

G-PROTEIN-COUPLED A₁ ADENOSINE RECEPTORS IN COATED VESICLES OF MAMMALIAN BRAIN: CHARACTERIZATION BY RADIOLIGAND BINDING AND PHOTOAFFINITY LABELLING

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Abstract—A₁ adenosine receptors in coated vesicles have been characterized by radioligand binding and photoaffinity labelling. Saturation experiments with the antagonist 8-cyclopentyl-1,3-³H]dipropyl-xanthine (³H]DPCPX) gave a K_d value of 0.7 nM and a B_{max} value of 82 ± 13 fmol/mg protein. For the highly A₁-selective agonist 2-chloro-*N*⁶-³H]cyclopentyladenosine (³H]CCPA) a K_d value of 1.7 nM and a B_{max} value of 72 ± 29 fmol/mg protein was estimated. Competition of agonists for ³H]DPCPX binding gave a pharmacological profile with *R-N*⁶-phenylisopropyladenosine (R-PIA) > CCPA > S-PIA > 5'-*N*-ethylcarboxamido-adenosine (NECA), which is identical to brain membranes. The competition curves were best fitted according to a two-site model, suggesting the existence of two affinity states. GTP shifted the competition curve for CCPA to the right and only one affinity state similar to the low affinity state in the absence of GTP was detected. The photoreactive agonist 2-azido-*N*⁶-¹²⁵I-p-hydroxyphenylisopropyladenosine (¹²⁵I]AHPIA) specifically labelled a single protein with an apparent molecular weight of 35,000 in coated vesicles, which is identical to A₁ receptors labelled in brain membranes. Therefore, coated vesicles contain A₁ adenosine receptors with similar binding characteristics as membrane-bound receptors, including GTP-sensitive high-affinity agonist binding. Photoaffinity labelling data suggest that A₁ receptors in these vesicles are not a processed receptor form. These results confirm that A₁ receptors in coated vesicles are coupled to a G-protein, and it appears that the A₁ receptor systems in coated vesicles and in plasma membranes are identical.

Key words: Adenosine receptors, coated vesicles, G-protein, radioligand, photoaffinity labelling, brain membranes.

INTRODUCTION

COATED vesicles are organelles which serve as vehicles for selective transport of macromolecules between different cell compartments [1, 2]. They have been shown to play a role in the regulation of the function of a variety of

receptors, e.g. for growth factors, hormones and cell nutrients. Ligands for these receptors are internalized along with their receptors in a process called receptor-mediated endocytosis (for review see [3-5]). Some reports have also shown the presence in coated vesicles of receptors that are coupled to guanyl nucleotide-binding proteins (G-proteins), like opioid receptors [6], β -adrenergic receptors [7], muscarinic cholinergic receptors [8] and A₁ adenosine receptors [9], indicating that these receptors might be internalized via a similar mechanism.

Receptor sequestration and down-regulation are regulatory processes which have been extensively investigated for β -adrenergic receptors [10, 11]. The exact mechanism of receptor sequestration or internalization is unclear so

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Abbreviations: AHPIA—2-azido-*N*⁶-p-hydroxyphenylisopropyladenosine; CCPA—2-chloro-*N*⁶-cyclopentyladenosine; DPCPX—8-cyclopentyl-1,3-dipropylxanthine; MES—2-(*N*-morpholino)ethanesulphonic acid; NECA—5'-*N*-ethylcarboxamidoadenosine; PIA—*N*⁶-phenylisopropyladenosine; SDS-PAGE—sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

far, but the presence of β -adrenergic receptors and other members of the family of G-protein-coupled receptors suggests that coated vesicles may be involved in their translocation and redistribution. Furthermore, it has recently been shown that tyrosine residues located in the cytoplasmic tail of β_2 -adrenergic receptors are involved in agonist-induced down-regulation of the receptor [12]. The direct implication of a cytoplasmic tyrosine for endocytosis via coated vesicles which has been suggested for a number of receptors [2, 13] also points to a potential role of coated vesicles in the regulation of G-protein-coupled receptors.

In addition to the lack of clear-cut evidence for a direct participation of coated vesicles in the regulation of G-protein-coupled receptors, differences have appeared between the G-protein-coupled receptor systems characterized in coated vesicles. A_1 adenosine receptors have been shown to be still functionally coupled to adenylate cyclase [9], while β -adrenergic receptors seem to be uncoupled from adenylate cyclase [7]. Opioid receptors in coated vesicles exhibited GTP-sensitive agonist binding [6]. Agonist binding to muscarinic acetylcholine receptors, however, has been demonstrated to be insensitive to GTP [8], indicating that these receptors are uncoupled from a G-protein.

These reported differences between receptor systems in coated vesicles, which work according to similar principles in plasma membranes, led us to investigate high- and low-affinity agonist binding, including regulation by GTP, to A_1 adenosine receptors in coated vesicles in more detail; this enabled us to further establish G-protein coupling of A_1 receptors in coated vesicles. For this purpose, competition of agonists for the A_1 selective antagonist radioligand 8-cyclopentyl-1,3- 3 H-dipropylxanthine (3 H]DPCPX) [14] was measured. In addition, saturation experiments with the new agonist 2-chloro- N^6 - 3 H]cyclopentyladenosine (3 H]CCPA), which has been shown to be suitable to detect A_1 receptors that are present only in low density [15], were performed. Comparison with binding data from bovine brain membranes [16] and photoaffinity

labelling data showed that A_1 receptors in coated vesicles were similar to plasma membrane receptors and confirmed the presence of G-protein-coupled A_1 receptors in coated vesicles.

MATERIALS AND METHODS

Materials

3 H]CCPA was purchased from Du Pont-New England Nuclear (Dreieich, F.R.G.) and 3 H]DPCPX from Amersham Buchler (Braunschweig, F.R.G.). 2-Azido- N^6 -p-hydroxyphenylisopropyladenosine (AHPIA) was iodinated to give [125 I]AHPIA with a specific radioactivity of about 74 TBq/mmol [17], CCPA was synthesized as previously described [18]. All other chemicals were of the highest purity available.

Preparation of membranes and coated vesicles

Membranes from rat brain were prepared as described earlier [19]. Coated vesicles from bovine brain have been prepared as described recently [9]. The same procedure was used for the isolation of rat brain coated vesicles. Briefly, whole brains from 25–30 rats (≈ 35 g) were homogenized in isolation buffer [10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES)/NaOH, 100 mM KCl, 1 mM EGTA, 0.5 mM MgCl₂, 0.1 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride, 0.02% NaN₃, 7.3 mU/ml bacitracin, pH 6.5] at a ratio of 1:2 (w/v). After differential and density centrifugations the crude coated vesicles were loaded onto a Sephacryl S-1000 column (1.6 \times 70 cm) equilibrated in isolation buffer. Coated vesicles were collected from peak II by centrifugation at 100,000 g for 30 min. This preparation yielded 1–1.5 mg of coated vesicles.

Radioligand binding

Binding of 3 H]DPCPX was performed according to Lohse *et al.* [14] and binding of 3 H]CCPA was performed as described recently [15]. In competition experiments, 1 nM 3 H]DPCPX with about 100 μ g protein in a total volume of 250 μ l was used. Nonspecific binding of 3 H]DPCPX and 3 H]CCPA was determined in the presence of 10 μ M *R-N*⁶-phenylisopropyladenosine (R-PIA) and 1 mM theophylline, respectively. Adenosine deaminase was present in all binding assays at a concentration of 0.2 U/ml. Each value was determined in duplicate. Data were analysed by nonlinear curve-fitting with the program SCTFIT as described [14].

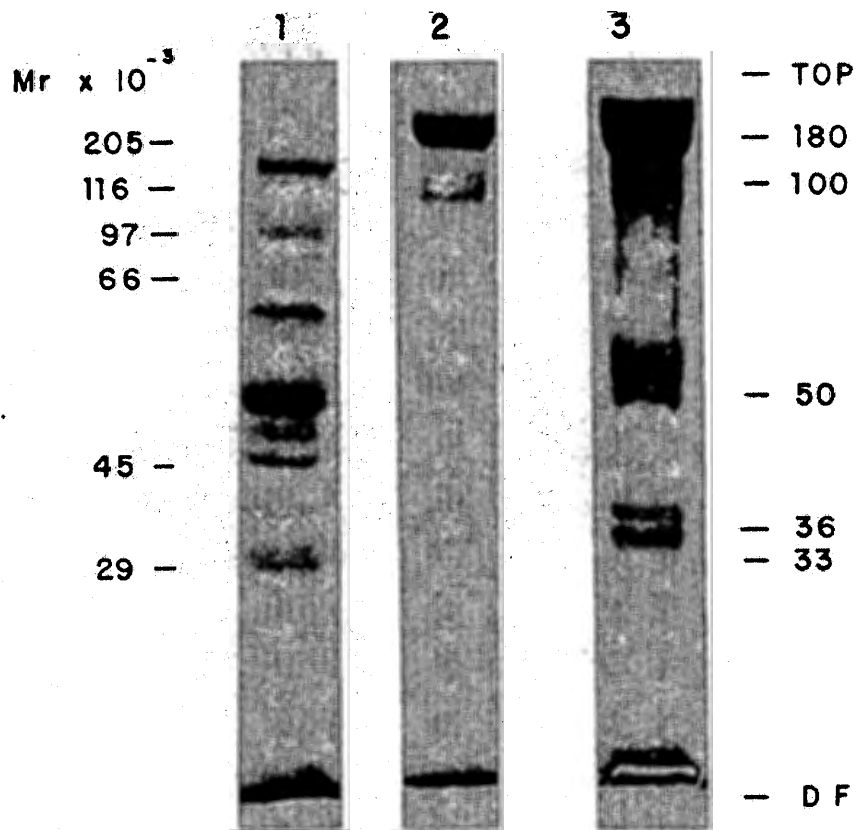


FIG. 1. SDS-PAGE of coated vesicles purified on Sephacryl S-1000. Two-hundred micrograms of protein was loaded onto a 10% gel and the gel was stained with Coomassie Blue. Lane 1: rat brain membranes; lane 2: coated vesicles from rat brain; lane 3: coated vesicles from bovine brain.

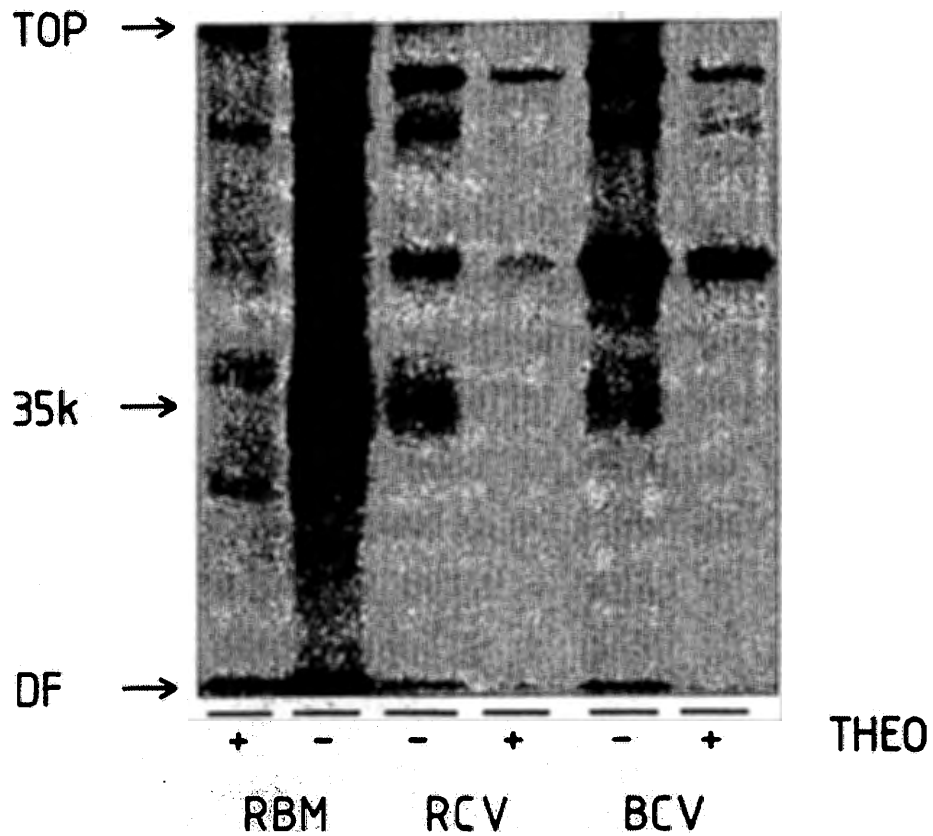


FIG. 5. Photoaffinity labelling of A_1 adenosine receptors in coated vesicles from bovine and rat brain. A_1 receptors were photoaffinity labelled as outlined in Materials and Methods. In coated vesicles from bovine (BCV) and rat brain (RCV) labelling of a band with a molecular weight of 35,000 is inhibited in the presence of 1 mM theophylline (THEO), indicating specific labelling. For comparison, labelling of rat brain membranes (RBM) is shown.

Photoaffinity labelling

Photoaffinity labelling of A₁ receptors in brain membranes was performed with [¹²⁵I]AHPIA at a concentration of about 200 pM as described [17, 20]. In brief, membranes (100 μg protein) or coated vesicles (200–400 μg protein) were incubated for 2–3 h with [¹²⁵I]AHPIA (500,000 c.p.m.) in a total volume of 750 μl in the dark in the presence and absence of 1 mM theophylline to define nonspecific labelling. Then membranes were UV-irradiated and prepared for SDS-PAGE on 10% gels. The gels were stained with Coomassie Blue and then dried for autoradiography [17].

RESULTS

Highly purified coated vesicles have been obtained by density gradient centrifugation and Sephacryl S-1000 gel filtration from bovine and rat brain. Figure 1 shows the distinguished protein pattern of coated vesicles (lane 2) compared to the microsomal membrane fraction (lane 1) from rat brain. It is also shown that the coated vesicle preparation from rat brain (lane 2) gave results comparable to the

TABLE 1. SATURATION DATA FOR THE ANTAGONIST [³H]DPCPX AND THE AGONIST [³H]CCPA

Radioligand	K _d value (nM)	B _{max} value (fmol/mg)
[³ H]DPCPX	0.69 (0.34–1.41)	82 ± 13
[³ H]CCPA	1.67 (0.72–3.85)	72 ± 29

Shown are mean values from three experiments, K_d values with 95% confidence limits and B_{max} values ± S.E.M.

well-established preparation of coated vesicles from bovine brain (lane 3; see also [9]).

Coated vesicles from bovine brain membranes bound the A₁ receptor-selective antagonist [³H]DPCPX with high affinity and in a saturable manner (Fig. 2). The saturation experiments gave a K_d value of 0.69 nM and a B_{max} value of 82 fmol/mg protein (Table 1). This affinity is similar to the K_d value of 0.22 nM determined in bovine brain membranes [16]. Competition of several agonists for [³H]DPCPX binding resulted in biphasic competition curves,

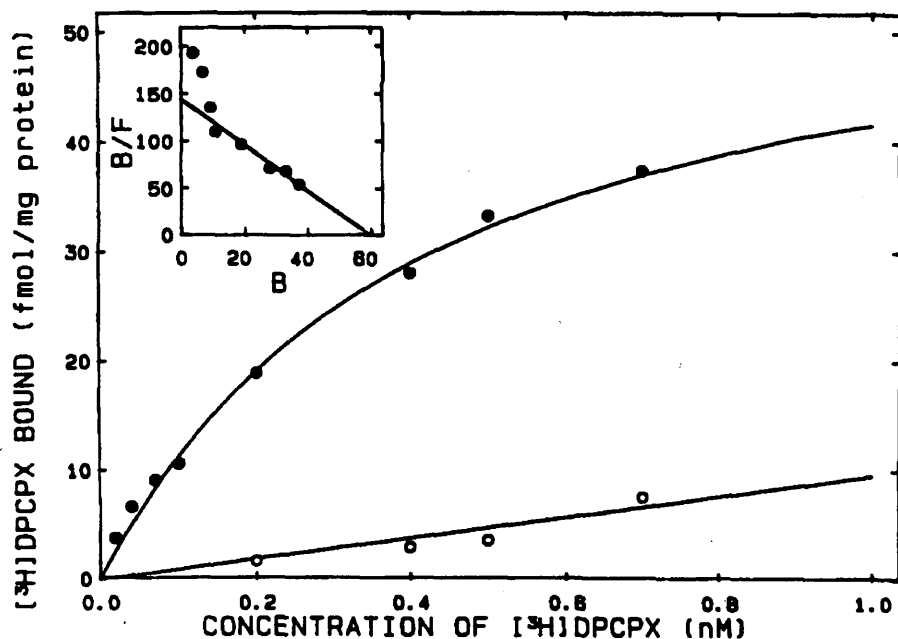


FIG. 2. Saturation of [³H]DPCPX binding to coated vesicles from bovine brain. Specific (●) and nonspecific binding (○) of the antagonist [³H]DPCPX are shown from a representative experiment. Nonlinear curve fitting gave a K_d value of 0.4 nM and a B_{max} value of 59 fmol/mg protein. A Scatchard plot of the data is shown in the inset.

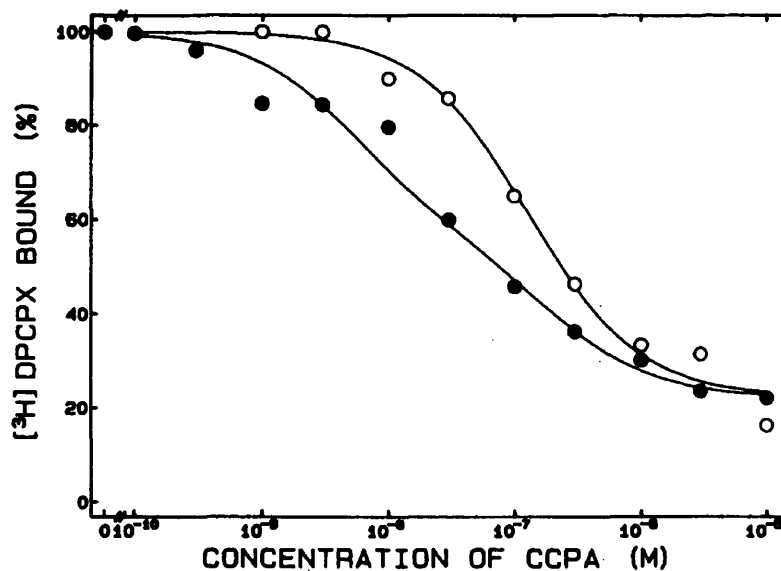


FIG. 3. Competition of CCPA for [^3H]DPCPX binding to coated vesicles from bovine brain in the presence or absence of GTP. Shown is total binding of [^3H]DPCPX in the absence (●) and in the presence of $100\ \mu\text{M}$ GTP (○) from a representative experiment. Nonlinear curve fitting of the control curve gave K_d values of 1.6 and 53 nM for the high- and low-affinity states, respectively, with 55% of the receptors being in a high-affinity state. A two-site fit was significantly better than a one-site fit ($P < 0.005$). In the presence of GTP only one affinity state was detected with a K_d value of 37 nM.

indicating high- and low-affinity binding of the agonists (Table 2). About 50% of the receptors are in the high- and low-affinity states, respectively. The rank order of potency for the agonists was R-PIA > CCPA > S-PIA > 5'-*N*-ethylcarboxamidoadenosine (NECA), with a marked stereoselectivity for the PIA diastereomers, which is typical for an A_1 adeno-

TABLE 2. COMPETITION OF AGONIST FOR [^3H]DPCPX BINDING TO A_1 RECEPTORS IN COATED VESICLES FROM BOVINE BRAIN MEMBRANES

Agonist	K_i (nM)	
	High affinity	Low affinity
R-PIA	0.04 (0.01–0.15)	8.8 (6.5–12.0)
CCPA	1.1 (0.47–2.45)	60 (32–116)
CCPA + GTP	—	38 (9–167)
S-PIA	3.6 (0.6–20.9)	261 (66–1031)
NECA	77 (17–343)	2733 (656–11,390)

Data are means from three to six separate experiments, K_i values are given with 95% confidence limits in parentheses.

sine receptor. In contrast to the classical pharmacological profile for the A_1 adenosine receptor in rat brain, R-PIA is more potent than CCPA and S-PIA is more potent than NECA. However, these results match the binding data for this receptor subtype in bovine brain membranes [16].

The presence of high- and low-affinity agonist binding suggests that the receptors are G-protein coupled. To substantiate further this result, agonist competition for [^3H]DPCPX binding was done in the presence and absence of GTP. Figure 3 shows competition curves for CCPA, with a biphasic curve in the absence of GTP. In the presence of GTP, the curve is shifted to the right and only one affinity state can be detected. The high- and low-affinity K_d values for CCPA in the absence of GTP are 1.1 and 60 nM, respectively (Table 2). The single-affinity state detected in the presence of GTP exhibits a K_d value of 38 nM (Table 2) and corresponds well to the low-affinity state found in the absence of GTP.

[^3H]CCPA was also used to show agonist

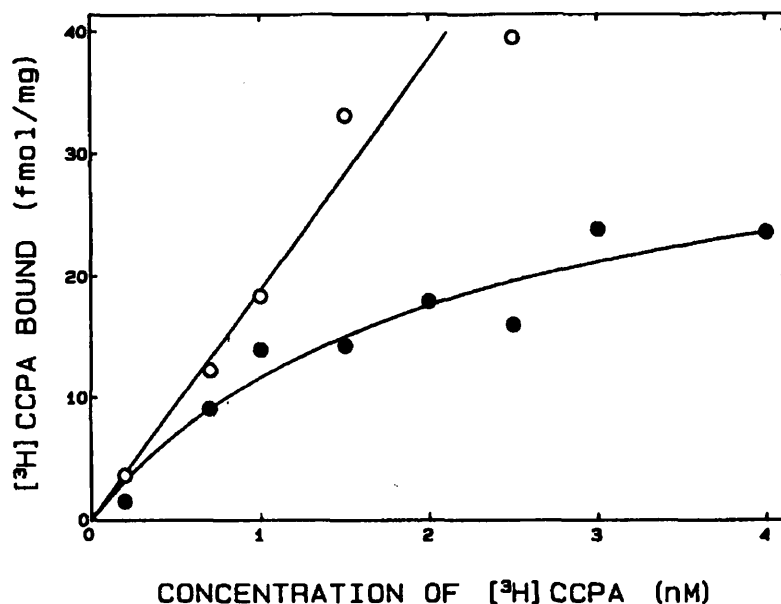


FIG. 4. Saturation of [³H]CCPA binding to coated vesicles from bovine brain. The specific (●) and nonspecific binding (○) of the agonist [³H]CCPA are shown from a representative experiment. A K_d value of 2 nM and a B_{max} value of 36 fmol/mg protein were calculated by nonlinear curve fitting. The inset shows the Scatchard plot of the data.

radioligand binding to A₁ receptors in coated vesicles. Figure 4 demonstrates that this new highly A₁-selective agonist showed saturable binding. A K_d value of 1.7 nM and a B_{max} value of 72 fmol/mg protein was determined from saturation experiments (Table 1).

To investigate possible structural differences between the A₁ adenosine receptor protein identified in coated vesicles compared to the plasma membrane receptor, the photoreactive adenosine derivative [¹²⁵I]AHPIA was used to label covalently the A₁ receptors of bovine and rat brain coated vesicles. The autoradiogram of a SDS-PAGE shows specific labelling of a broad band with an apparent molecular weight of 35,000 for both the bovine and rat coated vesicles (Fig. 5). The same band has been labelled in rat brain membranes (Fig. 5), and in bovine brain membranes [16], suggesting that A₁ receptors in membranes and coated vesicles are structurally identical. The photoincorporation of the agonist [¹²⁵I]AHPIA again indicates the presence of high-affinity A₁ receptors in a G-protein-coupled state.

DISCUSSION

In the present study, the presence of A₁ adenosine receptors in coated vesicles in a G-protein-coupled state has been confirmed. Agonist competition for [³H]DPCPX gave biphasic curves with high- and low-affinity binding of a series of agonists. About 50% of the receptors were found in the high-affinity state in the absence of GTP, while GTP shifted virtually all receptors to the low-affinity state. The GTP regulation of agonist binding is indicative for interaction of A₁ receptors with a G-protein. The detection of high-affinity binding of the A₁-selective agonist [³H]CCPA corresponds with these findings.

Opioid receptors in coated vesicles also exhibit GTP-sensitive agonist binding [6]. High-affinity agonist binding has been demonstrated for muscarinic acetylcholine receptors in coated vesicles from bovine brain, although the significance of this high-affinity state is unclear. It seems to be unrelated to a G-protein-coupled state, because no GTP sensitivity of agonist

binding has been found [8]. β -Adrenergic receptors in coated vesicles, on the other hand, are uncoupled from adenylate cyclase and agonist binding is GTP insensitive [7], although G_s is present in coated vesicles [21]. These differences may be explained by the different G-proteins which couple these receptors to an effector system. There is evidence for tighter coupling of A_1 receptors, and possibly other G_i -coupled receptors, to G_i compared to interaction of G_s -coupled receptors with G_s . The GTP regulation of agonist binding to A_1 adenosine receptors is preserved during solubilization [22, 23], and A_1 receptors co-purify with G-proteins [24], suggesting tight receptor-G-protein association. Solubilization of G_s -coupled β -adrenergic receptors in the absence of an agonist, on the other hand, induces loss of high-affinity agonist binding [25]. It is also conceivable that the microenvironment of receptors and G-proteins plays a role in their coupling and, thus, receptor-G-protein coupling for different receptors might be differently affected upon their translocation from plasma membranes into coated vesicles.

Although the pharmacological profile for A_1 receptors in coated vesicles determined in this study matches the profile found in bovine brain membranes [16], some differences were observed for the K_d values for CCPA and NECA. These agonists exhibited six- to seven-fold higher K_d values in coated vesicles compared to membranes. The same difference was observed for the K_d values for [3 H]CCPA. These differences may be explained by different microenvironments of the receptor proteins in coated vesicles vs membranes [26]. It is also not clear whether the G-protein composition in the coated vesicles is the same as in plasma membranes. It has been shown that A_1 adenosine receptors can interact with more than one G-protein [24]. Coupling of the receptor to different G-proteins could, therefore, also play a role in determining slightly different affinities for some agonists in coated vesicles vs membranes.

The density of receptors found in coated vesicles is only about 10% of the density found

in brain membranes (Table 1; [16]). It is, however, unlikely that the receptors found in coated vesicles represent a contamination of coated vesicles with plasma membranes. All major protein bands present in plasma membranes are virtually absent in the coated vesicle preparation (Fig. 1). Even if we assume a 1% contamination with plasma membranes this could only account for 10% of the A_1 receptors detected in coated vesicles. The functional significance of the receptors in coated vesicles could be based on a high turnover rate of the receptors at a low steady state concentration.

G-protein coupling of A_1 receptors in coated vesicles and, hence, high-affinity binding of agonists enabled us to characterize these receptors by photoaffinity labelling with the agonist [125 I]AHPIA [17]. Comparison of receptors labelled in bovine brain coated vesicles, rat brain coated vesicles and rat brain membranes, revealed that they all have an apparent molecular weight of 35,000. This suggests that the receptors in coated vesicles are not a processed form. Successful agonist photoaffinity labelling in both rat and bovine brain coated vesicles supports that A_1 receptors are G-protein-coupled in both species.

The data in this paper show that the A_1 receptor protein in coated vesicles of bovine and rat brain is virtually indistinguishable from receptors in the plasma membrane and thus structurally intact. High- and low-affinity agonist binding have been demonstrated and, in addition, agonist binding is GTP regulated. Taken together with the previous finding of A_1 receptor-mediated inhibition of adenylate cyclase in coated vesicles [9], our present results confirm that the entire A_1 receptor-effector system is present in these organelles. This may suggest that coated vesicles do indeed play a role in translocation and regulation of G-protein-coupled receptors.

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