British Journal of Pharmacology (1995) 115, 1415-1424

Synergy between the inositol phosphate responses to transfected human adenosine A_1-receptors and constitutive P_2-purinoceptors in CHO-K1 cells

Anne C. Megson, John M. Dickenson, *Andrea Townsend-Nicholson & [1]Stephen J Hill

Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, NG7 2UH and *Garvan Institute of Medical Research, St Vincent's Hospital, Sydney, Australia

1 The effect of adenosine A_1-receptor and P_2-purinoceptor agonists on [^{3}H]-inositol phosphate accumulation has been investigated in CHO-K1 cells transfected with the human adenosine A_1-receptor.

2 Adenosine receptor agonists stimulated [^{3}H]-inositol phosphate accumulation in CHO-K1 cells with a rank potency order of N^6-cyclopentyladenosine (CPA) > 5'-N-ethylcarboxamidoadenosine (NECA) > 2-chloroadenosine > N^6-2-(4-aminophenyl) ethyladenosine (APNEA). The responses to both CPA and APNEA were antagonized by the A_1 selective antagonist, 1,3-dipropylcyclopentylxanthine (DPCPX) yielding K_D values of 1.2 nM and 4.3 nM respectively.

3 ATP, UTP and ATPγS were also able to stimulate [^{3}H]-inositol phosphate accumulation in these cells with EC_{50} values of 1.9 μM, 1.3 μM and 5.0 μM respectively. 2-Methyl-thio-ATP was a weak agonist of this response (EC_{50} > 100 μM).

4 The [^{3}H]-inositol phosphate response to CPA was completely attenuated by pertussis toxin treatment (24 h; 100 ng ml^{-1}). In contrast, the responses to ATP, UTP and ATPγS were only reduced by *circa* 30% in pertussis toxin-treated cells.

5 The simultaneous addition of CPA and either ATP, UTP or ATPγS produced a large augmentation of [^{3}H]-inositol phospholipid hydrolysis. This was due to an increase in the maximal response and was significantly greater than the predicted additive response for activation of these two receptor systems. The synergy was not observed in pertussis toxin-treated cells.

6 No synergy was observed between the [^{3}H]-inositol phosphate responses to histamine and ATP in CHO-K1 cells transfected with the bovine histamine H_1-receptor. In these cells the response to histamine was completely resistant to inhibition by pertussis toxin treatment.

7 This study provides a clear demonstration of a synergy between pertussis toxin-sensitive and insensitive receptor systems in a model cell system which is an ideal host for transfected cDNA sequences. This model system should provide a unique opportunity to unravel the mechanisms underlying this example of receptor cross-talk involving phospholipase C.

Keywords: A_1-adenosine receptor; P_{2u}-purinoceptor; nucleotide receptor; inositol phosphates; CHO-K1 cells; synergy

Introduction

In a number of cell lines (e.g. the DDT_1MF-2 hamster vas deferens smooth muscle cell line and airway epithelial cells derived from cystic fibrosis patient), adenosine A_1-receptor activation has been shown to inhibit adenylyl cyclase and to stimulate phosphoinositide hydrolysis and subsequent release of intracellular calcium stores (Ramkumar *et al.*, 1990; White *et al.*, 1992; Gerwins & Fredholm, 1992a,b; Dickenson & Hill, 1993a; Rugolo *et al.*, 1993). These responses to adenosine A_1-receptor activation are all sensitive to inhibition by pertussis toxin treatment (Ramkumar *et al.*, 1990; Gerwins & Fredholm, 1992a,b; Dickenson & Hill, 1993b; White *et al.*, 1993; Rugolo *et al.*, 1993). Recent studies have shown that a number of phospholipase C β-isoforms can be activated by G-protein βγ-subunits (Camps *et al.*, 1992; Clapham & Neer, 1993; Wu *et al.*, 1993; Boyer *et al.*, 1994), including the enzyme present in DDT_1MF-2 smooth muscle cells (Dickenson *et al.*, 1995). Furthermore, expression of the carboxyl βγ-binding domain of β-adrenoceptor kinase (β-ARK) in Cos-7 cells has been shown to attenuate the activation of phospholipase C by G_i-linked M_2-muscarinic receptors and α_2-adrenoceptors, without affecting the inhibition of adenylyl cyclase which is mediated via $G_{i\alpha}$-subunits (Koch *et al.*, 1994). These data suggest that G_i-coupled receptors, such as the adenosine A_1-receptor, mediate inhibition of adenylyl cyclase activity via $G_{i\alpha}$-subunits and stimulate phosphoinositide hydrolysis via the action of $G_{i\beta\gamma}$-subunits.

Activation of adenylyl cyclase II can also be achieved via a $G_{i\beta\gamma}$-dependent mechanism (Federman *et al.*, 1992; Koch *et al.*, 1994). However, in this case the activation of adenylyl cyclase by βγ-subunits requires conditional activation of adenylyl cyclase II by $G_{\alpha s}$ (Federman *et al.*, 1992; Clapham & Neer, 1993). We, and others have recently shown that adenosine A_1-receptor-stimulation can augment the calcium responses elicited by histamine, bradykinin and ATP in DDT_1MF-2 cells (Gerwins & Fredholm, 1992a,b; Dickenson & Hill, 1993b). It is possible, therefore, that $G_{\beta\gamma}$-subunits derived from activated G_i-proteins may augment the activation of phospholipase by receptors which act through $G_{\alpha q}$.

The molecular sequence for the human adenosine A_1-receptor has now been established by molecular cloning techniques (Townsend-Nicholson, 1992; Libert *et al.*, 1992). Coupling of the adenosine A_1-receptor to inhibition of adenylyl cyclase (Townsend-Nicholson, 1992; Libert *et al.*, 1992) and mobilisation of intracellular calcium (Iredale *et al.*, 1994) has been observed in CHO-K1 cells stably transfected with the human A_1-receptor cDNA. These cells also contain an endogenous nucleotide receptor (P_{2U}-purinoceptor) which appears to be coupled to phospholipase C (Iredale & Hill, 1993). Interestingly, the calcium response to adenosine A_1-receptor agonists, but not that to nucleotide-receptor stimulation is sensitive to inhibition by pertussis toxin (Iredale *et al.*, 1994). In the present study, we have characterized the inositol phosphate responses to these two receptor systems and investigated the potential for synergistic interactions between them.

[1] Author for correspondence.

Methods

Cell culture

CHO-A1 cells (CHO-K1 cells transfected with the human brain adenosine A_1 receptor sequence, Townsend-Nicholson & Shine, 1992) or CHO-H1 cells (CHO-K1 cells transfected with the bovine histamine H_1 receptor sequence, Iredale *et al.*, 1993) were grown at 37°C in a humidified air/CO_2 atmosphere (90:10) in 75 cm^2 flasks (Costar). The cells were grown in Dulbecco's Modified Eagles Medium/Nutrient Mix F12 (1:1) supplemented with 2 mM L-glutamine and 10% foetal calf

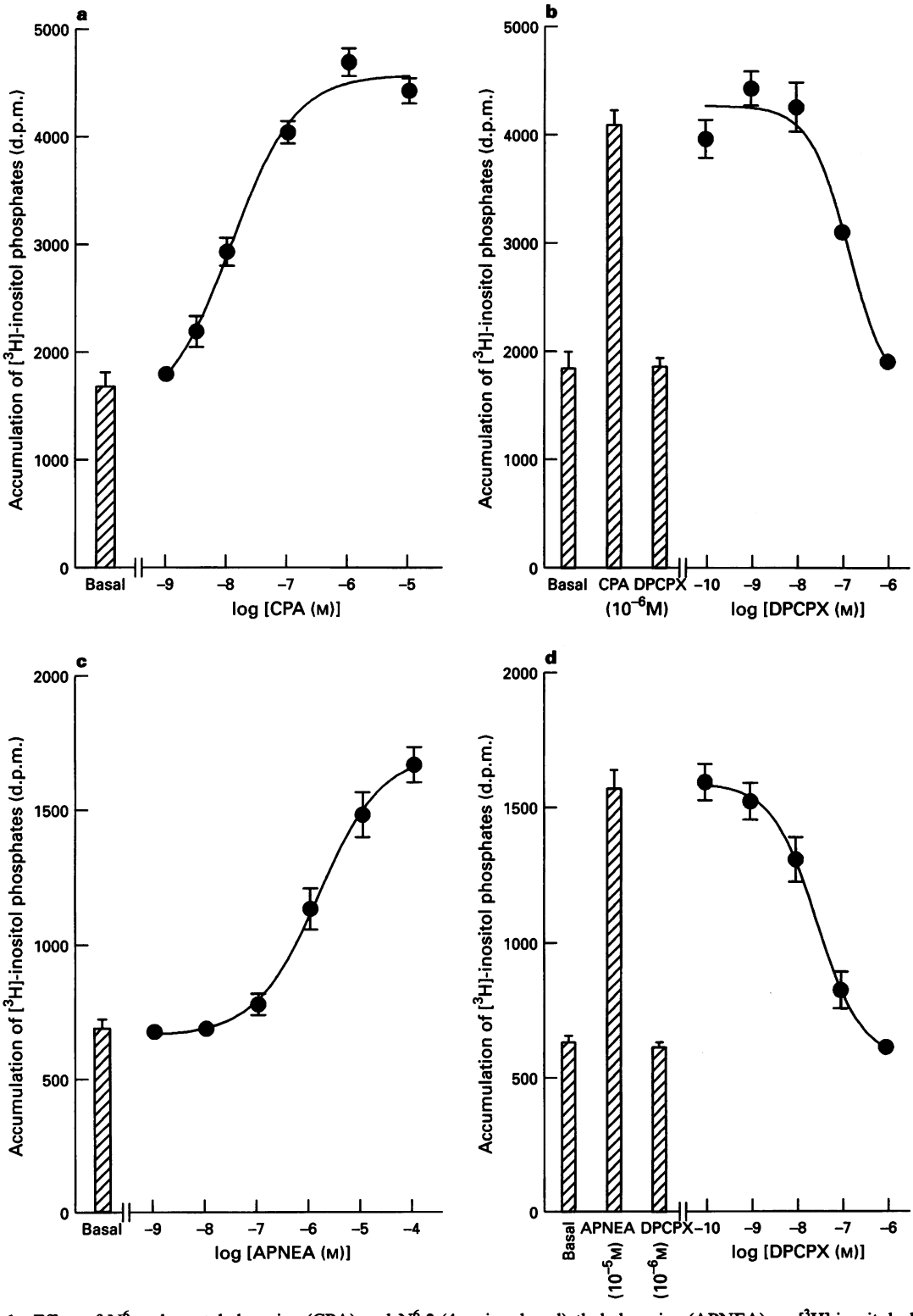

Figure 1 Effect of N^6-cyclopentyladenosine (CPA) and N^6-2-(4-aminophenyl)ethyladenosine (APNEA) on [^{3}H]-inositol phosphate accumulation in CHO-K1 cells (CHO-A1) transfected with the human adenosine A_1-receptor. Panels (a) and (c) show the concentration-response curves to CPA (a) and APNEA (c) in CHO-A1 cells. Panels (b) and (d) show the concentration-dependent effect of 1,3-dipropylcyclopentylxanthine (DPCPX) on the response to (b) 1 μM CPA or (d) 10 μM APNEA. Values represent mean ± s.e.mean of triplicate determinations in a single experiment. Similar data were obtained in 18 (a), 5 (b), 10 (c) and 2 (d) other experiments.

serum. The cells for assaying (either inositol phospholipid hydrolysis or [^{3}H]-cyclic AMP accumulation) were grown in 24 well cluster dishes (Costar) and all experiments were performed on confluent monolayers.

Inositol phospholipid hydrolysis

Confluent monolayer cultures were loaded for 24 h with [^{3}H]-*myo*-inositol (37 kBq/well) in 24 well cluster dishes in inositol-free DMEM containing 2 mM glutamine and 0.5% foetal calf serum. If required, pertussis toxin (PTX; 100 ng ml^{-1}) was added at this point. After being washed twice with 1 ml/well Hanks/HEPES buffer (pH 7.4) the cells were incubated for 30 min at 37°C in 290 µl/well Hanks/HEPES buffer containing 20 mM LiCl and, if required, antagonist drugs. Agonists were added in 10 µl medium and the incubation continued for 40 min (unless otherwise stated). Incubations were terminated by aspiration of the incubation medium and addition of 800 µl cold (–20°C) methanol/0.12 M HCl (1 : 1,v/v). The cells were left at –20°C for at least 2 h before isolating total [^{3}H]-inositol phosphates as described previously (White *et al.*, 1993). [^{3}H]-inositol phosphate levels were determined by liquid-scintillation counting.

Accumulation of [^{3}H]-cyclic AMP

Confluent cell monolayers were incubated for 2 h at 37°C with 1 ml of Hanks/HEPES buffer (pH 7.4) containing [^{3}H]-adenine (37 kBq/well). If required pertussis toxin (200 ng ml^{-1}) was added 4 h prior to loading. The cells were washed twice and then incubated in 1 ml Hanks/HEPES buffer containing the cyclic AMP phosphodiesterase inhibitor rolipram (100 µM) for 15 min at 37°C. Agonists were added in 10 µl medium and the incubation continued for 10 min. Incubations were terminated by the addition of 50 µl conc. HCl. [^{3}H]-cyclic AMP was then isolated by sequential Dowex-alumina chromatography as previously described (Donaldson *et al.*, 1988). To allow for percentage recovery correction the samples were spiked with [^{14}C]-cyclic AMP before being applied to the columns. After elution, the levels of [^{3}H]-cyclic AMP and [^{14}C]-cyclic AMP were determined by liquid-scintillation counting.

Data analysis

Agonist concentration-response curves were fitted to a logistic equation by the non-linear regression programme Inplot4 (GraphPad Software, San Diego) as described previously (Ruck *et al.*, 1990). The equation fitted was:

$$\text{Response} = \frac{E_{MAX} \times A^n}{(EC_{50})^n + A^n} \quad (1)$$

where E_{max} is the maximal agonist response, A is the agonist concentration and n is the Hill coefficient. Antagonist dissociation constants (K_D) were estimated by a modification of the method of Lazareno & Roberts (1987). Briefly, a concentration-response curve to an agonist was generated and a concentration (C) of the agonist under study chosen which gave a response greater than 50% of the maximal response. The concentration of antagonist (IC_{50}) required to reduce the response of this concentration (C) of agonist by 50% was then determined. The agonist concentration-response curve was fitted to a logistic equation as described above and a concentration of the agonist identified (C^1) which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_D was then determined from the following relationship:

$$C/C^1 = IC_{50}/K_D + 1 \quad (2)$$

Statistical analysis of agonist concentration-response curves obtained in the presence and absence of a fixed concentration of a second agonist (for another receptor; see Table 3 below) were analyzed by the programme, Allfit (NIH; DeLean *et al.*, 1978). Briefly, the two concentration-response curves were fitted simultaneously to the logistic equation (equation 1, above) using Allfit. Allfit was then used to test for differences in the concentration-response parameters (EC_{50}, E_{max} and n), obtained in the presence and absence of the second agonist, by investigation of the effect on the residual sums of squares of forcing them to be equal (DeLean *et al.*, 1978). In order to test for synergy, the analysis was repeated with the curve generated for the predicted additive response (obtained by adding the response to the fixed concentration of the second agonist to

Table 1 Concentration-response parameters for adenosine A_1-agonist stimulation of [^{3}H]-inositol phosphate accumulation in CHO-K1 cells transfected with the human adenosine A_1-receptor

Agonist	*-log EC_{50}* (M)	n	*% maximal response**	n
CPA	8.13 ± 0.12	(14)	105.1 ± 2.6	(14)
NECA	7.04 ± 0.22	(6)	126.3 ± 9.6	(3)
2-CA	6.23 ± 0.05	(6)	137.2 ± 8.8	(3)
APNEA	5.53 ± 0.18	(11)	128.1 ± 16.6	(5)
Adenosine	5.46 ± 0.17	(6)	50.5 ± 3.2	(3)

Concentration-response parameters were obtained from fitting each curve to the logistic equation as described under Methods. * The maximal responses are expressed as a percentage of the response to 1 µM CPA which was measured in the same experiment. Values represent mean ± s.e.mean in *n* separate experiments. CPA (N^6-cyclopentyladenosine); 2-CA (2-chloroadenosine); NECA (N-ethylcarboxamidoadenosine); APNEA (N^6-2-(4-amino-phenyl)ethyladenosine).

Table 2 Concentration-response parameters for P_2-purinoceptor agonist stimulation of [^{3}H]-inositol phosphate accumulation in CHO-K1 cells transfected with the human adenosine A_1-receptor

Agonist	*-log EC_{50}* (M)	n	*% maximal response**	n
UTP	5.87 ± 0.07	(12)	117.1 ± 1.4	(12)
ATP	5.72 ± 0.03	(6)	141.3 ± 4.4	(3)
ATPγS	5.31 ± 0.03	(6)	166.0 ± 1.8	(3)
2-MeSATP	< 4.00	(3)	44.3 ± 1.5#	(3)

Concentration-response parameters were obtained from fitting each curve to the logistic equation as described under Methods. * The maximal responses are expressed as a percentage of the response to 100 µM UTP which was measured in the same experiment. Values represent mean ± s.e.mean in *n* separate experiments. # Response to 100 µM 2-methylthio-ATP (2-MeSATP).

that obtained with each concentration of the first agonist alone; see for example Figure 3).

The constraint imposed by parameter sharing between curves normally increased the residual variance in addition to the number of degrees of freedom (number of data points minus the number of estimated parameters). The appropriateness of a particular constraint was then evaluated by analysis of residual variances using the following statistical test (Munson & Rodbard, 1980):

$$F = \frac{(R_1 - R_2)/(df_1 - df_2)}{R_2/df_2}$$

where R_2 and R_1 are the residual sums of squares and df_2 and df_1 are the degrees of freedom associated with the original fit and the analysis with shared parameters respectively. F represents the variance ratio with (df_1 - df_2) and df_2 degrees of freedom.

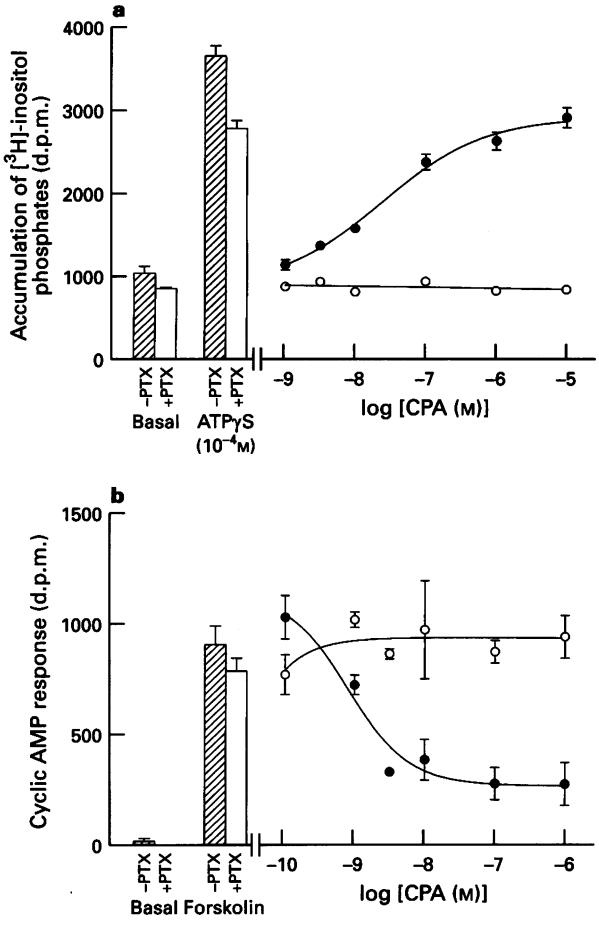

Figure 2 Effects of N^6-cyclopentyladenosine (CPA) on [^{3}H]-inositol phosphate accumulation (a) and [^{3}H]-cyclic AMP accumulation (b) in control and pertussis toxin-treated CHO-A1 cells. (a) Control cells (●) and cells treated with pertussis toxin (100 ng ml^{-1}; 24 h; PTX; ○) were incubated for 40 min in increasing concentrations of CPA in the presence of 20 mM LiCl. Also shown are the basal and adenosine 5'-(3-thio)triphosphate (ATγS, 100 μM) stimulated accumulations in the pertussis-toxin-treated cells (+PTX; open columns) or control cells (–PTX; hatched columns). Values represent mean±s.e.mean of triplicate determinations in a single experiment. Similar data were obtained in two other experiments. (b) Control cells (●) and cells treated with pertussis toxin (200 ng ml^{-1}; 4 h; ○) were incubated for 10 min with 10 μM forskolin in the presence of different concentrations of CPA. The histograms show the basal and 10 μM forskolin-stimulated accumulation in control cells (–PTX; hatched columns) and cells treated with pertussis toxin (+PTX; open columns). Values represent mean±s.e.mean of triplicate determinations in a single experiment. Similar data were obtained in four other experiments and the combined data are presented in the text.

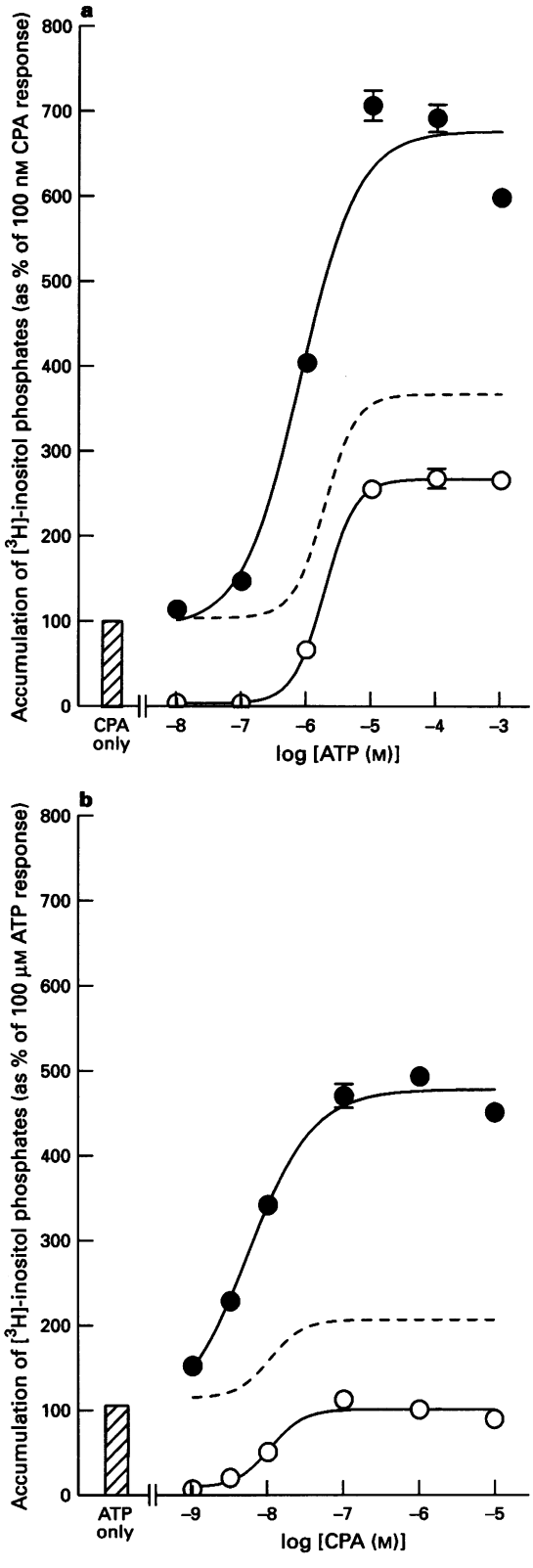

Figure 3 Synergy between N^6-cyclopentyladenosine (CPA) and adenosine 5'-triphosphate (ATP) in CHO-A1 cells. Panel (a) shows the concentration-response curve to ATP in the presence (●) or absence (○) of CPA (100 nM). To normalize responses from different experiments, data are expressed as a percentage of the response to 100 nM CPA alone (after subtraction of basal values). Panel (b) shows the concentration-response curve to CPA in the presence (●) or absence (○) of ATP (100 μM). Data are expressed as a percentage of the response to 100 μM ATP alone (after subtraction of basal values). Values represent mean±s.e.mean from three separate experiments. The dashed lines indicate the predicted additive responses to both agonists calculated as described under Methods.

Statistical analysis of differences between other mean data was performed by use of Student's unpaired t test. Unless otherwise stated, each value given in the text represents mean ± s.e.mean of n separate experiments.

Materials

Cell culture flasks and 24 well cluster dishes were from Costar. Dulbecco's Modified Eagles Medium/Nutrient Mix F12, L-glutamine, N^6-cyclopentyladenosine (CPA), 5'-N-ethylcarboxamidoadenosine (NECA), 2-chloroadenosine, adenosine, ATP, histamine, mepyramine and forskolin were obtained from Sigma (Poole, Dorset). Foetal calf serum was from Biological Industries (Glasgow, U.K.). [2-^3H]-*myo*-inositol (758.5 GBq mmol^{-1}) was supplied by New England Nuclear (Stevenage, Herts) and [2,8-^{3}H]-adenine (888 Gbq mmol^{-1}) and cyclic [8-^{14}C]-AMP (11.43 GBq mmol^{-1}) were from Amersham International (Aylesbury, Bucks). Inositol-free DMEM was obtained from Flow Laboratories (Irvine, U.K.), pertussis toxin from Porton Products Ltd (Maidenhead, Berks), rolipram from Schering A.G. (Berlin, Germany), UTP and adenosine 5'-(3-thio) triphosphate (ATPγS) from Boehringer Mannheim (Lewes, Sussex) and 2-methylthio ATP (2-MeSATP) and 1,3-dipropyl-cyclopentylxanthine (DPCPX) from RBI/Seamat (Herts). N^6-2-(4-aminophenyl)ethyladenosine (APNEA) was synthesized by Dr Andrew Boyd (Dept. of Pharmaceutical Sciences, University of Nottingham). All other chemicals were of analytical grade.

Results

[^3H]-inositol phosphate response to adenosine A_1-receptor stimulation

The A_1-receptor selective agonist N^6-cyclopentyladenosine (CPA), produced a 2.5 ± 0.1 fold (n = 19) increase in total [^{3}H]-inositol phosphate accumulation in CHO-K1 cells stably transfected with the human A_1-adenosine receptor (CHO-A1; Figure 1a). Similar or slightly larger maximal increases in [^{3}H]-inositol phosphate accumulation were also observed with N-ethylcarboxamidoadenosine (NECA), 2-chloroadenosine and N^6-2-(4-aminophenyl)ethyladenosine (APNEA; Figure 1c) (Table 1). In contrast, adenosine itself produced a maximal response substantially lower than that of CPA (Table 1). The rank order of potencies of these agonists for the human A_1-receptor-mediated stimulation of inositol phospholipid hydrolysis was CPA > NECA > 2-chloroadenosine > APNEA (Table 1), which is consistent with that reported for the A_1-receptor-mediated response in the hamster vas deferens smooth muscle line DDT$_1$MF-2 (White *et al.*, 1992). The responses to CPA (1 μM; Figure 1b) and APNEA (10 μM; Figure 1d) were both antagonized by the selective adenosine A_1-receptor antagonist, 1,3-dipropylcyclopentylxanthine (DPCPX) yielding apparent K_D values of 1.2 ± 0.3 nM (n = 6) and 4.3 ± 1.9 nM (n = 3) respectively.

[^3H]-inositol phosphate response to P_2-purinoceptor agonists

Consistent with our previous report of an endogenous P_{2u}-purinoceptor (or 'nucleotide receptor') coupled to mobilisation of intracellular free calcium in CHO-K1 cells (Iredale & Hill, 1993), ATP, ATPγS and UTP were able to stimulate [^{3}H]-inositol phosphate accumulation in CHO-A1 cells (Table 2). The EC_{50} values obtained for UTP (1.3 μM), ATP (1.9 μM) and ATPγS (5.0 μM) (Table 2) were similar to the values of 2.8 μM, 2.3 μM and 12 μM respectively, deduced from measurement of intracellular calcium levels (Iredale & Hill, 1993). 2-Methyl-S-ATP (0.1 mM) was a weak agonist of this response (EC_{50} > 100 μM) and produced a response of only 44.3 ± 1.5% (n = 3) of that produced by 0.1 mM UTP.

Pertussis toxin sensitivity

The [^{3}H]-inositol phosphate response to CPA was completely attenuated by prior treatment of CHO-A1 cells with pertussis toxin (100 ng ml^{-1}; 24 h) (Figure 2a). In marked contrast, the

Table 3 Concentration-response parameters for synergy between A_1- and nucleotide-receptor agonists mediating [^{3}H]-inositol phosphate accumulation in transfected CHO-K1 cells

		Simultaneous addition		*Concentration-response parameters for Agonist 1*	
Agonist 1	*Agonist 2* (Conc.)	(+/–)	*Incubation time* (Min)	E_{max} (% of response to agonist 2)#	*-log EC_{50}*
ATP	CPA (0.1 μM)	–	40	267.2 ± 27.8	5.73 ± 0.30
ATP	CPA (0.1 μM)	+	40	667.7 ± 24.3*	6.05 ± 0.08
UTP	CPA (0.1 μM)	–	40	168.0 ± 32.6	5.89 ± 0.60
UTP	CPA (0.1 μM)	+	40	759.8 ± 28.4**	6.30 ± 0.10
UTP	CPA (0.1 μM)	–	10	238.6 ± 13.1	5.92 ± 0.16
UTP	CPA (0.1 μM)	+	10	647.2 ± 11.0*	6.44 ± 0.06
ATPγS	CPA (0.1 μM)	–	40	506.8 ± 37.7	5.31 ± 0.17
ATPγS	CPA (0.1 μM)	+	40	1548.2 ± 36.2*	5.80 ± 0.06
CPA	ATP (0.1 mM)	–	40	95.5 ± 7.3	8.04 ± 0.15
CPA	ATP (0.1 mM)	+	40	457.6 ± 7.6*	8.22 ± 0.05
CPA	UTP (0.1 mM)	–	40	109.0 ± 5.4	8.10 ± 0.12
CPA	UTP (0.1 mM)	+	40	453.0 ± 4.7*	8.27 ± 0.03
CPA	UTP (0.1 mM)	–	10	63.9 ± 3.6	8.14 ± 0.14
CPA	UTP (0.1 mM)	+	10	248.5 ± 3.1*	8.39 ± 0.04

Concentration-response parameters were obtained from simultaneous fitting of each pair of curves to a logistic equation using the non-iterative procedure Allfit as described under Methods. Concentration-response curves to Agonist 1 were obtained in the presence or absence of a fixed concentration of Agonist 2 (added simultaneously where indicated). #Maximal responses (E_{max}) are expressed as a percentage of the response to Agonist 2 (alone) which was measured in the same experiment. Statistical analysis of the fitted parameters was performed by analysis of variance of the increase in the residual sums of squares obtained from simultaneous curve fitting of the two curves when the parameter under test was constrained to be the same for each curve (see Methods for further details). In the case of the maximal responses (E_{max}), the test was performed by comparing the E_{max} obtained from the simultaneous addition of agonist 1 and agonist 2 with the E_{max} predicted for simple additivity (i.e. E_{max} for agonist 1 alone plus 100%). (*) P < 0.001 and (**) P < 0.01 compared to simple additivity (ANOVAR of residual sums of squares; Allfit). There were no significant differences between Hill slopes and EC_{50} values for any of the pairs of curves analyzed. Values in the Table for E_{max} and -log EC_{50} represent mean and approximate s.e.mean derived from Allfit. Data used in the fitting procedure were the mean values obtained in three separate experiments, where each data point was obtained from triplicate determinations.

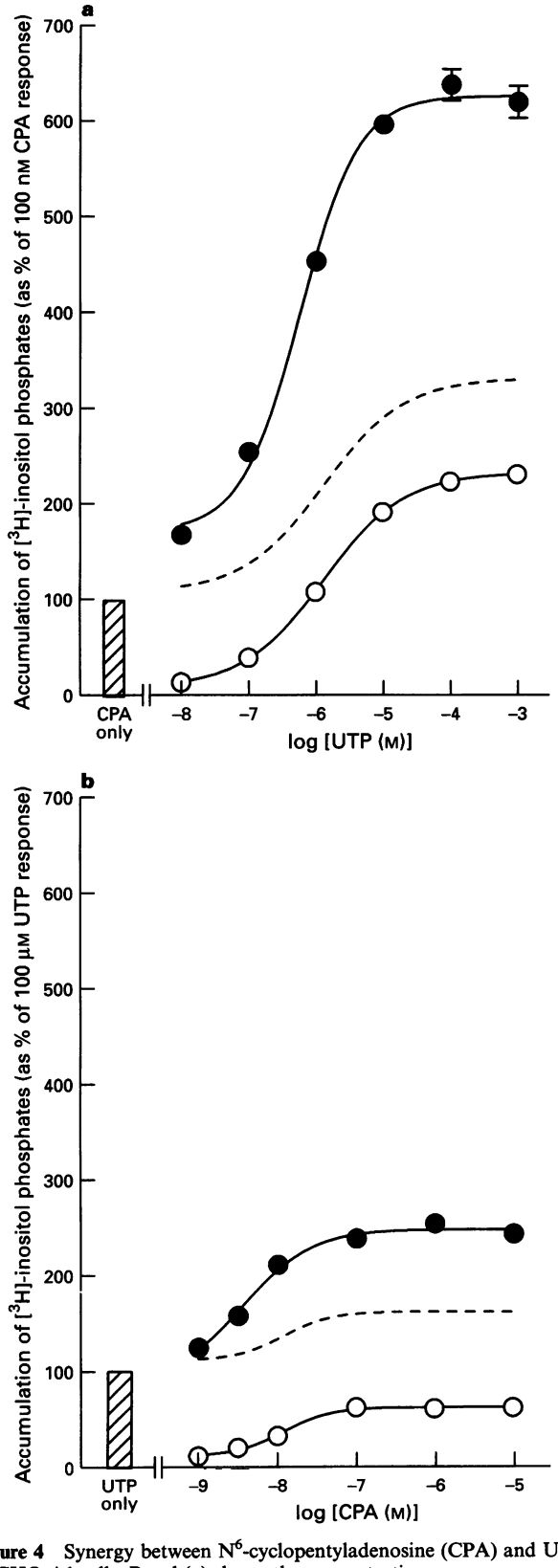

Figure 4 Synergy between N^6-cyclopentyladenosine (CPA) and UTP in CHO-A1 cells. Panel (a) shows the concentration-response curve to UTP in the presence (●) or absence (○) of CPA, (100 nM), with 10 min agonist stimulation. To normalize responses from different experiments, data are expressed as a percentage of the response to 100 nM CPA alone (after subtraction of basal values). Panel (b) shows the concentration-response curve to CPA in the presence (●) or absence (○) of UTP (100 μM), with 10 min agonist stimulation. Data are expressed as a percentage of the response to 100 μM UTP alone (after subtraction of basal values). Values represent mean ± s.e.mean from three separate experiments. The dashed lines indicate the predicted additive responses to both agonists calculated as described under Methods.

[^{3}H]-inositol phosphate responses to ATP (0.1 mM; 34.5 ± 2.1% inhibition; $n = 3$), ATPγS (0.1 mM; 28.6 ± 4.0% inhibition, $n = 5$; Figures 2a and 5) and UTP (1 mM; 34.0 ± 3.9% inhibition; $n = 3$) were only partially inhibited by this toxin. As expected, the ability of CPA to inhibit forskolin-stimulated [^{3}H]-cyclic AMP accumulation was completely prevented by pertussis toxin treatment (200 ng ml^{-1}; 4 h; Figure 2b). In the absence of pertussis toxin, CPA was able to produce a maximal inhibition of forskolin-stimulated [^{3}H]-cyclic AMP accumulation of 74.4 ± 4.7% (EC_{50} 3.1 ± 1.2 nM; $n = 6$; Figure 2b).

Synergy between A_1-adenosine receptors and P_2-purinoceptors

The simultaneous addition of CPA and ATP produced a greater than additive accumulation of total [^{3}H]-inositol phosphates in CHO-A1 cells (Figure 3; Table 3). For example, the maximal [^{3}H]-inositol phosphate response to ATP obtained in the presence of 0.1 μM CPA was 667.7 ± 24.3% (expressed as a percentage of the response to 0.1 μM CPA on its own). This was significantly greater ($P < 0.001$; analysis for variance of residual sums of squares; Allfit, see Methods) than the predicted additive response of 367.2 ± 27.8% (i.e. the E_{max} obtained in the absence of 0.1 μM CPA plus 100%; Table 3). A similar synergy was observed between CPA and UTP and CPA and ATPγS (Table 3; Figures 4 and 5).

The synergy between UTP and CPA was still evident when the agonist incubation time was reduced from 40 min to 10 min (Figure 4; Table 3). Figure 5 shows data which confirm that both the direct effect of CPA on [^{3}H]-inositol phosphate accumulation and the augmentation of P_{2u}-purinoceptor-mediated inositol phospholipid hydrolysis by the adenosine A_1-receptor agonist were sensitive to inhibition by pertussis toxin. It is clear that in pertussis toxin-treated cells, CPA is not able to elicit a direct [^{3}H]-inositol phosphate response or to augment the residual response to ATPγS (Figure 5).

Lack of an interaction between transfected histamine H_1-receptors and P_2-purinoceptors in CHO-K1 cells

To investigate whether the synergy observed between CPA and P_{2U}-purinoceptor agonists could be demonstrated in cells transfected with a receptor which does not couple to a pertussis

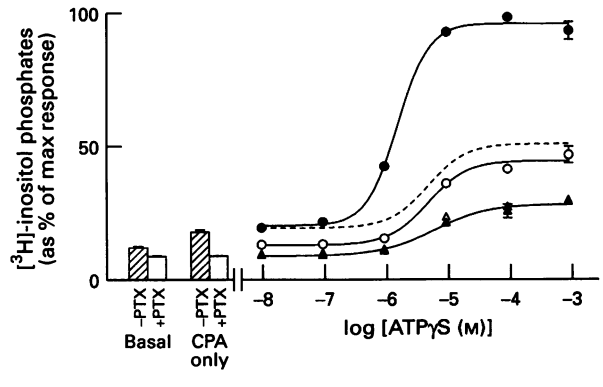

Figure 5 Effect of N^6-cyclopentyladenosine (CPA) and pertussis toxin (PTX) on adenosine 5′-(3-thio)triphosphate (ATPγS) stimulated accumulation of total [^{3}H]-inositol phosphates in CHO-A1 cells. Curves represent concentration-response curves to ATPγS alone (○); (●) ATPγS in the presence of CPA (100 nM); (△) ATPγS in pertussis toxin-treated cells (24 h; 100 ng ml^{-1}); (▲) ATPγS and CPA (100 nM) in pertussis toxin-treated cells. Also shown are the basal and CPA (100 nM) alone accumulations in the presence (open columns) or absence (hatched columns) of pertussis toxin. Values represent mean ± s.e.mean from three separate experiments. Dashed line represents the predicted additive response to CPA and ATPγS in control cells (i.e. not treated with pertussis toxin).

toxin-sensitive G-protein, a similar study of receptor interactions was performed in CHO-K1 cells transfected with the bovine histamine H_1-receptor (Iredale *et al.*, 1994). In these cells, histamine produced a large stimulation of [^{3}H]-inositol phosphate accumulation (17.7±1.2 fold over basal values; $n=16$; Figure 6a). The EC_{50} value obtained (250±40 nM; $n=16$; Figure 6) was similar to the value of 87 nM determined from studies of H_1-receptor-mediated increases in intracellular calcium in these cells (Iredale *et al.*, 1994). The inositol phosphate response to 1 μM histamine was antagonized by the selective H_1-receptor antagonist, mepyramine (Figure 6b) yielding an apparent K_D of 3.7±0.9 nM ($n=3$). This is similar to the values of 2.9 nM and 1.3 nM deduced previously in this cell line for mepyramine from inhibition of histamine-mediated calcium responses and [^{3}H]-mepyramine binding (Iredale *et al.*, 1993). The inositol phosphate response to histamine was largely insensitive to inhibition by pertussis toxin treatment (Figure 7). In these cells (CHO-H1), pertussis toxin treatment reduced the inositol phosphate response to 0.1 mM histamine by 9.2±4.4% ($n=4$) and that to 0.1 mM ATP by 21.5±6.4% ($n=3$).

The simultaneous addition of histamine and ATP, however, produced an accumulation of [^{3}H]-inositol phosphates which could be readily accounted for by simple additivity (Figure 8).

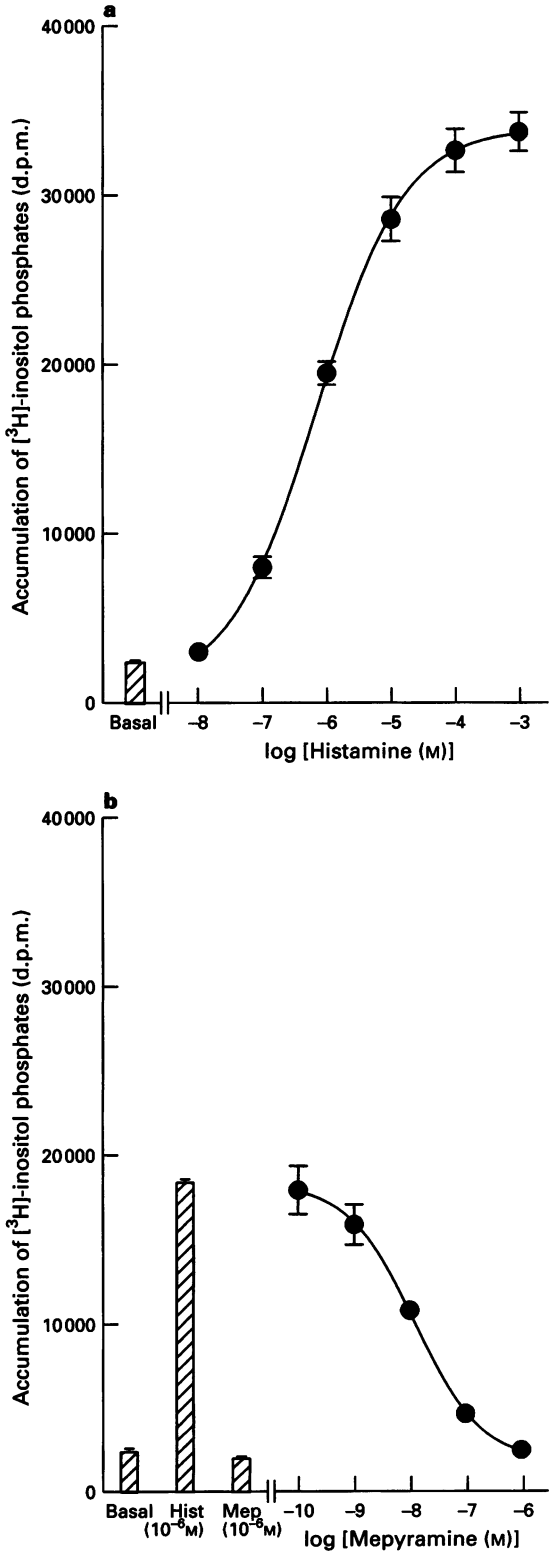

Figure 6 Effect of histamine on the accumulation of [^{3}H]-inositol phosphate accumulation in CHO-K1 cells transfected with the bovine histamine H_1-receptor (CHO-H1). Panel (a) shows the concentration-response curve to histamine (Hist) in CHO-H1 cells. Panel (b) shows the effect of increasing concentrations of mepyramine on the [^{3}H]-inositol phosphate response to 1 μM histamine. Values represent mean±s.e.mean of triplicate determinations in a single experiment. Similar data were observed in sixteen (a) and two (b) other experiments.

Discussion

We have previously demonstrated the coupling of the human adenosine A_1-receptor to mobilisation of intracellular calcium in CHO-K1 cells transfected with the human A_1-adenosine receptor cDNA (Iredale *et al.*, 1994). The present study confirms that adenosine A_1-receptor activation in these cells is also associated with a stimulation of inositol phospholipid hydrolysis. Thus, [^{3}H]-inositol phosphate accumulation can be potently stimulated by the A_1-selective agonist, CPA and a range of adenosine analogues. The rank order of potencies of these agonists for the human A_1-receptor-mediated stimulation of inositol phospholipid hydrolysis (CPA > NECA > 2-chloroadenosine > APNEA; Table 1) is consistent with that reported for the other A_1-receptor-mediated responses (White *et al.*, 1992; Collis & Hourani, 1993). Furthermore, the dissociation constants obtained for DPCPX from antagonism of the responses to either CPA (1.2±0.3 nM) or APNEA (4.3±1.9 nM) were similar to the value of 1 nM obtained from studies of [^{3}H]-DPCPX binding to cell membranes derived

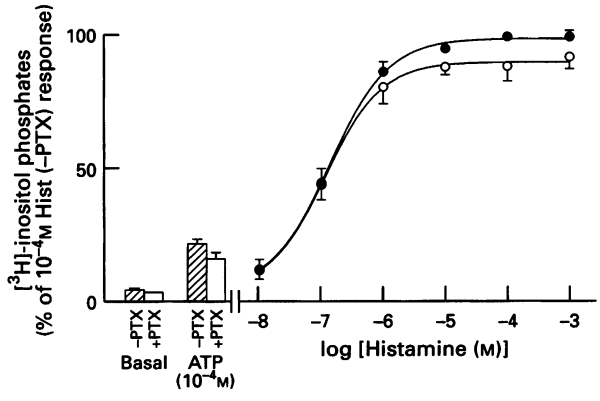

Figure 7 Effect of pertussis toxin on histamine-stimulated [^{3}H]-inositol phosphate accumulation in CHO-K1 cells transfected with the bovine histamine H_1-receptor (CHO-H1). Control cells (●) and cells treated with pertussis toxin (PTX; 100 ng ml^{-1}; 24 h; ○) were incubated for 40 min in increasing concentrations of histamine in the presence of 20 mM LiCl. Also shown are the basal and ATP (100 μM) stimulated accumulations in control cells (–PTX; hatched columns) or cells treated with pertussis toxin (+PTX; open columns). Values represent mean±s.e.mean from three separate experiments. Data are expressed as a percentage of the response to 0.1 mM histamine (in control cells) measured in each individual experiment.

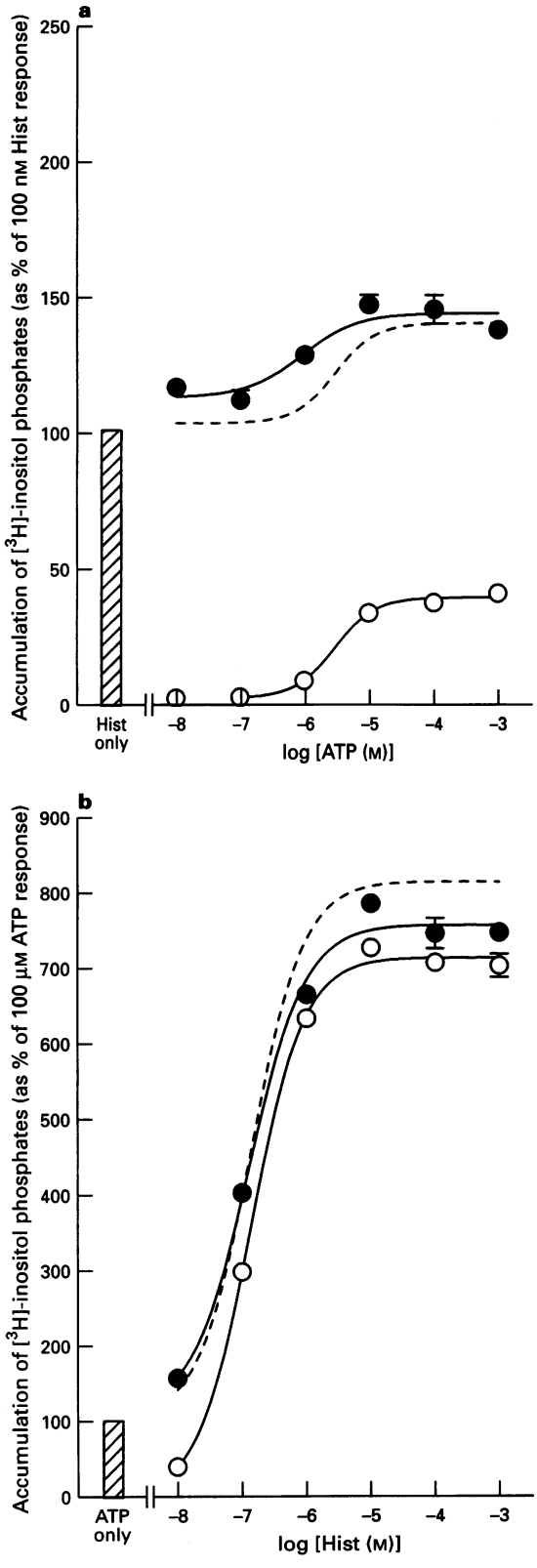

Figure 8 The effect on [^{3}H]-inositol phosphate accumulation of simultaneous application of histamine and ATP in CHO-H1 cells. Panel (a) shows the concentration-response curve to ATP in the presence (●) or absence (○) of histamine (Hist, 100 nM). To normalize responses from different experiments, data are expressed as a percentage of the response to 100 nM histamine alone (after subtraction of basal values). Panel (b) shows the concentration-response curve to histamine in the presence (●) or absence (○) of ATP (100 μM). Data are expressed as a percentage of the response to 100 μM ATP alone (after subtraction of basal values). Values represent mean ± s.e.mean from three separate experiments. The dashed lines indicate the predicted additive responses to both agonists.

from CHO-K1 cells transfected with the human adenosine A_1-receptor (Townsend-Nicholson & Shine, 1992; Iredale *et al.*, 1994). A similar effect of adenosine A_1-receptor stimulation on both adenylyl cyclase activity and phospholipase C has been observed recently in CHO-cells transfected with the rat A_1-receptor cDNA (Freund *et al.*, 1994).

The adenosine A_1-receptor-mediated stimulation of [^{3}H]-inositol phosphate accumulation in CHO-K1 cells was sensitive to complete inhibition by pertussis toxin treatment indicating a role for G_i or G_0 proteins in this response. A similar sensitivity to inhibition by pertussis toxin was also observed for the adenosine A_1-receptor-mediated stimulation of inositol phosphate accumulation and calcium mobilisation in the hamster vas deferens smooth muscle cell line DDT_1MF-2 (Gerwins & Fredholm, 1992a,b; Dickenson & Hill, 1993b; White *et al.*, 1993). If G_i or G_o proteins are involved in these calcium and inositol phosphate responses to adenosine A_1-receptor stimulation in these cell lines, then it is very likely that the βγ subunits of these proteins (rather than α_i or α_o) mediate the activation of phospholipase C, since recent studies have shown that a number of phospholipase C β-isoforms can be activated by G-protein βγ-subunits (Camps *et al.*, 1992; Clapham & Neer, 1993; Wu *et al.*, 1993; Boyer *et al.*, 1994), including the enzyme present in DDT_1MF-2 smooth muscle cells (Dickenson *et al.*, 1995). Consistent with a role for $G_{\beta\gamma}$ subunits in the activation of phospholipase C by G_i or G_o-coupled receptors, it has been demonstrated that expression of the carboxyl βγ-binding domain of β-adrenoceptor kinase (β-ARK) in Cos-7 cells can attenuate the activation of phospholipase C by G_i-linked M_2-muscarinic receptors and α_2-adrenoceptors, without affecting the inhibition of adenylyl cyclase which appears to be mediated via $G_{i\alpha}$-subunits (Koch *et al.*, 1994). These data suggest that G_i-coupled receptors, such as the adenosine A_1-receptor, mediate inhibition of adenylyl cyclase activity via $G_{i\alpha}$-subunits and stimulate phosphoinositide hydrolysis via the action of $G_{i\beta\gamma}$-subunits. An alternative possibility is that the inhibition of adenylyl cyclase is also mediated via G_o derived βγ subunits acting on adenylyl cyclase I (Tang & Gilman, 1992). Whatever the exact mechanism, it is notable that the EC_{50} for CPA-mediated inhibition of cyclic AMP accumulation (3 nM; Figure 2b) is lower than that obtained for stimulation of [^{3}H]-inositol phosphate accumulation (7.4 nM; Table 1) and calcium mobilisation (15 nM; Iredale *et al.*, 1994).

In addition to an apparently direct effect of adenosine A_1-receptors on inositol phosphate accumulation and calcium mobilisation in DDT_1MF-2 cells (Dickenson & Hill, 1993b; White *et al.*, 1993), we and others have recently shown that adenosine A_1-receptor-stimulation can augment the calcium responses elicited by histamine, bradykinin and ATP in this cell line (Gerwins & Fredholm, 1992a,b; Dickenson & Hill, 1993b). It is interesting, therefore, that activation of the transfected human adenosine A_1-receptor in CHO-K1 cells is able to interact synergistically with purine nucleotides in activating the accumulation of [^{3}H]-inositol phosphates.

We have previously shown that CHO-K1 cells possess a nucleotide receptor (P_{2U}-purinoceptor) which can mediate release of calcium from intracellular stores (Iredale & Hill, 1993). This nucleotide receptor has been reported to be linked to both phospholipase C activation and calcium mobilisation in a number of cell lines (Fine *et al.*, 1989; Brown *et al.*, 1991; Murrin & Boarder, 1992; Iredale *et al.*, 1992; Pavenstadt *et al.*, 1992). In the present study we have shown that ATP and UTP both stimulate [^{3}H]-inositol phosphate accumulation in CHO-K1 cells which have been prelabelled with [^{3}H]-*myo*-inositol. The pharmacological characteristics of this response were very similar to those that we have previously reported for the mobilisation of intracellular calcium by these agents (Iredale & Hill, 1993). The inositol phosphate responses to these purine and pyrimidine nucleotides are much less sensitive to inhibition by pertussis toxin treatment than those produced by adenosine-A_1-receptor agonists. Thus, the responses to ATP, UTP

and ATPγS were only reduced by *circa* 30% by 24 h treatment with 100 ng ml^{-1} pertussis toxin (conditions which completely attenuate the equivalent response to CPA).

The simultaneous addition of CPA and P_{2U}-purinoceptor agonists produced a much greater than additive accumulation of total [^{3}H]-inositol phosphates. This synergy was as a consequence of a change in the maximal responses to CPA and ATP, UTP or ATPγS (Table 3) and it was notable that there were no significant changes in the EC_{50} values for any of the agonists involved in the synergistic response (Table 3). The synergy was also clearly evident at short (10 min) incubation periods. However, in cells which had been pretreated with pertussis toxin, the synergy between CPA and purine nucleotides was lost. This is consistent with the synergy occurring between receptors coupled to pertussis-toxin-sensitive and insensitive G-proteins as has been observed in DDT_1MF-2 cells (Gerwins & Fredholm 1992a,b; Dickenson & Hill, 1993). In order to determine whether a similar synergy could be demonstrated between two receptors which couple to pertussis-toxin insensitive G-proteins (i.e. G_Q), experiments were also conducted in CHO-K1 cells which had been transfected with the cDNA for the bovine histamine H-receptor (Iredale *et al.*, 1994).

In these CHO-H1 cells, histamine produced a large stimulation of [^{3}H]-inositol phosphates, with pharmacological characteristics similar to those previously reported for H_1-receptor-mediated calcium mobilisation in this cell line (Iredale *et al.*, 1994). The response to histamine was insensitive to inhibition by pertussis toxin and was not amplified by conjoint activation of the constitutive P_2-purinoceptor. Thus, just as we have previously observed in DDT_1MF-2 cells, there was no evidence for any synergy between histamine H_1 and P_2-purinoceptors (Dickenson & Hill, 1993b).

The greater sensitivity to pertussis toxin of the response to ATP in CHO-A1 cells (compared with that to histamine in CHO-H1 cells), raises the possibility that hydrolysis of ATP to adenosine (and the potential for synergy between A_1-adenosine and P_{2U}-purinoceptors) is contributing to the overall response to ATP in CHO-A1 cells. Alternatively, since the response to UTP also had a small pertussis toxin-sensitive component, it is possible that the subsequent stimulation of calcium mobilisation in these cells leads to a release of adenosine from the CHO-K1 cells (which in turn amplifies the P_{2U}-receptor-mediated response). This would be consistent with the lack of effect of pertussis toxin on the histamine H_1-receptor-mediated response in the CHO-H1 cells which have not been transfected with the human adenosine A_1-receptor cDNA. However, the fact that some 20% of the response to ATP in CHO-H1 cells is also sensitive to pertussis toxin, suggests that some other mechanism may be operating.

In conclusion the results of the present study have shown that the human transfected adenosine A_1-receptor can stimulate [^{3}H]-inositol phosphate accumulation in CHO-K1 cells via the intermediacy of a pertussis-toxin-sensitive G-protein (i.e. G_i or G_o). In addition, the simultaneous activation of the transfected adenosine A_1-receptor and the constitutively expressed P_{2U}-purinoceptor produced a large augmentation of the inositol phospholipid hydrolysis. This synergy was not observed between two G_Q-coupled receptors in CHO-K1 cells transfected with the histamine H_1-receptor. The demonstration of a synergy between pertussis toxin-sensitive and insensitive receptors in a model cell system (such as CHO-K1), which is a suitable host for transfected cDNA sequences, provides a unique opportunity to unravel the mechanisms underlying this important example of receptor cross-talk.

We thank the Wellcome Trust for financial support (Grant Reference 038757/z/93/z/1.5).

References

BOYER, J.L., GRABER, S.G., WALDO, G.L., HARDEN, T.K. & GARRISON, J.C. (1994). Selective activation of phospholipase C by recombinant G-protein α- and βγ-subunits. *J. Biol. Chem.*, **269**, 2814–2819.

BROWN, H.A., LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (1991). Evidence that UTP and ATP regulate phospholipase C through a common 5′-nucleotide receptor in human airway epithelial cells. *Mol. Pharmacol.*, **40**, 648–655.

CAMPS, M., CAROZZI, A., SCHNABEL, P., SCHEER, A., PARKER, P.J. & GIERSCHIK, P. (1992). Isozyme-selective stimulation of phospholipase C-β_2- by G-protein βγ-subunits. *Nature*, **360**, 684–689.

CLAPHAM, D.E. & NEER, E.J. (1993). New roles for G-protein βγ-dimers in transmembrane signalling. *Nature*, **365**, 403–406.

COLLIS, M.G. & HOURANI, S.M.O. (1993). Adenosine receptor subtypes. *Trends Pharmacol. Sci.*, **14**, 360–366.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-responses curves. *J. Neurochem.*, **235**, E97–E102.

DICKENSON, J.M., CAMPS, M., GIERSCHIK, P. & HILL, S.J. (1995). Activation of phospholipase C by G-protein βγ subunits in DDT_1MF-2 cells. *Eur. J. Pharmacol.*, **266**, 393–398.

DICKENSON, J.M. & HILL, S.J. (1993a). Adenosine A_1-receptor stimulated increases in intracellular calcium in the smooth muscle cell line DDT_1MF-2. *Br. J. Pharmacol.*, **108**, 85–92.

DICKENSON, J.M. & HILL, S.J. (1993b). Intracellular cross-talk between receptors coupled to phospholipase C via pertussis toxin-sensitive and pertussis toxin-insensitive G-proteins in DDT_1MF-2 cells. *Br. J. Pharmacol.*, **109**, 719–724.

DONALDSON, J., BROWN, A.M. & HILL, S.J. (1988). Influence of rolipram on the cyclic 3′,5′-adenosine monophosphate response to histamine and adenosine in slices of guinea-pig cerebral cortex. *Biochem. Pharmacol.*, **37**, 715–723.

FEDERMAN, A.D., CONKLIN, B.R., SCHRADER, K.A., REED, R.R. & BOURNE, H.R. (1992). Hormonal stimulation of adenylyl cyclase through Gi-protein βγ subunits. *Nature*, **356**, 159–161.

FINE, J., COLE, P. & DAVIDSON, J.S. (1989). Extracellular nucleotides stimulate receptor-mediated calcium mobilization and inositol phosphate production in human fibroblasts. *Biochem. J.*, **263**, 371–376.

FREUND, S., UNGERER, M. & LOHSE, M.J. (1994). A_1 adenosine receptors expressed in CHO-cells couple to adenylyl cyclase and to phospholipase C. *Naunyn-Schmied. Arch. Pharmacol.*, **350**, 49–56.

GERWINS, P. & FREDHOLM, B.B. (1992a). ATP and its metabolite adenosine act synergistically to mobilize intracellular calcium via the formation of inositol 1,4,5-trisphosphate in a smooth muscle cell line. *J. Biol. Chem.*, **267**, 16081–16087.

GERWINS, P. & FREDHOLM, B.B. (1992b). Stimulation of adenosine A_1 receptors and bradykinin receptors, which act via different G proteins, synergistically raises inositol 1,4,5-trisphosphate and intracellular free calcium in DDT_1MF-2 smooth muscle cells. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 7330–7334.

IREDALE, P.A., ALEXANDER, S.P.H. & HILL, S.J. (1994). Coupling of the transfected human brain A_1 adenosine receptor to calcium mobilisation in CHO cells by a pertussis toxin-sensitive mechanism. *Br. J. Pharmacol.*, **111**, 1252–1256.

IREDALE, P.A., FUKUI, H. & HILL, S.J. (1993). High, stable expression of the bovine histamine H_1-receptor coupled to $[Ca^{2+}]i$ mobilisation in CHO-K1 cells. *Biochem. Biophys. Res. Commun.*, **195**, 1294–1300.

IREDALE, P.A. & HILL, S.J. (1993). Increases in intracellular calcium via activation of an endogenous P_2-purinoceptor in cultured CHO-K1 cells. *Br. J. Pharmacol.*, **110**, 1305–1310.

IREDALE, P.A., MARTIN, K.F., ALEXANDER, S.P.H., HILL, S.J. & KENDALL, D.A. (1992). Inositol 1,4,5-trisphosphate generation and calcium mobilisation via activation of an atypical P_2 receptor in the neuronal cell line, N1E-115. *Br. J. Pharmacol.*, **107**, 1083–1087.

KOCH, W.J., HAWES, B.E., INGLESE, J., LUTTRELL, L.M. & LEFKOWITZ, R.J. (1994). Cellular expression of the carboxyl terminal of a G protein-coupled receptor kinase attenuates Gβγ-mediated signalling. *J. Biol. Chem.*, **269**, 6193–6197.

LAZARENO, S. & ROBERTS, F.F. (1987). Measuring muscarinic antagonist potency using phosphoinositide breakdown in rat cortex slices. *Br. J. Pharmacol.*, **92**, 677P.

LIBERT, F., VAN SANDE, J., LEFORT, A., CZERNILOFSKY, A., DUMONT, J.E., VASSART, G., ENSINGER, H.A. & MENDLA, K.D. (1992). Cloning and functional characterization of a human A_1 adenosine receptor. *Biochem. Biophys. Res. Commun.*, **187**, 919–926.

MUNSON, P.J. & RODBARD, S. (1980). LIGAND: a versatile computerised approach for characterisation of ligand-binding systems. *Anal. Biochem.*, **107**, 220–239.

MURRIN, R.J.A. & BOARDER, M.R. (1992). Neuronal 'nucleotide' receptor linked to phospholipase C and phospholipase D? Stimulation of PC12 cells by ATP analogues and UTP. *Mol. Pharmacol.*, **41**, 561–568.

PAVENSTADT, H., SPATH, M., SCHLUNCK, G., NAUCK, M., FISHER, R., WANNER, C. & SCHOLLMEYER, P. (1992). Effect of nucleotides on the cytosolic free calcium activity and inositol phosphate formation in human glomerular epithelial cells. *Br. J. Pharmacol.*, **107**, 189–195.

RAMKUMAR, V., BARRINGTON, W.W., JACOBSON, K.A. & STILES, G.L. (1990). Demonstration of both A_1 and A_2 adenosine receptors in DDT_1MF-2 smooth muscle cells. *Mol. Pharmacol.*, **37**, 149–156.

RUCK, A., MILLNS, P., KENDALL, D.A. & HILL, S.J. (1990). Expression of β_2-adrenoceptors mediating cyclic AMP accumulation in astroglial and neuronal cell lines derived from the rat CNS. *Biochem. Pharmacol.*, **40**, 2371–2375.

RUGOLO, M., MASTRCOLA, T., WHORLE, C., RASOLA, A., GRUENERT, D.C., ROMEO, G. & GALIETTA, L.J.V. (1993). ATP and A_1 adenosine receptor agonists mobilize intracellular calcium and activate K^+ and Cl^- currents in normal and cystic fibrosis airway epithelial cells. *J. Biol. Chem.*, **268**, 24779–24784.

TANG, W.J. & GILMAN, A.G. (1992). Adenylyl cyclases. *Cell*, **70**, 869–872.

TOWNSEND-NICHOLSON, A. & SHINE, J. (1992). Molecular cloning and characterisation of a human brain A_1 adenosine receptor cDNA. *Mol. Brain Res.*, **16**, 365–370.

WHITE, T.E., DICKENSON, J.M., ALEXANDER, S.P.H. & HILL, S.J. (1992). Adenosine A_1-receptor stimulation of inositol phospholipid hydrolysis and calcium mobilisation in DDT_1 MF-2 cells. *Br. J. Pharmacol.*, **106**, 215–221.

WHITE, T.E., DICKENSON, J.M. & HILL, S.J. (1993). Histamine H_1-receptor-mediated inositol phospholipid hydrolysis in DDT_1 MF-2 cells: agonist and antagonist properties. *Br. J. Pharmacol.*, **108**, 196–203.

WU, D., KATZ, A. & SIMON, M.I. (1993). Activation of phospholipase C β2 by the α and βγ subunits of trimeric GTP-binding protein. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 5297–5301.

(Received February 17, 1995
Revised April 4, 1995
Accepted May 9, 1995)