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The effects of saponin on the binding and functional properties of the human adenosine A_1 receptor

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1 Experiments with adenosine deaminase suggest that adenosine is present in membrane preparations from CHO cells bearing adenosine A_1 receptors.

2 Pretreatment of the membranes (ca 0.6 mg protein ml^{-1}) with the permeabilizing agent saponin ($100\,\mu g\,ml^{-1}$) or addition of saponin ($10\,\mu g\,ml^{-1}$) to the membranes (0.02-0.08 mg protein ml^{-1}) in the assay, generates homogeneous low affinity agonist binding curves in the presence of GTP and an increased function, assessed by agonist stimulation of [^{35}S]-GTPγS binding. The affinity constants for the binding of an agonist and an antagonist are not affected by this saponin treatment. Saponin facilitates the interaction of guanine nucleotides with receptor G-protein complexes, possibly by removing a permeability barrier to access of G-proteins by GTP. However, adenosine is still present in the binding assays after saponin treatment.

3 The agonist binding properties of the human A_1 receptor have been characterized. In saponin pretreated membranes, 80-90% of the A_1 receptors are capable of forming agonist-receptor-G protein complexes in the absence of GTP. These complexes have a 300-600 fold higher affinity than uncoupled receptors for N^6-cyclohexyladenosine.

4 A very slow component is observed in the association and dissociation kinetics of the agonist [^{3}H]-N^6-cyclohexyladenosine ([^{3}H]-CHA) and in the association but not dissociation kinetics of the antagonist [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-DPCPX). The slow association component of [^{3}H]-DPCPX is essentially absent when incubations are carried out in the presence of GTP. The slow dissociation component of [^{3}H]-CHA binding is rapidly disrupted by GTP.

5 It is hypothesized that long-lasting adenosine-receptor-G protein complexes are present in the CHO membrane preparations. The existence of these complexes, resistant to the action of adenosine deaminase but sensitive to GTP, may rationalize the observed kinetics and the increase in ^{3}H-antagonist binding produced by GTP which has been observed in essentially all studies of A_1 receptors and has been ascribed previously to precoupling of A_1 receptors to G-proteins in the absence of agonists.

Keywords: Saponin; adenosine; adenosine A_1 receptors; receptor-G-protein coupling

Introduction

Investigations of the binding properties of A_1 adenosine receptors in membrane preparations from mammalian tissues have been complicated by the presence of endogenous adenosine during the assay. Nanomolar concentrations of adenosine have been measured and it has been postulated that the adenosine is released from small sealed vesicles present in both membrane and even soluble preparations (Schiemann *et al.*, 1990; Prater *et al.*, 1992). In all but the earliest reported binding studies, adenosine deaminase has been added to the incubations in order to increase radioligand binding by elimination of the endogenous adenosine (Linden, 1989).

Saponins permeabilize different membrane structures at different concentrations. At low concentrations, they have been widely used to permeabilize the plasma membrane of different cells (see for example Burgess *et al.*, 1983; Wassler *et al.*, 1987). Higher concentrations of saponins also permeabilize intracellular membrane vesicles (Willingham *et al.*, 1978; Wassler *et al.*, 1987).

During the course of attempts to permeabilize putative vesicular structures in preparations of CHO cell membranes, we discovered that saponin could simplify the binding properties of the transfected human A_1 adenosine receptor and facilitate the characterization of its binding and functional properties. A preliminary account of some of these data has been published (Cohen *et al.*, 1994).

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Methods

Cell culture

Cells were grown in 1:1 mixture of DMEM and HAMS F-12 media containing 10% new born calf serum, 2 mM L-glutamine and geneticin (0.25 mg ml^{-1}) at 37°C in 5% CO_2. Cells were subcultured twice weekly at a ratio of 1:10 and once weekly the cells were transferred to large 24 x 24 cm plates.

Membrane preparation

Cells were washed with PBS and scraped from the plates in 5 ml of ice-cold homogenization buffer (20 mM Tris, 10 mM EDTA, pH 7.4, +saponin 0-1 mg ml^{-1}). Cells were disrupted in a Polytron homogenizer (setting 6) at 4°C, twice for 5 s at a 30 s interval. Plasma membranes and the cytosolic fraction were separated by centrifugation at 100,000 *g* for 15 min. The membrane pellet was resuspended in buffer (20 mM Tris, 0.1 mM EDTA, pH 7.4), again disrupted in a Polytron (5 s) and the homogenate centrifuged at the same speed for 15 min. The pellet was resuspended in binding assay buffer at approximately 3 mg protein ml^{-1} and stored in 0.5 ml aliquots at -70°C. Protein determinations were performed with the Bio-Rad protein assay, using BSA as a standard. The yield of protein was approximately 3 mg protein/plate.

Radioligand binding

All binding assays were performed in triplicate in a final volume of 1 ml. For the saturation studies, the radioligand concentrations varied from 0.02-6 nM for [^{3}H]-DPCPX and

0.1–35 nM for both [^{3}H]-CHA and [^{3}H]-PIA. For the competition studies, various concentrations (0.1 nM–300 μM) of agonist were included in the assay with 0.2 nM [^{3}H]-DPCPX as radioligand. Both competition and saturation studies were performed with cell membranes that were untreated or previously treated with 3 u ml^{-1} adenosine deaminase for 30 min at 25°C. Studies were also performed both in the absence and presence of GTP (1 mM) where appropriate. Binding assays were initiated by the addition of membranes at a final protein concentration of 40–80 μg ml^{-1} in assay buffer (NaCl 100 mM, HEPES 20 mM, $MgCl_2$ 10 mM, pH 7.4). Reactions were continued for 1 h at 25°C. Non-specific binding was determined by the addition of 3 mM theophylline.

For the measurement of association kinetics, a reverse-time course strategy was used to allow batchwise processing of the assays by filtration (Hulme & Birdsall, 1992). An analogous approach was used in the dissociation kinetics measurements. Furthermore, the radioligand (0.2 nM) was always incubated with the membranes for precisely 1 h before initiation of dissociation of the radioligand by dilution in assay buffer containing 3 mM theophylline. This protocol ensured that slow association kinetics did not interfere with the estimation of the dissociation rate constants.

Bound and free ligand were separated by filtration through Whatman GF/B filter strips which were presoaked in 0.3% polyethyleneimine for 1 h. This treatment resulted in lower nonspecific binding. Filtration was performed on a cell harvester (Brandel); filters were washed 3 times with 5 ml aliquots of ice-cold water, transferred to liquid scintillation vials and 5 ml of scintillation fluid was added. Samples were counted after they were stored at room temperature for at least 6 h to permit the glass fibre filters to become uniformly translucent.

Binding studies with [^{35}S]-GTPγS

The incubation mixture for measuring [^{35}S]-GTPγS binding contained in a total volume of 1 ml: buffer (NaCl 100 mM, HEPES 20 mM, $MgCl_2$ 10 mM, pH 7.4), 10 μM GDP, 0–10 μM agonist, 20 μg ml^{-1} membrane protein (pretreated, as appropriate, with 3 u ml^{-1} adenosine deaminase for 30 min at 25°C) and 0.1 nM [^{35}S]-GTPγS. The experiment was carried out in triplicate for 30 min at 25°C. Incubations were terminated by rapid filtration of the samples through glass fibre filters (Whatman GF/B), previously soaked in ice-cold water, followed by 3 × 5 ml washes in ice-cold water.

Materials

All cell culture reagents were obtained from Gibco laboratories. Chinese Hamster Ovary cells stably transfected with the human A_1 receptor (CHO-A_1 cells) were a kind gift from Glaxo Group Research Ltd, Greenford, UK; 8-cyclopentyl-1,3-dipropyl-2,3-[^{3}H]-(N)-xanthine ([^{3}H]-DPCPX), N^6-cyclohexyl-2,8-[$^3H_2$]-adenosine ([^{3}H]-CHA) and [^{35}S]-GTPγS were from DuPont NEN. (−)-N^6-**R**-[G-^{3}H]-2-phenylisopropyladenosine ([^{3}H]-PIA) was obtained from Amersham International. N^6-cyclohexyladenosine (CHA) was from Research Biochemicals Inc, Natick, Mass, U.S.A. Adenosine deaminase (ADA) and saponin were from Sigma.

Data analysis

Unless otherwise stated, data are presented as (mean ± s.e.mean, n = number of independent experiments). For $n = 2$, the estimated error is shown as the range/2. Binding data were analysed by non-linear least squares analysis using the appropriate models and the programmes Enzfitter and Sigmaplot. A two-site model was used to analyse agonist inhibition curves. Parameters obtained by this form of analysis are numerically within 10% of the equivalent parameters obtained by fitting the data to the ternary complex model of De Lean *et al.* (1980) (Jones *et al.*, 1995).

Results

Adenosine is present in binding assays using CHO-A_1 membranes

The addition of adenosine deaminase to the incubation increased the binding of a low concentration (0.2 nM) of the radiolabelled antagonist [^{3}H]-DPCPX by 190 ± 10% ($n = 5$). In the presence of GTP (1 mM) the enhancement of binding was still present but was of a much smaller magnitude (20 ± 7%) ($n = 5$). These results are in accord with adenosine being present in the assay at a concentration at which it binds to the receptors strongly in the absence of GTP and relatively weakly in the presence of GTP.

Effects of saponin pretreatment in the binding of [^{3}H]-DPCPX in the presence and absence of ADA and GTP

CHO-A_1 cell membranes were pretreated with various concentrations of saponin during the course of preparation of the membranes. It was anticipated that saponin would permeabilize vesicular structures and remove or prevent the formation of adenosine in the assay. This change would be manifest as an attenuation of the stimulatory effects of ADA on [^{3}H]-DPCPX binding in the presence of and, particularly, in the absence of GTP.

The data (Table 1) show that the binding of [^{3}H]-DPCPX under all conditions increased with the saponin pretreatment. However the enhancement of [^{3}H]-DPCPX binding by ADA measured in the presence of GTP was unaffected and that measured in the absence of GTP was only partially attenuated at the highest saponin concentrations. This change could have been due to a lowering of endogenous adenosine levels or to a perturbation of the agonist binding properties of the A_1 receptor by the saponin pretreatment.

Effects of saponin pretreatment on the binding of CHA to A_1 receptors

The binding of the agonist CHA was measured in competition experiments with [^{3}H]-DPCPX in the absence and presence of ADA and GTP. The membranes had been pretreated with 0–1000 μg ml^{-1} saponin. The results are presented in Table 2 and Figure 1.

Control samples, those not treated with saponin, gave inhibition curves that were complex and could be fitted well to a 2-site model but not to a 1-site model. The effect of ADA was to increase the proportion and affinity of the high affinity binding component. As found with all G-protein coupled receptors, GTP decreased the fraction of the high affinity component but a high affinity binding component was still observed in the presence of GTP.

Table 1 Effect of saponin pretreatment on the binding of [^{3}H]-DPCPX (0.2 nM) to CHO-A_1 membranes, measured in the presence or absence of ADA and GTP

[Saponin] (mg ml^{-1})	*−ADA −GTP*	*+ADA −GTP*	*−ADA +GTP*	*+ADA +GTP*
0	26 ± 2	75 ± 3	82 ± 7	[100]
0.03	31 ± 2	84 ± 1	86 ± 2	112 ± 4
0.1	24 ± 2	85 ± 2	87 ± 4	112 ± 3
0.3	46 ± 4	113 ± 3	98 ± 8	117 ± 3
1.0	59 ± 6	123 ± 4	92 ± 10	123 ± 10

Data are the results of 5 experiments, all performed at the same protein concentration (30 μg ml^{-1}), and have been normalized to the control value [100] of the binding of [^{3}H]-DPCPX in the presence of ADA and GTP (10^{-3}M). Additional experimental details are described in the Methods section.

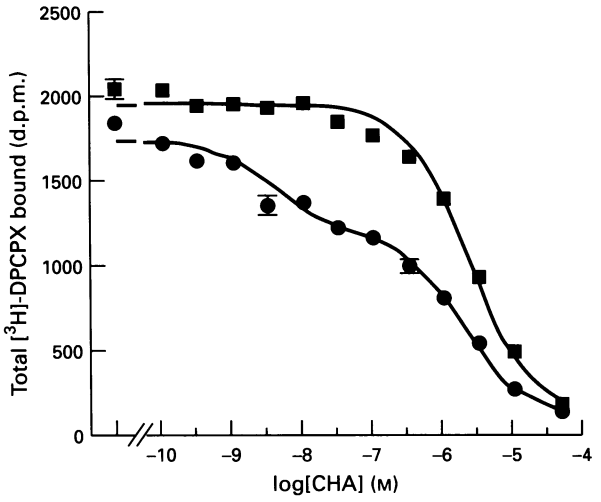


Figure 1 Effect of saporin pretreatment on the competititon by CHA of [^{3}H]-DPCPX binding to human A_1 adenosine receptors. Membranes were prepared with a pretreatment of saponin at the following concentrations: 0 μg ml^{-1} (●), 100 μg ml^{-1} (■). Binding assays were carried out by competition with a fixed concentration of [^{3}H]-DPCPX (0.2 nM) in the presence of 1 mM GTP using membranes that were previously treated with 3 u ml^{-1} ADA for 30 min at 25°C. Data points are the means of triplicate determinations of one experiment. The error bars generally fall within the data points. Curves are computer generated best fits to a 1-site or 2-site model, as appropriate. Parameter estimates from two to five experiments are summarized in Table 2.

A dramatic change in the CHA binding properties of the A_1 receptors in the presence of GTP was detected in membranes treated with 0.1–1 mg ml^{-1} saponin (Figure 1, Table 2): the inhibition curves became simple mass action curves. However, no change was detected in the complex CHA-inhibition curves in the absence of GTP (Table 2).

The affinity constant for the low affinity component was unchanged by pretreatment with up to 100 μg ml^{-1} saponin but showed a tendency to decrease in membranes pretreated with 0.3 and 1 mg ml^{-1} saponin. This decrease in agonist affinity is in accord with the observed decrease in inhibition produced by endogenous adenosine at these saponin concentrations (Table 1). High affinity agonist binding, monitored by [^{3}H]-CHA (1 nM), was also unaffected by pretreatment of the membranes with 30 and 100 μg ml^{-1} saponin but decreased at higher saponin concentrations (Figure 2). The high and low affinity states of the A_1 receptor therefore seem to be affected by saponin in a qualitatively similar manner.

Effect of the presence of saponin in the assay on the binding of CHA

The results of analogous experiments to those depicted in Figure 1 and Table 2 in which saponin was included in the binding assay rather than during the preparation of the membranes are summarized in Table 3. There was the same conversion of a heterogeneous CHA binding curve, measured in the presence of ADA and GTP, to a homogeneous binding curve when 10–100 μg ml^{-1} saponin was present in the assay. No decrease in the affinity constant of CHA for the low affinity state of the receptor was noted at these saponin concentrations. [^{3}H]-DPCPX binding was also relatively unaffected by 10–100 μg ml^{-1} saponin in the assay (97±6% of control, $n=6$).

Effects of saponin treatment on receptor function

In order to confirm that saponin pretreatment, at concentrations up to 100 μg ml^{-1}, did not have a detrimental effect on agonist-receptor-G protein coupling and activation, [^{35}S]-GTPγS functional assays were performed (Figure 3, Table 4). As was observed in the binding studies, a 30 μg ml^{-1} saponin pretreatment had little effect but 100 μg ml^{-1} produced a substantial increase in both basal binding and the stimulation produced by high concentrations of CHA. At higher concentrations of saponin (>100 μg ml^{-1}) basal binding decreased and the agonist potency decreased 5 fold but the stimulation (expressed as d.p.m. above basal) was unchanged relative to that found at 100 μg ml^{-1} saponin (Table 4).

In parallel experiments a similar increase in the stimulated

Table 2 Effect of saponin pretreatment on the competition by CHA of [^{3}H]-DPCPX binding to human A_1 adenosine receptors in the presence and absence of GTP and ADA

Condition	*[Saponin]* (μg ml^{-1})	*FR_1*	*log*K_{app1}	*log*K_{app2}
+ADA +GTP				
	0	0.33±0.02	8.31±0.04	5.65±0.02
	30	0.28±0.03	8.41±0.27	5.58±0.06
	100	0	–	5.58±0.02
	300	0	–	5.43±0.01
	1000	0	–	5.35±0.02
+ADA −GTP				
	0	0.79±0.04	8.48±0.05	5.90±0.20
	100	0.88±0.02	8.31±0.06	5.71±0.08
−ADA +GTP				
	0	0.27±0.01	7.40±0.21	5.68±0.08
	100	0	–	5.60±0.03
−ADA −GTP				
	0	0.50±0.08	7.65±0.13	5.83±0.13
	100	0.68±0.04	7.60±0.10	5.67±0.10

Binding assays were carried out by competition with a fixed concentration of [^{3}H]-DPCPX (0.2 nM) using membranes that were previously treated with or without 3 u ml^{-1} ADA for 30 min at 25°C. Binding assays were carried out in the absence or presence of 1 mM GTP. The values listed in the table are means±s.e.mean of 2 to 5 separate experiments each performed in triplicate. logK_{app1} and logK_{app2} are the log of the apparent affinity constants of the high and low affinity states respectively of the A_1 receptor obtained by a 2-site analysis of the inhibition curve; FR_1, represents the fraction of the high affinity state.

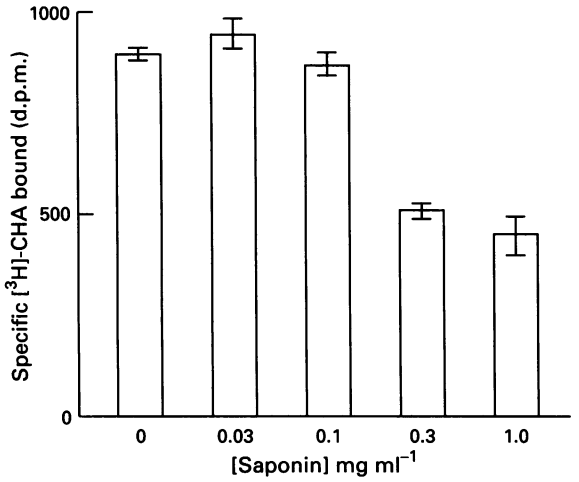


Figure 2 The effect of saponin pretreatment on [^{3}H]-CHA binding: membranes, pretreated with the stated concentrations of saponin, were preincubated with 3 u ml^{-1} ADA for 30 min at 25°C and then incubated with [^{3}H]-CHA (1 nM) for 1 h at 25°C. The data are the means ± s.e.mean of 4 independent experiments.

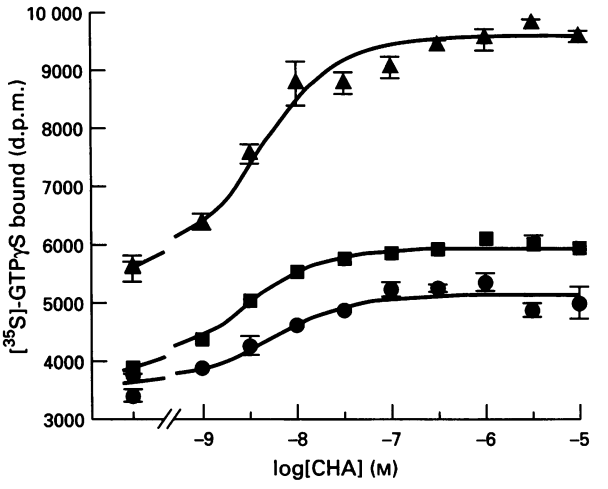


Figure 3 The effect of saponin pretreatment on CHA-stimulated binding of [^{35}S]-GTPγS to G proteins; CHO – A_1 membranes were prepared with a pretreatment of saponin at the following concentrations: 0 μg ml^{-1} (●), 30 μg ml^{-1} (■), 100 μg ml^{-1} (▲). Membranes were treated with 3 u ml^{-1} ADA for 30 min at 25°C prior to assay. [^{35}S]-GTPγS binding assays were performed as described in Methods. Data points are the means of triplicate determinations of one experiment. The error bars generally fall within the data points. The curves depict the fits to a 1-site model. Parameter estimates from four experiments are summarized in Table 4.

response was found when 10–25 μg ml^{-1} saponin was present in the assay although smaller increases in basal levels of binding were observed (Table 5). At 100 μg ml^{-1} saponin in the assay a decrease in the magnitude of the response and the CHA potency was observed, as was found in membranes pretreated with 300 and 1000 μg ml^{-1} saponin.

It seems that the presence of 10 μg ml^{-1} saponin in the binding and functional assays may be broadly equivalent to pretreating the membranes with 100 μg ml^{-1} saponin. In all subsequent characterization experiments, CHO-A_1 membranes pretreated with 100 μg ml^{-1} saponin were used.

Characterization of the binding properties of human A_1 receptors

The above studies show that saponin pretreatment (100 μg ml^{-1}) simplifies the binding properties of A_1 receptors in the presence of GTP and increases receptor function without affecting agonist binding or potency. The binding properties of the human A_1 receptor have been examined by kinetic, saturation and competition studies. All experiments were carried out on saponin pretreated membranes and the results, where appropriate, were compared with those of untreated membranes.

Kinetics of radioligand binding

The dissociation of the antagonist radioligand [^{3}H]-DPCPX from A_1 receptors was monophasic (Figure 4a) with a k_{-1} of 0.11 ± 0.01 min^{-1} which was unaffected by the experiment being carried out in the presence of GTP (data not shown).

The dissociation of [^{3}H]-CHA (Figure 4b) was biphasic, consisting of a slow component with k_{-1} of 0.03 ± 0.01 min^{-1} and a very slow pseudoirreversible component. However, as shown in Figure 4, both the slow and very slow components were converted to a fast component with k_{-1} of ⩾0.5 min^{-1} in the presence of 1 mM GTP. An analogous pseudoirreversible component was detected in dissociation studies with [^{3}H]-PIA (data not shown).

The association of both a radiolabelled agonist and an antagonist to the A_1 receptor was complex. In the absence of GTP the rate of approach to equilibrium of [^{3}H]-DPCPX (0.2 nM) consisted of a fast component with a rate constant (k_{app}) of 0.18 ± 0.01 min^{-1} (n = 5) and a slow component whose rate constant was less well defined (k_{app} <0.01 min^{-1} n = 5, Figure 5). The k_{app} of the fast component of [^{3}H]-DPCPX binding was unaffected by the presence of either ADA or GTP (mean value for all conditions, 0.18 ± 0.01, n = 11). However the slow component was essentially abolished if the binding was carried out in the presence of GTP (Figure 5). An index of the slow component is the ratio of binding of [^{3}H]-DPCPX at 160 min and 20 min. In the presence of GTP, the ratio was 1.16 ± 0.02, (n = 5) which was significantly different (P <0.05, single tailed t test) from the ratio found in the absence of GTP (1.38 ± 0.06, n = 3). The ratio found in the presence of GTP was close to the ratio for a simple bimolecular association process (1.08 ± 0.03) which

Table 3 Competition of CHA for [^{3}H]-DPCPX binding to human A_1 adenosine receptors in the presence of various concentrations of saponin in the assay

[Saponin] (μg ml^{-1})	FR_1	$log K_{app1}$	$log K_{app2}$
0	0.34 ± 0.01	8.37 ± 0.06	5.67 ± 0.06
10	0	–	5.51 ± 0.13
25	0	–	5.38 ± 0.03
100	0	–	5.57 ± 0.01

Binding assays were carried out using membranes that were previously treated with 3 u ml^{-1} ADA for 30 min at 25°C. Binding of [^{3}H]-DPCPX was measured in the presence of 1 mM GTP and various concentrations of saponin. The values listed in the table are means ± range/2 of 2 separate experiments each performed in triplicate.

was calculated utilizing the observed dissociation rate constant of [^{3}H]-DPCPX, its concentration and its affinity constant. This suggests that, in the presence of GTP, the binding of [^{3}H]-DPCPX to A_1 receptors in membranes pretreated with saponin can be described to a reasonable approximation by a simple bimolecular kinetic scheme. The association of [^{3}H]-CHA (2 nM) also consisted of a component with k_{app} of 0.07 ± 0.01 min^{-1} and a slow component with a k_{app} that was also not well defined ($n=3$, Figure 6). This was also observed for [^{3}H]-PIA (data not shown). In both cases of association and dissociation, the rate constants which could be determined agreed well with those determined in experiments using membranes not treated with saponin (data not shown).

Saturation analysis

[^{3}H]-CHA, [^{3}H]-PIA and [^{3}H]-DPCPX in the presence of ADA showed saturable binding to a homogeneous population of high affinity A_1 receptors in saponin pretreated membranes with B_{max} values of 1.10 ± 0.05, 1.11 ± 0.08 and 1.15 ± 0.05 pmol mg^{-1} of protein and K_D values of 4.3, 3.3 and 0.91 nM respectively (Table 6). It was not possible to quantitate the binding parameters of [^{3}H]-CHA, [^{3}H]-PIA and [^{3}H]-DPCPX in the absence of ADA and GTP because the inhibitory actions of endogenous adenosine resulted in low levels of binding.

Further parameter estimates from saturation studies using [^{3}H]-DPCPX in the presence of GTP and the presence and absence of ADA could be obtained, and are summarized in Table 6. In the presence of ADA, the addition of GTP resulted in a small increase in the B_{max} from 1.15 ± 0.05 pmol mg^{-1} protein to 1.26 ± 0.03 pmol mg^{-1} protein but left the K_D unchanged. In the absence of ADA, the B_{max} was essentially unchanged, again indicating that in the presence of GTP, endogenous adenosine does not alter binding.

CHA binding properties in competition experiments under various conditions

CHA- [^{3}H]-DPCPX competition experiments were performed on saponin pretreated (100 μg ml^{-1}) membranes in the presence and absence of ADA and GTP. The results are illustrated in Figure 7 and Table 2. In the presence of GTP ($\pm$ADA), all [^{3}H]-DPCPX-CHA inhibition curves on saponin pretreated membranes are well described by simple mass action curves (Figure 7) with a mean log $K_{app} = 5.59 \pm 0.02$ ($n=5$), log $K_{corr} = 5.67 \pm 0.10$ (where $K_{app} = IC_{50}^{-1}$ and log K_{corr} represents the affinity constant corrected for receptor occupancy by the radioligand or other competing ligand). In the absence of GTP (ADA), this low affinity site (mean log $K_{app} = 5.69 \pm 0.06$; log $K_{corr} = 5.77 \pm 0.14$) is also present as a minor component together with a high affinity component. In the absence of ADA the low affinity component comprises $32 \pm 4\%$ of the observable [^{3}H]-DPCPX binding. In the presence of ADA this

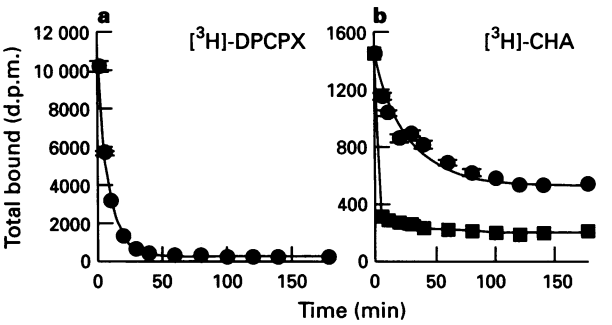


Figure 4 The dissociation kinetics of [^{3}H]-DPCPX and [^{3}H]-CHA binding to A_1 adenosine receptors: dissociation of [^{3}H]-DPCPX (a) was measured in the absence (●) of GTP and dissociation of [^{3}H]-CHA (b) was measured both in the absence (●) and presence (■) of 1 mM GTP. CHO – A_1 membranes were equilibrated with [^{3}H]-DPCPX (0.5 nM) or [^{3}H]-CHA (2 nM) for 1 h at 25°C after which dissociation was initiated by diluting the incubation 10 fold with buffer containing theophylline (final concentration of 3 mM) with or without GTP as appropriate. Binding was determined as described under Methods. Data points are the means of triplicate determinations in one of three experiments. The error bars generally fall within the data points. The data are fitted to single exponential functions.

Table 4 The effect of saponin pretreatment on CHA-stimulated binding of [^{35}S]-GTPγS to CHO-A_1 membranes

Saponin pretreatment (μg ml^{-1})	*Basal binding* (% control)	*Stimulation* (% basal)	*–log EC_{50}*
0	[100]	44 ± 2	8.55 ± 0.13
30	120 ± 8	46 ± 4	8.17 ± 0.29
100	178 ± 16	63 ± 4	8.23 ± 0.06
300	123 ± 6	82 ± 8	7.68 ± 0.03
1000	115 ± 4	79 ± 7	7.71 ± 0.04

Experiments were performed as described in the legend to Figure 2. The values listed in the table are means ± s.e.mean of 3 or 4 experiments each performed in triplicate. Data of individual experiments are normalized to the basal binding found with no saponin pretreatment. (100% = 3180 ± 160 d.p.m., $n=4$).

Table 5 CHA-stimulated binding of [^{35}S]-GTPγS to CHO-A_1 membranes in the presence of various concentrations of saponin

[Saponin] (μg ml^{-1})	*Basal binding* (% control)	*Stimulation* (% basal)	*–log EC_{50}*
0	100	43 ± 3	8.55 ± 0.14
10	118 ± 4	70 ± 1	7.90 ± 0.38
25	101 ± 7	79 ± 15	7.61 ± 0.26
100	65 ± 4	39 ± 14	7.24 ± 0.09

[^{35}S]-GTPγS binding assays were carried out using membranes that were previously treated with 3 u ml^{-1} ADA for 30 min at 25°C. Experimental conditions were as described under Methods but in the presence of various concentrations of saponin. The values listed in the table are means ± range/2 of 2 separate experiments each performed in triplicate. Data of individual experiments were normalized to the basal binding found with no saponin treatment (100% ≡ 2950 ± 30 d.p.m.).

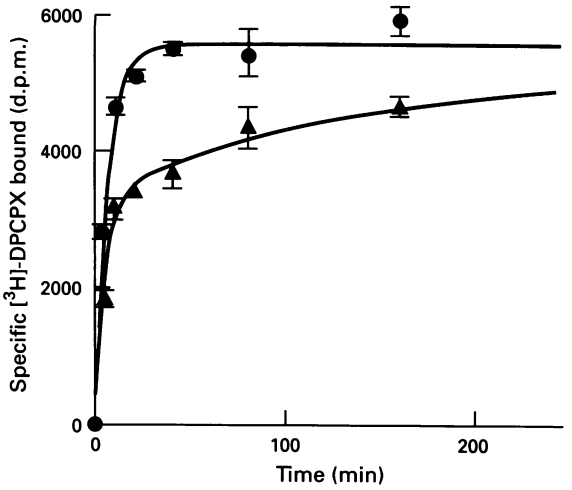


Figure 5 Association of [^{3}H]-DPCPX to A_1 adenosine receptors in the presence or absence of GTP (1 mM): binding assays were carried out on membranes previously treated with 3 u ml^{-1} ADA for 30 min at 25°C. The binding of [^{3}H]-DPCPX (0.2 nM) was measured as a function of time in the presence (●) or absence (▲) of GTP (1 mM). The curve through the closed circles (+GTP) is the best fit mono-exponential function with a rate constant of 0.14 ± 0.01 min^{-1} and maximum value of 5450 ± 120 d.p.m. The curve through the data in the absence of GTP (▲) represents a best fit bi-exponential function with rate constants of 0.17 ± 0.04 min^{-1} and 0.008 ± 0.010 min^{-1} and amplitudes of 3310 ± 480 d.p.m. and 1830 ± 370 d.p.m. respectively. The quoted errors are estimates of parameter errors provided by the programme (Enzfitter) and illustrate the ill-defined value of the slow rate constant.

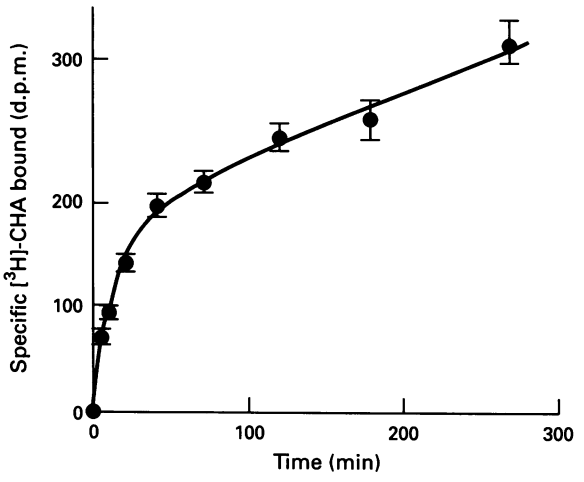


Figure 6 Association of [^{3}H]-CHA to A_1 adenosine receptors: binding assays were carried out on membranes previously treated with 3 u ml^{-1} ADA for 30 min at 25°C and using 2 nM [^{3}H]-CHA. The curve through the data points is the best fit bi-exponential function with rate constants of 0.08 ± 0.01 min^{-1} and $(7 \pm 6) \times 10^{-4}$ min^{-1} with amplitudes of 170 ± 10 d.p.m. and 990 ± 670 d.p.m. respectively. The quoted errors are estimates of the parameter errors provided by the programme (Enzfitter) and illustrate the ill-defined estimate of both the rate constant and maximum amplitude of the slow component.

Table 6 Affinity constant and B_{max} values for ligand binding to saponin pretreated human A_1 adenosine receptors

Ligand	*Conditions*	*log*K_A	B_{max} (pmol mg^{-1})
[^{3}H]-CHA	+ADA−GTP	8.37 ± 0.02	1.10 ± 0.05
[^{3}H]-PIA	+ADA−GTP	8.48 ± 0.06	1.11 ± 0.08
[^{3}H]-DPCPX	+ADA−GTP	9.04 ± 0.04	1.15 ± 0.05
[^{3}H]-DPCPX	+ADA+GTP	9.04 ± 0.02	1.26 ± 0.03
[^{3}H]-DPCPX	−ADA+GTP	8.97 ± 0.06	1.32 ± 0.01

Saturation studies were performed as described in the Methods, in the presence or absence of ADA (3 u ml^{-1}) and GTP (1 mM). The values listed are the means ± s.e.mean of three separate experiments each performed in triplicate. The affinity constants are expressed as log K_A.

component falls to $12 \pm 2\%$ of the observable [^{3}H]-DPCPX binding (Table 2). This is significantly lower than the value ($21 \pm 4\%$) found in membranes not treated with saponin ($P < 0.05$, unpaired t test).

The high affinity binding component has different apparent affinity constants in the absence and presence of ADA; log $K_{app} = 7.60 \pm 0.10$ and 8.31 ± 0.06 respectively. The log K_{corr} value for CHA of 8.39 ± 0.14 in the presence of ADA agrees well with the directly measured value of 8.37 ± 0.02 (Table 5) as would be expected. Similarly, the percentage of high affinity CHA sites relative to total [^{3}H]-DPCPX sites (measured +ADA, −GTP) measured in competititon and saturation experiments are in reasonable agreement ($88 \pm 2\%$ versus $96 \pm 7\%$ respectively).

In the absence of ADA and GTP, the log K_{app} value for the high affinity CHA component (7.60 ± 0.10) is much lower than that found in the presence of ADA. This difference results from the competitive effect of endogenous adenosine on CHA binding which is sufficient to produce a 5 fold shift in the K_{app} value (0.7 log unit) of the high affinity CHA binding component. Similarly the absence of ADA results in a 4–5 fold decrease in [^{3}H]-DPCPX binding to high affinity CHA binding sites (Figure 7 and legend). This effect results from the competition between [^{3}H]-DPCPX and endogenous adenosine for the high affinity agonist binding sites.

The low affinity binding sites for CHA observed in the absence of GTP are not affected to a detectable extent by ADA (Table 2, Figure 1). This is in agreement with the small effect of ADA on [^{3}H]-DPCPX binding measured in the presence of GTP.

Discussion

The experiments with adenosine deaminase suggest that adenosine is present in binding assays utilising CHO cell membranes. This is seen most clearly as a 200% increase in the binding of the antagonist [^{3}H]-DPCPX produced by the addition of adenosine deaminase (ADA) which metabolizes the adenosine and thus relieves the 'tonic' inhibition (Table 2). When the assays are carried out in the presence of GTP the binding of [^{3}H]-DPCPX is higher and the enhancement in [^{3}H]-DPCPX binding in the presence of ADA is only 20%. The differential effects of ADA on [^{3}H]-DPCPX binding in the absence and presence of GTP reflects the high affinity (and hence high potency to inhibit [^{3}H]-DPCPX binding) of adenosine in forming adenosine-receptor-G protein complexes in the absence of GTP versus the disruption of these complexes by GTP to form low affinity adenosine-receptor complexes.

The high affinity agonist-receptor-G protein complexes can

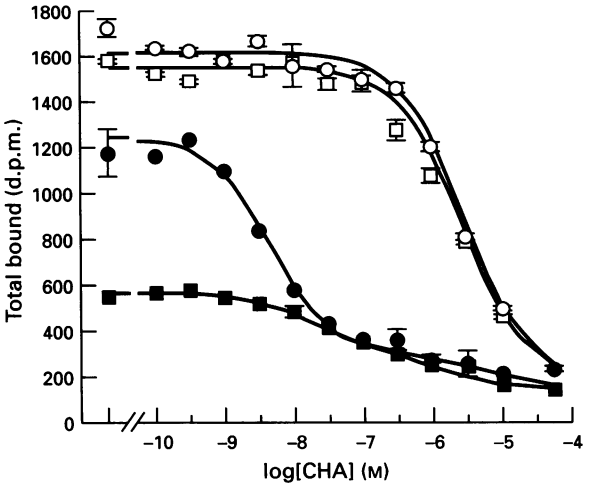


Figure 7 Competition by CHA for [^{3}H]-DPCPX binding to CHO A_1 membranes pretreated with 100 μg ml^{-1} saponin in the presence and absence of GTP and ADA: binding assays were carried out by competition against a fixed concentration of [^{3}H]-DPCPX (0.2 nM) using membranes that were untreated (□, ■) or previously treated (○, ●) with 3 u ml^{-1} ADA for 30 min at 25°C. Incubation conditions are as described under Methods. Binding of [^{3}H]-DPCPX was measured in the absence (filled symbols) or presence (open symbols) of 1 mM GTP. Data points are the means of triplicate determinations in one of three experiments. The error bars generally fall within the data points. Curves are computer generated best fits to a 1-site or 2-site model where appropriate and give the following parameter estimates:

		FR_l	$log K_{app1}$	$log K_{app2}$
−ADA	−GTP	0.55	7.83	6.05
	+GTP	–	–	5.61
+ADA	−GTP	0.87	8.32	5.39
	+GTP	–	–	5.60

be detected readily in CHA-[^{3}H]-DPCPX competition experiments (Table 2), as can the conversion of the high affinity state of receptor to a low affinity state by GTP. Similarly, the inhibitory effect of the endogenous adenosine is seen as a 6–7 fold decrease in apparent affinity of CHA for the high affinity state of the receptor when measurements are made in the absence of ADA (Table 2). Interestingly the apparent affinity constant of CHA for the low affinity (uncoupled) state of the receptor is unaffected by ADA. Hence the endogenous adenosine is present at levels which affect only the binding of agonists to the high affinity state and not the low affinity state of the receptor.

We have investigated the use of the permeabilizing agent, saponin, to reduce the levels of endogenous adenosine or inhibit adenosine generation in membrane fragments from CHO cells. As found in studies which have used prolonged washing procedures or detergent treatment (Schiemann *et al.*, 1990; Prater *et al.*, 1992), saponin pretreatment of the membranes does not remove the endogenous adenosine. However saponin treatment of membranes does result in the induction of a complete conversion of the high affinity state of the receptor to the low affinity state by GTP and increased coupling of the receptor to the G-protein in the absence of GTP (Table 2, Figures 1, 7). Associated with the increased coupling/uncoupling is an enhanced receptor function as monitored by agonist stimulation of [^{35}S]-GTPγS binding (Tables 4, 5, Figure 3).

One procedure is to treat the membranes with 100 μg ml^{-1} saponin during homogenization of the CHO cells. Subsequent washing steps will presumably reduce the saponin concentration. This procedure does not appear to result in a significant perturbation of the binding kinetics or affinities of the ligands tested. The small effects of saponin ($\leq$10%) on binding of [^{3}H]-DPCPX and CHA (Tables 1, 2 and Figure 2) suggest that saponin does not produce substantial changes in the measured number of binding sites.

It is also possible to carry out binding and functional assays in the presence of 10 μg ml^{-1} saponin. Although this is a tenfold lower concentration than that used for the membrane pretreatment, the protein/saponin ratios are in fact comparable in the two saponin treatment procedures. As this ratio is important in determining the actions of surface active agents, it is not surprising that both methods give comparable results.

The simplest interpretation of the effect of saponin on A_1 adenosine receptors is that in CHO cell membranes there are G-proteins which cannot be accessed by GTP. The effect of saponin may be to remove this permeability/access barrier, allow all agonist-receptor-G-proteins to be uncoupled by GTP, and to increase the agonist stimulated (and basal) binding of [^{35}S]-GTPγS in the functional assays.

The findings in this paper are in accord with the increase in [^{35}S]-GTPγS binding to G-proteins, observed on treating purified atrial membranes with alamethacin, an antibiotic which forms pores in lipids and changes the structure of lipid bilayers (Hilf & Jakobs, 1992). Similarly, alamethicin has recently been reported to increase ^{3}H-agonist binding to adenosine A_{2a} receptors (Luthin *et al.*, 1995).

In a number of receptor systems, incomplete formation of a low affinity agonist state in the presence of GTP or GTP analogue has been observed (Hill coefficients <1) (e.g. Matsui *et al.*, 1995). It may be that in these systems, as well as in CHO-A_1 cells, there are G-proteins which are inaccessible to GTP.

In general, the binding data on A_1 receptors form a consistent set. The low affinity agonist state, irrespective of the conditions of the assay, has the same affinity constant for the agonist CHA. Similarly the properties of high affinity state, in terms of its affinity for CHA and the number of sites are the same, irrespective of the binding being measured directly in a saturation experiment or indirectly in a competition experiment.

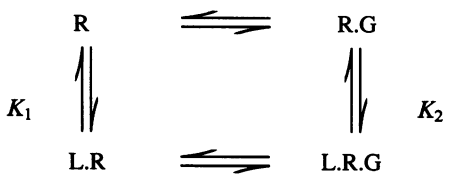
We now have a system which, in the presence of ADA and agonist, can exist as 88–96% high affinity (coupled) state in the absence of GTP and *ca* 100% low affinity (uncoupled) state (Tables 1, 5, 6) in the presence of GTP. In addition it should be noted that there is one of the largest differences in the affinity of high efficacy agonists (300–600 fold for CHA, adenosine and PIA (Tables 2, 6 and Cohen *et al.*, unpublished)) between the two states of any G-protein coupled receptor. Both the features may be useful in the detailed analysis of aspects of receptor-G-protein coupling.

In binding studies of G-protein coupled receptors (e.g. β-adrenoceptors and muscarinic receptors) it is commonly found that there is an apparent incomplete receptor-G protein coupling in the presence of agonist: the Hill slopes of ^{3}H-antagonist/agonist competition curves are less than 1. Models of receptor binding, for example the ternary complex model, have had to postulate that the concentration of G proteins is less than that of the receptor in order to rationalize these binding data (De Lean *et al.*, 1980; Wong *et al.*, 1986). This postulate is at variance with the general finding that levels of individual G-proteins in membranes can be considerably higher than those of the receptors to which they can couple and furthermore that one receptor can activate catalytically several G-protein molecules (Hilf *et al.*, 1989; Lazareno *et al.*, 1993). It may be that, for many receptor systems, there is a population of receptors (very small in the case of CHO-A_1 receptors) which for certain reasons are not capable of coupling to G-proteins. If so, the remaining part of the system may not be subject to the [R] $\geq$ [G] restriction order to explain the binding properties. In other words, the ^{3}H-antagonist/agonist competition curves consist of two separate components, (i) a minor population of receptors which does not couple to G-proteins and (ii) the remaining receptors which are capable of freely and reversibly interacting with a larger population of G-proteins according to the ternary complex model. This interpretation in terms of

non-interacting pools of receptors may explain why many receptors, including A_1 receptors, have agonist inhibition curves with minimum Hill slopes of less than 0.7, a finding that is incompatible with the predictions of a simple ternary complex model (Wong *et al.*, 1986).

There are three other unusual findings of interest which require interpretation. Firstly, the association kinetics of [^{3}H]-CHA and [^{3}H]-DPCPX have a very slow component (Figures 5, 6) which, in the case of [^{3}H]-DPCPX, is not present when the assay is carried out in the presence of GTP (Figure 5). Secondly, the off-rate kinetics of [^{3}H]-CHA and [^{3}H]-PIA show two components, a slow component, ($t_{½}$ ~20 min) and a pseudoirreversible component. However, both components represent agonist-receptor-G-protein complexes as GTP enhances their off-rates. The molecular explanation for the existence of two components is not known but could for example result from the A_1 receptor coupling to more than one G-protein and thus generating two kinetically different complexes. Alternatively there could be a slow isomerisation process of an agonist-receptor-G-protein. A very slow dissociation component has also been observed in autoradiographic studies of A_1 receptors (Parkinson & Fredholm, 1992). The third finding is that GTP enhances the binding of the antagonist [^{3}H]-DPCPX even in the presence of ADA. The effect is to increase the B_{max} without affecting the antagonist affinity constant.

These phenomena have been observed in this study and in previous A_1 receptor binding studies (e.g. Stiles, 1988; Leung *et al.*, 1990; Klotz *et al.*, 1990; Parkinson & Fredholm, 1992; Prater *et al.*, 1992). The latter effect has been explained previously in terms of A_1 receptors, R, being precoupled to G-proteins, G, to form R.G and, in that state, having a lower affinity than the uncoupled receptor for the antagonist, L ($K_1 > K_2$, Model 1). However, this model does not explain the data.



Model 1

If R is in equilibrium with R.G (and/or L.R is in equilibrium with L.R.G) then the effect of GTP would be to increase the affinity of L without changing the B_{max} and to change the dissociation rate constant of L. This is not observed.

If R and L.R are not in equilibrium with R.G and L.R.G respectively during the time frame of the binding experiment but do equilibrate in the presence of GTP, then it might be possible to observe a GTP-induced increase in B_{max} without affecting the K_d of L or its dissociation rate. This would only be true if $K_1 >> K_2$ and the concentration of L was such that only insignificant amounts of L.R.G were generated ($K_2[L] << 1$). Because of these multiple restrictions this model is somewhat implausible.

A more reasonable explanation is that, by analogy with the CHA and PIA kinetic studies, adenosine can form slowly reversible and pseudoirreversible adenosine-receptor-G-protein complexes. These complexes could be generated during the preparation of the membranes or even (because of the pseudo irreversible nature of the binding process) in the presence of ADA. Such complexes would not bind a radioligand, agonist or antagonist, until adenosine dissociated.

Dissocation of adenosine could be very slow in the absence of GTP, explaining the observed slow association component in the ^{3}H-agonist or [^{3}H]-DPCPX kinetic assays. In the presence of GTP, dissociation of adenosine would be fast and effectively generate extra receptor sites which would be detected as an increase in B_{max} but not affinity of [^{3}H]-DPCPX. There is no necessity to postulate A_1 receptor precoupling to G-proteins in the absence of agonist. Finally, because adenosine, as long-lived adenosine-receptor-G-protein complexes, is not metabolized by ADA it is not surprising that GTP induces increases in [^{3}H]-DPCPX binding even in the presence of ADA. This phenomenon, sometimes described as that of a 'locked agonist' has been reported for other receptors (e.g. Severne *et al.*, 1987).

In conclusion, saponin treatment of membranes does not remove endogenous adenosine. It does appear to allow a more ready access of guanine nucleotides to G-proteins capable of coupling to A_1 receptors. This procedure may facilitate the quantitative analysis and interpretation of receptor-G-protein coupling by eliminating an 'inaccessibility factor'. The presence of long-lived adenosine-receptor-G-protein complexes may explain a number of unusual findings that have been reported in A_1 adenosine receptor binding studies.

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