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Interaction of Purified Bovine Brain A_1-Adenosine Receptors with Guanine Nucleotide-Binding Proteins of Human Platelet Membranes following Reconstitution

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SUMMARY

A_1-adenosine receptors and associated guanine nucleotide-binding proteins (G proteins) have been co-purified from bovine cerebral cortex by agonist affinity chromatography [*J. Biol. Chem.* **264**:14853–14859 (1989)]. In this study we have reconstituted purified bovine brain A_1 receptors into human platelet membranes that contain A_2- but no detectable A_1-adenosine receptors. The recovery of reconstituted receptors was assessed from the binding of the antagonist radioligand [^{125}I]3-(4-amino-3-iodo)phenethyl-1-propyl-8-cyclopentyl-xanthine and ranged from 32 to 84%. Coupling of reconstituted A_1 receptors to platelet G proteins was evaluated by measurement of the high affinity binding of an agonist radioligand, ^{125}I-aminobenzyladenosine, to receptor-G protein complexes and by stereospecific photoaffinity labeling of a 35,000-Da receptor polypeptide with the agonist photoaffinity label ^{125}I-azidobenzyladenosine. Fifty percent of receptors reconstituted into platelet membranes bound agonists with high affinity, indicative of coupling to platelet G proteins. Reconstituted A_1 receptors bound various ligands with affinities characteristic of A_1 receptors of bovine brain. Although platelets contain both pertussis toxin-sensitive and -insensitive G proteins, reconstituted high affinity agonist binding was almost completely abolished by treatment of platelet membranes with guanosine 5′-3-*O*-(thio)triphosphate, pertussis toxin, *N*-ethylmaleimide, or heparin. Following reconstitution, A_1 receptors could be resolubilized in complexes with platelet G proteins. The data suggest that marked species differences in the binding affinity of ligands to adenosine receptors result from differences in the receptors rather than membrane structure or G proteins and, further, that A_1 receptors couple selectively and tightly to pertussis toxin-sensitive G proteins.

Adenosine receptors are involved in the control of a number of physiologic processes (reviewed in Ref. 1). Activation of adenosine receptors reduces cardiac contractility, platelet aggregation, lipolysis, insulin and prolactin secretion, and neuronal activity. These responses are mediated through at least two types of well defined adenosine receptors, which have different structure-activity profiles. A_1 receptors, which are abundant in brain and fat, selectively bind N^6-substituted adenosine analogs (2) and 8-cyclopentylxanthines (3, 4). Agonist and antagonist radioligands with high selectivity for A_1 receptors have been described. These include ^{125}I-labeled radioligands such as the agonist ^{125}I-ABA (5), the antagonist ^{125}I-BW-A844U (4), and the agonist photoaffinity label ^{125}I-azidobenzyladenosine (6, 7). Because radioligands with selectivity for A_1 receptors do not bind to platelet membranes (8) and because submicromolar concentrations of N^6-substituted adenosine analogs do not influence platelet function (9), it appears that platelets do not contain A_1 receptors. Platelets do contain A_2 receptors, which are coupled to stimulation of platelet adenylate cyclase and inhibition of platelet aggregation (10, 11).

A_1-adenosine receptors appear to couple to multiple G proteins, because in various tissues activation of these receptors has been found to produce several different effects, including inhibition of adenylate cyclase (1), activation or inhibition of phospholipase C (Refs. 12 and 13 and references therein), activation of potassium channels (14), and inactivation of Ca^{2+} channels (15). A common feature of all of these G protein-mediated responses is their sensitivity to inactivation by *Bordetella pertussis* toxin (13, 16–18) and NEM (19, 20). It is not known whether the toxin sensitivity of A_1-adenosine receptors occurs because these receptors interact selectively with pertussis toxin-sensitive G proteins. Thus, one goal of this study was

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ABBREVIATIONS: ^{125}I-ABA, [^{125}I]N^6(3-iodo,4-amino)benzyladenosine; G proteins, guanine nucleotide-binding proteins; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CHAPS,3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; ^{125}I-BW-A844U, [^{125}I]3-(4-amino,3-iodo)phenethy-8-cylopentylxanthine; PIA, N^6-phenylisopropyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; 8-PST, 8-*p*-sulfophenyltheophylline; GTPγS, guanosine-5′-*O*-(3-thil)triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

to determine whether reconstituted A_1 receptors also interact with pertussis toxin-insensitive G proteins. An interesting property of A_1-adenosine receptors is that they can be solubilized as complexes with G proteins (Ref. 19 and references therein). A second goal of this study was to determine whether such stable complexes would form between A_1-adenosine receptors and foreign G proteins. The approach used was to solubilize reconstituted receptors and examine the detergent extract for the presence of receptor-G protein complexes.

A_1 receptors of various species differ markedly in their affinities for ligands. In general, bovine receptors bind both agonist and antagonist ligands with much higher affinities than receptors of other species (4, 21, 22). Such differences could be the result of variations in the structure of receptors themselves or they could result from species differences in membrane structure or G proteins. A third goal of this study was to determine whether bovine receptors retain their very high affinity for ligands following reconstitution into human membranes.

Experimental Procedures

Chemicals. Carrier-free $Na^{125}I$ (2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA); 8-PST from RBI (Natick, MA); CHAPS, NEM, HEPES, Tris, GTP, theophylline, adenosine deaminase, DTT, polyethylene glycol, phenylmethylsulfonyl fluoride and leupeptin from Sigma Chemical Co. (St. Louis, MO); (*R*)-PIA, (*S*)-PIA, and GTPγS from Boehringer Mannheim, (West Germany); asolectin from Associated Concentrates (Long Island, NY); and heparin from Elkins-Sinn Inc. (Cherry Hill, NJ). The syntheses of ABA, BW-A844U, ^{125}I-ABA, ^{125}I-BW-A844U, and ^{125}I-azidobenzyladenosine has been described (4, 7, 19).

Preparation of platelet membranes. Platelet membranes used in reconstitution experiments were prepared from outdated platelets obtained from the University of Virginia blood bank. The partially purified platelets were centrifuged at 200 × *g* for 10 min to remove a small number of contaminating red blood cells. Platelets in the supernatant were pelleted and washed twice by centrifugation at 18,000 × *g* for 10 min in buffer composed of 50 mM Tris·HCl, pH 7.5, 20 mM EGTA, and 150 mM NaCl. The final pellet was resuspended in lysis buffer (5 mM Tris·HCl, pH 7.5, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin) and frozen at −20° overnight. After thawing, the platelets were homogenized (Dounce, 10 passes) and centrifuged at 70,000 × *g* for 1 hr. The pellet was resuspended in 5 mM HEPES (pH 7.5), 10% w/v sucrose, and 1 mM DTT, and stored in small aliquots at −70°.

Preparation of A_1-adenosine receptors. Receptors were solubilized from bovine cerebral cortical membranes using CHAPS and purified by affinity chromatography over aminobenzyladenosine-AG-202 affinity columns, essentially as described (19). The B_{max} for binding of ^{125}I-BW-A844U to purified receptors used for reconstitution ranged between 167 and 446 pmol/mg of protein (371–991-fold enriched over crude soluble receptors). Receptors were eluted with 0.1–0.2 mM NEM and stored frozen at −20° in 10 mM HEPES (pH 7.4), 1 mM EDTA, 0.1 mM benzamidine (buffer A), supplemented with 100 mM NaCl, 10% glycerol, 0.1–0.2% CHAPS, and 0.01% soybean phospholipids (asolectin). The use of NEM to elute receptors completely and irreversibly inactivated G protein α-subunits, which co-purified with receptors (19).

Reconstitution of receptors into platelet membranes. Frozen purified receptors were thawed and 1 mM DTT was added to quench residual NEM. Reconstitution of these receptors into platelet membranes was typically performed as follows: purified receptors in buffer B (buffer A plus 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT) were incubated with 1–3.3 mg/ml platelet membranes in a total volume of 1–2.4 ml. An equal volume of a polyethylene glycol 8,000 solution in buffer B was added such that the final polyethylene glycol concentration was 12%. After incubation at 21° for 10 min, the membranes were diluted to 25 ml in ice-cold buffer B and incubated for 20–30 min before being centrifuged at 70,000 × *g* for 1 hr. The pellets were resuspended in buffer A. In control experiments, platelet membranes were subjected to the reconstitution protocol in the absence of receptors.

ADP-ribosylation of platelet membranes. Following reconstitution, platelet membranes were ADP-ribosylated with pertussis toxin in order to inactivate susceptible platelet G proteins. Forty micrograms of platelet membranes in buffer A containing 230 fmol of reconstituted receptors (^{125}I-BW-A844U binding sites) were incubated at 37° with various concentrations (2.5–100 μg/ml) of pertussis toxin in the presence of 25 mM Tris·HCl, pH 8.0, 10 mM thymidine, 10 mM DTT, 1 mM ATP, 0.1 mM GTP, 2.5 mM $MgCl_2$, and 1 mM NAD, in a final volume of 400 μl. The reactions were terminated after 30 min by dilution to 25 ml with buffer A, followed by centrifugation at 70,000 × *g* for 1 hr to pellet the membranes. The pellets were resuspended in buffer A for use in binding assays (see below).

Pretreatment of platelet membranes with NEM. In some experiments, platelet membranes (2 mg/ml) were treated with NEM before reconstitution. Membranes were washed once by centrifugation at 70,000 × *g* for 1 hr in buffer A to remove traces of DTT and resuspended in the same buffer. The washed membranes were treated with various concentrations (20–200 μM) of NEM at 37° for 20 min, diluted 2-fold in buffer A containing 2 mM DTT (to quench NEM), pelleted by centrifugation at 16,000 × *g* for 1 hr, washed once, and resuspended in buffer A. One-milliliter aliquots of these platelet membranes (1 mg of protein) were each incubated with 210 or 300 fmol (in two different assays) of receptors (^{125}I-BW-A844U binding sites) for reconstitution, as described above.

Photoaffinity labeling. Reconstituted receptors were further characterized by photoaffinity labeling with the A_1 receptor agonist ^{125}I-azidobenzyladenosine (6, 7). Samples for photolabeling contained 240 fmol of reconstituted receptors (^{125}I-BW-A844U binding sites) in 0.5 mg of platelet membranes, 2.5 units of adenosine deaminase, 5 mM $MgCl_2$, 10^6 cpm of ^{125}I-azidobenzyladenosine, and either no competing ligand or (*R*)-PIA (0.5 μM), (*S*)-PIA (0.5 μM), or 8-PST (100 μM) and were incubated in a final volume of 0.5 ml at 21° for 2 hr. Identical incubations were made with platelet membranes that were subjected to the conditions of reconstitution in the absence of receptors. At the end

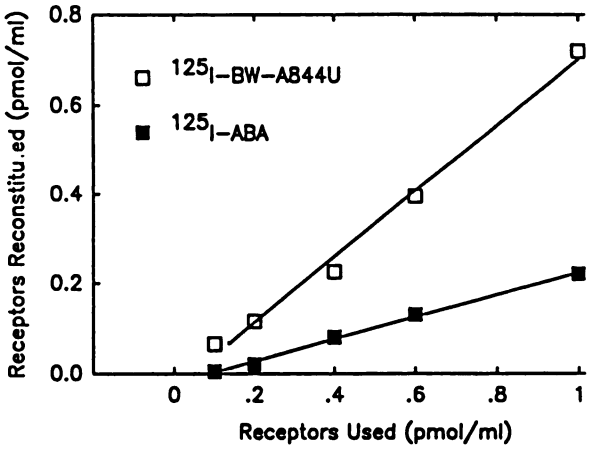

Fig. 1. Reconstitution of A_1-adenosine receptors into platelet membranes. Various amounts of purified receptors (0.1–1.0 pmol of ^{125}I-BW-A844U binding sites) were incubated with 2 mg of platelet membranes for reconstitution, as described in Experimental Procedures. Reconstituted receptors were detected with ^{125}I-BW-A844U (0.33 nM) and with ^{125}I-ABA (0.43 nM). The latter radioligand will selectively bind high affinity agonist binding sites. Each point represents the mean of triplicate determinations.

[1] R. Munshi and J. Linden. Unpublished data.

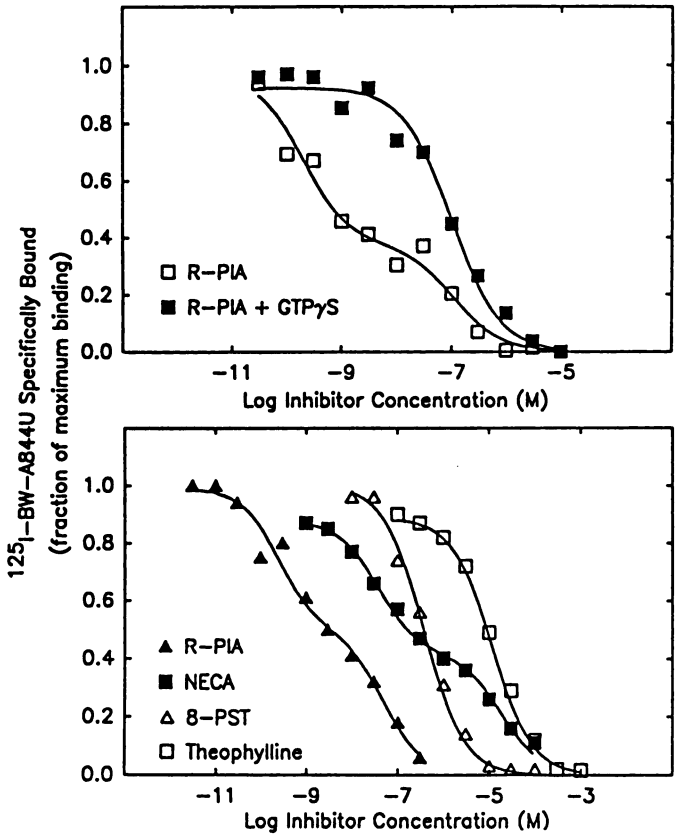

Fig. 2. Competition by various ligands with ^{125}I-BW-A844U for binding to reconstituted A_1 receptors. A, ^{125}I-BW-A844U (0.21–0.45 nM) binding to reconstituted receptors (20–35 fmol) was determined in the presence of various concentrations of (*R*)-PIA or (*R*)-PIA plus 100 μM GTPγS, in a final volume of 100 μl. Each point represents the mean of triplicate determinations. The competition curves were optimally fit to a two-site [(*R*)-PIA] or a single site [(*R*)-PIA plus GTPγS] binding model. K_i determinations (means ± standard errors) of three different experiments are as follows: K_H = 0.15 ± 0.046 nM, K_L = 35 ± 3 nM, and f_h = 0.52 ± 0.06 [(R)-PIA]; K_i = 23 ± 5 nM [(*R*)-PIA plus GTPγS]. B, Platelet membranes containing 20–30 fmol of reconstituted receptors were incubated with 0.22–0.4 nM ^{125}I-BW-A844U and various concentrations of competing ligands, in a final volume of 100 μl. Each point represents the mean of triplicate determinations. (*R*)-PIA and NECA competition curves were optimally fit to a two-site binding model and 8-PST and theophylline to a single-site model. Binding parameters are summarized in Table 1.

of 2 hr, photolabeled receptors were transferred into 50 ml of buffer A containing 5 mM $MgCl_2$ and centrifuged at 70,000 × *g* for 1 hr. The pellets were resuspended in 1 ml of buffer A containing 5 mM $MgCl_2$ and the membrane suspensions were exposed to UV light for 15 min (7) and then centrifuged at 16,000 × *g* for 1 hr. The pellets were dissolved in Laemmli's buffer (23) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 1-mm thick 10% polyacrylamide gels. The gels were stained with Coomasie blue stain and dried, and the radioactive polypeptides were visualized by autoradiography, using Kodak XK-1 film in DuPont X-ray cassettes with Cronex intensifying screens at −70°. All steps starting from incubation of the photolabel until photolysis were carried out in dim light.

Solubilization of reconstituted receptors from platelet membranes. In order to determine whether A_1-adenosine receptors couple tightly to platelet G proteins, platelet membranes containing the reconstituted receptors were solubilized in the detergent CHAPS by a method similar to that described for solubilization of the bovine brain receptor-G protein complexes (19). Platelet membranes (12.2 mg) containing 18.3 pmol of reconstituted high affinity ^{125}I-ABA binding sites or the same amount of platelet membrane protein subjected to the conditions of reconstitution without receptors were pelleted at 70,000

TABLE 1

Comparison of inhibitory constants of (*R*)-PIA, NECA, 8-PST, and theophylline in competition with ^{125}I-BW-A844U for binding to A_1-adenosine receptors of bovine brain membranes, soluble receptors, and reconstituted receptors

K_H and K_l represent dissociation constants at agonist high and low affinity binding sites, respectively, and f_H is the fraction of receptors in high affinity state.

Inhibitor	Membrane receptors[a]	Soluble receptors[b]	Reconstituted receptors
Agonists			
(*R*)-PIA			
K_H (nM)	0.074 ± 0.01	0.37 ± 0.03	0.17 ± 0.005
K_L (nM)	3.8 ± 0.8	69.7 ± 11.4	28.6 ± 1.6
f_H	0.62 ± 0.03	0.52 ± 0.04	0.52 ± 0.02
NECA			
K_H (nM)	0031 ± 12	400 ± 215	19.2 ± 7.2
K_L (nM)	3,220 ± 740	9,400 ± 3,800	9,700 ± 240
f_H	0.58 ± 0.01	0.40 ± 0.1	0.46 ± 0.02
Antagonists			
8-PST, K_i (nM)	1,300	510 ± 130	240 ± 44
Theophylline, K_i (nM)		18,900 ± 6,200	8,200 ± 1,400

[a] Unpublished data and data from Ref. 4.
[b] Data from Ref. 19.

× *g* for 1 hr, resuspended in 1.8 ml of buffer A containing 2 mM $MgCl_2$, 1 mM DTT, and 1% CHAPS (a detergent to protein ratio of 1.5:1), and kept on ice for 20 min before ultracentrifugation at 100,000 × *g* for 90 min. The supernatants containing the soluble membranes were frozen at −20° before use in radioligand binding assays.

Radioligand binding assays. Binding incubations in buffer A containing 2.5 units of adenosine deaminase, 50,000–160,000 cpm of radioligand, and either 5 mM $MgCl_2$ (^{125}I-ABA) or 500 mM NaCl (^{125}I-BW-A844U), in a final volume of 100 μl, were terminated at the end of 2 hr at 21° by filtration over Whatman GF/C glass fiber filters (reconstituted receptors) or G/B filters pretreated with 0.3% polyethyleneimine (purified soluble receptors) (24), using a modified cell harvester (Brandel). Nonspecific binding was measured in the presence of 10 μM (*R*)-PIA. The dried filter papers were counted in a Beckman 5500 γ-counter at a counting efficiency of 70%.

Protein analyses. The protein content of samples or bovine serum albumin standards was measured using a sensitive fluorescence method (25). The protein content of purified receptors was determined by a modification of the above method, as described (19).

Data analyses. Binding parameters (K_D, B_{max}, and K_l) were fit by Marquardt's nonlinear least squares interpolation to equations for one or multiple binding sites, as described (4), and the appropriate number of affinity states was selected by use of *F* tests evaluated at $p < 0.001$. To calculate binding parameters, the concentrations of radioligands and competing ligands were corrected to account for their fractional binding to receptors.

Results

Reconstitution of A_1-adenosine receptors into platelet membranes. It was observed in preliminary experiments that when purified A_1-adenosine receptors were reconstituted into platelet membranes, specific binding of the A_1-selective antagonist ^{125}I-BW-A844U could be detected. No specific binding was detected when receptors were excluded during reconstitution. We examined the effect of addition of increasing amounts of purified receptors on the amount of receptors reconstituted into platelet membranes. When various concentrations of receptors ranging between 0.1 and 1.0 pmol of ^{125}I-BW-A844U binding sites were used for reconstitution, each into 2 mg of platelet membranes, the amount of ^{125}I-BW-A844U binding recovered increased linearly with the amount of receptors added

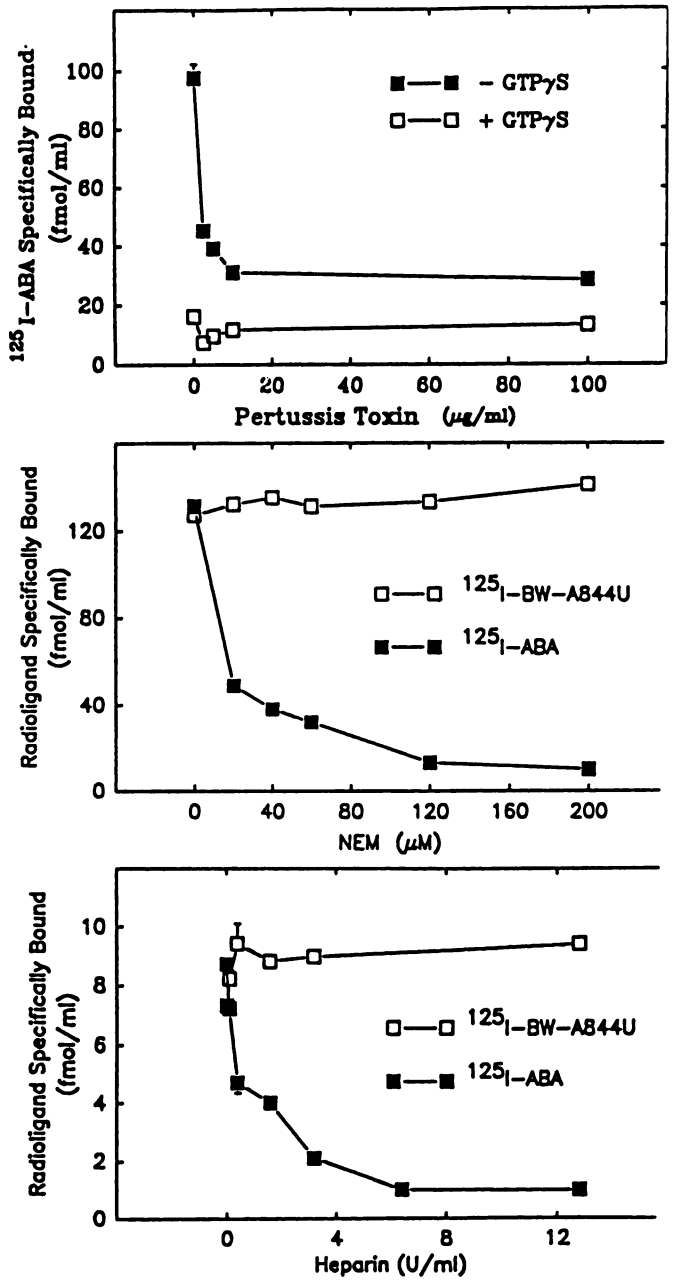

Fig. 3. Effect of pertussis toxin-catalyzed ADP-ribosylation, NEM, and heparin on radioligand binding to reconstituted A_1 receptors. A, Aliquots of reconstituted receptors were treated with various concentrations of pertussis toxin (as detailed in Experimental Procedures) before use in binding assays with ^{125}I-ABA (0.3 nM), in the presence or absence of 10 μM GTPγS. The concentration of pertussis toxin required to produce half-maximal inhibition of binding was <2.5 μg/ml in two different assays. B, Platelet membranes were treated with various concentrations of NEM, following which each membrane fraction was incubated with the same amount of receptor (210 fmol) for reconstitution, as described in Experimental Procedures. The concentration of NEM required to produce half-maximal inhibition of ^{125}I-ABA (0.28 nM) binding was <20 μM in two different assays. The concentration of ^{125}I-BW-A844U used was 0.22 nM. C, Reconstituted receptors were incubated with ^{125}I-ABA (0.14 nM) or ^{125}I-BW-A844U (0.34 nM) in the presence of various concentrations of heparin as shown. The concentration of heparin that produced half-maximal inhibition of ^{125}I-ABA binding was 1 unit/ml in two different assays. All points represent means of duplicate determinations with standard errors either as shown or smaller than the size of the symbol.

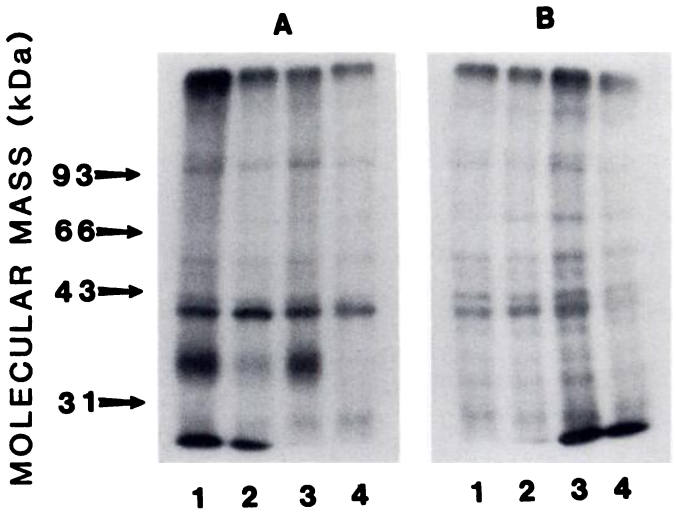

Fig. 4. Photoaffinity labeling of A_1-adenosine receptors following their reconstitution into platelet membranes. ^{125}I-Azidobenzyladenosine (0.56 nM) was incubated either with reconstituted receptors (A) or with platelet membranes that were subjected to the conditions of reconstitution in the absence of receptors (B), as described in Experimental Procedures. In each case separate incubations were made either without (*lane 1*) or with 0.5 μM (*R*)-PIA (*lane 2*), 0.5 μM (*S*)-PIA (*lane 3*), or 100 μM 8-PST (*lane 4*).

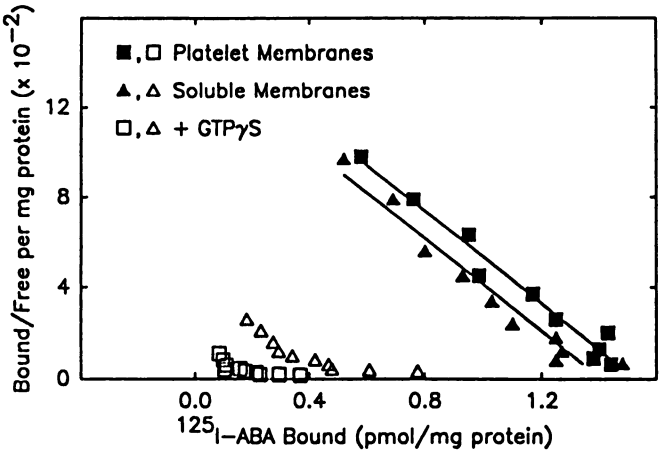

Fig. 5. Co-solubilization of reconstituted A_1-adenosine receptors and platelet G proteins. Scatchard plots of high affinity ^{125}I-ABA binding to reconstituted A_1 receptors and CHAPS-solubilized reconstituted A_1 receptor-G protein complexes are shown. The solubilization protocol is described in Experimental Procedures. Reconstituted receptors were diluted before binding assays such that the final protein concentration was 68 μg/ml (platelet membranes) and 96 μg/ml (soluble membranes). Thirty-three percent of receptor-G protein complexes measured with the agonist ^{125}I-ABA were solubilized in the high affinity state. The B_{max} values of reconstituted receptors before and after solubilization were 1.5 ± 0.03 and 1.4 ± 0.04 pmol/mg of protein, respectively. Corresponding K_D values were 0.097 ± 0.008 and 0.1 ± 0.01 nM, respectively. Addition of GTPγS greatly reduced high affinity binding to both populations of receptors.

(Fig. 1). The recovery of receptors in the membranes ranged between 60 and 77%. These data provided tentative evidence of reconstitution of purified A_1 receptors into platelet membranes. However, these data do not prove that the receptors were reconstituted into platelet membranes, because restoration of binding could be attributed to aggregation of receptors into vesicles that are segregated from platelet membranes. To prove that receptors had become incorporated into platelet membranes, we sought evidence of receptor coupling to platelet

G proteins. Antagonists do not discriminate between various affinity states of a receptor that are produced by the interaction of receptors with G proteins, whereas agonists bind with higher affinity to receptors coupled with G proteins (4). NEM-treated purified receptors did not bind the agonist ^{125}I-ABA with high affinity, i.e., little specific binding could be detected if concentrations of the radioligand below 1 nM were used. Reconstitution of purified receptors into platelet membranes restored some high affinity agonist binding. This is shown in Fig. 1 as a linear increase in the amount of high affinity ^{125}I-ABA (0.43 nM) binding to platelet membranes following reconstitution of increasing amounts of NEM-treated purified receptors. Based on these data, we concluded that at least some purified receptors coupled to native platelet G proteins following reconstitution.

Quantitation of receptor coupling to platelet G proteins. The fraction of receptors in the high affinity state can be determined by measurement of agonist competition for antagonist radioligand binding. Also, it is known that, in membranes, most A_1 receptors are converted to a low affinity state by the addition of guanine nucleotides (4, 7). All of the receptors reconstituted into platelet membranes were converted to a low affinity state by the addition of GTPγS (Fig. 2A). The fraction of receptors in the high affinity state was found to be about half when (*R*)-PIA or another agonist, NECA, were used in competition assays. On the other hand, the antagonists 8-PST and theophylline each bound with single affinities (Fig. 2B). A comparison of the dissociation constants and fraction of receptors in the high affinity state in bovine membranes, solubilized bovine receptors, and reconstituted receptors (Table 1) indicates that the properties of both agonist and antagonist binding sites are similar in these various preparations. The low affinity binding sites of (*R*)-PIA and NECA could not be attributed to A_2 receptors, because no specific binding of ^{125}I-BW-A844U could be detected to control platelet membranes (not shown). These data provide evidence of coupling of about 50% of reconstituted receptors to native platelet G proteins.

Uncoupling of reconstituted receptors from platelet G proteins by pertussis toxin, NEM, and heparin. The property of guanine nucleotides to uncouple A_1 receptors from G proteins is mimicked by ADP-ribosylation catalyzed by pertussis toxin (13, 17, 18). Treatment of platelet membranes with various concentrations of pertussis toxin in the presence of NAD caused a dose-dependent uncoupling of receptor-G protein complexes, detected by the loss of GTPγS-sensitive ^{125}I-ABA binding to reconstituted receptors (Fig. 3A). The half-maximal inhibition of agonist binding occurred at <2.5 μg/ml pertussis toxin and the maximum inhibition was achieved by 10 μg/ml. Even a 10-fold increase in the concentration of pertussis toxin to 100 μg/ml did not produce complete inhibition of ^{125}I-ABA binding, i.e., a minor component of GTPγS-sensitive reconstituted agonist binding was insensitive to pertussis toxin (Fig. 3A). It is possible that some pertussis-sensitive G proteins are in compartments that are not accessible to the toxin. These data indicate that most but perhaps not all A_1 receptors couple to pertussis toxin-sensitive platelet G proteins following reconstitution.

The functional coupling of pertussis toxin-sensitive G proteins to A_1-adenosine receptors is known to be inhibited as a result of alkylation of α-subunits by NEM, which irreversibly abolishes high affinity agonist binding (19, 20). We reasoned that, if platelet G proteins to which the A_1 receptors couple following reconstitution are sensitive to NEM, then inactivation of these G proteins by treatment of platelet membranes with NEM before reconstitution should prevent the formation of receptor-G protein complexes. This would result in reconstitution of antagonist but not the high affinity agonist binding sites. Fig. 3B shows that treatment with NEM had little effect on the binding of the antagonist ^{125}I-BW-A844U to reconstituted receptors but it produced a dose-dependent inhibition of high affinity binding of the agonist ^{125}I-ABA (Fig. 3B). The inhibitory potency of NEM is comparable to that reported for rat (7, 26) and bovine[1] brain membrane A_1-adenosine receptors. These data (i) support the suggestion that A_1-adenosine receptors couple to NEM-sensitive native platelet G proteins following reconstitution and (ii) are consistent with the suggestion that pertussis toxin-sensitive G proteins can also be inactivated by NEM (20).

As a third method of uncoupling reconstituted receptors from platelet G proteins we utilized heparin, which has been shown to uncouple platelet α_2-adrenergic receptors from G proteins (27). Heparin also appeared to uncouple reconstituted A_1-adenosine receptors from G proteins, because it abolished high affinity agonist binding without inhibiting antagonist binding (Fig. 3C).

Photoaffinity labeling of reconstituted receptors. The molecular mass of purified receptors did not change following their reconstitution into platelet membranes. This was evident from photoaffinity labeling studies in which ^{125}I-azidobenzyladenosine specifically labeled a single polypeptide of 35,000-Da molecular mass in platelet membranes following reconstitution (Fig. 4). The photolabeling was specific, because it was inhibited by the antagonist 8-PST and stereospecifically by the agonist PIA (Fig. 4). In the same assay, no specific photolabeling was observed in platelet membranes subjected to the conditions of reconstitution in the absence of receptors. The agonist photoaffinity label was used at a concentration (0.56 nM) that binds predominantly to high affinity sites (7). The fact that receptors were photoaffinity labeled under these conditions provides further evidence for the coupling of the receptors to platelet G proteins following reconstitution.

Solubilization of A_1-adenosine receptor-platelet G protein complexes in CHAPS following reconstitution. An interesting feature of A_1-adenosine receptors is their ability to remain coupled to G proteins following solubilization in detergents. Platelet membrane proteins were solubilized with the detergent CHAPS following reconstitution of receptors. Whereas the detergent extract from control platelet membranes (subjected to the conditions of reconstitution in the absence of receptors) did not reveal any GTPγS-sensitive ^{125}I-ABA binding sites, such binding sites were detected in detergent extracts of platelet membranes containing reconstituted receptors (Fig. 5). This suggests that, as in native bovine brain membranes. A_1 receptor coupling to G proteins of platelet membranes is stable to solubilization in CHAPS. Thirty-three percent of reconstituted high affinity ^{125}I-ABA binding sites were solubilized. The specific activities and affinities of reconstituted receptors for ^{125}I-ABA in membranes and in CHAPS extracts were similar. In both cases ^{125}I-ABA binding was similarly reduced by the addition of GTPγS (Fig. 5).

Discussion

A_1-adenosine receptors have been purified from bovine brain and reconstituted into human platelet membranes. Purified

receptors did not bind the agonist radioligand ^{125}I-ABA with high affinity, because receptors and G proteins were coeluted from an agonist affinity column in the presence of NEM, which functionally uncouples the two proteins. In similar protocols, restoration of the coupling of rat brain μ-opioid receptors uncoupled by NEM (28) of human leukemia cell fMet-Leu-Phe receptors uncoupled by pertussis toxin (29) following reconstitution with G proteins has been demonstrated.

Although this is the first report of reconstitution of adenosine receptors, such incorporation of a hydrophobic protein into plasma membranes is not an unexpected phenomenon. However, it is noteworthy that bovine brain A_1 receptors couple very well with foreign G proteins, so well in fact that the reconstituted receptor-G protein complexes can be resolubilized intact. Tight coupling of detergent-solubilized A_1 receptors and G proteins has been observed in bovine and rat brain (Ref. 19 and references therein) and porcine atria (30). A tight coupling of rat cardiac muscarinic (31), rat striatal D_1-dopaminergic (32), and rat liver vasoactive intestinal peptide receptors (33) to G proteins also has been reported.

Five lines of evidence suggest that A_1-adenosine receptors couple to native platelet G proteins following reconstitution. (i) High affinity ^{125}I-ABA binding to NEM-pretreated purified receptors was restored following reconstitution into platelet membranes. (ii) GTPγS, pertussis toxin, and heparin all inhibited restoration of the high affinity agonist binding. (iii) When reconstitution of these receptors was attempted into platelet membranes that had also been pretreated with NEM to inactivate platelet G proteins, there was no restoration of high affinity ^{125}I-ABA binding. (iv) ^{125}I-Azidobenzyladenosine, which specifically photolabels only receptor-G protein complexes (7), labeled a polypeptide with a molecular mass of 35,000 Da in platelet membranes following reconstitution. This is identical to the molecular mass of crude and purified bovine brain A_1-adenosine receptors (19). (v) Reconstituted receptors bound the agonists (*R*)-PIA and NECA with two affinity states in competition with ^{125}I-BW-A844U, and the potency order of binding [(*R*)-PIA > NECA] is characteristic of bovine brain A_1-adenosine receptors (19).

Solubilized receptors have lower affinity for ligands than receptors in native membranes or those reconstituted into platelet membranes. This is likely due to the organizational structure imposed by the membrane and/or structural contributions from specific lipids. The property of bovine A_1 receptors to bind both agonist and antagonist ligands with high affinity relative to other species is maintained following reconstitution into human platelet membranes. This suggests that the high affinity is due to the receptors *per se* rather than to the properties of membranes or G proteins with which they interact.

Because heparin abolished agonist but not the antagonist binding to reconstituted adenosine receptors, it would seem that the effect of heparin is to uncouple reconstituted A_1 receptors from G proteins, probably through a direct interaction with G proteins. A decrease in the affinity of adrenaline but not [^{3}H]rauwolscine (antagonist) binding to α_2-adrenergic receptors in human platelet membranes treated with heparin has been reported (27). Heparin also inhibited adrenaline-stimulated GTP hydrolysis and reversed the GTP- and adrenaline-mediated inhibition of forskolin-stimulated adenylate cyclase activity in platelet membranes (27). Heparin produced a slight increase in antagonist binding to reconstituted (Fig. 3C) and bovine brain membrane[1] A_1-adenosine receptors. An increase in antagonist radioligand binding to A_1 receptors of rat (7, 26, 34) and bovine[1] brain membranes in the presence of GTP or NEM has also been observed. Therefore, heparin may act like GTP or NEM to prevent interaction between hormone receptors and G proteins (27).

G_i has been implicated in the hormonal inhibition of adenylate cyclase (35). A_1-adenosine receptors and α_2-adrenergic receptors couple to adenylate cyclase through G_i, causing inhibition of the enzyme activity in bovine brain (36) and human platelet membranes (27, 37), respectively. It is, therefore, possible that the A_1-adenosine receptor-mediated inhibition of platelet adenylate cyclase would occur following reconstitution of the receptors. Under conditions where epinephrine (10 μM) inhibited forskolin-stimulated adenylate cyclase in platelet membranes reconstituted with purified A_1 receptors by 35–40%, we have been unable to consistently demonstrate a significant inhibition of platelet adenylate cyclase in response to A_1 receptor agonists (not shown). As in this study with A_1-adenosine receptors, reconstitution of turkey erythrocyte β-adrenergic receptors into human erythrocyte membranes restored guanine nucleotide-sensitive agonist binding but failed to reconstitute receptor-mediated regulation of adenylate cyclase (38). It is possible that reconstituted receptors couple selectively to G proteins that have poor accessibility to the catalytic subunit of adenylate cyclase. NEM does not appear to affect the properties of these receptors, inasmuch as their radioligand binding characteristics are similar before and after reconstitution. However, NEM could modify the structure of the receptor in such a way that the receptor binds to but fails to activate G proteins. Also, reconstitution *per se* may disturb the ability of receptors to interact with adenylate cyclase.

All of the actions of adenosine that are mediated by A_1 receptors are inhibited in tissues and membranes pretreated with pertussis toxin (13, 17, 18). Thus, current evidence suggests that A_1 receptors couple selectively to pertussis toxin- or NEM-sensitive G proteins. The ability to co-purify A_1-adenosine receptors and G proteins over agonist affinity columns (19) provides a useful means of determining which G proteins interact with the receptor. The fact that all G proteins that co-purify with the receptor are inactivated by pertussis toxin or NEM supports the notion that toxin- or NEM-sensitive G proteins are preferentially capable of interacting with the A_1 receptor. However, it is possible that A_1 receptors interact with other G proteins that do not form stable high affinity receptor-G protein complexes following solubilization in detergent. Such G proteins would not adhere to an agonist affinity column that selectively binds receptor-G protein complexes and, hence, would not be identified as interacting with A_1 receptors. The results of this study provide additional evidence that A_1 receptors interact selectively with pertussis toxin- and NEM-sensitive G proteins.

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