Br. J. Pharmacol. (1994), **111**, 1252–1256 © Macmillan Press Ltd, 1994

Coupling of a transfected human brain A_1 adenosine receptor in CHO-K1 cells to calcium mobilisation via a pertussis toxin-sensitive mechanism

Philip A. Iredale, Stephen P.H. Alexander & [1]Stephen J. Hill

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 The presence of A_1 adenosine receptors in CHO-K1 cells transfected with the human brain A_1 sequence was confirmed by ligand binding studies using 8-cyclopentyl-[^{3}H] 1,3-dipropylxanthine ([^{3}H]-DPCPX).

2 Alterations in intracellular calcium ($[Ca^{2+}]_i$) were measured with the calcium-sensitive dye, fura-2.

3 N^6-cyclopentyladenosine (CPA), the selective A_1 agonist, and 5'-N-ethylcarboxaminoadenosine (NECA), a relatively non-selective adenosine receptor agonist, elicited rapid, biphasic increases in $[Ca^{2+}]_i$ which involved both mobilisation from intracellular stores and calcium entry.

4 The calcium response to CPA was significantly inhibited by the selective A_1 antagonist DPCPX. The non-selective adenosine receptor, xanthine amino congener (XAC), was less potent.

5 The calcium response to CPA was completely prevented by pretreatment of the cells with pertussis toxin implicating the involvement of G_i in the receptor-mediated response.

6 In summary, we present evidence for the coupling of transfected human brain A_1 adenosine receptors in CHO-K1 cells to mobilisation of $[Ca^{2+}]_i$ via a pertussis toxin-sensitive G protein.

Keywords: A_1 adenosine receptor; transfection; intracellular calcium; pertussis toxin; fura-2

Introduction

Adenosine receptors have been divided into three major subtypes (A_1, A_2 and A_3) on the basis of agonist structure-activity relationships and the use of a range of xanthine and non-xanthine antagonists (Daly *et al.*, 1986; Bruns *et al.*, 1987; Jacobson *et al.*, 1992; van Galen *et al.*, 1992; Zhou *et al.*, 1992; Carruthers & Fozzard, 1993; Gurden *et al.*, 1993). The A_2-adenosine receptor has been further divided into high and low affinity subtypes, A_{2a} and A_{2b} through the use of the A_{2a}-selective agonist, CGS 21680 (Daly *et al.*, 1983; Bruns *et al.*, 1986; Abbracchio *et al.*, 1993). A_2 adenosine receptors are positively coupled to adenylyl cyclase via a G_s-protein, while A_1 adenosine receptors are generally negatively linked to adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation via a pertussis toxin-sensitive G-protein (Van Calker *et al.*, 1978; Gerwins & Fredholm, 1992). Preliminary data obtained with cells expressing cDNA for the putative A_3-adenosine receptor indicate that this receptor may also regulate cyclic AMP formation in a negative fashion (Zhou *et al.*, 1992).

The molecuar sequences for A_1 and A_{2a} receptors have been cloned from a variety of sources, including dog (Maenhaut *et al.*, 1990; Libert *et al.*, 1991), rat (Mahan *et al.*, 1991; Chern *et al.*, 1992) and man (Furlong *et al.*, 1992; Townsend-Nicholson & Shine, 1992). Putative A_{2b} receptors have also been cloned from rat (Stehle *et al.*, 1992) and human (Pierce *et al.*, 1992) tissues although the lack of a selective ligand for the A_{2b} subtype makes conclusive identification difficult.

The A_1 adenosine receptor is thought to be a promiscuous receptor in that it is reported to couple to inhibition of adenylyl cyclase activity, modulation of ion channels and modulation of phosphoinositide turnover (van Galen *et al.*, 1992). For example, in the hamster vas deferens smooth muscle cell line, DDT_1 MF-2, A_1 receptors couple to inhibition of adenylyl cyclase activity (Gerwins *et al.*, 1990), stimulate phosphoinositide turnover (White *et al.*, 1992) and mobilise intracellular calcium ($[Ca^{2+}]_i$) (Dickenson & Hill, 1993a) via a pertussis toxin-sensitive G-protein (Dickenson & Hill, 1993b).

The human brain A_1 adenosine receptor expressed in CHO-K1 cells has been reported to inhibit forskolin-stimulated cyclic AMP accumulation (Townsend-Nicholson & Shine, 1992). The aim of this investigation was to determine whether this transfected receptor was also able to stimulate mobilisation of $[Ca^{2+}]_i$.

[1] Author for correspondence.

Methods

Cell culture

CHO-NAX2 (CHO-K1 cells transfected with the human brain A_1 adenosine receptor sequence, Townsend-Nicholson & Shine, 1992) cells were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagles Medium/Nutrient Mix F12 (1:1, with glutamine) containing 10% foetal calf serum without antibiotics.

Calcium measurements

$[Ca^{2+}]_i$ was measured as previously described (Iredale *et al.*, 1992). Briefly, the monolayers from two near-confluent flasks were detached with trypsin/EDTA (trypsin, 0.5 g; EDTA 0.2 g and NaCl, 0.85 g per litre) and resuspended in a simple saline HEPES buffer (composition mM: $CaCl_2$ 2, NaCl 145, glucose 10, KCl 5, $MgSO_4$ 1 and HEPES 10, pH 7.45). This was followed by incubation with fura-2 acetoxy methyl ester (5 μM; Calbiochem/Novobiochem) at 37°C (in the presence of 5% foetal calf serum) for 20 min and for a further 5 min following a three fold dilution (to ensure maximum hydrolysis of ester to the acid form). At the end of this loading period, excess dye was removed by centrifugation, the cells resuspended in fresh buffer (without serum), and left at room temperature until use. Each time course was preceded by a rapid spin in a microcentrifuge followed by resuspension in fresh buffer (2 ml).

All experiments were carried out using a Perkin Elmer LS 50 Spectrometer, with excitation ratioing between 340 and 380 nm, recording at 500 nm. The time course for each calcium measurement was 200 s with drugs added in 10 μl aliquots.

Calibration

At the end of each time course, ionomycin (20 μM; Calbiochem/Novabiochem) was added followed by EGTA (6.25 mM, pH greater than 8.5) in order to calculate R_{max} and R_{min}. Autofluorescence was determined in a separate cuvette following the addition of manganese (5 mM) after the ionophore ionomycin (20 μM). From these values and those obtained with fura-2 free acid, $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz *et al.* (1985).

Pertussis toxin experiments

Culture media was removed from two confluent flasks and replaced with fresh media containing pertussis toxin (200 ng ml^{-1}). The cells were incubated for 4 h before experiments were carried out.

[³H]-DPCPX binding

Ligand binding analysis in CHO-K1 cells was performed as previously described (Iredale *et al.*, 1993). Cells from 1 or 2 confluent 175 cm^2 flasks were detached with PBS/EDTA (composition mM: NaCl 138, KCl 2.7, $Na_2HPO_4.2H_2O$ 12.9, KH_2PO_4 1.5, pH 7.45). Membranes were prepared by resuspending the cells in ice-cold Tris-EDTA buffer (50 mM: 1 mM), followed by homogenization in a glass-teflon homogenizer (approximately 7 strokes) and centrifugation at 20,000 *g* for 15 min. The resulting pellet was resuspended in 500 μl of Tris-EDTA buffer. Samples (10 μl) were incubated in the absence (total binding) or presence (non-specific binding) of theophylline (5 mM) with increasing concentrations of 8-cyclopentyl-[³H] 1,3-dipropylxanthine ([³H]-DPCPX) in Tris-EDTA buffer containing adenosine deaminase (1 unit ml^{-1}) and Triton X-100 (0.01%) for 90 min at room temperature (assay volume of 200 μl). The incubation was stopped by rapid filtration and washing with ice-cold Tris-EDTA buffer (3 times, approximate volume 10 ml) over Whatman GF/B filters (pre-soaked for 1 h in 0.3% polyethylenemine to reduce non-specific binding) using a Brandel MR24 cell harvester. The bound [³H]-DPCPX was quantified by liquid scintillation spectrometry.

An estimate of the protein content of each sample was made using the method of Bradford (1976) following digestion in NaOH (0.5 M).

Statistics and data analysis

EC_{50} and IC_{50} (concentrations of drug producing 50% of maximal stimulation or inhibition) values were obtained by computer-assisted curve fitting by use of the computer programme InPlot (GraphPad, California, U.S.A.). Curves were also fitted to radioligand binding data using the same programme. Calcium data were captured using the ICBC programme supplied by Perkin-Elmer and imported to the graphics programme Sigma-Plot (Jandel, California, U.S.A.).

Statistical significance was determined by Student's unpaired *t* test ($P<0.05$ was considered statistically significant). The *n* in the text refers to the number of separate experiments.

Materials

CHO-NAX2 cells were a generous gift from Dr Andrea Townsend-Nicholson (Garvan Institute, Sydney, Australia). Cell culture flasks were obtained from Costar with Dulbecco's Modified Eagles Medium/Nutrient Mix F12 and foetal calf serum from Biological Industries (Glasgow, U.K.). Fura-2 AM and ionomycin were from Calbiochem/Novabiochem (Nottingham, U.K.). 8-Cyclopentyl-1,3-dipropylxanthine, xanthine amino congener, 5'-N-ethylcarboxaminoadenosine, N⁶-cyclopentyladenosine were supplied by RBI/Sexmat (Herts, U.K.), adenosine deaminase, theophylline, nickel chloride and Triton X-100 by Sigma (Poole, U.K.), 8-cyclopentyl-[³H] 1,3-dipropylxanthine by NEN Dupont (Stevenage, U.K.) and pertussis toxin and trypsin/EDTA by Gibco (Uxbridge, U.K.).

Results

[³H]-DPCPX binding

Saturation assays using increasing concentrations of [³H]-DPCPX to membranes of CHO-NAX2 cells revealed a K_d of 1.2 ± 0.3 nM ($n = 3$) and a B_{max} of 200 ± 44 fmol mg^{-1} protein ($n = 3$; Figure 1).

The calcium response to N⁶-cyclopentyladenosine

Addition of N⁶-cyclopentyladenosine (CPA; 100 nM) to populations of fura-2-loaded CHO NAX2 cells resulted in a rapid increase in $[Ca^{2+}]_i$ of 274 ± 39 nM above basal from a resting value of 103 ± 14 nM ($n = 5$; Figure 2a). This initial rise in $[Ca^{2+}]_i$ was accompanied by a secondary, maintained response (111 ± 17 nM above basal, measured at 120 s; $n = 5$; $P<0.01$ compared with basal) which was abolished by omission of extracellular calcium or the presence of nickel ions (Ni^{2+} 1 mM; Table 1 and Figure 2). The CPA response was concentration-dependent with an EC_{50} of 15 ± 1 nM ($n = 3$; Figure 2). A similarly-sized maximal increase in $[Ca^{2+}]_i$ was observed after addition of 5'-N-ethylcarboxaminoadenosine (NECA; Figure 3b and data not shown; EC_{50} 7 ± 2 nM, $n = 3$).

Antagonism of the adenosine receptor response

In order to verify the adenosine receptor subtype involved, the effects of antagonists on the observed increases in $[Ca^{2+}]_i$ were assessed. The cells were pre-incubated with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 25 nM) and xanthine amino congener (XAC; 25 nM) for 10 min. Both agents significantly inhibited the calcium response to a sub-maximal concentration of CPA (60 nM), however DPCPX was more effective (39 ± 3%, 66 ± 6% ($n = 5$) of controls respectively; Figure 4a). Further, a full concentration-response inhibition

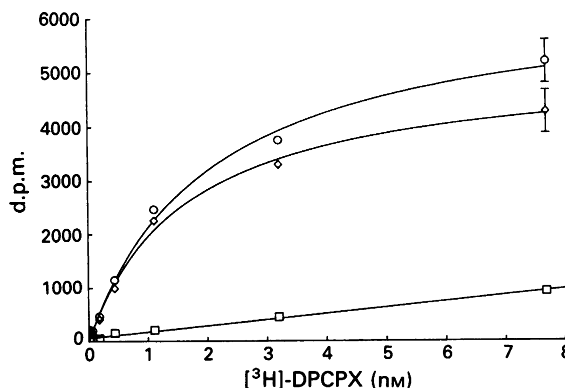

Figure 1 Binding of 8-cyclopentyl-[³H] 1,3-dipropylxanthine ([³H]-DPCPX) to CHO-NAX2 membranes. The graph shows saturation isotherms (total (O), non-specific (□) and specific (◇) binding shown). Data points represent single experiments carried out in quadruplicate. The experiment was carried out twice more with similar results.

curve revealed an IC_{50} for DPCPX of 26 ± 1 nM ($n = 3$) (Figure 4b). The K_d for this response, as calculated by the method of White *et al.* (1992) was 2.5 ± 0.2 nM ($n = 3$).

Pertussis toxin sensitivity

Pretreatment of the cells with pertussis toxin (200 ng ml^{-1} for 4 h) completely prevented the response to CPA (100 nM; Figure 5a; $n = 6$). However, the calcium rise following stimulation of the endogenous P_{2U} purinoceptor (Iredale & Hill, 1993) was unaffected (100 ± 1% of controls, $n = 6$; Figure 5b).

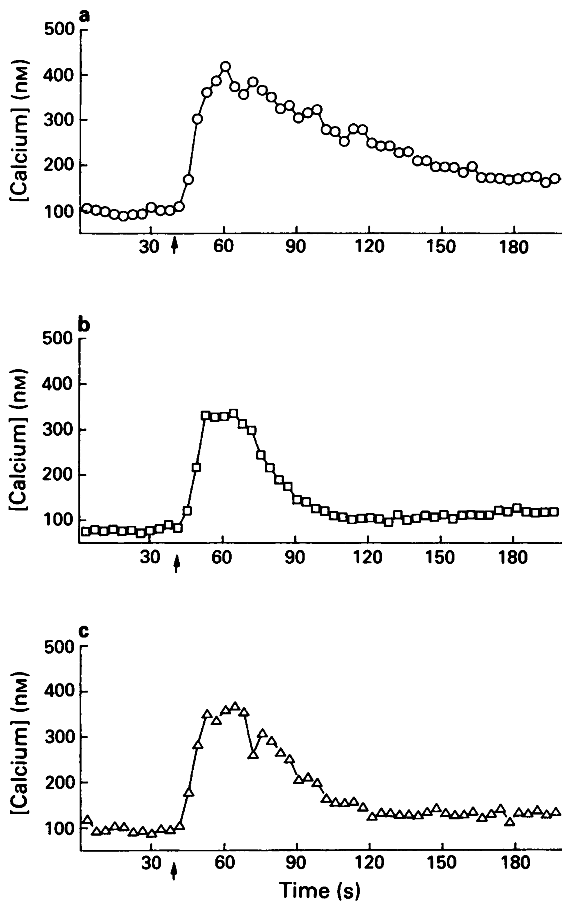

Figure 2 The effect of N^6-cyclopentyladenosine (CPA, 100 nM) on fura-2-loaded CHO-NAX2 cells in the presence (a) and absence of (b) 2 mM extracellular calcium and in the presence of the inorganic calcium channel blocker, Ni^{2+} (1 mM; c). Each graph shown is typical of three others.

Table 1 The effect of the omission of extracellular calcium and Ni^{2+} on the responses to N^6-cyclopentyladenosine (CPA)

Agonist		*Basal*	*Peak*	*120 s*
	+ Ca^{2+}	103 ± 14	377 ± 51	214 ± 28**
CPA	− Ca^{2+}	77 ± 7	320 ± 52	94 ± 8
	+ Ni^{2+}	80 ± 9	403 ± 44	101 ± 14

The data are the $[Ca^{2+}]_i$ ($n = 5$; except Ni^{2+}, $n = 4$) of cells challenged with CPA (100 nM) in the presence and absence of extracellular calcium and following pre-incubation for 5 min with the inorganic calcium channel blocker, Ni^{2+} (1 mM). Those responses still significantly above basal at 120 s are shown with asterisks (**$P < 0.01$, Student's unpaired t test).

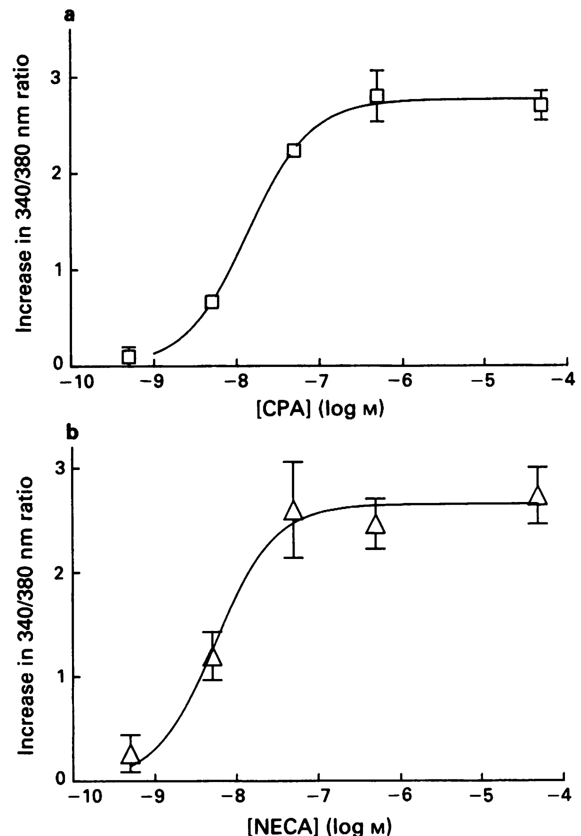

Figure 3 Concentration-response curves to N^6-cyclopentyladenosine (CPA, a) and 5′-N-ethylcarboxaminoadenosine (NECA, b). Data are means ± s.e.mean of three separate determinations.

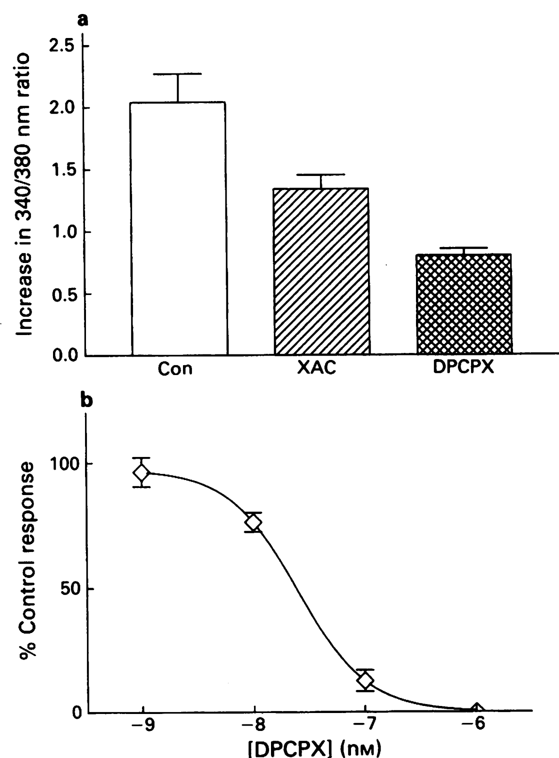

Figure 4 Panel (a) shows the effects of the adenosine antagonists, xanthine amino congener (XAC, 25 nM) and 8-cyclopentyl 1,3-dipropylxanthine (DPCPX, 25 nM), on the calcium response to N^6-cyclopentyladenosine (CPA, 60 nM). The cells were pre-incubated with each antagonist for 10 min prior to stimulation with CPA. Panel (b) shows the concentration-response relationship for DPCPX. Aliquots of cells were pre-incubated for 10 min with increasing concentrations of DPCPX and then challenged with CPA (100 nM). Data are means ± s.e.mean of three separate determinations.

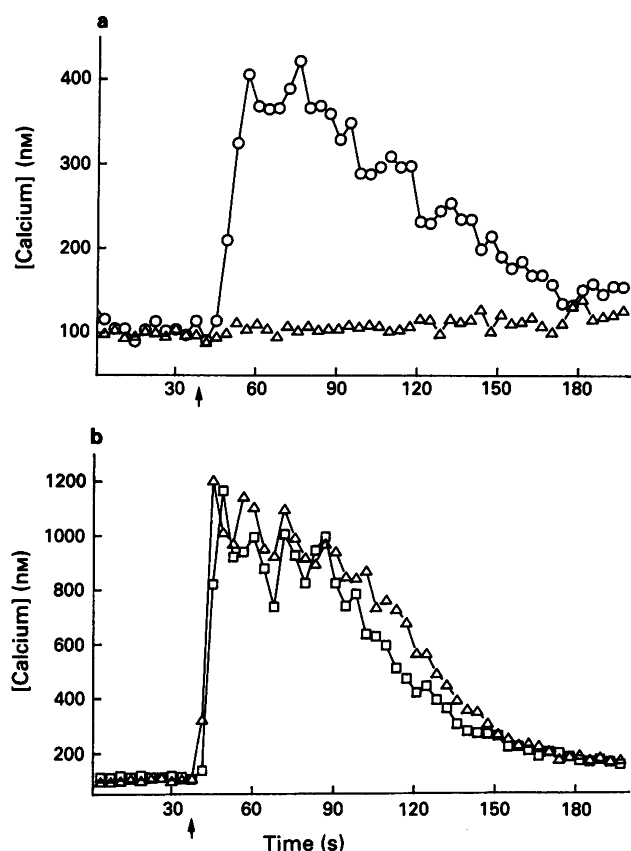

Figure 5 The effect of pertussis toxin on the calcium responses to (a) N^6-cyclopentyladenosine (CPA, 100 nM): (○) control; (△) pertussis toxin pretreated and (b) ATP (100 μM) (□) control; (△) pertussis toxin pretreated. The cells were pre-incubated with 200 ng ml^{-1} for 4 h as described in the text. Each graph shown is typical of five others.

Discussion

The present paper describes the coupling of the human brain A_1 adenosine receptor to phosphoinositide turnover and calcium mobilisation in CHO-K1 cells transfected with the A_1 adenosine receptor cDNA.

Saturation assays using the highly selective A_1 adenosine receptor radioligand, [^{3}H]-DPCPX (van Galen *et al.*, 1992) confirmed the presence of the A_1 receptor subtype in membranes derived from CHO-NAX2 cells. The determined K_d is in close agreement with the value of 0.96 nM reported by Townsend-Nicholson & Shine (1992) in the same cell line and also with the native human brain A_1 adenosine receptor (Ferkany *et al.*, 1986).

Stimulation of populations of fura-2-loaded cells revealed a concentration-dependent increase in $[Ca^{2+}]_i$ following stimulation with both CPA and NECA suggesting receptor coupling to phosphoinositide C. Both agonists showed similar potencies. The responses were biphasic, with an initial rapid increase in $[Ca^{2+}]_i$ which returned to a secondary, maintained plateau (data for NECA not shown). Results from a number of different cell lines have revealed that some agonist responses are biphasic, involving an initial transient release of calcium from intracellular stores followed by a secondary sustained influx across the plasma membranes resulting in a maintained plateau (Baird *et al.*, 1989; Jacob *et al.*, 1990). This plateau is maintained as long as the agonist occupies the receptor. The characteristics of the responses to both CPA and NECA are consistent with this theory. Furthermore, omission of calcium, or inclusion of the inorganic calcium channel blocker Ni^{2+} in the extracellular medium, prevented the formation of the plateau phase, providing further evidence that influx of calcium forms part of the overall response.

The binding data clearly indicate the presence of an A_1 adenosine receptor in CHO-NAX2 cells. The fact that the observed increases in $[Ca^{2+}]_i$ are a consequence of stimulation of this receptor can be deduced mainly from the effects of the antagonists used. The similar potency observed for CPA and NECA is a further characteristic of the human brain A_1 adenosine receptor identified by radioligand binding experiments (Ferkany *et al.*, 1986). The response to CPA (a selective A_1 receptor agonist; van Galen *et al.*, 1992) was potently inhibited following pre-incubation with DPCPX, a highly selective A_1 adenosine receptor antagonist (van Galen *et al.*, 1992). XAC, a high affinity but relatively non-selective adenosine receptor antagonist (van Galen, 1992) was less potent than DPCPX at inhibiting the CPA response (Figure 3). These data clearly suggest that the observed increase in $[Ca^{2+}]_i$ is a consequence of stimulation of the transfected A_1 adenosine receptor, most likely via stimulation of phosphoinositidase C as has been reported in DDT_1 MF2 cells (White *et al.*, 1992). In human brain slices, adenosine receptor activation fails to elicit phosphoinositide turnover (Kendall & Firth, 1990). However, the presence of adenosine analogues leads to an inhibition of histamine-stimulated [^{3}H]-inositol phosphates accumulation, similar to the phenomenon observed in mouse cerebral cortical slices (Kendall & Hill, 1988). The receptor which mediates this modulatory response appears to be an A_1 adenosine receptor. Thus, the current study implies that a transfected receptor couples to a second messenger system to which it fails to couple under native conditions. Examples of A_1 adenosine receptors which couple to phosphoinositide turnover under native conditions are relatively sparse, e.g. in guinea-pig myometrium (Schiemann & Buxton, 1991) and DDT1 MF-2 cells (White *et al.*, 1992). There are however, other instances of the promiscuity of transfected receptors (Milligan, 1993).

We have previously shown that CHO-K1 cells contain an endogenous P_{2U}-purinoceptor (Iredale & Hill, 1993) which is coupled to increases in $[Ca^{2+}]_i$ (mobilisation and entry). Pretreatment of the cells with pertussis toxin inhibited the calcium response to CPA but had no effect on the response to ATP. These data suggest that the transfected A_1 adenosine receptor is not coupled to the same G protein as the P_{2U}-purinoceptor. The lack of pertussis toxin-sensitivity of the latter receptor response suggests that it belongs to the G_q-family. It has previously been shown that the transfected A_1 adenosine receptor in CHO-NAX2 cells is negatively linked to adenylyl cyclase (Townsend-Nicholson & Shine, 1992). One possibility is, therefore, that the G protein involved is coupled to both adenylyl cyclase and phosphoinositidase C, as has been suggested for the A_1 adenosine receptor found in DDT_1 MF2 cells (Gerwins & Fredholm, 1992). This hypothesis would certainly explain the sensitivity to pertussis toxin. One interesting possibility is that the receptor-mediated calcium response might be as a consequence of G_i-derived $\beta\gamma$ subunits activating phosphoinositidase C and not due to direct coupling of the A_1 adenosine receptor to the enzyme (Dickenson & Hill, 1993b). $\beta\gamma$ subunits have been shown to stimulate phosphoinositidase C in cytosolic fractions obtained from HL-60 cells (Camps *et al.*, 1992) and therefore this remains a distinct possibility.

In summary, we have shown that the A_1 adenosine receptor transfected into CHO-K1 cells couples to phosphoinositidase C and is therefore linked to mobilisation of $[Ca^{2+}]_i$. Agonist stimulation is pertussis toxin-sensitive suggesting the involvement of G_i in the mechanism, although whether the enzyme is directly coupled or is activated by $\beta\gamma$ subunits remains to be elucidated.

We thank the MRC (P.A.I.), Wellcome Trust (S.P.H.A.) & Nuffield Foundation for financial support. We are very grateful to Drs Andrea Townsend-Nicholson and John Shine at the Garvan Institute, Sydney, Australia for the supply of the A_1 adenosine receptor transfected CHO-K1 cells.

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(Received October 7, 1993
Revised November 22, 1993
Accepted December 8, 1993)