Identification of the A2 adenosine receptor binding subunit by photoaffinity crosslinking

WILLIAM W. BARRINGTON*, KENNETH A. JACOBSON†, ALAN J. HUTCHISON‡, MICHAEL WILLIAMS‡, AND GARY L. STILES§

ABSTRACT  A high-affinity iodinated agonist radioligand for the A2 adenosine receptor has been synthesized to facilitate studies of the A2 adenosine receptor binding subunit. The radioligand 125I-labeled PAPA-APEC (125I-labeled 2-[4-(2-[4-(aminophenyl)methylcarbonylaminooethyl]amino-5'-N-ethylcarboxamidoadenosine} was synthesized and found to bind to the A2 adenosine receptor in bovine striatal membranes with high affinity (Kd = 1.5 nM) and A2 receptor selectivity. Competitive binding studies reveal the appropriate A2 receptor pharmacologic potency order of 5'-N-ethylcarboxamidoadenosine (NECA) >> (--)N1-[R]-1-methyl-2-phenylethyl]adenosine (R-PIA) > (--)N1-[S]-1-methyl-2-phenylethyl]adenosine (S-PIA). Adenylate cyclase assays, in human platelet membranes, demonstrate a dose-dependent stimulation of cAMP production. PAPA-APEC (1 μM) produces a 43% increase in cAMP production, which is essentially the same degree of increase produced by 5'-N-ethylcarboxamidoadenosine (the prototypic A2 receptor agonist). These findings combined with the observed guanine nucleotide-mediated decrease in binding suggest that PAPA-APEC is a full A2 agonist. The A2 receptor binding subunit was identified by photoaffinity-crosslinking studies using 125I-labeled PAPA-APEC and the heterobifunctional crosslinking agent N-succinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (SAPAH). After covalent incorporation, a single specifically radiolabeled protein with an apparent molecular mass of 45 kDa was observed on NaDodSO4/PAGE/autoradiography. Incorporation of 125I-labeled PAPA-APEC into this polypeptide is blocked by agonists and antagonists with the expected potency for A2 receptors (see above) and is decreased in the presence of 10−4 M guanosine 5'-[β,γ-imido]triphosphate. Photoaffinity crosslinking of the A1 adenosine receptor binding subunit with 125I-labeled 8-[4-[2-[4-aminoethyl]acetylaminooethyl]carbonylmethylxophenyl]-1,3-dipropyl-xanthine (PAPAXAC) (an A1 selective photoaffinity probe) in the same tissue reveals a 38-kDa peptide that exhibits the appropriate A1 receptor pharmacology. 125I-labeled PAPA-APEC, therefore, has identified the A2 receptor binding subunit as a 45-kDa protein that is unique and distinct from the A1 binding subunit.

Adenylate cyclase-coupled adenosine receptors are divided into two main subtypes (1). The A1 subtype is inhibitory to adenylate cyclase and exhibits the agonist potency order of (--)N1-[R]-1-methyl-2-phenylethyl]adenosine (R-PIA) > 5'-N-ethylcarboxamidoadenosine (NECA) > (--)N1-[S]-1-methyl-2-phenylethyl]adenosine (S-PIA). The development of a large number of selective high-affinity radioligands and photoaffinity and affinity probes for this receptor subtype has led to the rapid accumulation of information concerning the structure, function, and regulation of the A1 adenosine receptor (1–9). In contrast, the A2 adenosine receptor is stimulatory to adenylate cyclase and exhibits the distinctly different agonist potency order of NECA > R-PIA > S-PIA. A2 adenosine receptors are distributed in a wide range of tissues including: brain, where they modulate neurotransmitter release; platelets, where they inhibit aggregation and adhesion; and smooth muscle cells, where they may mediate vasodilation (1). Available radioligands for the A2 receptor have suffered from a number of problems including low affinity, poor specificity, and little selectivity for the A2 receptor. This dearth of useful probes has lead to an almost complete lack of information on the structure, function, and regulation of this physiologically important receptor subtype.

We now report on the development of 125I-labeled 2-[4-(2-[4-(aminophenyl)methylcarbonylaminooethyl]amino-5'-N-ethylcarboxamido-adenosine (125I-PAPA-APEC) (Fig. 1), which was synthesized from the methyl ester of the highly A2 selective agonist, COS 21680 (A.J.H., R. L. Webb, H. H. Oei, G. R. Ghai, M. B. Zimmerman, M.W., unpublished data and ref. 10) (2-[p-(2-carboxyethyl)phenethyl]amino-5'-N-ethylcarboxamido-adenosine) by a functionalized conger approach (11). This is a high-affinity (Kd = 1.5 nM), iodinated radioligand for the A2 adenosine receptor. Utilizing this radioligand and the heterobifunctional crosslinking agent N-succinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (SAPAH), we have determined that the A2 binding subunit in bovine striatal membranes resides on a peptide with an apparent molecular weight of 45 kDa and is distinct from the labeled A1 adenosine receptor binding subunit. This radioligand should provide a powerful tool for further elucidation of the structure, function, and regulation of the A2 adenosine receptor.

EXPERIMENTAL PROCEDURES

Materials. Chloramine-T, cAMP, creatine phosphokinase, dATP, Heps, MgCl2, phosphocreatine, and Tris were obtained from Sigma. Adenosine deaminase, guanosine 5'-[β,γ-imido]triphosphate (p[NH]ppG), guanosine 5'-[γ-thio]triphosphate, R-PIA, and S-PIA were purchased from Boehringer Mannheim. NECA was a generous gift from Ray Olsson (University of South Florida). SANPAH and N-hydroxysuccinimidy 4-azidosalicylic acid (NHS-ASA) were

Abbreviations: CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; CPA, N,N'-cyclohexyladenosine; p[NH]ppG, guanosine 5'-[β,γ-imido]triphosphate; NECA, 5'-N-ethylcarboxamido-adenosine; NHS-ASA, N-hydroxysuccinimidy 4-azidosalicylic acid; PAPA-APEC, 2-[4-(2-[4-aminophenyl)methylcarbonylaminooethyl]amino-5'-N-ethylcarboxamido-adenosine; R-PIA, (--)N1-[R]-1-methyl-2-phenylethyl]adenosine; S-PIA, (++)N1-[S]-1-methyl-2-phenylethyl]adenosine; SANPAH, N-succinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate.
purchased from Pierce. [α-32P]ATP was purchased from DuPont-New England Nuclear, and Na32P (carrier free) was obtained from Amersham. All other reagents were of the highest available grade and were purchased from standard sources.

Preparation of Bovine Striatal Membranes. The striatal membrane preparation was fashioned after the rat striatal membrane preparation outlined by Bruns et al. (12). Briefly, the striatum was excised from fresh bovine brain, placed in 20 ml of ice-cold 50 mM Tris·HCl (pH 8.26 at 5°C), minced, and gently disrupted with a motor-drive Teflon pestle. This suspension was centrifuged at 43,000 × g for 10 min, resuspended in 20 ml of Tris buffer, and recentrifuged at 43,000 × g for an additional 10 min. The final pellet was then suspended in sufficient buffer to yield a final protein concentration of 200 mg of wet striatum per ml and stored at −70°C. Frozen membranes were found to remain stable for at least 1 month at this temperature.

Synthesis of 125I-PAPA-APEC. The detailed synthesis of PAPA-APEC will be reported elsewhere. Briefly, the parent compound PAPA-APEC was iodinated by a chloramine-T method (4, 13). The 125I-PAPA-APEC was completely separated from the starting materials by an isocratic HPLC protocol using a Waters C18 Bondapak column and a mobile phase composed of 60% (vol/vol) methanol and 40% (vol/vol) 20 mM ammonium formate. The purified radioligand was assumed to have a specific activity of 2200 Ci/mmol (1 Ci = 37 GBq).

Ligand Binding Studies. A 1-ml aliquot of frozen striatal membrane was thawed at room temperature and suspended in 9 ml of buffer containing 50 mM Hepes and 10 mM MgCl2 (hereafter referred to as 50/10 buffer), adenosine deaminase (0.2 unit/ml), and 50 nM CPA (final concentration), all adjusted to pH 7.2. Initial studies demonstrated that a low concentration of 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) in the incubation buffer dramatically reduced nonspecific binding. Consequently, all the binding and crosslinking studies were performed in the presence of 0.01% CHAPS.

Binding studies were performed in a 250-μl reaction volume consisting of 150 μl (75 μg of protein) of the above membrane suspension, 50 μl of diluted ligand, and 50 μl of either water or competing ligand. The ligand/membrane suspension was incubated at 37°C for 1 hr with continuous shaking. The mixture was then rapidly filtered over glass filters [Schleicher & Schuell, no. 32, treated for 1 hr with 0.3% polyethylenimine (PEI)] and washed three times with 3-ml aliquots of ice-cold 50/10 buffer containing 0.05% CHAPS.

The filters were placed in polypropylene tubes and radioactivity was measured in a Packard γ counter.

Adenylate Cyclase Activity. Fresh human platelet membranes were used in adenylate cyclase assays since only the A2 subtype of the adenosine receptor is expressed in this tissue. The platelet membranes were prepared and adenylate cyclase activity was assayed as outlined by Newman et al. (14) in a total reaction volume of 50 μl. Platelet membranes were suspended in solution of 75 mM Hepes (pH 7.4), 12.5 mM MgCl2, and 1 mM dithiothreitol, and the regeneration mixture consisted of 5 × 10−7 M GTP, 1 μCi of [α-32P]ATP, 25 units of creatine phosphokinase, 5 mM creatine phosphate, 10−4 M dATP, 10−6 M cAMP, and 10−4 M papaverine. The assay was begun by adding 20 μl of platelet membranes to 20 μl of regeneration mixture and 10 μl of water (basal activity) or the indicated ligand concentration (10−11 M to 10−5 M). The reaction mixture was then incubated at 30°C for 15 min (linear portion of activity curve) and the [32P]cAMP was separated by the method of Salomon et al. (15).

Photofunctional Crosslinking. A 1-ml aliquot of frozen striatal membranes was thawed and suspended in 9 ml of 50/10 buffer (pH 7.2) with adenosine deaminase (0.4 unit/ml). The suspension was incubated at 37°C for 15 min and centrifuged at 43,000 × g for 5 min, and the remaining pellet was suspended in 15 ml of 50/10 buffer containing 0.01% CHAPS and adenosine deaminase (0.2 unit/ml). A volume of 2 ml was used for labeling and consisted of 1.6 ml of the above membrane suspension (=500 μg of protein per/ml), 0.4 ml of water (control) or competing ligand, and a final concentration of 2 nM radioligand. After a 1-hr incubation at 37°C, the membranes were washed once with ice-cold 50/10 buffer containing 0.03% CHAPS, centrifuged at 43,000 × g for 5 min, washed again with 50/10 buffer containing 0.01% CHAPS and, after a final centrifugation, were resuspended in 2 ml of 50/10 buffer containing 0.01% CHAPS. This suspension was then allowed to react with the crosslinking agent SANPAH as described (2, 5). In brief, 25 μl of SANPAH (0.5 mg/ml of Me2SO) was added to the membrane/ligand suspension and allowed to react at room temperature for 5 min. At the end of this time the suspension was poured into a Petri dish (on ice) and UV-irradiated for 4 min at a distance of 1 cm. The photolyzed suspension was then washed once with 50/10 buffer containing 0.05% CHAPS and once with plain 50/10 buffer before being prepared for NaDodSO4/PAGE.

NaDodSO4/PAGE. Electrophoresis was performed according to the standard methods outlined by Laemmli (16) in homogeneous gels of 12% polyacrylamide gel. Samples were solubilized for 45 min in sample buffer containing 10% (wt/vol) NaDodSO4, 10% (vol/vol) glycerol, 25 mM Tris·HCl, and 5% (vol/vol) 2-mercaptoethanol, adjusted to pH 6.8 at 25°C. After electrophoresis, the gels were dried and exposed to Kodak XAR-5 film with dual intensifying screens for 48–72 hr.

Protein Determinations. The protein content of samples was determined by the method of Bradford (17) using bovine serum albumin as a standard.

Analysis of Data. Saturation binding, competitive binding, and adenylate cyclase activity dose–response curves were all analyzed by a nonlinear least squares technique that has been described and validated (18). All averages are expressed as mean ± SD and statistical significance was determined by a Student’s t test analysis where appropriate.

RESULTS AND DISCUSSION

Characterization of PAPA-APEC Binding. Saturation binding experiments with 125I-PAPA-APEC in bovine striatal
membranes demonstrate saturable high-affinity binding (Fig. 2A) with a dissociation constant \( K_d \) of 1.5 ± 0.5 nM (mean ± SD) and a receptor density \( B_{max} \) of 222 ± 95 fmol/mg of protein \((n = 4)\). The guanine nucleotides guanosine 5'-[β,γ-imido]triphosphate (p[NH]ppG) and guanosine 5'-[γ-thio]triphosphate consistently demonstrated an ≈10% decrease in specific binding at a concentration of \( 10^{-4} \) M. The agonist potency order observed in competitive binding experiments (Fig. 2B) is indicative of \( A_2 \) receptor pharmacology with NECA \((IC_{50} = 86 ± 41 \text{ nM})\) being more potent than R-PIA \((IC_{50} = 1350 ± 423 \text{ nM})\), which is more potent than S-PIA \((IC_{50} = 16,000 ± 4480 \text{ nM})\), which is more potent still than the antagonist theophylline \((IC_{50} = 31,600 ± 4240 \text{ nM})\).

All of these experiments were initially performed in the presence of 50 nM N6-cyclopentyladenosine (CPA), a highly \( A_1 \) selective ligand, to eliminate the possibility that PAPA-APEC might also bind to the \( A_1 \) receptor (12) since bovine striatum contains both \( A_1 \) and \( A_2 \) receptors. Studies that examined \( [\text{H}] \)NECA binding in striatal membranes suggested that \( A_2 \) binding can be “unmasked” in these preparations by performing the incubations in the presence of CPA (19). \(^{125}\)I-PAPA-APEC binding, however, yielded statistically identical \((P > 0.9)\) binding parameters both in the presence \((K_d = 1.5 ± 0.5 \text{ nM}; B_{max} = 222 ± 95 \text{ fmol/mg})\) and absence \((K_d = 1.6 ± 0.7 \text{ nM}; B_{max} = 203 ± 77 \text{ fmol/mg})\) of 50 nM CPA, indicating that \(^{125}\)I-PAPA-APEC is highly selective for the \( A_2 \) receptor.

This selectivity was confirmed by competitive binding studies in bovine striatal membranes that utilized PAPA-APEC as a competitor for the binding of a 0.5 nM concentration of \(^{3}\)H-labeled xanthine amine congener (11) (at this concentration \(^{3}\)H-labeled xanthine amine congener is an \( A_1 \)-selective radioligand). These experiments failed to demonstrate any competition for \( A_1 \) receptor binding at PAPA-APEC concentrations \(<1000 \text{ nM}\). This finding, combined with our observed \( K_d \) of 1.5 nM for \(^{125}\)I-PAPA-APEC binding to the \( A_2 \) receptor indicates that PAPA-APEC is at least 500-fold selective for the \( A_2 \) receptor.

Previous studies of various adenosine analogues (20) and the observed effects of guanine nucleotides suggest that PAPA-APEC (which is a substituted adenosine analogue) should be an \( A_2 \) receptor agonist and stimulate cAMP production. Platelet membranes were employed to assess cAMP generation since adenylate cyclase is more responsive in this tissue than in striatal membranes. Although agonists are known to be less potent at the platelet \( A_2 \) adenosine receptor than the striatal \( A_2 \) adenosine receptor (21–23), the lack of inhibitory platelet \( A_1 \) receptors more than compensated for the decrease in platelet \( A_2 \) receptor affinity and led us to use platelet membranes in our cyclase assays. These assays demonstrated the expected \( A_2 \) receptor agonist-mediated stimulation of cAMP production with an \( EC_{50} \) of \( \sim 150 \text{ nM} \) \((n = 3)\). PAPA-APEC exhibited a 43% increase above basal activity \((28.3 \text{ pmol per mg of protein per min})\) at \( 10^{-6} \) M whereas the same concentration of NECA resulted in a 35% increase over basal cAMP activity. The apparent discrepancy between the \( K_d \) for binding in brain tissue and the \( EC_{50} \) for cyclase stimulation in platelet membranes can be attributed to tissue differences and is consistent with the differences seen by other investigators studying these same systems (21).

Although \(^{125}\)I-PAPA-APEC binding clearly exhibits the expected \( A_2 \) receptor pharmacology, we wished to exclude the possibility that we could be binding to the adenosine (nucleoside) transporter protein. We addressed this question by conducting competitive binding experiments with \((S)-(4-nitrobenzyl)-6-thioinosine (NBMPR), which is a potent inhibitor of adenosine transporter binding. \((S)-(4-nitrobenzyl)-6-thioinosine has been reported to have an inhibition constant \((K_i)\) for the transporter protein in the 1–10 nM range for a wide variety of mammalian tissues (24). Computer analysis of competitive binding curves using \(^{125}\)I-PAPA-APEC in bovine striatal membranes indicated that \((S)-(4-nitrobenzyl)-6-thioinosine had an \( IC_{50} \) of 27,200 nM, which makes it very unlikely that there is binding to the adenosine transporter protein in these studies.

Photoaffinity Crosslinking of the \( A_2 \) Binding Subunit. The presence of both \( A_1 \) and \( A_2 \) receptor subtypes in bovine striatal membranes allowed a direct comparison between the \( A_2 \) and \( A_2 \) adenosine receptor binding subunits. In these membranes, photoaffinity crosslinking of \(^{125}\)I-labeled 8-[4-[2-(4-aminophenyl)acetamido]ethyl]carbonylmethylxoyphenyl]-1,3-dipropylxanthine (PAPAXAC) (an \( A_1 \)-selective photoaffinity crosslinking antagonist) with SANPAH identifies the 38-kDa \( A_1 \) receptor binding subunit (4) (Fig. 3A, lane 1) and several nonspecifically labeled proteins. This band is distinct from the 45-kDa band seen in lanes 2–4 (Fig. 3A) that shows the photoaffinity crosslinking of \(^{125}\)I-PAPA-APEC with SANPAH. The 45-kDa band exhibits the appropriate \( A_2 \)
pharmacology with 1 μM NECA (lane 3) being more potent than 1 μM R-PIA (lane 4) in inhibiting labeling. A less-prominent band at 55 kDa seen in lanes 2–4 was not consistently seen. Although this band is decreased in lane 3 (Fig. 3A), the magnitude of this decrease in intensity corresponds most closely to the decrease in background activity and not the presence of the competing ligand. This fact is more obvious in Fig. 3B where the 55-kDa band is clearly present in all lanes and supports our belief that the 55-kDa band is a nonspecifically labeled protein.

The possibility that proteolytic degradation of a higher molecular weight polypeptide resulted in the observed specific 45-kDa band led us to prepare fresh striatal membranes in the presence of a combination of proteinase inhibitors [10⁻⁴ M phenylmethylsulfonyl fluoride/5 mM EDTA/10⁻⁴ M benzamidine/soybean trypsin inhibitor (5 μg/ml)] shown to inhibit proteolysis in other receptor systems (25). The full pharmacology of the ¹²⁵I-PAPA-APEC-crosslinked receptor in membranes prepared in the presence of these proteinase inhibitors is shown in Fig. 3B and is identical to the results obtained when untreated membranes are labeled. Fig. 3B is typical of five experiments and demonstrates that the radio-labeled 45-kDa band exhibits all the appropriate A₂ receptor pharmacology. By using 5 mM theophylline to define nonspecific binding and by excising and measuring radioactivity in the section of the polyacrylamide gel that corresponds to the 45-kDa band, we found an average 47% decrease in labeling with 1 μM NECA, a 36% decrease with 1 μM R-PIA, and an 8% decrease with 1 μM S-PIA. Additionally, photoaffinity crosslinking was decreased ≈10% in lane 6 by the presence of 10⁻⁴ M p[NH]ppG. These findings and the clear presence of the 55-kDa band in all lanes of Fig. 3B suggest that the 45-kDa band is the A₂ adenosine receptor binding subunit and that this band is not the result of the proteolytic digestion of a higher molecular weight protein.

The high degree of A₂ selectivity noted in the saturation binding experiments was also confirmed by photoaffinity crosslinking. An identical distribution and pharmacology of labeled protein was observed in the bovine striatal membranes that were photoaffinity crosslinked in the presence of 50 nM CPA (data not shown). Furthermore, a 38-kDa band (the A₁ receptor binding subunit) was never observed in any of the ¹²⁵I-PAPA-APEC-labeled lanes, which provides additional evidence of the A₂ adenosine receptor selectivity of this ligand.

Although the results are straightforward, it is important to realize that several limitations are inherent in all photoaffinity crosslinking experiments. These limitations have been reviewed elsewhere (26) and include: (i) the labeling of nonreceptor proteins, (ii) the low efficiency of photoincorporation, and (iii) the possibility of “exolabeling” adjacent proteins yielding bands that display the correct pharmacology but provide an erroneous estimate of the apparent molecular mass. Each of these potential problems has been addressed.

The labeling of nonreceptor nonspecific proteins does occur to a small degree in this system and is seen most prominently as a 55-kDa band in Fig. 3. Fortunately, the degree of nonreceptor labeling and molecular mass of the band does not overlap the regions of interest and, thus, has no effect on our results.

The efficiency of photoincorporation was determined by excising and counting the radioactivity in the appropriate region of the gel that corresponds to the 45-kDa bands in Fig. 3A, lanes 2 and 3. Measuring radioactivity in those regions demonstrated that from 2.2% to 2.7% of the specifically bound ligand was incorporated into that band. These results are in excellent agreement with the results seen utilizing SANPAH as the crosslinker with other radioligands (2, 5).

The possibility of “exolabeling” adjacent nonreceptor proteins as a consequence of the long carbon chain that joins the two functional ends of the SANPAH molecule was addressed by repeating the photoaffinity crosslinking with a different crosslinking agent, NHS-ASA. This agent contains the same two functional groups as the SANPAH molecule but
has an intramolecular chain that is five carbons and one nitrogen shorter than SANPAH and, thus, should decrease the extent of exolabeling if any was present. The NHS-ASA crosslinking experiments (results not shown) demonstrated the same 45-kDa specifically labeled protein (at a much lower efficiency of incorporation) seen in the SANPAH experiments and suggest that exolabeling is not a factor in this photoaffinity labeling system. Furthermore, crosslinking with NHS-ASA failed to show the 55-kDa band and provides further evidence that the 55-kDa band represents a nonspecifically labeled protein.

CONCLUSIONS

This study has demonstrated that the agonist radioligand \(^{125}\text{I}\)-PAPA-APEC binds specifically, selectively, and with high affinity to a protein that exhibits all the appropriate \(A_2\) adenosine receptor pharmacology. Furthermore, this radioligand can be covalently photoincorporated into the \(A_2\) receptor binding subunit with the crosslinking agent SANPAH, thus labeling a single specific 45-kDa protein as shown by NaDodSO\(_4\)/PAGE that also exhibits the appropriate \(A_2\) pharmacology. We believe that these photoaffinity crosslinking experiments demonstrate the labeling of the \(A_2\) receptor binding subunit and that \(^{125}\text{I}\)-PAPA-APEC provides a powerful tool for further studies of the \(A_2\) adenosine receptor.

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