British Journal of Pharmacology (2004) 143, 705–714
www.nature.com/bjp

Coupling of the human A_1 adenosine receptor to different heterotrimeric G proteins: evidence for agonist-specific G protein activation

[1]Yolande Cordeaux, [2]Adriaan P. IJzerman & *,[1]Stephen J. Hill

[1]Institute of Cell Signalling, Medical School, University of Nottingham, Nottingham NG7 2UH and [2]Leiden/Amsterdam Centre for Drug research, Division of Medicinal Chemistry, Leiden, Netherlands

1 The present study investigates the effect of varying ligand structure on the ability of agonists to activate guanine nucleotide-binding proteins of the Gi, Gs and Gq families *via* the A_1 adenosine receptor.

2 In CHO cells expressing this receptor, inhibition or potentiation of forskolin-stimulated cAMP accumulation was used as an end point to measure the activation of Gi and, in Pertussis toxin (PTX)-treated cells, Gs, respectively. Stimulation of inositol phosphate accumulation in PTX-treated cells was used as an index of Gq activation.

3 CPA (N^6-cyclopentyladenosine), NECA (5′-*N*-ethyl-carboxyamidoadenosine) and eight analogues of these ligands presented a range of guanine nucleotide-binding protein (G-protein)-activating profiles. Some ligands could only activate Gi (e.g. 2′deoxyCPA), some primarily Gi and Gs (and only weakly Gq) (e.g. 3′deoxyCPA), highlighting the importance of the ribose hydroxyls in agonist activation of multiple G proteins. CHA (N^6-cyclohexyladenosine) activated Gi, Gs and Gq, but was more efficacious than CPA in activating Gs.

4 The NECA analogues 5′-*N*-cyclopropyl-carboxamidoadenosine, 5′-*N*-cyclobutyl-carboxamidoadenosine and 5′-*N*-cyclopentyl-carboxamidoadenosine (CPeCA) also activated all three G proteins, although their ability to activate Gs and Gq (relative to CPA) was reduced with increasing substituent size, such that CPeCA produced only a small stimulation (at 100 μM) at Gq, but was a full agonist, relative to CPA, at Gi and Gs.

5 This study suggests that the A_1 adenosine receptor can adopt agonist-specific conformations, arising from small changes in ligand structure, which lead to the differential activation of Gi, Gs and Gq.

British Journal of Pharmacology (2004) **143,** 705–714. doi:10.1038/sj.bjp.0705925

Keywords: Adenosine A_1 receptor; agonist trafficking; cyclic AMP; guanine nucleotide-binding proteins; phosphoinositol hydrolysis; receptor conformation; second messengers

Abbreviations: CBuCA, 5′-*N*-cyclobutyl-carboxyamidoadenosine; CHA, N^6-cyclohexyladenosine; CPA, N^6-cyclopentyladenosine; CPCA, 5′-*N*-cyclopropyl-carboxyamidoadenosine; 8CPCPA, 8-cyclopentyl-N^6-cyclopentyladenosine; CPeCA, 5′-*N*-cyclopentyl-carboxyamidoadenosine; 2′dCPA, 2′-deoxy-N^6-cyclopentyladenosine; 3′dCPA, 3′-deoxy-N^6-cyclopentyladenosine; G protein, guanine nucleotide-binding protein; [^{3}H]DPCPX, 8-cyclopentyl-[^{3}H]1,3-dipropylxanthine|8MeCPA, 8-methylamino-N^6-cyclopentyladenosine; NECA, 5′-*N*-ethyl-carboxyamidoadenosine; PTX, Pertussis toxin

Introduction

The adenosine A_1 receptor is a member of the guanine nucleotide-binding protein (G-protein)-coupled receptor superfamily (Libert *et al*., 1992; Olah & Stiles, 1995). The receptor is widely distributed throughout the body and has been implicated in the control of heart rate, renal blood flow, lipolysis and ischaemic injury (Ralevic & Burnstock, 1998). Traditionally, it is described as a Gi-coupled receptor, whose activation leads to the inhibition of adenylyl cyclase and calcium channels, the activation of potassium channels (Olah & Stiles, 1995; Figler *et al*., 1996; Srinivas *et al*., 1997) and, *via* the release of active Gi betagamma subunits, the activation of phospholipase C (Gerwins & Fredholm, 1992; Dickenson & Hill, 1998).

Recently, we have demonstrated that, in addition to members of the Gi family, this receptor can also couple to Pertussis toxin (PTX)-insensitive G proteins of the Gs and Gq families (Cordeaux *et al*., 2000). Thus, activation of the adenosine A_1 receptor can also lead to a stimulation of adenylyl cyclase and to a PTX-resistant accumulation of inositol phosphates (Cordeaux *et al*., 2000). Interestingly, the ability of this receptor to activate PTX-insensitive G proteins depended on the level of receptor expression and on the agonist present (Cordeaux *et al*., 2000). While the agonists CPA (N^6-cyclopentyladenosine) and NECA (5′-*N*-ethyl-carboxyamidoadenosine) were equiefficacious in activating Gi, NECA was approximately twice as efficacious as CPA in

*Author for correspondence; E-mail: stephen.hill@nottingham.ac.uk
Advance online publication: 9 August 2004

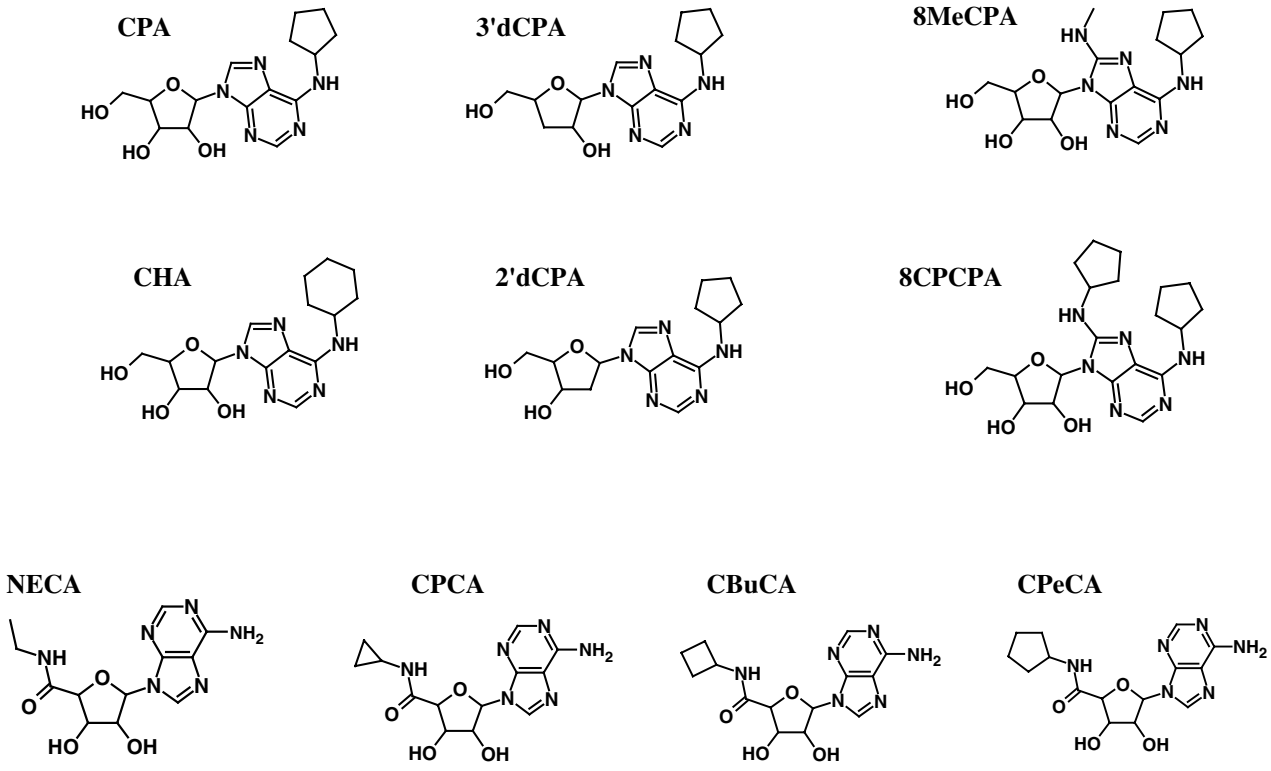

Figure 1 Chemical structures of CPA and NECA analogues. (Top two rows) CPA and analogues: CPA, CHA, 3′dCPA, 2′dCPA, 8MeCPA and 8CPCPA . (Bottom row) NECA and analogues: NECA, CPCA, CBuCA and CPeCA.

activating Gs and Gq (Cordeaux *et al*., 2000). These differences in relative efficacy could be measured both at the level of effector activation and, more directly, at the level of the G protein itself (by measuring [^{35}S]GTPγS binding to G proteins immunoprecipitated with subtype-specific antibodies). Hence, the adenosine A_1 receptor can be added to the growing number of G-protein-coupled receptors that activate multiple G proteins, in an agonist-specific manner, for example, $5HT_{2C}$ serotonin (Berg *et al*., 1998), CB_1 cannabinoid (Bonhaus *et al*., 1998), β_2 adrenoceptor (Wenzel-Seifert & Seifert, 2000) and $P2Y_{11}$ receptors (White *et al*., 2003).

Early studies, in which agonists were shown to 'bias' the signalling of receptors to effector pathways, gave rise to the terms 'differential strength of signal' (to describe the activation of two effectors by a 'strong' agonist and only one effector by a 'weaker' agonist) and 'agonist trafficking' (where two or more agonists activate multiple effector pathways but with reversed orders of potency or relative efficacy) (Kenakin, 1995). Subsequent studies have provided a large body of evidence to suggest that such agonist-dependent receptor signalling is due to the formation of agonist-specific receptor conformations (for a recent review see Kenakin, 2003). Studies that use a range of compounds to address this behaviour can often identify elements of ligand structure that may favour or limit the activation of specific G proteins. Conversely, site-directed mutagenesis of the receptor may also identify key sites of receptor–ligand interaction required for stabilising the coupling of receptor to particular G proteins.

For example, *via* α2 adrenoceptors, catecholamines can activate Gi and Gs, while phenolamines only activate Gi (Airriess *et al*., 1997). In addition, while catecholamines are more potent at increasing intracellular calcium than in decreasing cAMP, for non-catecholamines the reverse is true (Kukkonen *et al*., 2001). For peptide ligands acting at neurokinin-1 receptors, differential activation of Gs and Gq is observed between substance P analogues with various constrained methionine residues (Sagan *et al*., 1999). Recently, agonist trafficking has been observed at neurotensin NTS1 receptors (Skrzydelski *et al*., 2003). At this receptor, EISAI-1 was more potent than neuromedin N in stimulating cAMP accumulation, while neuromedin was more potent than EISAI-1 in stimulating inositol phosphate accumulation. The relatively low potency of EISAI-1 at inositol phosphate accumulation could be attributed to the presence of an ethyl group at its COOH end, since an analogue lacking this motif was more potent in this assay than either EISAI-1 or neuromedin N (Skrzydelski *et al*., 2003).

Having demonstrated that CPA and NECA differentially activate G proteins *via* the adenosine A_1 receptor, the present study aimed to explore the effects of varying the structure of these two ligands on their ability to activate multiple G proteins. Here, we use a series of CPA analogues (modified in the C8-position or lacking a ribose hydroxyl group, Figure 1) and a series of NECA analogues (with sequentially increasing size of cycloalkyl group in the 5′-*N*-position, Figure 1) and examine their ability to activate Gi, Gs and Gq.

Methods

Cell culture

Cells expressing the human A_1 adenosine receptor were cultured as described previously (Cordeaux *et al*., 2000).

Briefly, CHO cells were grown in 75 cm^2 flasks (SLS) in Dulbecco's modified Eagle's medium/nutrient F12 (1 : 1) supplemented with 2 mM L-glutamine and 10% ($v\,v^{-1}$) foetal calf serum. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere until confluency, and were subcultured using trypsin (0.05% ($w\,v^{-1}$))/EDTA (0.02% ($w\,v^{-1}$)) solution. Cells for [^{3}H]inositol phosphate and [^{3}H]cyclic AMP determinations were grown in 24-well cluster dishes (Corning). Cells for ^{3}H-labelled radioligand binding assays were grown in 96-well white view plates (Corning).

Preparation of cell homogenates

CHO cells were cultured in 225 cm^2 flasks (Corning), as described above. Confluent cells were washed with 10 ml of Dulbecco's phosphate-buffered saline solution (Sigma Chemical Co, Dorset, U.K.) containing 5 mM EDTA (PBS/EDTA). Cells were detached in 10 ml of PBS/EDTA using a cell scraper and centrifuged at $3000 \times g$ for 10 min. The resulting pellet was resuspended in 10 ml ice-cold PBS/EDTA and homogenised using a Dounce homogeniser (40 strokes). The cell suspension was then centrifuged at $20{,}000 \times g$ for 15 min. Cell pellets were resuspended in PBS/EDTA (at approximately 1 mg protein ml^{-1}) and stored in 1 ml aliquots at −80°C until used in [^{35}S]GTPγS binding assays. Protein determinations were made using the method of Bradford (1976) using bovine serum albumin as the standard.

Radioligand binding assays

Cells were grown to confluency in white 96-well cluster dishes (Corning). Saturation binding assays were performed in Hank's buffered salt solution (Sigma Chemical Co) containing 20 mM HEPES (pH 7.4) (HHB). Cells were incubated, in triplicate, with increasing concentrations of [^{3}H]DPCPX (8-cyclopentyl-[^{3}H]1,3-dipropylxanthine) (0.05–20 nM) in the presence (nonspecific binding) or absence (total binding) of 5 mM theophylline in a total volume of 200 μl HHB. After 60 min at 37°C, the incubation was terminated by rapid aspiration of buffer and washing cells with ice-cold HHB (3×200 μl). Buffer was removed by aspiration and replaced with 200 μl MICROSCINT™ 20 (Packard, Bioscience, Gronigen, Netherlands). Plates were left overnight at room temperature (in the dark) before counting by liquid-scintillation spectrometry (using a TopCount). Competition binding assays were performed in HHB in a total volume of 200 μl. Cells were incubated, in triplicate, with [^{3}H]DPCPX (1-4 nM) and increasing concentrations of agonist (10^{-10}–10^{-4}). Non-specific binding was defined as that observed in the presence of 5 mM theophylline. After 60 min at 37°C, incubations were terminated and radioactivity determined as described above. Protein determinations were by the method of Lowry using bovine serum albumin as the standard (Lowry *et al*., 1951).

[^{35}S]GTPγS binding

Cell homogenates (50 μg) were incubated in triplicate in Tris-buffered saline (50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, 0.001% Triton X-100, pH 7.4) in the presence of adenosine deaminase (1 U ml^{-1}), GDP (10 μM), [^{35}S]GTPγS (100 pM) and increasing concentrations of agonist (total volume 1 ml). After 1 h at 25°C, the incubation was terminated by rapid filtration using a Brandel MR24 cell harvester and washing with ice-cold PBS (0.14 M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 5 mM Na_2HPO_4), 4×4 ml (over Whatman GF/B filters (presoaked for 1 h in 0.3% polyethylenimine to reduce nonspecific binding). Filters were transferred to scintillation vial inserts and 4 ml of Emulsifier-Safe scintillator (Packard) added. The filters were left overnight at room temperature before liquid-scintillation spectrometry.

Measurement of [^{3}H]cyclic AMP accumulation

Confluent cell monolayers were incubated for 2 h at 37°C with 500 μl of HHB containing [^{3}H]adenine (37 kBq $well^{-1}$). The cells were washed twice and then incubated in 1 ml $well^{-1}$ HHB containing the cyclic AMP phosphodiesterase inhibitor, rolipram (10 μM) for 15 min at 37°C. Agonists were added (in 10 μl of medium) 5 min prior to the incubation with 3 μM forskolin (10 min). Incubations were terminated by the addition of 50 μl concentrated HCl. [^{3}H]cyclic AMP was isolated by single column alumina chromatography as described previously (Alvarez & Daniels, 1990). After elution, the levels of [^{3}H]cyclic AMP were determined by liquid-scintillation counting. In experiments designed to measure stimulation of adenylyl cyclase (*via* Gs), cells were incubated overnight with PTX (100 ng ml^{-1}).

Measurement of [^{3}H]inositol phosphate accumulation

Confluent cell monolayers were loaded for 24 h with [^{3}H]-*myo*-inositol (37 kBq $well^{-1}$) in 24-well cluster dishes in inositol-free DMEM containing 1% foetal calf serum. Prelabelled cells were then washed once with 1 ml $well^{-1}$ Hanks/HEPES buffer (pH 7.4) and incubated at 37°C for 30 min in HHB in the presence of 20 mM LiCl (290 μl $well^{-1}$). Agonists were then added in 10 μl of medium and the incubation continued for 40 min at 37°C. Incubations were terminated by aspiration of the incubation medium and the addition of 1 ml cold (−20°C) methanol/0.12 M HCl (1 : 1 ($v\,v^{-1}$)). Cells were left a minimum of 2 h at −20°C before isolation of total [^{3}H]inositol phosphates in the supernatant of the disrupted cell monolayers by anion exchange chromatography. A measure of 800 μl aliquots of the supernatant were neutralised by the addition of 135 μl 0.5 M NaOH, 1 ml 25 mM Tris-HCl (pH 7.0) and 3.1 ml distilled water and added to columns of Dowex 1 anion exchange resin ($\times$ 8, 100–200 mesh, chloride form). [^{3}H]inositol and [^{3}H]glycerophosphoinositol were removed with 20 ml of distilled water and 10 ml of 25 mM ammonium formate, respectively. Total [^{3}H]inositol phosphates were then eluted with 3 ml of 1 M HCl and the columns regenerated with 10 ml 1 M HCl followed by 20 ml distilled water. To each sample, 10 ml of Emulsifier-Safe scintillator (Packard) was added and radioactivity was quantified by scintillation counting in the gel phase (scintillator plus, Packard). In experiments designed to measure phospholipase C activation *via* Gq, cells were incubated overnight with PTX (100 ng ml^{-1}).

Data analysis

EC_{50} and IC_{50} (concentrations of drug producing 50% of the maximal stimulatory or inhibitory response) and pEC_{50} (negative logarithm of the EC_{50}) values were obtained by computer-assisted curve fitting (to a sigmoidal dose response) using the computer program PRISM (GraphPAD, California,

U.S.A.). The same program was also used to perform nonlinear regression analysis for fitting data from saturation experiments (one site binding hyperbola). Data from competition experiments were fit using nonlinear regression analysis (one site competition). IC_{50} values obtained (concentration of drug required to inhibit 50% of specific [^{3}H]DPCPX binding) were used to calculate K_i values for each ligand, using the Cheng–Prusoff equation (Cheng & Prusoff, 1973) (from which pK_i values were subsequently calculated as the negative logarithm of the K_i value). Statistical significance was determined by Student's unpaired *t*-test, unless otherwise stated. All data are presented as mean ± s.e.m. The *n* in the text refers to the number of separate experiments performed.

Chemicals

[2-^{3}H]*myo*-inositol, [2,8-^{3}H]adenine, [^{35}S]GTPγS and 8-cyclopentyl-[^{3}H]1,3-dipropylxanthine were from NEN Dupont (Herts, U.K.). Adenosine deaminase was from Boehringer Mannheim/Roche. Forskolin, rolipram, theophylline, Triton X-100, GDP, NECA, CPA. 5′-*N*-cyclopropyl-carboxyamidoadenosine (CPCA) and N^6-cyclohexyladenosine (CHA) were purchased from Sigma Chemical Co (Dorset, U.K.). PTX was obtained from Merck, Darmstadt, Germany. Dulbecco's modified Eagle's medium/nutrient mix F-12 (1 : 1) and foetal calf serum were from Sigma Chemical Co (Dorset, U.K.). All other chemicals were of analytical grade. 2′-Deoxy-N^6-cyclopentyladenosine (2′dCPA), 3′-deoxy-N^6-cyclopentyladenosine (2′dCPA), 8-methylamino-N^6-cyclopentyladenosine (8MeCPA) and 8-cyclopentyl-N^6-cyclopentyladenosine (8CPCPA) were synthesised as described previously (van der Wenden *et al*., 1995; Roelen *et al*., 1996). 5′-*N*-cyclobutyl-carboxyamidoadenosine (CBuCA), 5′-*N*-cyclopentylcarboxyamidoadenosine (CPeCA) were synthesised as described previously (de Zwart *et al*., 1999).

Results

Radioligand binding assays in whole cells

In CHO cells, expressing the human A_1 adenosine receptor, the binding of the radioligand [^{3}H]DPCPX was saturable and best described by a model of one binding site with a K_d of 2.0 nM

Table 1 Ligand binding affinities, maximal responses and potencies of CPA and NECA analogues at multiple effector pathways of the A_1 adenosine receptor

	Ligand binding pK_i	*[^{35}S]GTPγS (% CPA)*	*↓ adenylyl cyclase (% Fsk inhibition) Gi*	*↑ adenylyl cyclase + PTX (%stimulation over Fsk) Gs*	*Inositol phosphate accumulation (% CPA) Gi + Gq*	*Inositol phosphate accumulation + PTX (% CPA) Gq*
CHA	5.88 ± 0.07	7.00 ± 0.19 (100 ± 6)	8.49 ± 0.07 (94 ± 5)	6.42 ± 0.11 (160 ± 18)	6.49 ± 0.08 (148 ± 1)	6.15 ± 0.25 (195 ± 27)
CPA	6.17 ± 0.02	7.40 ± 0.28 (100)	8.84 ± 0.13 (98 ± 1)	6.56 ± 0.07 (115 ± 10)	6.84 ± 0.19 100	6.69 ± 0.14 (100)
3′dCPA	5.08 ± 0.02	5.43 ± 0.19 (105 ± 13)	7.50 ± 0.15 (87 ± 9)	5.14 ± 0.37 (75 ± 16)	5.75 ± 0.24 (38 ± 8)	< 4.00 *(8 ± 4)*
2′dCPA	*< 4.00*	*< 4.00* (54 ± 4)	5.59 ± 0.14 (96 ± 3)	ND (2 ± 3)	4.43 ± 0.53 (18 ± 5)	ND (2 ± 2)
8MeCPA	*4.86 ± 0.02*	4.98 ± 0.15 (89 ± 11)	7.06 ± 0.13 (95 ± 1)	5.20 ± 0.23 *(71 ± 12)*	5.60 ± 0.24 (43 ± 6)	< 4.00 *(18 ± 4)*
8CPCPA	*5.16 ± 0.05*	*4.68 ± 0.12* (61 ± 10)	6.55 ± 0.06 (85 ± 5)	< 4.00 *(86 ± 16)*	< 4.00 *(32 ± 6)*	< 4.00 *(53 ± 11)*
NECA	5.77 ± 0.04	6.79 ± 0.12 (108 ± 4)	8.59 ± 0.14 (90 ± 12)	6.18 ± 0.05 (235 ± 33)	6.76 ± 0.11 (149 ± 11)	6.30 ± 0.11 (211 ± 26)
CPCA	5.79 ± 0.01	6.72 ± 0.31 (92 ± 4)	8.58 ± 0.01 (95 ± 4)	6.23 ± 0.06 (188 ± 26)	6.01 ± 0.06 (171 ± 1)	5.76 ± 0.12 (233 ± 23)
CBuCA	5.74 ± 0.08	6.82 ± 0.14 (99 ± 1)	8.14 ± 0.05 (98 ± 2)	6.25 ± 0.19 (154 ± 26)	6.18 ± 0.18 (125 ± 8)	5.52 ± 0.03 (209 ± 32)
CPeCA	*4.71 ± 0.06*	5.18 ± 0.28 (92 ± 9)	7.00 ± 0.11 (89 ± 4)	5.30 ± 0.13 (136 ± 16)	5.32 ± 0.42 (61 ± 6)	< 4.00 (22 ± 3)

Values given are pK_i (for ligand binding) or pEC_{50} values (for all other assays) expressed as mean value ± s.e.m. from three or more independent experiments, performed in triplicate. Data where $n > 3$ are as follows: adenylyl cyclase stimulation by 2′dCPA $n = 6$, 8MeCPA $n = 6$, 8CPCPA $n = 4$, CPA $n = 9$ and CPeCA $n = 9$. Inositol phosphate accumulation (control) by 3′dCPA $n = 4$, NECA $n = 4$, CPA $n = 4$, IP accumulation + PTX; NECA $n = 4$, CPA $n = 4$. Values within parentheses are maximal responses, expressed either as a percentage of that obtained with 100 μM CPA ([^{35}S]GTPγS and inositol phosphate accumulation) or as a percentage of that obtained with 3 μM forskolin (increase and decrease in adenylyl cyclase activity). Values in italics denote approximate pEC_{50} or % maximal effect (obtained at 100 μM), in cases where concentration response curves could not be fully defined. ND (as for 2′dCPA) refers to cases where experiments were performed but no significant stimulation could be measured, even at 100 μM.

($pK_d = 8.70 \pm 0.07$, $n = 3$) and a maximum density of 3.05 ± 0.17 pmol mg protein^{-1} ($n = 3$). In competition binding assays, agonist binding was best described by a model of one binding site (of low affinity) for all ligands (see Table 1). Within the CPA analogues, removal of the 3′- or 2′-hydroxyl groups reduced ligand affinity by approximately 10- or 100-fold, respectively. Both substitutions in the C8-position (Figure 1) also reduced affinity by approximately 10-fold. The NECA analogues CPCA and CBuCA had similar micromolar affinity as NECA, only CPeCA showing a reduction in affinity, compared to NECA (unpaired t-test; $P < 0.01$).

Inhibition of [^{3}H]cAMP accumulation

All of the CPA and NECA analogues caused a concentration-dependent inhibition of forskolin-stimulated [^{3}H]cAMP accumulation, and were all full agonists, relative to CPA (the maximum efficacy of 8CPCPA was not significantly different from that of CPA; $P > 0.05$, Student's t-test). The potencies with which agonists effected this response ranged from nanomolar (e.g. CPA) to micromolar (e.g. 2′dCPA) (Table 1). For the CPA analogues, the rank order of potency for this response was similar to that of ligand binding affinity (CHA $\approx$ CPA > 3′dCPA > 8MeCPA $\approx$ 8CPCPA > 2′dCPA). Similarly, the potencies with which NECA analogues inhibited cAMP accumulation were of the same rank order (NECA $\approx$ CPCA > CBuCA > CPeCA) as ligand binding affinity.

[^{35}S]GTPγS binding

The activation of G proteins by A_1 adenosine receptors can also be monitored directly by measuring an increase in [^{35}S]GTPγS binding, following agonist stimulation. Owing to the rate of guanine nucleotide exchange, this assay detects primarily the activation of G proteins of the Gi/o family (Milligan, 2003), allowing agonist responses to be measured before the stage of adenylyl cyclase inhibition. Indeed, in membranes of CHO cells, expressing the A_1 adenosine receptor, NECA-stimulated [^{35}S]GTPγS binding is totally inhibited by preincubating the cells overnight with 100 ng ml^{-1} PTX (Figure 2c). All agonists stimulated [^{35}S]GTPγS binding to $CHOA_1$ cell membranes, to some extent (Figure 2). The maximum response of 2′dCPA and 8CPCPA could not be achieved at the concentration of 100 μM. Although these ligands cannot be defined as partial agonists in this assay, since the concentration–response curves have not reached a plateau, this observation is in contrast with the fully defined maximal responses observed with the inhibition of adenylyl cyclase. This may reflect a degree of amplification or receptor reserve in the [^{3}H]cAMP accumulation assay. For the remaining ligands, the concentration–response curves reached a plateau, and they could be defined as full agonists, relative to CPA in stimulating [^{35}S]GTPγS binding. The ratios of agonist potency, in stimulating [^{35}S]GTPγS binding, compared to binding affinity, varied between agonists (Table 1). The K_i/EC_{50} ratio was greatest for CPA (17) and similar for CHA, NECA, CPCA and CBuCA (13,10, 9 and 12, respectively). The CPA analogues (3′dCPA, 2′dCPA, 8MeCPA and 8CPCPA) and the largest NECA analogue CPeCA, however, had K_i/EC_{50} ratios that were less than 3.

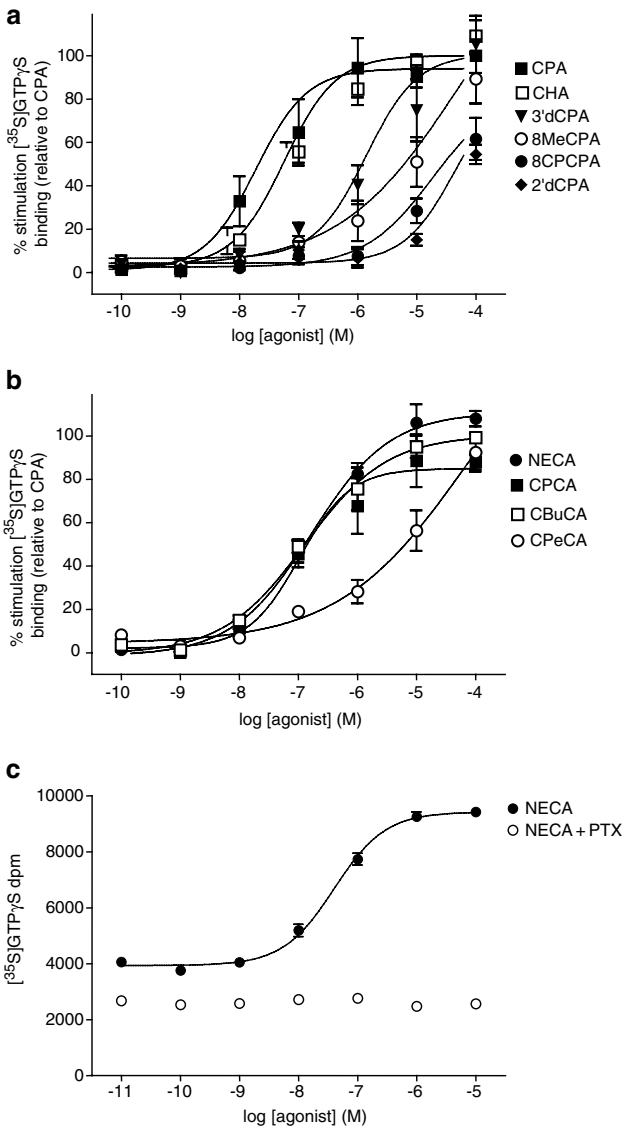

Figure 2 Stimulation of [^{35}S]GTPγS binding to $CHOA_1$ cell membranes by CPA and NECA analogues. Data (a and b) are expressed as a percentage of the maximal stimulation achieved with 100 μM CPA and represent mean ± s.e.m. from three independent experiments, performed in triplicate. (a) Stimulation by CPA and CPA analogues. (b) Stimulation by NECA and NECA analogues. (c) Stimulation of [^{35}S]GTPγS binding is blocked by preincubating cells overnight with 100 ng ml^{-1} PTX. Data are representative of three independent experiments, performed in triplicate.

Stimulation of [^{3}H]cAMP accumulation

In PTX-treated $CHOA_1$ cells, several agonists potentiated forskolin-stimulated [^{3}H]cAMP accumulation, although with much lower potencies than for inhibition of adenylyl cyclase (Figure 3). In particular, the NECA analogues all produced a robust stimulation of Gs (Table 1, Figure 3b), with NECA and CPCA being significantly more efficacious than CPA ($P = 0.02$ and 0.01, respectively, paired t-test, $n = 3$). The NECA analogue CBuCA, however, was not significantly more efficacious than CPA ($P > 0.05$), although it is of note that even CPeCA was as efficacious as CPA in this assay (maximal efficacies of 136 ± 16 and 115 ± 10% over forskolin, respectively, $n = 9$). 2′dCPA, 8MeCPA and 8CPCPA failed to

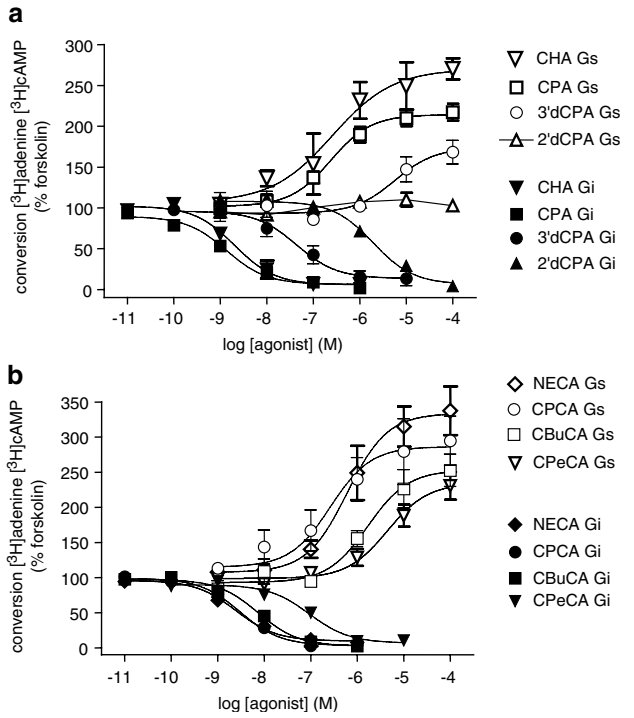

Figure 3 Dual inhibition and stimulation of cAMP accumulation by CPA and NECA analogues in CHO cells expressing the human A_1 adenosine receptor. Stimulation of [^{3}H]cAMP accumulation (from [^{3}H]adenine) is expressed as a percentage of that obtained with 3 μM forskolin. Each point represents mean $\pm$ s.e.m. obtained from three or more independent experiments (for n number see Table 1), performed in triplicate in either control conditions (filled symbols) or in cells treated overnight with 100 ng ml^{-1} PTX (empty symbols). (a) Inhibition (filled symbols) and potentiation (empty symbols) of forskolin-stimulated cAMP accumulation by CHA, CPA, 3′dCPA and 2′dCPA. (b) Inhibition (filled symbols) and potentiation (empty symbols) of forskolin-stimulated cAMP accumulation by NECA, CPCA, CBuCA and CPeCA. Note the scale on the Y-axis for stimulation of cAMP accumulation by NECA analogues (b) compared to CPA (a).

produce a clear concentration-dependent response; 2′dCPA had no effect at all (the percentage of [^{3}H]adenine converted to [^{3}H]cAMP observed with 100 μM 2′dCPA; 0.67 $\pm$ 0.05 was not significantly different from that with forskolin alone; 0.72 $\pm$ 0.09, $n = 6$) and 8MeCPA and 8CPCPA produced only a modest response at 100 μM. 3′dCPA did stimulate cAMP accumulation, but did not produce the same level of stimulation as CPA, at 100 μM (58 $\pm$ 1% CPA maximum). CHA, however, was significantly more efficacious than CPA (160 $\pm$ 18% over forskolin, $n = 3$ compared to 115 $\pm$ 10% over forskolin, $n = 9$; $P < 0.05$, unpaired t-test) (Figure 3a). We were assured that this stimulation of cAMP accumulation (in PTX-treated cells) could be attributed to the activation of the A_1 adenosine receptor, since in native CHOK1 cells (pretreated with PTX), NECA did not potentiate forskolin-stimulated adenylyl cyclase ($n = 4$, data not shown). Likewise, NECA failed to inhibit forskolin-stimulated adenylyl cyclase in CHOK1 cells ($n = 4$, data not shown).

Stimulation of [^{3}H]inositol phosphates accumulation

The CPA and NECA analogues also stimulated inositol phosphate accumulation in these cells, although with much

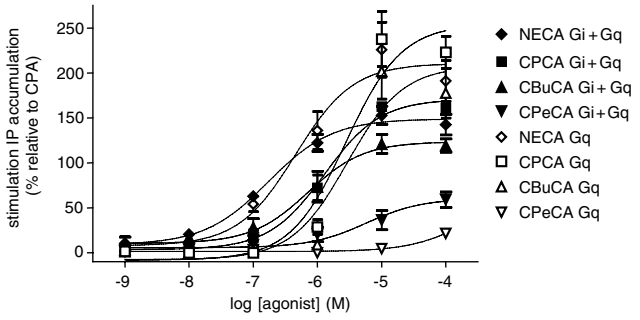

Figure 4 Stimulation of inositol phosphates accumulation by NECA analogues in the absence (Gi + Gq) and presence (Gq) of PTX. Data show stimulation of inositol phosphates accumulation obtained with NECA, CPCA, CBuCA and CPeCA, expressed as a percentage of the maximal stimulation obtained with 100 μM CPA. Each point represents mean $\pm$ s.e.m. obtained from either four (NECA) or three (CPCA, CBuCA and CPeCA) independent experiments, performed in triplicate in either control conditions (filled symbols) or in cells treated overnight with 100 ng ml^{-1} PTX (empty symbols).

lower potencies than for inhibiting adenylyl cyclase (Table 1). The reduced potency is as expected for a response-mediated predominantly by Gi betagamma subunits. Some ligands produced a partial response, relative to CPA, or a response that was not fully defined at 100 μM (responses produced at 100 μM, relative to that obtained with CPA were as follows: 3′dCPA, 38 $\pm$ 8%; 2′dCPA, 18 $\pm$ 5%; 8MeCPA, 43 $\pm$ 6%; and 8CPCPA, 32 $\pm$ 6%). 8CPCPA only produced a response when administered at 100 μM. NECA and its analogues produced a robust response, although maximum efficacy of the analogues was reduced with increase in substituent size (Figure 4), such that NECA and CPCA were more efficacious than CPA (maximum responses significantly greater than that of CPA; 149 $\pm$ 11 and 171 $\pm$ 1% CPA response, respectively, t-test; $P < 0.05$), CBuCA was a full agonist, relative to CPA (maximum response not significantly greater than that of CPA) and CPeCA was, in fact, a partial agonist (61 $\pm$ 6% CPA maximal response, $n = 3$, $P < 0.05$, t-test).

Stimulation of [^{3}H]inositol phosphates accumulation in PTX-treated cells

For the CPA analogues, activation of PLC in PTX-treated cells (presumably *via* Gq) was the pathway at which they were least effective. Only CPA and CHA elicited a dose-dependent response, with the least potency of all the functional assays (Table 1). 2′dCPA was ineffective altogether (stimulation (relative to 100 μM CPA) with 100 μM 2′dCPA was 2 $\pm$ 2%) and 3′dCPA, 8MeCPA and 8CPCPA only produced a response when administered at 100 μM. NECA and its analogues, however, produced a robust stimulation of Gq (Figure 4), with the notable exception of CPeCA, which caused only a small stimulation (when administered at 100 μM; 22 $\pm$ 3% CPA maximal response). Indeed, while NECA, CPCA and CBuCA were significantly more efficacious than CPA ($P < 0.05$, unpaired t-tests, $n = 4$, 3 and 3, respectively), CPeCA was significantly less efficacious than CPA ($P < 0.001$, unpaired t-test, $n = 3$).

Discussion

The present study examines the activation of multiple effector pathways by the A_1 adenosine receptor and the role that ligands play in the selective activation of G proteins. Further to our initial observations that NECA can couple the receptor to Gs and Gq proteins more efficiently that CPA, we now identify ligands that are much less efficacious than CPA in activating Gs and Gq, or ineffective altogether. Thus, identifying a spectrum of G-protein-activating potential for this receptor, which depends on the agonist present.

Initially, competition binding assays were performed in whole cells to obtain agonist affinities at the receptor in similar conditions to those employed in the various effector activation assays. The resulting data for all agonists were best described by a single, low-affinity site, which most likely represents binding to the receptor in the uncoupled form. The measurement of [^{35}S]GTPγS binding revealed the ability of agonists to activate G proteins that are predominantly of the Gi family. The agonists' ratios of K_i (ligand binding) and EC_{50} (in [^{35}S]GTPγS binding) values were varied, but provided an index of overall efficacy; they could be grouped as 'high ratios', for ligands whose efficacy in each assay was equal to or greater than that of CPA (ratios 9–17 or CPA, CHA, NECA, CPCA and CBuCA) and 'low ratios' (less than 3) for ligands that either failed to produce a response in further assays or produced only a small responses, at high concentrations (3′dCPA, 2′dCPA, 8MeCPA, 8CPCPA and CPeCA). Not surprisingly, the relationship between agonist potencies in the [^{35}S]GTPγS binding assay correlated well with those achieved at the subsequent step of adenylyl cyclase inhibition (Table 1). In the [^{35}S]GTPγS binding assay, however, agonists were of low potency and in some cases did not produce a maximal response at 100 μM. This is likely due in part to the design of the assay, which optimises the signal-to-noise ratio by employing a high concentration of GDP, and partly by being upstream of the signal amplification and receptor reserve associated with adenylyl cyclase inhibition.

Indeed, inhibition of adenylyl cyclase was readily achieved by agonists acting at the A_1 adenosine receptor; most agonist potencies were in the nanomolar range and all agonists were 'full', relative to CPA. In contrast, the stimulation of inositol phosphates accumulation (primarily *via* Gi betagamma subunits) was less robust, agonists were approximately 10- to 400-fold less potent in this assay compared to adenylyl cyclase inhibition, and in some cases appeared to be partial agonists, relative to CPA. Since both Gi betagamma and Gq alpha subunits may contribute to this response, this observation may be due to a low receptor reserve for the former component and/or differential activation of the second component.

In cells pretreated with PTX, most agonists caused an amplification of forskolin-stimulated cAMP accumulation, indicative of receptor coupling to Gs. Agonist potencies were approximately 1000-fold less than those for adenylyl cyclase inhibition, that is, in the micromolar range. Some ligands were only partial agonists, relative to CPA, and others unable to couple the receptor to this response at all (e.g. 2′dCPA). Agonists also stimulated PTX-resistant inositol phosphate accumulation, indicative of Gq coupling. This response was the poorest; agonists potencies being the lowest for all the assays and with the fewest number of ligands eliciting a dose-dependent response. Hence, if the agonists' behaviour is the result of eliciting 'differential strength of signal' (e.g. 2′dCPA producing a very weak signal; coupling only to Gi), the receptor can be described as coupling to the different G proteins with the following rank order of 'preference': Gi > Gs > Gq. The agonists could be described as having the following (approximate) decreasing rank order of 'strength' (based on efficacies at Gi, Gs and Gq): NECA ≈ CPCA > C-BuCA ≈ CHA > CPA > CPeCA > 8CPCPA ≈ 3′dCPA ≈ 8MeC-8MeCPA · 2′dCPA. In fact, most of the data could be described by agonists activating the receptor with different strengths of signal, such that only the 'strongest' would be able to activate Gq, the weakest only Gi and those ligands activating Gi and Gs, with 'strengths' somewhere in between.

However, the profile of G-protein activation by the ligand CPeCA does not fit into this pattern. Compared to ligands with similar efficacy at other assays (i.e. 8MeCPA at Gi and Gq or CPA at Gi and Gs), it appears to be selectively poor in activating Gq, or surprisingly good at activating Gs. This is shown clearly when comparing the profiles of CPeCA with that of CPA for all three G proteins (Figure 5). It is not unexpected that CPeCA is a full agonist, relative to CPA (and NECA) in the assay of Gi, since there is substantial signal amplification in this assay and even the weakest of ligands in other assays (e.g. 2′dCPA) are 'full'. What is clear from the assays measuring Gs and Gq activation is that the relative efficacies of CPeCA, CPA and NECA are different. At Gs, CPeCA is a full agonist, relative to CPA, while they are both partial agonists, relative to NECA. If this were due to 'differential strength of signal', then one would expect a similar pattern of relative efficacies at Gq, that is, both CPA and CPeCA to be partial agonists (relative to NECA), to the same extent. The response with CPeCA at Gq is only achieved at a concentration of 100 μM, which seems to suggest that CPeCA is selectively weak at Gq. Its efficacy, however, cannot be defined due its low potency in this assay. How this selective G-protein activation arises is not clear, but it is possible that the receptor exists in agonist-specific conformations that determine the receptor's ability to couple to G proteins. Just which elements of agonist structure may be involved in this 'propensity to couple' will now be discussed with regard to the CPA and NECA analogues used.

CPA is often considered as the 'classical' A_1 adenosine receptor agonist; its N^6-substitution conveying good selectivity and potency for this receptor (Trivedi *et al*., 1990). In this study, it is the most potent of all the agonists, in each of the assays, although not always the most efficacious. CHA differs from CPA by one carbon atom at the N^6-substituent and, not surprisingly, has similar potency to CPA in activating each of the G proteins (Table 1). In stimulating Gs, however, CHA was significantly more efficacious than CPA (160 ± 18% over forskolin, $n = 3$ compared to 115 ± 10% over forskolin, $n = 9$; $P < 0.05$, unpaired t-test). Exactly how this change in the N^6-position could affect formation of the R–Gs tandem is not clear, although mutagenesis studies have highlighted key residues in the receptor that interact with this family of ligands. Transmembrane domains 1–4 were identified as important for agonist and antagonist binding at A_1 adenosine receptors (Rivkees *et al*., 1995), and would thus be a logical location for interaction with the N^6-domain that denotes A_1-specificity. It has since been proposed that the N^6-moiety is positioned towards the top of third transmembrane domain (Rivkees *et al*., 1999) since there are several residues in this

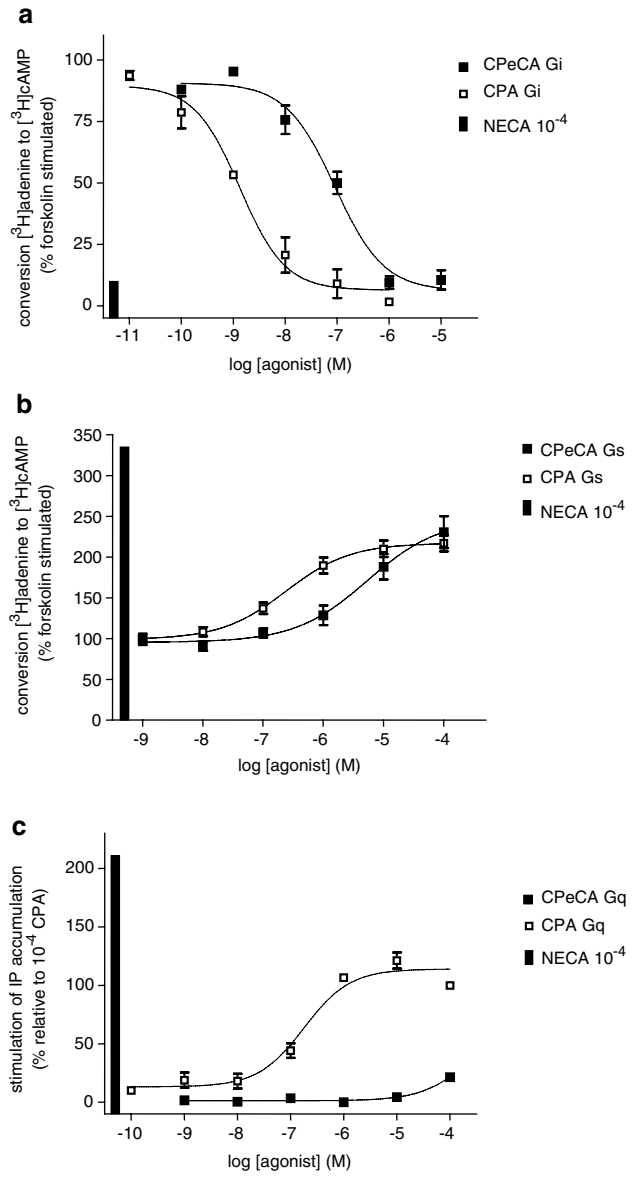

Figure 5 Comparison of G protein activation by CPA and CPeCA in CHO A_1 cells. In each assay, points represent mean ± s.e.m. obtained from three or more independent experiments (for *n* number see Table 1), performed in triplicate. Column represents maximal response obtained with 100 μM NECA. (a) Inhibition of cAMP accumulation (Gi), expressed as a percentage of that obtained with 3 μM forskolin. (b) Potentiation of cAMP accumulation (Gs), in PTX-treated cells, also expressed as a percentage of that obtained with 3 μM forskolin. (c) Stimulation of accumulation of inositol phosphates in PTX-treated cells (Gq), expressed relative to that obtained with 100 μM CPA.

region (e.g. Leu88, Thr91 and Gln92) that are particularly important for the binding for N^6-substituted agonists. As the Leu88 to Ala88 mutation had the largest effect on the ligand with the most hydrophobic N^6-moiety (R-PIA), it was proposed that Leu88 interacts with hydrophobic N^6-substituents (Rivkees *et al*., 1999). It is possible then that a hydrophobic interaction with the leucine residue differs between CPA and CHA, and may lead to the stabilisation of different receptor conformations.

The deoxyribose analogues of CPA (3′dCPA and 2′dCPA) were compromised, relative to CPA, in terms of binding affinity, potency and in some cases efficacy (Table 1). The importance of the ribose moiety and, more specifically, the hydroxyl groups in conveying high affinity and agonist efficacy has been known for some time. Removal of the ribose completely from CPA produces an A_1 adenosine receptor antagonist (Barrett *et al*., 1993), as does removal of both the 2′ and 3′ hydroxyls from CHA (Lohse *et al*., 1988). The individual removal of the 2′ or 3′ hydroxyls from CPA produces partial agonists for the receptor (van der Wenden *et al*., 1995); removal of the 2′ hydroxyl having the most detrimental effect. In the activation of G proteins of the Gi/o family, 2′dCPA and 3′dCPA are partial agonists; 2′dCPA being the weakest, particularly in activating Go (Lorenzen *et al*., 1998).

In the present study, the removal of the 3′- and 2′-hydroxyl groups led to approximately a 10- or 100-fold reduction, respectively (compared to CPA), in either affinity or potency (Table 1). Most dramatically, removal of the 2′ hydroxyl abolished agonist-mediated activation of Gs and Gq, while removal of the 3′ hydroxyl reduced Gs activation and almost abolished Gq activation. Thus, the 2′- and 3′-hydroxyl groups have a key role in denoting agonist efficacy at multiple G proteins.

A molecular model for the adenosine A_1 receptor, based on the structure of rhodopsin, aligns the ribose group with the bottom half of TM3 and TM7 (Rivkees *et al*., 1999), in agreement with previous reports highlighting the importance of residues Ser94, Thr277 and His278 in agonist binding and function (Townsend-Nicholson & Schofield, 1994; Barbhaiya *et al*., 1996). Thus, it is possible that the hydroxyl groups form an important interaction with the conserved serine residue at position 94. It is tempting to compare the importance of the 2′ and 3′ hydroxyls on G-protein coupling at the A_1 adenosine receptor with the well-established role of hydroxyls on catecholamines and phenolamines in activating multiple G proteins *via* (interaction with serine residues on) adrenoceptors, octopamine and dopamine receptors (Cox *et al*., 1992; Robb *et al*., 1994; Woodward *et al*., 1996; Airriess *et al*., 1997; Wiens *et al*., 1998; Rudling *et al*., 1999; Cordeaux *et al*., 2001; Peltonen *et al*., 2003). However, although the A_1 adenosine receptor has a pair of conserved serines (at positions 93 and 94) that might similarly interact with the 2′ and 3′ hydroxyls, mutation of the former residue had little effect on agonist binding (Barbhaiya *et al*., 1996). It would therefore be of interest to study the role of these serines on receptor coupling to multiple G proteins.

The C8-substituted CPA analogues were synthesised as potential A_1 adenosine partial agonists of possible therapeutic use as antilipolytic agents with reduced cardiovascular side effects (Roelen *et al*., 1996). Indeed, they have very little effect on heart rate in rats (Schaick *et al*., 1997), while maintaining the same antilipolytic activity as CPA (Schaick *et al*., 1998). In the present study, 8MeCPA and 8CPCPA showed reduced affinity and potency compared to CPA, the greatest reduction being with the largest ligand (8CPCPA). Although full agonists in inhibiting adenylyl cyclase, both ligands were either partial agonists at other assays, or of such low potency that a maximal response was not achieved with 100 μM. Therefore, unlike 2′dCPA, these agonists can couple the receptor to Gs and Gq, but do so with very low potency.

The series of NECA analogues address the role of the 5′-N-substituent on relative efficacy of NECA at PTX-resistant G

proteins. The two smallest ligands in the series CPCA and CBuCA had similar binding affinities and potencies in each of the assays as NECA, the largest ligand CPeCA, however, showed at least a 10-fold reduction in affinity and potency, suggesting that while well tolerated, substitutions at this position have a specific size limitation. Within the cycloalkyl series, in the assays measuring activation of Gs and Gq, there is a sequential reduction in efficacy with increase in substituent size. For Gs activation, this is not enough to bring the efficacy of CPeCA below that of CPA (see Table 1). At Gq, the effect of substituent size is clearest; while CPCA and CBuCA (like NECA) are more efficacious than CPA, CPeCA is much less efficacious than CPA. Hence, a small change in ligand structure can cause a pathway-specific reduction in efficacy. The interaction of the 5′ substitution of NECA has been ascribed to the threonine residue at position 277 in the seventh transmembrane domain (Townsend-Nicholson & Schofield, 1994; Tucker *et al*., 1994), and it is possible that this and other interactions are reduced with the increase in ligand hydrophobicity.

It is clear from the present study that, in addition to ligands with greater efficacy than CPA in activating Gs and Gq, there are ligands that are less efficacious than CPA at these G proteins. Moreover, in addition to previous comparisons with only NECA and CPA, ligand-specific activation of G proteins has also been observed between agonists with very small changes in structure (e.g. one carbon added or removed, one hydroxyl group removed). Thus, we provide evidence that the A_1 adenosine receptor has a diverse G-protein-activating profile that depends on the agonist present. We do not yet have evidence for 'agonist trafficking', since we have not observed the definitive reversal of potency or efficacy. Unfortunately, we lack sufficient partial agonists of high enough potency at Gs and Gq, for their maximal responses to be defined. Although the majority of the data could be explained by 'differential strength of signal' and a single receptor conformation, it is difficult to reconcile the G-protein activation profile of some agonists, particularly CPeCA, with this model.

We thank the Wellcome Trust for financial support (ref 066817/z/02/z)

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(Received April 28, 2004
Revised June 2, 2004
Accepted June 24, 2004)