand prepared in 1 M NaCl (for agonist binding, NaCl was omitted). The radioligand concentrations were held constant at 150 pM and diluted with various concentrations of unlabeled ligands between 0.1 and 100 nM. Initial estimates of B_{max} and K_D values were derived by Scatchard[18] analysis. The untransformed data were fit to single-site model by the procedure of Marquadt.[19]

Photoaffinity Labeling Experiments. Membranes (140–275 μg of protein/100 μL) in 10 mM HEPES buffer (pH 7.4) containing 1 mM EDTA, 0.1 mM benzamidine, 10 μg/mL phenylmethanesulfonyl fluoride, 5 units/mL adenosine deaminase (buffer A) and antagonist photoaffinity label (0.5–1 nM) were incubated for 2 h in the dark at 21 °C. At the end of the incubation period, membranes were diluted with buffer A containing 1 M NaCl. When [^{125}I]AzBA was used, NaCl was replaced with 2.5 mM $MgCl_2$. Once binding reached equilibrium, the membranes were washed by centrifugation. The membranes were placed in 12-well tissue culture plates (Costar) on ice and exposed to 365-nm ultraviolet light (Blak-Ray lamp Model XX-15) for 10 min. After photoactivation, the membranes were pelleted by centrifugation at 25000*g* for 20 min and dissolved in 120 μL of SDS-PAGE denaturing buffer containing 6% (v/v) 2-mercaptoethanol.

SDS-PAGE. Gel electrophoresis was performed according to the method of Laemmli[20] with a 3% stack and a 10% polyacrylamide resolving gel (16 cm × 14 cm). One hundred microliters of the denatured samples were electrophoresed with a constant current of 30 mA/gel. The gels were stained for protein with 0.1% coomassie brilliant blue and destained with 10% glacial acetic acid/10% methanol. After drying, the gels were exposed to Kodak XAR-5 film in Du Pont X-ray cassettes with Cronex enhancing screens at −70 °C for 16–72 h.

Registry No. Ib, 112533-72-9; Ic, 112575-66-3; IIa, 112533-55-8; IIb, 112575-62-9; IIc, 112575-63-0; IIIb, 112575-64-1; IIIc, 112595-98-9; IVb, 112575-65-2; IVc, 112575-67-4; [^{125}I]-AzBA, 106719-48-6; [^{125}I]-ABA, 95523-14-1.

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