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Characteristics of an Adenosine A_1 Binding Site in Human Placental Membranes[1]

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Binding sites were solubilized from human placental membrane using 1.5% sodium cholate and were assayed using polyethylene glycol precipitation. These soluble binding sites had properties of an adenosine A_1 binding site. 2-[^{3}H]Chloroadenosine and *N*-[^{3}H]-ethylcarboxamidoadenosine (NECA) binding were time dependent and reversible. Scatchard plots indicate two classes of binding sites with K_d values of 6 and 357 nM for 2-chloro[8-^{3}H]adenosine and 0.1 and 26 nM with [^{3}H]NECA. The specificity of [^{3}H]NECA binding was assessed by the ability of adenosine analogs to compete for binding sites. Using this approach the estimated IC_{50} values were 60 nM for N^6-((*R*)-1-methyl-2-phenylethyl)adenosine (*R*-PIA), 160 nM for *S*-PIA, 80 nM for NECA, and 20 nM for 2-chloroadenosine. Binding of [^{3}H]NECA to the soluble sites is inhibited to 48% of the control value by 100 μM guanylyl-5'-imidodiphosphate (Gpp(NH)p). The IC_{50} value for NECA binding to the soluble binding site was increased from 80 nM to 1500 by Gpp(NH)p. There was a shift of binding affinity from a mixture of high and low affinity to only low affinity with 100 μM Gpp(NH)p. Despite these alterations a NECA prelabeled molecular species of 150 kDa did not decrease in molecular weight upon the addition of 100 μM Gpp(NH)p during high-performance liquid chromatography on a Superose 12 column. Other evidence to support the concept of preferential solubilization and assay of a small population of A_1 binding sites was obtained. Following solubilization adenosine A_2-like binding sites could be detected only in reconstituted vesicles. The existence of small amounts of A_1 binding sites in intact human placental membranes was directly demonstrated using the A_1 agonist ligand N^6-[^{3}H]cyclohexyladenosine and the A1 antagonist ligand 8-[^{3}H]cyclopentyl-1,3-dipropylxanthine. JAR choriocarcinoma cells have "A_2-like" membrane binding sites. In contrast to placental membranes, only A_2-like binding sites could be solubilized from JAR choriocarcinoma cells. These observations indicate that human placental membranes contain adenosine A_1 binding sites in addition to A_2-like binding sites. These sites are guanine nucleotide sensitive, but do not shift to a lower molecular weight form upon assumption of a low affinity state.

The diverse biological activity of adenosine has been recognized for many years (1). These actions are associated with two cell surface receptors, referred to as A_1 or A_2, which have been defined by effects upon adenylate cyclase (2-6) and by binding studies (7-13). This system has simi-

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larities to other classical hormone receptor systems which are coupled to adenylate cyclase (2-4, 13-15).

We and others have reported binding of 2-[^{3}H]chloroadenosine and 5'-*N*-[^{3}H]ethylcarboxamidoadenosine ([^{3}H]NECA)[3] to sites which are not affected by guanine nucleotides (12, 16). These sites resemble A_2 receptors in having higher affinity for NECA and 2-chloroadenosine than for other nucleosides. However, these sites have little or no affinity for several adenosine agonists and antagonists that are known to have significant affinities at both A_1 and A_2 receptors. Because these sites differ from classical adenosine receptors, we refer to them as "A_2-like" binding sites.

In order to further probe the significance of these adenosine binding sites, we have studied binding of [^{3}H]NECA to sodium cholate-solubilized extracts of human placental membranes. These extracts revealed evidence for an adenosine A_1 binding site which we have characterized.

MATERIALS AND METHODS

Materials. Sodium cholate, Chaps, 2-chloroadenosine, 3-isobutyl-1-methylxanthine, theophylline, caffeine, 5'-methylthioadenosine, guanylyl-5'-imidodiphosphate (Gpp(NH)p), leupeptin, antipain, chymostatin, pepstatin A, polyethylene glycol (6000-8000 molecular weight), and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., St. Louis, Missouri. N^6-Cyclohexyladenosine, NECA, *S*-PIA, 1,3-diethyl-8-phenylxanthine, and pentostatin (2'-deoxycoformycin) were gifts from Warner-Lambert Co., Ann Arbor, Michigan. *R*-PIA was obtained from Boehringer-Mannheim Biochemicals, Indianapolis, Indiana. Triton X-100 was from Eastman Kodak Co., Rochester, New York. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was purchased from Burroughs Wellcome Co., Research Triangle Park, North Carolina. A Superose 12 HR 10/30 column and molecular weight standards were obtained from Pharmacia, Uppsala, Sweden. 8-Cyclopentyl-1,3-dipropylxanthine (CPX; also known as PD 116,948), an A_1 selective antagonist, was synthesized at Warner-Lambert/Parke-Davis, as was [^{3}H]CPX (116 Ci/mmol). The compound 2-chloro[8-^{3}H]adenosine (10 Ci/mmol) was obtained from Moravek, Brea, California; ([2,8-^{3}H]adenine)-5'-*N*-ethylcarboxamidoadenosine (20 Ci/mmol) and ([2,8-^{3}H]adenine)-N^6-cyclohexyladenosine ([^{3}H]CHA) (34 Ci/mmol) were obtained from New England Nuclear, Boston, Massachusetts. Bio-Rad protein reagent was obtained from Bio-Rad Laboratories, Richmond, California. JAR choriocarcinoma cells were generously provided by Dr. Raymond Ruddon of the Department of Pharmacology, The University of Michigan. Other reagents were the best grade commercially available.

Membrane preparation. Placental membranes were prepared as reported previously with a concentration of 10 to 18 mg/ml (12). These membranes were homogenized in 50 mM Tris-HCl, pH 7.5, 2.5 mM magnesium chloride, and 2.5 mM $CaCl_2$ (incubation buffer) which contained 1.5% sodium cholate. The mixture was incubated for 15 min at 22°C and centrifuged at 48,000*g* for 2 h. The supernatant was decanted and filtered through a Millipore Millex-GS 0.22-μm filter unit. The detergent extract was stored at −70°C and used within 3 days.

JAR cells, a human placental choriocarcinoma cultured cell line, were grown in monolayer using Dulbecco's medium in roller bottles. The confluent cells were harvested by scraping the surface. Cells were washed twice in 250 mM sucrose, 1 mM $MgCl_2$, and 5 mM Tris-HCl, pH 7.5, and lysed by bomb cavitation for 60 min at 750 to 1000 pounds per square inch in the same buffer at 4°C. The membranes were spun at 48,000*g* for 20 min. Membranes were then used for binding assays or solubilized using the same procedure as for placental membranes with the addition of proteolytic inhibitors.

Binding assays. Binding studies of the membranes for A_2-like binding sites using 2-[^{3}H]chloroadenosine were performed as described (12). With [^{3}H]NECA similar conditions were used except that only 100 μg protein was added and radioligand concentration was 10 or 20 nM. Eighty to ninety percent specific binding is observed under these conditions. The membrane bound A_1 binding site was assayed by incubating 1 mg protein in 2 ml 50 mM Tris-HCl, pH 7.7, containing 1 nM [^{3}H]CHA or 0.1 nM [^{3}H]CPX for 60 min at 25°C. The binding was measured by vacuum filtration on Whatman GF/B filters as previously described (17, 18).

The soluble binding assay was performed in triplicate in a total volume of 850 μl containing 0.8 to 1.5 mg soluble protein, 20 or 40 nM 2-[^{3}H]chloroadenosine or 20 nM [^{3}H]NECA, and incubation buffer. Sodium cholate was usually 0.15% in the assay mixture. The mixture was incubated at 22°C for 2 h with 2-chloro-

[3] Abbreviations used: NECA, 5'-*N*-ethylcarboxamidoadenosine; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid; Gpp(NH)p, guanylyl-5'-imidodiphosphate; PIA, phenylisopropyladenosine; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; CHA, N^6-cyclohexyladenosine.

adenosine or 2.5 h with NECA. Duplicate sets of incubation mixtures had 1 mM 2-chloroadenosine or NECA added. At the end of the incubation period γ globulin was added to a final concentration of 0.2% and polyethylene glycol was added to a final concentration of 12.5%. One minute after the addition of polyethylene glycol, 3 ml of incubation buffer containing 12.5% polyethylene glycol was added to each tube, which was then rapidly filtered on Whatman GF/B filters using a vacuum pump. The filters were washed three times with 3 ml of the buffer-polyethylene glycol solution and dried under vacuum for 5 min. Total filtration time is about 60 s. Filters were placed in vials containing 7 ml aqueous counting scintillant (Amersham) and allowed to stand overnight. Radioactivity was measured by liquid scintillation spectrometry using an LKB Rackbeta. Specific binding is defined as the cpm of total 2-[^{3}H]chloroadenosine or [^{3}H]NECA binding minus cpm of the tubes with 1 mM 2-chloroadenosine or NECA. For soluble receptor binding specific binding amounted to 50–80% of total counts bound.

Similar results were obtained using 6.7-ml Isolab columns containing Sephadex G-25 (Pharmacia), which were spun at 1800g in a centrifuge for 2 min to separate bound versus free radioactivity. In this assay the total volume of the incubation mixture was reduced to 300 μl of which 275 μl was applied to the column.

Gel permeation chromatography. Soluble placental membrane extract (10 mg) was prelabeled by incubation at 22°C for 2 h with 0.5 μM [^{3}H]NECA without or with 1 mM chloroadenosine or 100 μM Gpp(NH)p in a total volume of 300 μl. The sample was desalted rapidly using the small Sephadex G-25 column described above, filtered, and injected into an analytical Superose 12 HR 10/30 column equilibrated with 100 mM NaCl, 1% sodium cholate, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 50 mM Tris-HCl, pH 7.4. The column was eluted at 0.5 ml/min and fractions of 0.5 ml were collected.

Protein assay. Protein was measured by the Bradford method using bovine serum albumin as standard (19).

Calculations. The interaction of 2-[^{3}H]chloroadenosine or [^{3}H]NECA with the soluble receptor was analyzed by the method of Scatchard (20) using the LIGAND binding analysis program (21, 22) run on a VAX 11/730 computer. BMDP software was used for the analyses.

The apparent K_d for 2-[^{3}H]chloroadenosine was also calculated from kinetic data (23). The binding reaction was considered to be pseudo-first order because it is reversible and because ligand concentration (20–40 nM) was much greater than receptor concentration (0.2 nM). The second-order rate constant k_1 was calculated from the relationship

$$k_1 = (k_{ob} - k_2)/(\text{2-chloroadenosine}).$$

The observed forward rate constant, k_{ob}, was determined from

$$k_{ob} \cdot t = \ln(B_{max}/(B_{max} - B)),$$

where B_{max} equals total specific binding at equilibrium and B equals binding at time t. The first-order rate constant, k_2, was calculated from the relationship

$$k_2 \cdot t = \ln(B/B_{max}).$$

The K_d was then estimated from the relationship of $K_d = k_2/k_1$. The existence of multiple binding sites makes these estimates only a rough average of these different sites.

[^{3}H]CHA and [^{3}H]CPX binding results were analyzed as described (17, 18).

RESULTS

Optimal Conditions for Solubilization and Assay

Sodium cholate eluted the largest amount of soluble receptor as compared to 1% Chaps and Triton X-100. Comparison of 0.5 to 2.0% sodium cholate indicated optimal activity at 1.5%. The addition of proteolytic inhibitors, 10 μg/ml antipain, 10 μg/ml pepstatin, 0.01 μg/ml lima bean trypsin inhibitor, along with 10% sucrose provided an optimal yield, but did not modify binding characteristics.

A temperature of 22°C was optimal for assay. The addition of sodium cholate above a concentration of 0.15% inhibited binding. In the precipitation step, 12.5% polyethylene glycol and 1.5 mg/ml bovine γ globulin gave the maximum specific binding. The addition of the proteolytic inhibitors described above to the assay mixture itself did not increase the binding.

Overall, the binding assay detected 12 to 20% of the starting membrane binding activity with 2-[^{3}H]chloroadenosine, but only 2 to 4% with [^{3}H]NECA.

Properties of Soluble Binding Site

The soluble binding site was initially characterized to establish the properties of a receptor including time-dependent association and dissociation, saturability of binding sites, and pharamacologic speci-

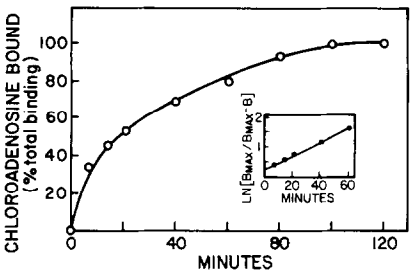

FIG. 1. Time dependence of 2-[^{3}H]chloroadenosine binding. One milligram of soluble membrane protein was incubated with 40 nM 2-[^{3}H]chloroadenosine for up to 120 min and 23°C. Binding was assayed by polyethylene glycol precipitation, as described under Materials and Methods. 2-[^{3}H]Chloroadenosine binding to placental microsomes reached equilibrium at approximately 120 min with a $T_{1/2}$ of 17 min. The observed forward rate constant was 0.022 min^{-1}.

ficity of agonist and antagonist binding. Adenosine A_1 receptors are characterized by agonist K_d values in the nanomolar range (2-6, 8-11, 13). The agonist *R*-PIA is more potent in its binding properties than the agonist NECA. In contrast, the adenosine A_2 binding site is a lower affinity site with K_d values in the micromolar range. NECA binding is more potent than *R*-PIA binding (2-6, 12, 13).

Binding kinetics. Binding of 2-[^{3}H]chloroadenosine to soluble protein reached equilibrium at 120 min with a $T_{1/2}$ of 17 min (Fig. 1). The observed apparent forward rate constant was 0.022 min^{-1} giving a second-order apparent forward rate constant (k_1) of 3.4×10^5 min^{-1} M^{-1}.

At equilibrium the addition of 10 μM 2-chloroadenosine resulted in a slow displacement of 2-[^{3}H]chloroadenosine which was still not complete at 133 min (Fig. 2). The apparent first-order rate constant (k_2) for the reversal of 2-[^{3}H]chloroadenosine binding was 8.5×10^{-3} min^{-1}. The apparent K_d calculated from the rate constant was 25 nM for the soluble receptor.

Dissociation studies were also performed with [^{3}H]NECA. At equilibrium the addition of 1 mM 2-chloroadenosine resulted in a slow displacement of [^{3}H]NECA which was not yet complete at 40 min (Fig. 3A). More rapid and complete dissociation occurred with the addition of 100 μM Gpp(NH)p. The first-order rate constant for the reversal of [^{3}H]NECA binding was 0.014 min^{-1} without and 0.042 min^{-1} with 100 μM Gpp(NH)p. To examine for a change in binding site conformation during incubation, we performed the same experiment after only a 30-min incubation. A substantial change in the dissociation was observed with two phases of dissociation. One phase was a rapid 40% reversal of binding within 2.5 min (Fig. 3B). The second component was extremely slow. The rapid reversal phase was increased to 70% reversal of binding within 2.5 min with 100 μM Gpp(NH)p.

Saturability and affinity of agonist binding sites. Saturation of binding sites with 2-[^{3}H]chloroadenosine occurred with 160 fmol of ligand bound or 0.18 pmol/mg protein (Fig. 4). Analysis of the data by Scatchard plot gave a nonlinear relationship with calculated K_d values of 6 nM for 30% of binding sites and 357 nM for 70% of binding sites using a two-site model (Fig. 4).

Saturation of binding sites with [^{3}H]-NECA occurred with 200 fmol of ligand bound or 0.23 pmol/mg (Fig. 5). Analysis of the data by Scatchard plot gave a nonlinear relationship with estimated K_d values of 0.1 nM for 5.5% of binding and 26 nM

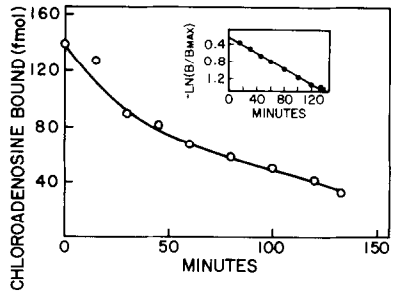

FIG. 2. Reversibility of 2-[^{3}H]chloroadenosine binding. The soluble placental membrane fractions (1 mg protein) were incubated with 40 nM 2-[^{3}H]chloroadenosine for 120 min at 23°C in an assay as described in Fig. 1. At equilibrium, the addition of 10 μM 2-chloroadenosine resulted in a displacement of 2-[^{3}H]chloroadenosine which was still not complete at 133 min. The apparent first-order rate constant (k_2) for the reversal of 2-[^{3}H]chloroadenosine binding was 8.5×10^{-3} min^{-1} (inset).

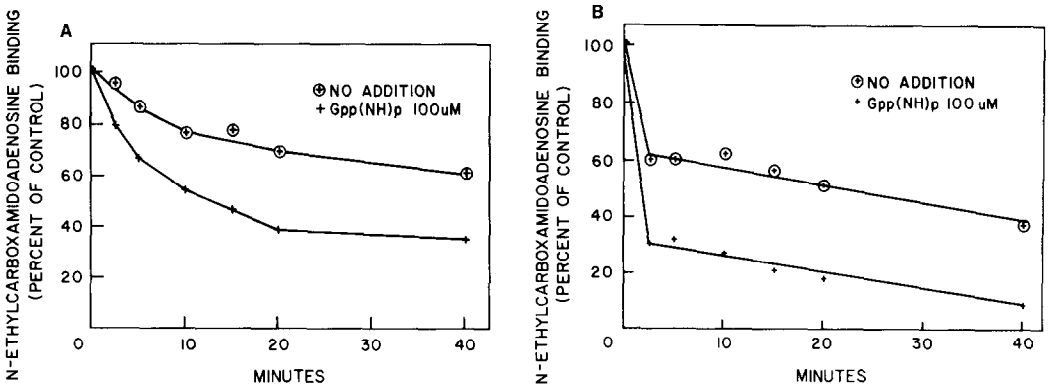

FIG. 3. Reversal of [^{3}H]NECA binding to soluble adenosine binding sites. Soluble extracts of placental membrane were preincubated with 20 nM [^{3}H]NECA and assayed as described in Fig. 1. The binding was determined after either 150 min (A) or 30 min (B) preincubation. Baseline binding ranged from 54 to 184 pmol/incubation medium. Dissociation was then induced with the addition of 1 mM NECA with or without 100 μM Gpp(NH)p. Binding was determined at the times indicated.

for 94.5% of binding using a two-site model (Fig. 5). The small population of high-affinity sites was not well defined by the data.

Specificity of agonist and antagonist binding. The ability of adenosine analogs to compete for binding sites on soluble protein was studied (Fig. 6). The IC_{50} of nonradioactive NECA versus [^{3}H]NECA was 80 nM (Fig. 6), which is quantitatively similar to the K_d value above of 26 nM. Using [^{3}H]-NECA we examined competition by other adenosine analogs. 2-Chloroadenosine was the most potent agonist (Fig. 6). *R*-PIA was more potent than NECA and *S*-PIA. 1,3-Diethyl-8-phenylxanthine was the most potent antagonist. Similar observations were made with 2-[^{3}H]chloroadenosine as the ligand. These relative binding

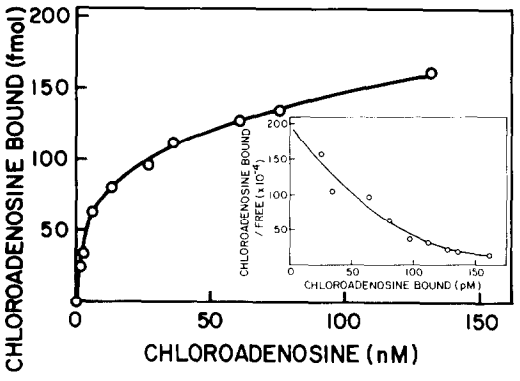

FIG. 4. Concentration dependence of 2-[^{3}H]chloroadenosine binding. One milligram of soluble membrane protein was incubated with 0 to 140 nM 2-[^{3}H]-chloroadenosine for 150 min at 23°C. Binding was assayed by polyethylene glycol precipitation as described under Materials and Methods. Saturation of binding sites occurred with 160 fmol of ligand bound. Analysis of the data by Scatchard plot indicates two classes of binding sites with K_d values of 6 and 357 nM (inset) by a computer-derived fit to a two-site model.

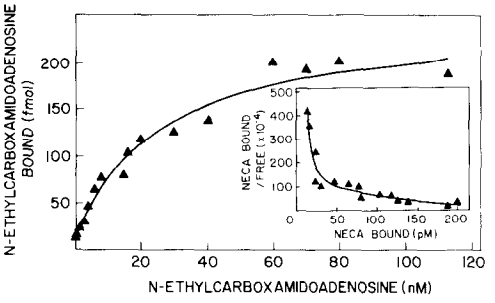

FIG. 5. Concentration dependence of [^{3}H]NECA binding. Studies were performed as described in Fig. 4 using NECA 0 to 115 nM. Saturation of binding sites occurred with 200 fmol of ligand bound. Analysis of the data by Scatchard plot (inset) indicates two classes of binding sites with K_d values of 0.1 and 26 nM. The former value must be considered a rough estimate, since there was a great deal of variability in the binding assay at concentrations below 5 nM. The lines plotted are a computer-derived fit to a two-site model.

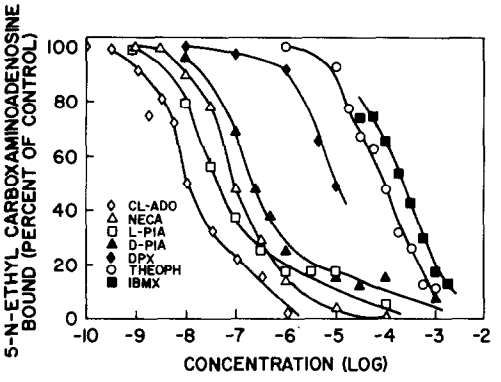

FIG. 6. Inhibition of [^{3}H]NECA binding by adenosine receptor ligands. Agonists and antagonists were incubated with 0.2 nM soluble receptor, 8 to 20 nM [^{3}H]NECA, and 1 mg soluble protein extract for 150 min under the conditions described in Fig. 1. Baseline binding ranged from 54 to 184 pmol/tube. Each point represents two to six experiments performed in triplicate. (CL-ADO, chloroadenosine; NECA, *N*-ethylcarboxamidoadenosine; L-PIA, *R*-phenylisopropyladenosine; D-PIA, *S*-phenylisopropyladenosine; DPX, diethylphenylxanthine; THEOPH, theophylline; IBMX, isobutylmethylxanthine).

potencies are similar to A_1 binding sites (8–11, 13).

IC_{50} values for deoxycoformycin and EHNA, adenosine deaminase inhibitors, were 40 and 180 nM, respectively. These inhibition curves were steep and occurred in less than one log unit of ligand concentration. A similar relationship has recently been observed in rat striatum (17). This relationship is virtually identical to the pattern observed in the depletion of unlabeled ligand. This occurs when the K_d of the unlabeled ligand is much lower than the labeled ligand and a limiting IC_{50} value is reached (24). If this is the case, then the true K_d values may be several orders of magnitude less than the IC_{50} values that we observed. In addition, in order to examine the possibility that there may be a high-affinity form of adenosine deaminase in the soluble membrane preparation, we assayed the enzyme with 0 to 1 mM NECA. There was no consistent inhibition by NECA suggesting that binding to adenosine deaminase was unlikely.

A relatively high IC_{50} value of 25 μM for nitrobenzylthioinosine, a potent inhibitor of the nucleoside transport protein, suggests that the NECA binding studied is distinct from the transport protein, which has a K_d for nitrobenzylthioinosine of about 1 nM.

Regulation by Guanine Nucleotides

Signal transduction is frequently mediated by guanine nucleotide regulatory proteins. Standard methods may be used to obtain evidence for a guanine nucleotide regulatory protein associated with the soluble binding site (14, 15). First, receptors associated with guanine nucleotide regulatory proteins often show both high- and low-affinity states of binding. With such receptors the addition of guanine nucleotide shifts the binding sites to a low-affinity state and accelerates the dissociation of the agonist. Finally, the addition of guanine nucleotide causes dissociation of the guanine nucleotide regulatory protein from the receptor itself and leads to a lower molecular weight form of the receptor. However, in some inhibitory receptors such a shift does not occur. We used these methods to examine for interaction of the soluble binding site with the guanine nucleotide regulatory protein.

[^{3}H]NECA binding to membrane-bound sites was not decreased by Gpp(NH)p up to 100 μM. In contrast 100 μM Gpp(NH)p inhibited [^{3}H]NECA binding to soluble sites to 48% of control value (Table I). Gpp(NH)p was the most potent guanine nucleotide inhibitor used (Table I). The IC_{50} for [^{3}H]NECA binding increased from 80 to 1500 nM with 100 μM Gpp(NH)p (Fig. 7). Analysis of the inhibition curves indicates that the soluble preparation has two affinity states with K_d values of 0.06 and 6.6 μM and comprising 13 and 87% of the binding sites in each affinity state, respectively. The addition of 100 μM Gpp(NH)p shifts all the receptors to a low-affinity state with a K_d of 2.9 μM. This type of guanine nucleotide sensitivity has been observed with adenosine A_1 receptors in either the membrane or the soluble state (8, 25–31).

Our dissociation studies support a role for sensitivity to guanine nucleotides as

TABLE I

INHIBITION OF [^{3}H]NECA BINDING BY Gpp(NH)p

Guanine nucleotide (μM)	Concentration (μM)	Binding (percent of control value)	
		Membrane	Soluble
None		100	100
GMP	100	—	84
GDP	100	—	87
GTP	100	—	81
Gpp(NH)p	1	100	100
Gpp(NH)p	10	100	87
Gpp(NH)p	100	100	48

Note. [^{3}H]NECA was incubated with membranes or soluble extract and 0 to 100 μM guanine nucleotide as described under Materials and Methods. The baseline specific binding for membranes was 1980 and 717 fmol/tube for [^{3}H]NECA at 40 and 10 nM, respectively. The baseline specific binding for soluble binding was 54 to 184 fmol/tube. The results are expressed as the mean values of two or three experiments performed in triplicate.

well (14, 15). After only a 30-min incubation, there is a rapid phase of dissociation. This is enhanced by the addition of 100 μM guanine nucleotide with dissociation of 70% of the binding within 2.5 min. This rapid dissociation induced by guanine nucleotide is compatible with a low-affinity state and is similar to that observed for the membrane adenosine A_1 receptor in other studies (14, 15).

The molecular basis for guanine nucleotide sensitivity was examined by studying the chromatographic properties of the agonist prelabeled binding site with and without guanine nucleotide. The binding site was prelabeled with 0.5 μM [^{3}H]NECA, 0.5 μM [^{3}H]NECA, and 100 μM Gpp(NH), or 0.5 μM [^{3}H]NECA plus 1 mM chloroadenosine. Gel permeation chromatography was performed in a buffer containing 1% sodium cholate (Fig. 8). The site prelabeled with or without guanine nucleotide had a similar elution profile. Virtually all the binding was removed by the addition of chloroadenosine.

Two major peaks of activity were evident. One peak eluted between 669 and 240 kDa. The lower molecular peak coeluted with aldolase and had an estimated molecular weight of 150,000. The relative quantity of the two peaks could be manipulated by changing the amount of detergent. When chromatography was performed with the elution buffer containing only 0.15% sodium cholate, the higher molecular weight form predominated and the fractions containing it had an opaque appearance. This suggests the possibility of aggregation or vesicle formation.

The observations are presumed to reflect the adenosine A_1 binding site, since the elution time was in excess of 20 min. Adenosine A_2-like binding is virtually totally dissociated within 1 min and would not show any binding at the peak elution time (12, 16).

Distinction from Adenosine A_2 Binding Sites

Since our previous studies of placental membranes provided evidence for adenosine A_2-like binding sites, we were suprised that the soluble binding sites had the properties of an adenosine A_1 receptor. We

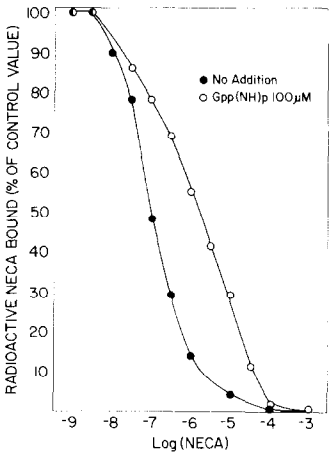

FIG. 7. Effect of Gpp(NH)p on [^{3}H]NECA binding to soluble binding sites. One milligram of soluble protein extract was incubated with 20 nM [^{3}H]NECA with or without 100 μM Gpp(NH)p for 150 min under the conditions described in Fig. 1. The IC_{50} for this agonist binding is 80 nM. With the addition of 100 μM Gpp(NH)p the IC_{50} increased from 80 nM to 1500 μM.

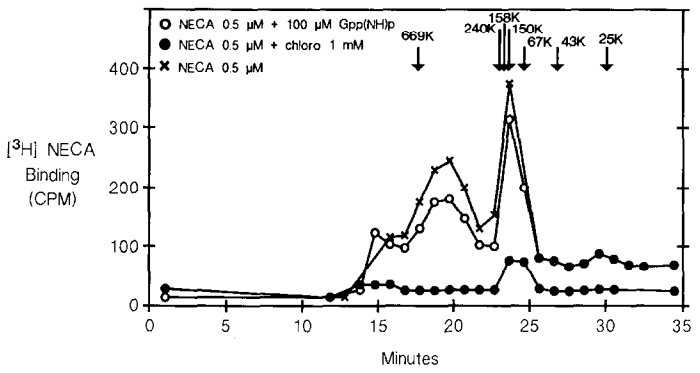

FIG. 8. Elution profile of adenosine A_1 binding site on Superose 12 column. Soluble placental membrane extract (10 mg) was prelabeled by incubation with 0.5 μM [^{3}H]NECA without or with 1 mM chloroadenosine or 100 μM Gpp(NH)p in a total volume of 300 μl. The sample was desalted rapidly on a small Sephadex G-25 column, filtered, and injected into a analytical Superose 12 HR10/30 column equilibrated with 100 mM NaCl, 1% sodium cholate, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.4. The column was eluted at 0.5 ml/min. Two peaks of radioactivity were detected, which were inhibited by 1 mM chloroadenosine. Gpp(NH)p did not modify the profile. Molecular weight standards were eluted as indicated by the arrows. These included thyroglobulin (669,000), catalase (240,000), alcohol dehydrogenase (158,000), aldolase (150,000), bovine serum albumin (67,000), ovalbumin (43,000), and α-chymotrypsinogen (25,000). There is no molecular weight adjustment for detergent effects upon the adenosine binding site. Therefore, all estimates must be considered apparent molecular weight values.

performed additional studies to prove whether adenosine A_1 binding sites were indeed present in the soluble extracts. Binding sites were incubated with *N*-ethylmaleimide which selectively inactivates adenosine A_1 receptors but not adenosine A_2 receptors (32, 33). Next placental membrane binding was studied using an adenosine A_1 selective agonist and antagonist. The binding properties of these two compounds were examined in detail with competition binding assays using agonists and antagonists. Finally, we solubilized placental membranes and reconstituted them into phospholipid vesicles. The reconstituted binding sites were examined for pharmacologic properties using rapid vacuum filtration to allow detection of adenosine A_2-like binding sites. The results of these experiments are described below.

Placental membranes were treated with 1 mM *N*-ethylmaleimide and made soluble (32, 33). Assays at 23°C indicated 67% inhibition with *N*-ethylmaleimide treatment, while there was no inhibition of binding at 4°C (Table II). Assays at 23°C would tend to measure adenosine A_1 binding sites optimally, while assays at 4°C would favor the measurement of adenosine A_2-like binding sites. This suggests that a major component of the soluble receptor assayed at 23°C is a high-affinity adenosine A_1 binding site, since 1 mM *N*-ethylmaleimide inactivates adenosine A_1 but not A_2 receptors (32, 33).

Placental membranes were examined for evidence of an A_1 binding site with an assay optimized for the adenosine A_1 receptor. Using 1 mg protein per assay, small amounts of specific binding of [^{3}H]CHA (A_1-selective agonist) and [^{3}H]CPX (A_1-selective antagonist) were detected. Both ligands showed about 200 cpm of specific binding and an additional 100 to 300 cpm of nonspecific binding. These levels of specific binding, although much less than those observed in rat brain (17, 18), nevertheless were consistent enough to allow reliable affinity determinations for adenosine agonists and antagonists (Table III). Affinities of compounds in both binding assays were indicative of an A_1 adenosine binding site. Both CHA and CPX had several-fold lower

TABLE II

INHIBITION OF SOLUBLE PLACENTAL MEMBRANE ADENOSINE BINDING BY *N*-ETHYLMALEIMIDE

Assay condition	[^{3}H]NECA binding (fmol/mg)	
	No addition	*N*-Ethylmaleimide (1 mM)
23°C	75 (66-83)	25 (21-29)
4°C	12 (8-17)	23 (23)

Note. Placental membranes (2 to 4 mg/ml) were incubated in 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.4, with or without 1 mM *N*-ethylmaleimide for 15 min at 37°C. At the end of the incubation the membranes were diluted with an equivalent volume of buffer containing 1 mM dithiothreitol and washed once with the incubation buffer. Untreated membranes were diluted and washed once with incubation buffer. The membranes were resuspended to 18 mg/ml in 1.5% sodium cholate, homogenized, and prepared as described under Materials and Methods. The soluble membrane extracts were assayed at either 23°C for 150 min or 4°C for 40 min. The results are the mean and range of these experiments performed in triplicate.

affinity than that previously reported in rat brain (17, 18). Therefore, the low binding of [^{3}H]CHA and [^{3}H]CPX in human placental membranes compared to rat brain was due to a combination of lower receptor density and lower affinity of the human adenosine A_1 receptor. A several-fold reduced affinity for CHA and CPX is also seen in human frontal cortex (R. F. Bruns and J. H. Fergus, unpublished observations).

JAR cells, human choriocarcinoma cells of placental origin, were assayed for evidence of a membrane-bound adenosine A_2-like binding site. This approach offered the advantage of a single cell type to study as compared to human placenta which contains a number of different cell types. Agonist potencies were similar to those observed in human placental membranes (Table IV). Binding studies of the membranes using [^{3}H]CHA and [^{3}H]CPX revealed no evidence for adenosine A_1 binding sites. Next the membranes were made soluble and restudied. No increase in affinity was evident, since the agonist IC_{50} values remained approximately the same as the membrane values. In addition, both membrane and soluble adenosine binding sites were resistant to guanine nucleotide related inhibition of binding. This result is different from that with soluble placental membranes.

TABLE III

AFFINITIES OF ADENOSINE AGONISTS AND ANTAGONISTS IN COMPETITION WITH BINDING OF [^{3}H]CHA (A_1 AGONIST) AND [^{3}H]CPX (A_1 ANTAGONIST) TO HUMAN PLACENTAL MEMBRANES

Compound	K_i (nM)	
	[^{3}H]CHA	[^{3}H]CPX
Agonists		
CPA	5.9 ± 0.4[a]	6.6 ± 0.1
CHA	7.3 ± 0.9	12.0 ± 3.5
R-PIA	7.9 ± 2.1	24 ± 10
NECA	11.0 ± 1.7	80 ± 46
S-PIA	67 ± 19	450 ± 50
Antagonists		
CPX	5.2 ± 0.4	6.4 ± 1.1
PD 113,297	230 ± 93	310 ± 100

Note. Binding of 1 nM [^{3}H]CHA (63,000 cpm) and 0.1 nM [^{3}H]CPX (38,000 cpm) was carried out in 2 ml 50 mM Tris-HCl, pH 7.7, at 25°C for 60 min with 1 mg human placental membrane protein. Nonspecific binding was determined by addition of 1 mM theophylline for [^{3}H]CHA binding and 100 μM N^6-cyclopentyladenosine for [^{3}H]CPX binding. Specific and nonspecific binding averaged respectively 171 and 125 cpm for [^{3}H]CHA and 205 and 300 cpm for [^{3}H]CPX. Concentration-inhibition curves were generated using six or more concentrations of competitor, and results shown are means ± interexperimental standard error for three or more experiments. IC_{50} values were determined by nonlinear least-squares curve-fitting to the Langmuir isotherm, and were converted to K_i values by multiplying by 0.880 for [^{3}H]CHA binding and 0.984 for [^{3}H]CPX binding according to the Cheng-Prusoff equation (45). CPX is 8-cyclopentyl-1,3-dipropylxanthine, and PD 113,297 is *N*-(3-(dimethylamino)propyl)-4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)benzenesulfonamide.

[a] Mean plus or minus the standard error of the mean.

TABLE IV

KINETIC STUDIES OF LIGAND BINDING TO PLACENTAL AND JAR CELL ADENOSINE BINDING SITES

Compound	IC_{50} (μM)	
	Placental membrane	JAR membrane
NECA	0.3	0.3
2-Chloroadenosine	2	1
R-PIA	>1000[a]	>1000[a]
3-Isobutyl-1-methylxanthine	30	30
Theophylline	200	180

Note. [^{3}H]NECA (10 to 20 nM was incubated with 100 μg membrane protein and 6 to 8 concentrations of competing ligands. The baseline specific binding was 1840 to 2670 fmol/mg protein for placental membranes and 800 to 1350 fmol/mg protein for JAR membranes. The results are expressed as the mean values of two to three experiments performed in triplicate.

[a] Highest concentration used in the experiment.

Finally, we have solubilized human placental membranes and reconstituted these proteins into phospholipid vesicles (34). Assays were performed using vacuum filtration at 4°C. Under these conditions up to 60% of the starting membrane binding activity is recovered in the vesicles. The binding properties of the vesicles are entirely identical to the membrane bound adenosine A_2-like binding previously described (12). The IC_{50} values observed are as follows: NECA, 300 nM; *R*-PIA, >1 mM; theophylline, 200 μM; and isobutylmethylxanthine, 20 μM. This indicates that adenosine A_2-like binding sites are present in soluble placental membrane extracts, but could not be assayed adequately using the polyethylene glycol precipitation method.

DISCUSSION

Our initial studies with the human placental adenosine binding site detected a low-affinity site in the membrane (12). We now report a high-affinity binding site in the placental membrane and in the soluble form whose properties resemble an A_1 binding site (8–11, 13, 25–31) by the potency order of agonists and antagonists.

We performed additional experiments to verify that the binding site properties in the soluble state are related to a small quantity of adenosine A_1 binding sites. The inhibition of the placental soluble binding at 23°C with 1 mM *N*-ethylmaleimide, the direct evidence for an adenosine A_1 binding site in the placental membranes, and the lack of increased affinity of JAR cell adenosine A_2-like binding sites upon being made soluble support the existence of adenosine A_1 binding sites in the soluble extracts. In addition, only a small proportion of total A_2-like binding sites could be detected by our soluble binding assay, which uses 23°C and slow vacuum filtration of 60 s duration following polyethylene glycol-induced precipitation. This is related to the properties of A_2-like binding sites which dissociate 60% of ligand in 20 s (12, 16) and the usual assay conditions which require 4°C rather than 23°C. Finally, up to 60% of starting placental membrane A_2-like binding sites have been recovered recently by reconstituting soluble receptors into phospholipid vesicles, thoroughly removing detergents, and assaying by standard vacuum filtration (34). Recent studies revealing multicomponent competition curves in placental membranes are best explained by the existence of an adenosine A_1 binding site in addition to the adenosine A_2-like binding site (35). It is not clear which of the different cellular components of the human placenta including endothelial cells, smooth muscle cells, blood cells, fibroblasts, chorionic cells, and trophoblastic cells, is the source of the A_1 binding. Adenosine A_1 and A_2 binding sites have also been observed together in cultured rabbit cortical collecting renal tubular cells (36).

The properties of soluble adenosine A_1 binding sites are elucidated by our experiments. The apparently low yield of soluble binding sites is related to their original comparison with the total quantity of membrane bound adenosine A_2 binding sites. In addition, the human placental adenosine A_1 binding site maintains a high (**RN**) and low (**R**) affinity state in the soluble state as demonstrated by the two-site model fit for the agonist competition

curves and the sensitivity of agonist dissociation to guanine nucleotide inhibition. Furthermore, different interconvertable states of the receptor are suggested by the variable dissociation properties of the binding site related to the duration of preincubation. This has previously been observed with glucagon and opioid receptors (37, 38).

These properties of adenosine A_1 binding sites differ from the catecholamine receptors which usually become soluble as the **R** alone (14, 15). In fact, the **RN** state has been proposed to account for properties of the rat brain soluble adenosine A_1 receptor (26, 27). High-performance liquid chromatography of the prelabeled soluble A_1 binding site indicates an apparent molecular weight of 150,000. There is no decrease in molecular weight upon the addition of guanine nucleotide. Since the soluble A_1 binding site is guanine nucleotide sensitive, this suggests that the adenosine A_1 site may remain in the **RN** state as previously proposed in the rat brain (26, 27). In addition, this observation implies that the adenosine A_1 binding site may assume a low-affinity state without dissociating from its guanine nucleotide regulatory protein. High-affinity binding in the soluble state suggestive of an **RN** conformation has been observed in other **Ni** coupled receptors including brain muscarinic and α-2 receptors (39, 40). Considering the known subunit molecular weight of the A_1 binding site of 35,000 to 38,000 (41-43) and the molecular weight of **Ni** of about 90,000 (44) we speculate that the 150,000 molecular weight species may consist of one **Ni** and one or two adenosine A_1 binding site subunits.

Our studies of the human placental soluble receptors have important implications. First, placenta has predominantly A_2-like binding sites, but our initial experiments with soluble receptors optimized small quantities of an A_1 binding site buried among a surplus of A_2 binding sites in the placental membranes. This pitfall can occur in any homogenized organ composed of more than one cell type and should always be considered in studies of solubilized receptors. A low recovery in the soluble state and a shift in properties are important clues for this type of problem. Second, we have demonstrated in tissue outside the brain that the A_1 binding site retains guanine nucleotide sensitivity in the soluble state. However, these kinetic changes are not accompanied by guanine nucleotide-mediated decreases in molecular weight. Further research is necessary to clarify the properties of adenosine A_1 and A_2-like binding sites.

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