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Separation of solubilized A_2 adenosine receptors of human platelets from non-receptor [^{3}H]NECA binding sites by gel filtration

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Summary. Human platelet membranes were solubilized with the zwitterionic detergent CHAPS (3-[3-(cholamidopropyl)-dimethylammonio]-1-propanesulfonate) and the solubilized extract subjected to gel filtration. Binding of the adenosine receptor agonist [^{3}H]NECA (5′-N-ethylcarboxamidoadenosine) was measured to the eluted fractions. Two [^{3}H]NECA binding peaks were eluted, the first of them with the void volume. This first peak represented between 10% and 25% of the [^{3}H]NECA binding activity eluted from the column. It bound [^{3}H]NECA in a reversible, saturable and GTP-dependent manner with an affinity of 46 nmol/l and a binding capacity of 510 fmol/mg protein. Various adenosine receptor ligands competed for the binding of [^{3}H]NECA to the first peak with a pharmacological profile characteristic for the A_2 adenosine receptor as determined from adenylate cyclase experiments. In contrast, most adenosine receptor ligands did not compete for [^{3}H]NECA binding to the second, major peak. These results suggest that a solubilized A_2 receptor-G_s protein complex of human platelets can be separated from other [^{3}H]NECA binding sites by gel filtration. This allows reliable radioligand binding studies of the A_2 adenosine receptor of human platelets.

Key words: A_2 Adenosine receptor – Human platelets – Radioligand binding – Adenylate cyclase

Introduction

Adenosine, a naturally occurring nucleoside, interacts with specific membrane-bound adenylate cyclase-coupled receptors. Two subtypes of adenosine receptors have been described, the A_1 receptor mediating an inhibition of adenylate cyclase and the A_2 receptor mediating a stimulation of adenylate cyclase (Van Calker et al. 1978; Londos et al. 1980). Stimulation of A_1 adenosine receptors causes a variety of physiological responses including a depression of cardiac and neural functions; A_2 receptors mediate, for example, vasodilatation and inhibition of platelet aggregation (Daly 1982).

Whereas several useful radioligands have been developed for the A_1 subtype, radioligand binding experiments with the A_2 receptor have been hampered by the lack of a suitable radioligand (Schwabe 1985). [^{3}H]NECA has been introduced as a radioligand for A_2 receptors in liver and platelet membranes (Schütz et al. 1982a, b; Hüttemann et al. 1984). However, the binding properties of this ligand did not agree with the pharmacology of A_2 receptors: Only 10%–20% of [^{3}H]NECA bound to human platelet membranes could be displaced by N^6-substituted adenosine analogues such as R-PIA and N^6-cyclohexyladenosine, which are agonists at A_2 receptors. Since about 95% of [^{3}H]NECA binding could be displaced with unlabelled NECA it was assumed that the major part of the [^{3}H]NECA binding to human platelet membranes was to nonreceptor binding sites. Due to the high amount of non-receptor [^{3}H]NECA binding the A_2 part could not be well characterized.

More recently, the A_1-selective antagonist [^{3}H]8-{4-[([{(2-aminoethyl)amino}carbonyl]methyl)oxy]phenyl}-1,3-dipropylxanthine ([^{3}H]XAC, Jacobson et al. 1986) has been used as an A_2 receptor radioligand in human platelet membranes (Ukena et al. 1986). However, despite of its higher affinity (K_D 24 nmol/l) and an agreement of the binding data with results of adenylate cyclase experiments, [^{3}H]XAC does also not permit satisfactory binding studies of A_2 receptors since it shows high levels of filter binding.

In order to achieve a more detailed characterization of the human platelet A_2 receptor by radioligand binding we attempted to separate the receptor from the non-receptor binding sites by solubilization and gel filtration.

Materials and methods

Materials. [^{3}H]NECA and [α-^{32}P]ATP were obtained from New England Nuclear, Dreieich, FRG, and Amersham Buchler, Braunschweig, FRG, respectively. [α-^{32}P]ATP was applied onto a 0.5 × 5 cm Dowex 1X2 (chloride form, Serva, Heidelberg, FRG) column; the column was washed with 30 ml 0.03 mol/l HCl, and ATP was eluted with 10 ml 0.25 mol/l HCl. The eluate was neutralized with triethanolamine base, and 2 µl of 10 mmol/l ATP was added to enhance stability. The purified [α^{32}P]ATP was stored at −20° C until use.

Sepharose CL-6B was purchased from Pharmacia, Freiburg, FRG, and CHAPS from Sigma, Deisenhofen, FRG. Adenosine analogues and all other chemicals were obtained from the sources indicated earlier (Lohse et al. 1984; Klotz et al. 1986).

Methods. Human platelet membranes were prepared as described by Hoffman et al. (1982). The solubilization of human platelet membranes was done with CHAPS as described

Abbreviations: CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate; ClA, 2-chloroadenosine; CPA, N^6-cyclopentyladenosine; DPX, 1,3-diethyl-8-phenylxanthine; NECA, 5′-N-ethylcarboxamidoadenosine; PAA, 2-phenylaminoadenosine; PIA, N^6-phenylisopropyladenosine; XAC, 8-{4-[([{(2-aminoethyl)-amino}carbonyl]methyl)oxy]phenyl}-1,3-dipropylxanthine

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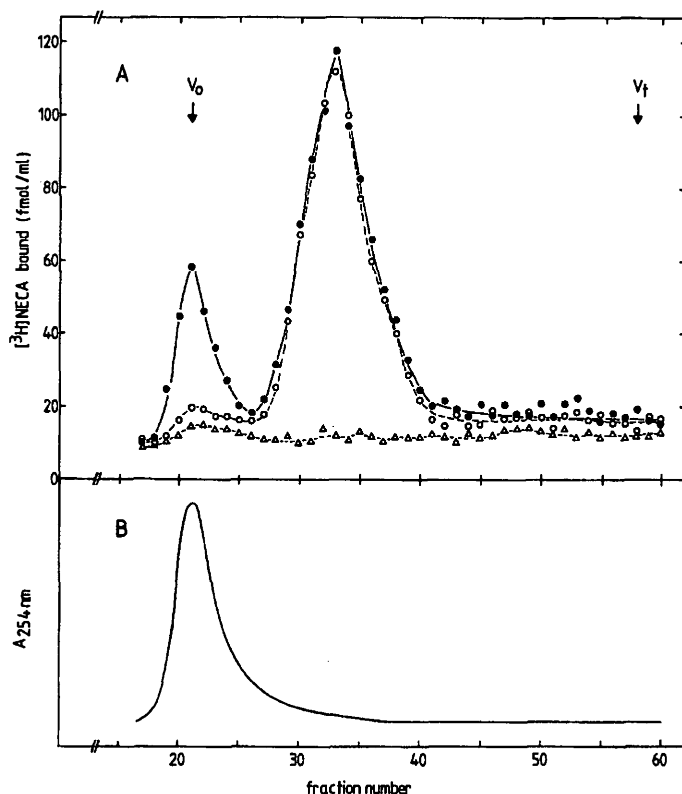

Fig. 1
A Elution profile of human platelet membrane extract from a Sepharose CL-6B column. CHAPS-extract from 40 mg membrane protein was loaded onto a 55 × 1.6 cm column and eluted with 50 mmol/l Tris-HCl buffer, pH 7.4, 10 mmol/l $MgCl_2$, 0.02% CHAPS. Fractions of 2.2 ml were collected and tested for [^{3}H]NECA binding: ●, total binding, ○, binding in the presence of 100 μmol/l R-PIA, △, binding in the presence of 100 μmol/l ClA. Data are not corrected for filter binding (corresponding to about 5 fmol/ml). B Absorbance at 254 nm (arbitrary units)

for A_1 receptors by Klotz et al. (1986): Membranes were centrifuged at 13,000 × *g* for 5 min and resuspended in 50 mmol/l Tris-HCl, pH 7.4, 10 mmol/l $MgCl_2$, containing 1% CHAPS. After centrifugation of the CHAPS-extracted membranes at 100,000 × *g* for 60 min, the supernatant was dialysed at 4° C against a 50-fold volume of 50 mmol/l Tris-HCl, pH 7.4, 10 mmol/l $MgCl_2$.

Two different Sepharose CL-6B columns (55 cm × 1.6 cm and 72 cm × 3.9 cm) were used for gel filtration at 4° C. The elution buffer was 50 mmol/l Tris-HCl, 10 mmol/l $MgCl_2$, 0.02% CHAPS. Fractions of 2.2 ml and 13 ml, respectively, were collected.

Binding assays with the eluate were done in 250 μl 50 mmol/l Tris-HCl, pH 7.4, 10 mmol/l $MgCl_2$, 0.02% CHAPS, containing 0.2 U/ml adenosine deaminase. [^{3}H]NECA with a specific activity of 20 Ci/mmol (New England Nuclear, Dreieich, FRG) was used in a final concentration of 10 nmol/l. To test the eluted fractions, 150 μl of each fraction (containing up to 600 μg protein/ml) were incubated with [^{3}H]NECA for 2 h at 0° C. For all other experiments pooled fractions were used which contained approximately 500 μg/ml in the first and approximately 30 μg/ml in the second [^{3}H]NECA binding peak. The incubation was terminated by filtration through glass fibre filters pretreated with polyethylenimine (Bruns et al. 1983; Klotz et al. 1986). Non-specific binding was defined by the presence of 100 μmol/l R-PIA; under standard conditions it amounted to 30% of total [^{3}H]NECA binding to the first peak (200 cpm and 600 cpm in typical experiments). In addition, non-specific binding was also defined by the presence of 100 μmol/l ClA; for the first peak this gave similar non-specific binding as with R-PIA, for the second peak non-specific binding was less than 10% of total (80 cpm and 1000 cpm). In saturation experiments, the specific radioactivity of [^{3}H]NECA at concentrations above 100 nmol/l was reduced to 2 Ci/mmol by addition of unlabelled NECA.

Binding of [^{3}H]NECA to human platelet membranes was done as described by Hüttemann et al. (1984).

The activity of human platelet adenylate cyclase was determined as described earlier (Klotz et al. 1985). The incubation medium contained in a total volume of 100 μl of 50 mmol/l Tris-HCl, pH 7.4, approximately 100,000 cpm [α-^{32}P]ATP, 100 μmol/l unlabelled ATP, 1 μmol/l GTP, 100 μmol/l cAMP, 1 mmol/l $MgCl_2$, 100 μmol/l EGTA, 500 μmol/l Ro 20-1724, 5 mmol/l creatine phosphate, 0.4 mg/ml creatine kinase, 2 mg/ml bovine serum albumin and 0.2 U/ml adenosine deaminase. Reactions were initiated by addition of membranes (50 μg protein) and carried out for 10 min at 37° C. Protein was determined according to Peterson (1977).

Equilibrium binding data were analyzed by non-linear curve-fitting using the program SCTFIT described by De Lean et al. (1982). Kinetic data were fitted as described by Lohse et al. (1984). All experiments were adequately fitted with a one-site model.

Results

Solubilization of platelet membranes and gel filtration over Sepharose CL-6B yielded two [^{3}H]NECA binding peaks (Fig. 1A). While the first peak representing between 10% and 25% of the total [^{3}H]NECA binding activity eluted with the void volume, the second peak containing the majority of [^{3}H]NECA binding activity eluted well behind the void volume (Fig. 1B). In both peaks [^{3}H]NECA binding was

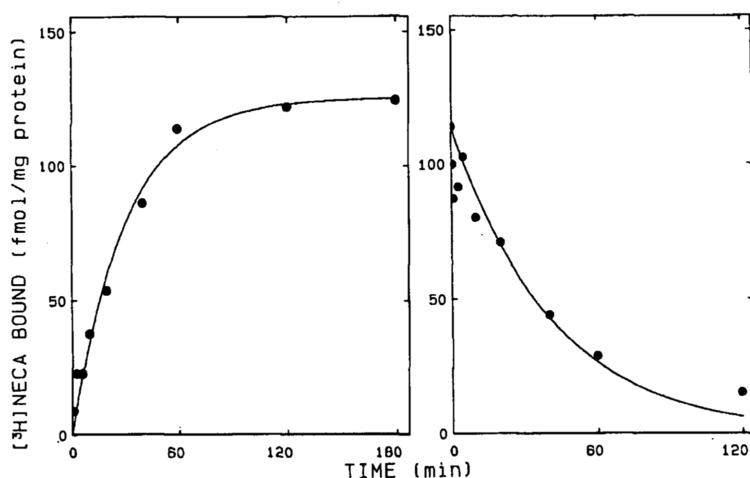

Fig. 2
Kinetics of specific [^{3}H]NECA binding to peak 1. Association and dissociation of [^{3}H]NECA were measured at 0°C. Dissociation was initiated by addition of 100 µmol/l XAC after 1 h of incubation. The data are means of three independent experiments and were fitted to mono-exponential equations

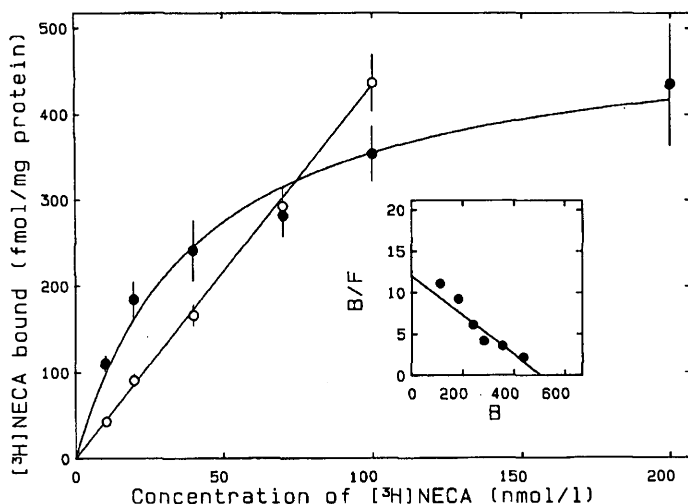

Fig. 3
Saturation of [^{3}H]NECA binding to peak 1. Data are means and SEMs of four independent experiments. ●, specific binding, ○, non-specific binding (100 µmol/l R-PIA). Nonlinear curve fitting gives a K_D-value of 46 nmol/l and a binding capacity (B_{max}) of 510 fmol/mg protein. The inset shows the Scatchard plot of the data with B, [^{3}H]NECA specifically bound (fmol/mg protein); F free [^{3}H]NECA (nmol/l)

displaced by 100 µmol/l ClA, while 100 µmol/l R-PIA displaced only [^{3}H]NECA binding to the first peak. Thus, more than 75% of the specific binding as defined by R-PIA eluted with the first peak. Fractions 20 to 24 (peak 1) and fraction 30 to 35 (peak 2) were pooled for further experiments.

Figure 2 shows the time course of [^{3}H]NECA binding to peak 1 at 0°C. Equilibrium was approximately reached after 90 min. Addition of a high concentration (100 µmol/l) of the adenosine receptor antagonist XAC caused dissociation of [^{3}H]NECA binding with a half life of 35 min. The rate constants $k_{+1} = 0.484 \times 10^6$ $1 \times mol^{-1} \times min^{-1}$ and $k_{-1} = 0.021$ min^{-1} give a kinetic K_D-value of 43 nmol/l. At 25°C binding occurred about ten times faster, but equilibrium binding was slightly lower. Peak 2 had more rapid kinetics; the association under the same conditions (0°C, 10 nmol/l radioligand) occurred with a half life of 40 s, and the dissociation after addition of 100 µmol/l unlabelled NECA had a half life of 30 s.

Specific binding of [^{3}H]NECA to the first peak was saturable in a single component (Fig. 3). A binding capacity (B_{max}) of 510 fmol/mg protein and a K_D of 46 nmol/l were estimated by non-linear curve-fitting. Nonspecific binding – defined by the presence of 100 µmol/l R-PIA – amounted to about 40% of the total [^{3}H]NECA binding at K_D. Saturation experiments to the second peak (Fig. 4) also showed a single component with a K_D-value of about 230 nmol/l and a binding capacity of 89,000 fmol/mg protein.

Competition experiments for [^{3}H]NECA binding were performed with different adenosine receptor ligands in order to characterize the two peaks. Table 1 compares the K_i-values of several agonists and antagonists determined from competition for [^{3}H]NECA binding to peak 1, peak 2 and membranes with the values obtained from adenylate cyclase experiments with platelet membranes. The K_i-values of the compounds in competing for the binding of [^{3}H]NECA to the first peak agree well with their affinities for the A_2 receptor as seen in adenylate cyclase experiments. This is true both for agonists and antagonists. In contrast, apart from NECA itself and ClA, most adenosine receptor ligands competed significantly for neither [^{3}H]NECA binding to the second peak nor [^{3}H]NECA binding to human platelet membranes.

Compounds that are inactive at A_2 adenosine receptors, such as adenine, inosine, the P-site agonist eritadenine and the nucleoside uptake blocker dipyridamole, did not compete for [^{3}H]NECA binding to the first peak in concentrations up to 100 µmol/l. In addition, these compounds did also not compete for [^{3}H]NECA binding to the second peak. Thus, it may be concluded that the specific [^{3}H]NECA binding to the first peak represents binding to the solubilized A_2 receptor.

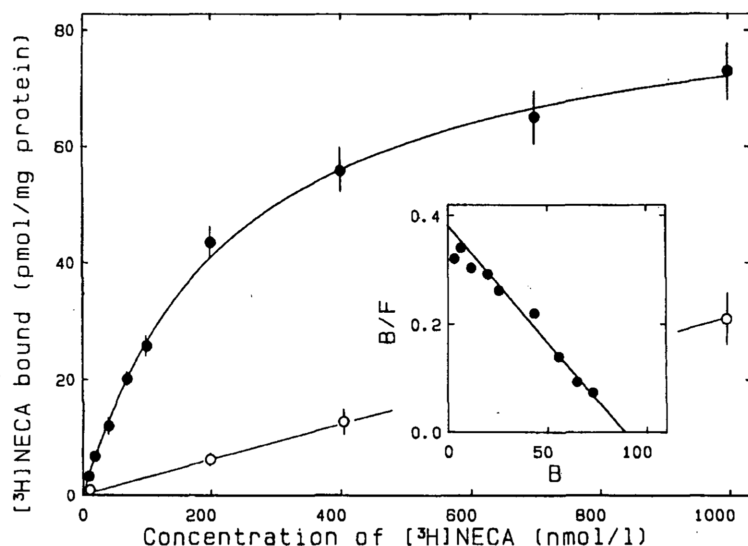

Fig. 4
Saturation of [^{3}H]NECA binding to peak 2. Data are means and SEMs of three independent experiments. ●, specific binding, ○, non-specific binding (100 μmol/l ClA). Non-linear curve fitting gives a K_D-value of 234 nmol/l and a binding capacity (B_{max}) of 89,000 fmol/mg protein. The inset shows the Scatchard plot of the data with B, [^{3}H]NECA specifically bound (pmol/mg protein); F free [^{3}H]NECA (nmol/l)

Table 1
Effects of adenosine receptor ligands on adenylate cyclase and on [^{3}H]NECA binding to peak 1, peak 2 and platelet membranes. Affinities for the A_2 receptor were determined in adenylate cyclase experiments as the EC_{50}-value for agonists and as the K_i-value from Schild plots for antagonists. Affinities for the [^{3}H]NECA binding sites are expressed as K_i-values. Data are geometric means from at least 3 independent experiments

Compound	Adenylate cyclase Platelet membranes	[^{3}H]NECA binding		
		Peak 1	Peak 2	Platelet membranes
	EC_{50} or K_i (nmol/l)	K_i (nmol/l)	K_i (nmol/l)	K_i (nmol/l)
Agonists				
NECA	190	70	330	500
PAA	350	130	8,800	6,200
R-PIA	1,300	1,700	> 100,000	> 100,000
ClA	1,900	1,000	4,100	6,300
CPA	3,000	2,400	45,000	> 100,000
S-PIA	19,000	10,000	> 100,000	> 100,000
Antagonists				
XAC	24	55	> 100,000	> 100,000
DPX	210	180	> 100,000	> 100,000
Theophylline	13,000	20,000	> 100,000	> 100,000

To investigate a remaining coupling of the solubilized A_2 receptor to the guanine nucleotide regulatory protein G_s, we tested the effects of GTP. [^{3}H]NECA binding to peak 1 was inhibited by GTP with an IC_{50}-value of 180 μmol/l; maximal inhibition (10 mmol/l GTP) was by 80% of specific binding. In dissociation experiments this inhibition by 10 mmol/l GTP was virtually complete after 15 s, indicating an allosteric mechanism of action.

Discussion

Two [^{3}H]NECA binding peaks could be separated by gel filtration of solubilized human platelet membranes. Several observations indicate that the first peak contains the solubilized A_2 adenosine receptor; [^{3}H]NECA binding to this peak was reversible, saturable and could be displaced by R-PIA and other A_2 receptor ligands. A number of adenosine receptor ligands showed almost the same rank order of potency as found for the A_2 receptor in adenylate cyclase assays with human platelet membranes. The second peak contains the non-receptor NECA binding sites of human platelet membranes: [^{3}H]NECA binding to this peak was not inhibited by R-PIA and most other A_2 receptor ligands. This resembles the binding of [^{3}H]NECA to human platelet membranes. In addition, the kinetics of [^{3}H]NECA binding to peak 2 and to platelet membranes (Hüttemann et al. 1984) are similarly rapid. The large proportion of [^{3}H]NECA binding sites in peak 2 represent the high amount of non-receptor binding of [^{3}H]NECA to platelet membranes. Separation of the A_2 receptor from these non-receptor binding sites by gel filtration allows radioligand binding studies to A_2 receptors with a reasonable ratio of specific to non-specific binding: under standard conditions non-specific binding to peak 1 (as defined by 100 μmol/l R-PIA) amounts to approximately 30% of total binding. The binding assays with human platelet membranes, however, showed 90% non-specific binding for [^{3}H]NECA (Hütte-

mann et al. 1984) and more than 75% for [^{3}H]XAC (Ukena et al. 1986).

The inhibition of [^{3}H]NECA binding by GTP suggests that the A_2 receptor remains at least in part coupled to the G_s-protein after solubilization. Similar results have been obtained with the A_1 receptor coupled to the inhibitory guanine nucleotide regulatory protein G_i (Gavish et al. 1982; Klotz et al. 1986). The IC_{50}-value of 180 µmol/l in inhibiting [^{3}H]NECA binding to the first peak is similar to the IC_{50}-value in inhibiting [^{3}H]PIA binding to solubilized A_1 receptors in the presence of 10 mmol/l $MgCl_2$ (140 µmol/l). In contrast to the results obtained with A_1 receptors, GTP did not completely abolish agonist binding to the solubilized A_2 receptor. This may indicate differences in coupling of the two adenosine receptor subtypes to their respective G-proteins.

In analogy to the A_1 receptor (Lohse et al. 1984), the effects of GTP would suggest the presence of a low agonist affinity state of the A_2 receptor. However, the linear Scatchard plot of the saturation experiment indicates that the affinity of such a state is too low to be detected by the binding assay used.

For the A_1 receptor there is a generally good correlation between agonist potencies in adenylate cyclase inhibition and their affinities for the low affinity state (Lohse et al. 1986). In contrast, our experiments show that for the A_2 receptor there is a good correlation of potencies in adenylate cyclase stimulation and the high affinity state as seen in the binding assay with [^{3}H]NECA. It remains to be seen, whether this is due to a different coupling mechanism as suggested above, or whether it is related to the higher concentration of GTP in A_1 receptor adenylate cyclase assays (10 µmol/l) compared to A_2 receptor assays (1 µmol/l).

The nature of the [^{3}H]NECA binding sites eluting as peak 2 remains to be elucidated. The high binding capacity suggests that it may represent a major protein of human platelet membranes. The relatively high-affinity of NECA and ClA for this peak suggest the presence of a purine binding domaine, which does, however, recognize neither N^6-substituted adenosine derivatives nor xanthines.

Binding assays to A_2 receptors with [^{3}H]NECA have been reported with membranes from corpus striatum after elimination of the A_1-component of [^{3}H]NECA binding with N-ethylmaleimide (Yeung and Green 1984) or CPA (Bruns et al. 1986) and with membranes of PC12 cells (Williams et al. 1987). These tissues appear to be particular in that they contain little if any non-receptor binding sites for [^{3}H]NECA. In membrane preparations from peripheral tissues, [^{3}H]NECA binds almost exclusively to non-receptor sites (Schütz et al. 1982a, b; Hüttemann et al. 1984). This is a major problem in the case of human platelet membranes, which have been used by many authors as the prototype source of A_2 adenosine receptors. However, after separation from these non-receptor binding sites the A_2 receptor of human platelets shows binding characteristics which are very similar to the striatal A_2 receptor, for which the subtype A_{2a} has been proposed (Bruns et al. 1986). This suggests that the A_2 receptor of human platelets is of the same high affinity A_{2a} subtype.

The results show that separation of the A_2 receptor from the non-receptor [^{3}H]NECA binding sites can be obtained by gel filtration which allows reliable binding studies of the A_2 receptor of human platelets.

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