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# Partial Separation of Platelet and Placental Adenosine Receptors from Adenosine $A_2$-Like Binding Protein

S. ZOLNIEROWICZ,[1] C. WORK, K. HUTCHISON, and I. H. FOX

*Human Purine Research Center, Departments of Internal Medicine and Biological Chemistry, Clinical Research Center, University Hospital, Ann Arbor, Michigan 48109-0108*

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## SUMMARY

The ubiquitous adenosine $A_2$-like binding protein obscures the binding properties of adenosine receptors assayed with 5′-*N*-[$^3$H]ethylcarboxamidoadenosine ([$^3$H]NECA). To solve this problem, we developed a rapid and simple method to separate adenosine receptors from the adenosine $A_2$-like binding protein. Human platelet and placental membranes were solubilized with 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. The soluble platelet extract was precipitated with polyethylene glycol and the fraction enriched in adenosine receptors was isolated from the precipitate by differential centrifugation. The adenosine $A_2$-like binding protein was removed from the soluble placental extract with hydroxylapatite and adenosine receptors were precipitated with polyethylene glycol. The specificity of the [$^3$H]NECA binding is typical of an adenosine $A_2$ receptor for platelets and an adenosine $A_1$ receptor for placenta. This method leads to enrichment of adenosine $A_2$ receptors for platelets and adenosine $A_1$ receptors for placenta. This provides a useful preparation technique for pharmacologic studies of adenosine receptors.

Adenosine is a biologically active purine nucleoside that binds to cell surface receptors (1). These extracellular adenosine receptors are classified as $A_1$ and $A_2$ on the basis of inhibition or stimulation of adenylate cyclase, respectively (2, 3). Signal transduction by adenosine receptors may also occur by potassium channels (4) and guanylate cyclase activation (5).

Radiolabeled agonists and antagonists pharmacologically define adenosine $A_1$ receptors, whereas no satisfactory adenosine $A_2$ receptor-specific radioligand is available (6). [$^3$H]NECA binds to the adenosine $A_2$ receptor, the adenosine $A_1$ receptor, and an adenosine $A_2$-like binding protein of unknown function (7–9). The coexistence of more than one adenosine binding site has been suggested by studies of human platelets, human placenta, rat pheochromocytoma PC-12 cells, rat liver, and rat striatum (7–14).

The adenosine $A_2$-like binding site is found on a number of tissues, including human placenta, human platelet, human neutrophil, human lymphocyte, and guinea pig lung (10, 15–18). This binding protein, a homodimer with a 98-kDa subunit, comprises up to 1% of the placental membrane protein and has a high degree of homology with murine, hamster, and avian stress-related proteins (19, 20). The adenosine $A_2$-like binding site has submicromolar affinity for NECA and a high binding density, ranging from 4.2 to 26 pmol/mg of protein (10, 12, 16, 17, 19, 21–23). This contrasts with adenosine $A_1$ and $A_2$ receptors, which have nanomolar affinity for NECA and less than 1 pmol/mg of protein density (24–27). The lack of binding by $N^6$-substituted adenosine analogs and $C^8$-substituted xanthine derivatives distinguishes the adenosine $A_2$-like binding site from the adenosine $A_1$ and $A_2$ receptors, which bind these analogs (9, 13, 24–27).

The adenosine $A_2$-like binding protein obscures the binding properties of the adenosine $A_2$ receptor in human platelets (13). To solve this problem, we have developed a rapid and simple method for separation of adenosine $A_2$ receptors from adenosine $A_2$-like binding protein. We have applied this method successfully to separate adenosine receptors contained in human placental membranes as well.

## Experimental Procedures

**Materials.** CHAPS and bicinchoninic acid were obtained from Pierce Chemical Co (Rockford, IL). NECA was obtained from Boehringer-Mannheim (Indianapolis, IN). [$^3$H]NECA (20–40 Ci/mmol) was

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[1] Postdoctoral fellow supported by United States Public Health Service Fogarty International Award 1-FOS-TW04102. Permanent address: Department of Biochemistry, Academic Medical School, 80-211 Gdansk, Poland.

**ABBREVIATIONS:** [$^3$H]NECA, 5′-*N*-[2,8-$^3$H]ethylcarboxamidoadenosine; XAC,, Xanthine amine congener, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine; DPX, 1,3-diethyl-8-phenylxanthine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PIA, phenylisopropyladenosine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Gpp(NH)p, guanylylimidodiphosphate; PEG, polyethylene glycol 8000; NECA, 5′-*N*-ethylcarboxamidoadenosine.

purchased from Amersham (Arlington Hts, IL). DPX and XAC were obtained from Research Biochemicals Inc. (Natick, MA). Glass fiber filters (GF/C) were from Whatman (Maidstone, England). Ecoscint A scintillation mixture was from National Diagnostics (Manville, NJ). Hydroxylapatite Bio-Gel HTP was obtained from Bio-Rad (Richmond, CA). All other reagents were obtained from Sigma Chemical Company (St. Louis, MO).

**Tissue preparation.** Human platelets were prepared as described (28) and frozen at −70°. Thawed lysates (100–200 ml at 20–25 mg of protein/ml) were centrifuged and the pellet was homogenized with a Dounce homogenizer in 150–200 ml of 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (buffer A), and centrifuged at 50,000 × *g* for 15 min. The pellet was washed as described two more times. The washed pellet was suspended in buffer A (80–140 ml, 20 mg of protein/ml) and extracted with an equal volume of 2% CHAPS in buffer A (final concentrations, 1% CHAPS and 10 mg of protein/ml), using a Dounce homogenizer (five strokes up and down). The mixture was stirred on ice for 15 min with a Teflon stirring bar. The suspension was spun at 50,000 × *g* (20,000 rpm) for 15 min in a Sorvall RC-5C centrifuge and the supernatant was collected. Clear CHAPS extract (145–250 ml) was precipitated by the addition of 50% PEG in buffer A to a final concentration of 10%. After stirring for 30 min on ice, the precipitate was centrifuged at 50,000 × *g* for 15 min as before. The PEG precipitate was homogenized with 20 ml of buffer A and spun at 1000 × *g* (3000 rpm) for 3 min in a Sorvall RC-5C centrifuge, and the supernatant was collected. The pellet was homogenized again in 20 ml of buffer A and spun for 3 min at 1000 × *g*. The pellet was discarded and the supernatant was collected and combined with the previous one. The combined supernatants were sedimented by spinning at 50,000 × *g* for 15 min and the pellet was resuspended in 10 ml of buffer A. The supernatant was used as a source of adenosine $A_2$-like binding protein. The resuspended pellet (10 ml) was again centrifuged at 1000 × *g* for 3 min. The supernatant was collected and spun at 50,000 × *g* for 15 min. The resulting pellet was resuspended in buffer A at approximately 6 mg of protein/ml and was used as a source of platelet adenosine $A_2$ receptor.

Fresh human placentas were prepared on ice. The blood was drained, the amnion and chorion were removed, and the tissue was rinsed with 0.9% NaCl. The chopped tissue was suspended in 20 mM imidazole-HCl, pH 7.0, 20 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 6 mM sodium azide (approximately 100 g of tissue in 400 ml of buffer), and homogenized at the highest speed, three times for 15 sec at 1-min intervals, in a Sorvall Omni-Mixer. The homogenate was centrifuged at 1000 × *g* (3000 rpm) for 10 min in a Sorvall RC-5C centrifuge and the supernatant was collected and centrifuged again at 50,000 × *g* (20,000 rpm) for 30 min. The membrane pellet obtained from one placenta was washed three times with 100–150 ml of buffer A and extracted with CHAPS as described for platelets (final concentrations, 1% CHAPS and 10 mg of protein/ml). The soluble placental extract (100–150 ml) was mixed with hydroxylapatite Bio-Gel HTP (1 g of hydroxylapatite/50 ml of extract) and stirred for 15 min on ice. Hydroxylapatite was centrifuged at 50,000 × *g* for 15 min and this procedure was repeated by adding fresh hydroxylapatite to the supernatant as before. The supernatant from the second treatment with hydroxylapatite (approximately 120 ml) was precipitated with 30 ml of 50% PEG, as described for platelets. The precipitate was suspended in 8–12 ml of buffer A at 6 mg of protein/ml and was used as a source of placental adenosine receptor. The adenosine $A_2$-like binding protein was eluted from hydroxylapatite with 10 ml of 0.5 M sodium phosphate in buffer A.

**Binding assay.** Adenosine receptors are identified by high affinity binding, with specific binding displaceable by 100 μM (*R*)-PIA (13). Adenosine $A_1$ and $A_2$ receptors are distinguished by typical features of agonist potency order for binding, as follows: $A_2$, NECA > (*R*)-PIA; and $A_1$, (*R*)-PIA > NECA (3, 29, 30). Adenosine $A_2$-like binding sites have low affinity binding and do not display specific binding with 100 μM (*R*)-PIA but do display displaceable binding with 100 μM NECA (13–15, 19, 31).

The binding of [$^3$H]NECA to membrane fractions or to binding sites solubilized from these tissues was measured in a total volume of 275 μl, containing 50 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, and 1 unit of adenosine deaminase/ml. In competition experiments, 7.5 nM [$^3$H]NECA was incubated with 300–600 μg of protein for adenosine receptors or 50–100 μg of protein for adenosine $A_2$-like binding protein. Incubation was performed at 25° for 90 min for adenosine receptor binding or on ice for 40 min for adenosine $A_2$-like binding. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/C glass-fiber filters that had been treated with 0.3% polyethyleneimine for at least 90 min (32). The incubation mixture was diluted with 3 ml of ice-cold incubation buffer and rapidly vacuum filtered, and the filter was washed three times with 3 ml of incubation buffer. Nonspecific binding was determined with 100 μM NECA or 100 μM (*R*)-PIA. The filter-bound radioactivity was counted in Ecoscint A. All assays were done in triplicate.

To identify adenosine $A_2$ receptors in platelet membranes, [$^3$H]NECA binding was performed, with nonspecific binding estimated separately using 100 μM NECA and 100 μM (*R*)-PIA (13). [$^3$H]NECA bound to the adenosine $A_2$-like binding protein is insensitive to displacement by 100 μM (*R*)-PIA. Accordingly, we used the ratio of [$^3$H]NECA radioactivity displaceable by 100 μM (*R*)-PIA to [$^3$H]NECA radioactivity displaceable by 100 μM NECA [(*R*)-PIA/NECA ratio] to indicate the relative abundance of adenosine $A_2$ receptors. We used the same method to assay adenosine $A_1$ receptors in human placental membranes and extracts.

**Protein assay.** Protein was estimated by the bicinchoninic acid method, using bovine serum albumin as a standard (33).

**Data analysis.** Saturation and competition curves were analyzed using a nonlinear least squares curve-fitting technique (GraphPAD; iSi Software). Using this method, curves fit best to a one-site model. Hill coefficients were calculated for each study. The values were always less than 1.3, supporting the existence of one site only.

## Results

**Platelet membranes.** To separate human platelet adenosine $A_2$ receptors from the adenosine $A_2$-like binding protein, we initially used gel filtration chromatography, as previously reported (13). We obtained a variable yield of receptors and found that $A_2$ receptors present in CHAPS extract precipitated during dialysis against 0.02% CHAPS. In addition, this method was impractical on a large scale.

In platelet membranes and CHAPS extract, the adenosine $A_2$-like binding protein predominates (Table 1). The binding is identical to the homogeneously purified adenosine $A_2$-like binding site (19). The PEG precipitate is relatively enriched for the (*R*)-PIA-displaceable binding (Table 1). The nonspecific binding, using 100 μM (*R*)-PIA, is 28% of the total binding. There is about 23% contamination with adenosine $A_2$-like binding sites. The final material obtained from human platelets had an 11.5-fold enrichment and a 9% yield of adenosine $A_2$ receptor. Our results from a number of such purifications show 9- to 14-fold enrichment and 7 to 11% yield.

The (*R*)-PIA-displaceable binding of [$^3$H]NECA to platelet PEG precipitate was saturable. A binding capacity ($B_{max}$) of 230 fmol/mg of protein and $K_d$ of 31 nM were estimated (Fig. 1). These data were fit to a one-site model. The specific binding of [$^3$H]NECA to platelet PEG precipitate was not sensitive to 100 μM Gpp(NH)p.

The pharmacologic properties of the PEG precipitate and supernatant were examined by equilibrium displacement studies (Table 2). The affinities of adenosine receptor agonists and

TABLE 1

**Partial purification of human platelet adenosine receptor**

Binding activity was estimated with 10 nM [$^3$H]NECA as a radioligand at 25°. The data are representative of five preparations. The differences among separate preparations were in the range of 20%.

| Step | Protein | Total binding activity | | Ratio[c] | Specific activity | | Purification | | Yield | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | (*R*)-PIA[a] | NECA[b] | | (*R*)-PIA[a] | NECA[b] | (*R*)-PIA[a] | NECA[b] | (*R*)-PIA | NECA |
| | *mg* | *pmol* | | | *fmol/mg* | | *fold* | | % | |
| Membranes | 2900 | 24.4 | 69.7 | 0.35 | 8.4 | 24.0 | 1 | 1 | 100 | 100 |
| CHAPS extract | 1365 | 8.4 | 18.3 | 0.46 | 6.2 | 13.4 | 0.7 | 0.6 | 34.4 | 26.3 |
| PEG precipitate | 37 | 3.6 | 4.7 | 0.77 | 97.3 | 127.0 | 11.5 | 5.3 | 9.2 | 6.7 |

[a] Binding was estimated using 100 μM (*R*)-PIA for nonspecific binding.
[b] Binding was estimated using 100 μM NECA for nonspecific binding.
[c] Ratio of binding estimated in *a* to binding estimated in *b*.

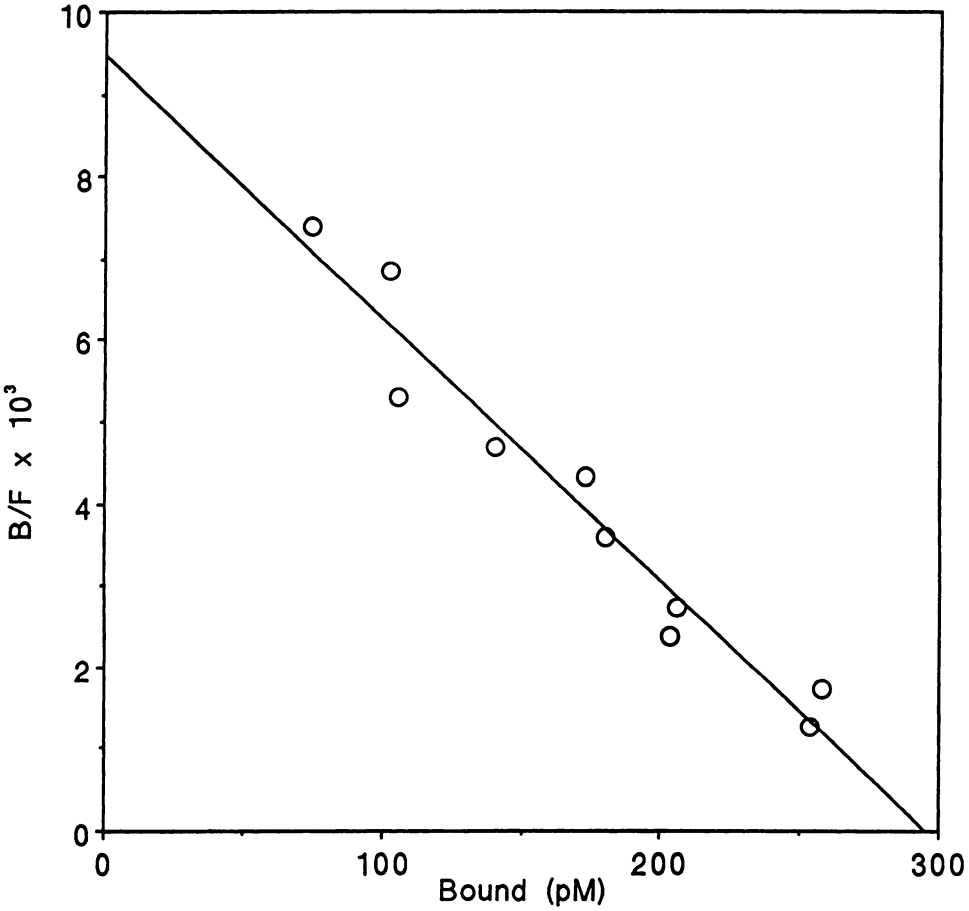


**Fig. 1.** Scatchard plot of [$^3$H]NECA binding to adenosine $A_2$ receptors. PEG-precipitated platelet membrane protein from CHAPS extract was studied by saturation analysis, using 10–200 nM [$^3$H]NECA, 100 μM (*R*)-PIA for determination of nonspecific binding, and 0.35 mg of protein. The $K_d$ was 31 nM and the $B_{max}$ was 230 fmol/mg.

antagonists were estimated by fitting to a one-site model. The fit of the data was not improved by a two-site model. The hierarchy of agonist and antagonist potency of the precipitate is compatible with the properties of an adenosine $A_2$ receptor. The displacement by XAC, DPX, and the $N^6$-substituted compounds, together with the high affinity distinguish this binding site from the adenosine $A_2$-like binding site. This agrees with previous observations (13) and with $EC_{50}$ values for platelet adenylate cyclase stimulation (10). The binding properties of the supernatant and platelet membranes are typical for the adenosine $A_2$-like binding protein (10, 13, 19).

**Placental membranes.** Placental membranes contain adenosine $A_2$-like binding protein and adenosine $A_1$ receptors (14, 15). Because JAR cells, a malignant placental cell line, contain adenosine $A_2$ receptors,[2] we examined whether placenta has adenosine $A_2$ receptors. The binding of placental membranes, CHAPS extract, and the hydroxylapatite eluate are typical for the adenosine $A_2$-like binding protein. This is shown by the $IC_{50}$ values, the hierarchy of ligand potencies, and the identity to the homogeneously purified protein (19) (Tables 3 and 4).

The PEG precipitate is relatively enriched for the (*R*)-PIA-displaceable binding and is only approximately 10% contaminated by adenosine $A_2$-like binding sites. The nonspecific binding, using 100 μM (*R*)-PIA, is 6% of the total binding. Scatchard analysis of [$^3$H]NECA binding to PEG-precipitated placental proteins yielded a $K_d$ of 2.7 and 5.9 nM, without and with 100 μM Gpp(NH)p, respectively (Fig. 2). In contrast to the platelet, the pharmacologic profile of agonist and antagonist binding measured by equilibrium displacement is compatible with the binding of an adenosine $A_1$ receptor (Table 4). The binding profile fit a one-site model. The high affinity binding of ligands, the relative agonist potencies, and the binding by $N^6$-substituted agonists XAC, and DPX, clearly distinguish this binding

TABLE 2

**Equilibrium displacement studies of adenosine receptors and adenosine $A_2$-like binding sites from human platelets**

Human platelet membranes were extracted and precipitated as described in Experimental Procedures. The PEG precipitate contains the adenosine $A_2$ receptor, whereas the supernatant contains the adenosine $A_2$-like binding sites. Assays were performed as described, using 7.5 nM [$^3$H]NECA and 0.5 mg of protein. Data represent mean values ± standard deviations from at least two experiments performed in triplicate.

| Compound | $IC_{50}$ | | |
|---|---|---|---|
| | PEG precipitate[a] | Supernatant[b] | Membrane[b] |
| | | μM | |
| NECA | 0.055 ± 0.002 | 0.49 ± 0.06 | 0.6 |
| 2-Chloroadenosine | 0.440 ± 0.136 | 2.18 ± 0.14 | 0.8 |
| | | >100[c] | 1000 |
| (*R*)-PIA | 0.510 ± 0.192 | | |
| (*S*)-PIA | 4.100 ± 2.270 | | |
| $N^6$-Cyclohexyladenosine | 5.600 ± 4.570 | | |
| XAC | 0.045 ± 0.012 | | |
| DPX | 0.220 ± 0.006 | | |
| 3-Isobutyl-1-methylxanthine | | | 9 |
| Theophylline | 12.62 ± 8.35 | | 282 |

[a] 100 μM (*R*)-PIA was used for nonspecific binding.
[b] 100 μM NECA was used for nonspecific binding. The membrane binding data were obtained from Ref. 34.
[c] There was no displacement by 100 μM (*R*)-PIA.

TABLE 3

**Partial purification of human placental adenosine receptor**

Binding activity was estimated with 7.5 nM [$^3$H]NECA as a radioligand at 25°. The data are representative of five preparations. The differences among separate preparations were in the range of 20%. Approximately 1200–1600 mg of membrane protein were obtained from an average human placenta.

| Step | Protein | Total binding activity | | Ratio[c] | Specific activity | | Purification | | Yield | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | (*R*)-PIA[a] | NECA[b] | | (*R*)-PIA[a] | NECA[b] | (*R*)-PIA[a] | NECA[b] | (*R*)-PIA | NECA |
| | mg | pmol | | | fmol/mg | | fold | | % | |
| Membranes | 1400 | 168 | 420 | 0.4 | 120 | 300 | 1 | 1 | 100 | 100 |
| CHAPS extract | 420 | 20.2 | 33.7 | 0.6 | 48 | 80 | 0.4 | 0.3 | 12.0 | 8.0 |
| CHAPS extract after hydroxylapatite | 116 | 16.8 | 18.7 | 0.9 | 145 | 161 | 1.2 | 0.5 | 10.0 | 4.4 |
| PEG precipitate | 76 | 15.1 | 16.8 | 0.9 | 199 | 221 | 1.7 | 0.7 | 8.9 | 4.0 |

[a] Specific binding estimated using 100 μM (*R*)-PIA for nonspecific binding.
[b] Specific binding estimated using 100 μM NECA for nonspecific binding.
[c] Ratio of binding estimated in *a* to binding estimated in *b*.

TABLE 4

**Equilibrium displacement studies of adenosine receptors and adenosine $A_2$-like binding sites from human placenta**

Human placental membranes were extracted and precipitated as described in Experimental Procedures. The PEG precipitate contains the adenosine $A_1$ receptor, whereas the hydroxylapatite eluate contains the adenosine $A_2$-like binding sites. Binding assays were performed as described, using 7.5 nM [$^3$H]NECA and 0.5 mg of protein. Data represent mean values ± standard deviations from at least two experiments performed in triplicate.

| Compound | $IC_{50}$ | | |
|---|---|---|---|
| | PEG precipitate[a] | Hydroxylapatite eluate[b] | Membrane[b] |
| | | μM | |
| NECA | 0.014 ± 0.003 | 0.41 ± 0.02 | 0.3 |
| 2-Chloroadenosine | 0.008 ± 0.001 | 2.33 ± 0.37 | 2.0 |
| (*R*)-PIA | 0.005 ± 0.002 | >100[c] | 1000 |
| (*S*)-PIA | 0.033 ± 0.011 | | |
| $N^6$-Cyclohexyladenosine | 0.021 ± 0.003 | | |
| XAC | 0.274 ± 0.048 | | |
| DPX | 2.10 ± 0.66 | | |
| 3-Isobutyl-1-methylxanthine | | | 30 |
| Theophylline | 27.65 ± 3.08 | | 200 |

[a] 100 μM (*R*)-PIA was used for nonspecific binding.
[b] 100 μM NECA was used for nonspecific binding. The membrane binding data were obtained from Work et al.[2]
[c] There was no displacement by 100 μM (*R*)-PIA.

site from the adenosine $A_2$-like binding site. There was no evidence for an adenosine $A_2$ receptor.

## Discussion

The adenosine $A_2$-like binding protein is found in membranes from human platelets, human placenta, cultured JAR choriocarcinoma cells, human neutrophils, human lymphocytes, rat liver, cultured pheochromocytoma PC-12 cells, and guinea pig lung (6, 7, 10, 12–18).[2] The function of this site beyond binding adenosine remains unclear. It is an acidic asymmetric protein with a subunit molecular mass of 98 kDa and a native molecular mass of 230 kDa (19).

The coexistence of the adenosine $A_2$-like binding protein with adenosine receptors (7, 12–14) makes the study of receptor

[2] C. Work, S. Zolnierowicz, K. Hutchison, M. Prasad, and I. H. Fox. Concurrence of adenosine $A_2$-like binding sites with adenosine receptors in mammalian cells. Manuscript in preparation.

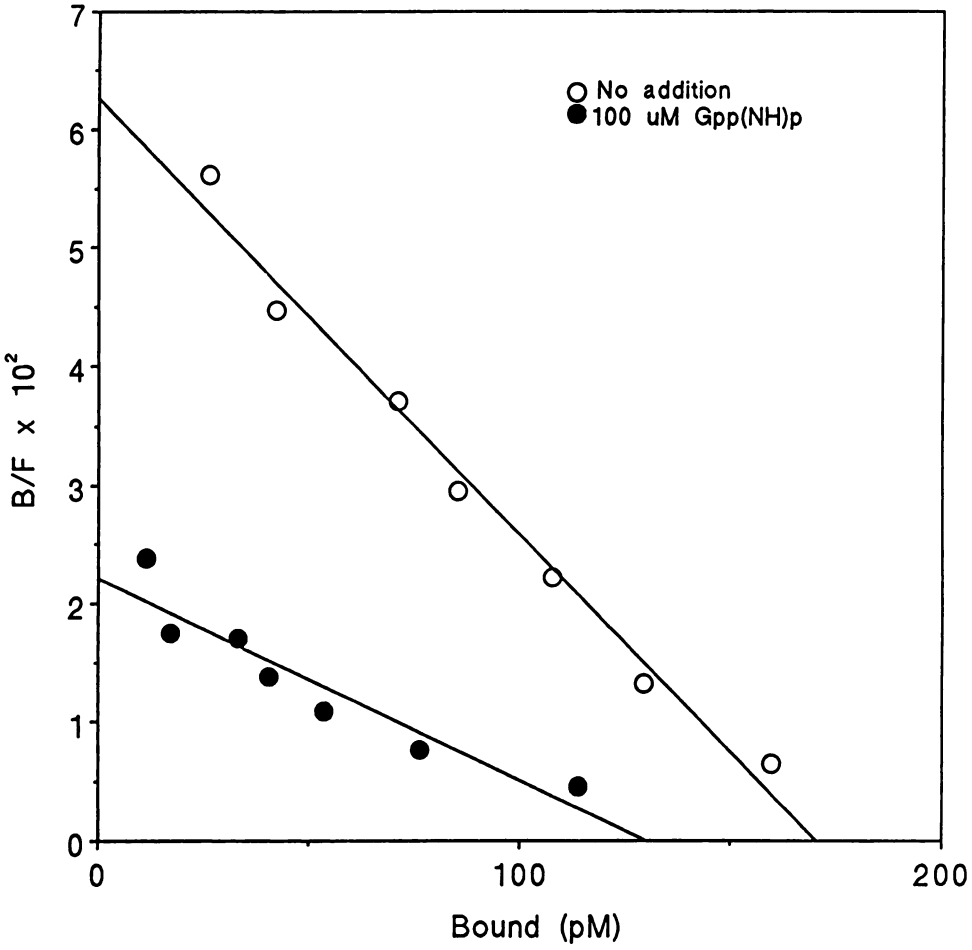


**Fig. 2.** Scatchard plot of [³H]NECA binding to adenosine $A_1$ receptors. PEG-precipitated placental membrane protein from CHAPS extract was studied by saturation analysis using 0.5–25 nM [³H]NECA, 100 μM (*R*)-PIA for determination of nonspecific binding, 0.6 mg of protein, and 0 or 100 μM Gpp(NH)p. The $K_d$ values were 2.7 and 5.9 nM without and with Gpp(NH)p, respectively. $B_{max}$ values were lower than expected, secondary to a loss of receptor binding during storage at 5°.

binding difficult. In human platelet membranes, the adenosine $A_2$ receptors activate adenylate cyclase (8, 12, 24), but binding studies with [³H]NECA are typical of the adenosine $A_2$-like binding protein and show virtually no displacement by the adenosine $A_2$ agonist (*R*)-PIA or the antagonist DPX (8, 12, 34, 35). During our assays of platelet membranes at 25°, there was high nonspecific binding used 100 μM (*R*)-PIA. The same problem occurred with the platelet adenosine $A_2$ receptor labeled with XAC (36). Our new method avoids high nonspecific binding by removing the majority of the adenosine $A_2$-like binding protein, using extraction of membranes with CHAPS and then PEG precipitation. This method allows the rapid batch preparation of large quantities of platelet membranes suitable for pharmacologic studies of the adenosine $A_2$ receptor.

We have applied our membrane extraction and precipitation method to human placenta to evaluate for evidence of adenosine $A_2$ receptors. Placenta has a large quantity of adenosine $A_2$-like binding protein, which was removed by hydroxylapatite (14, 15). No adenosine $A_2$ receptors were evident in the precipitated material. However, pharmacologic studies of agonist and antagonist binding and the sensitivity to guanine nucleotides were compatible with the existence of adenosine $A_1$ receptors (14, 24, 25, 37). This method allows the rapid batch preparation of large quantities of placental membranes suitable for pharmacologic studies of the adenosine $A_1$ receptor.

Our studies demonstrate a simple and rapid method for batch removal of adenosine $A_2$-like binding protein from human platelets and placenta. This will facilitate the study of the pharmacologic binding properties of the adenosine receptor in these tissues. Although it remains to be demonstrated that this method is useful in other tissues, this represents an important advance for the study of adenosine receptor binding properties, especially the adenosine $A_2$ type.

### Note Added in Proof

The term "adenotin" is now being applied to distinguish the adenosine $A_2$-like binding protein from adenosine receptors (20).

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**Send reprint requests to:** Irving H. Fox, Medical Research Department, Biogen, Inc., Fourteen Cambridge Center, Cambridge, MA 02142.