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Potentiation of adenosine A_1 receptor-mediated inositol phospholipid hydrolysis by tyrosine kinase inhibitors in CHO cells

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1 The effect of protein tyrosine kinase inhibitors on human adenosine A_1 receptor-mediated [^{3}H]-inositol phosphate ([^{3}H]-IP) accumulation has been studied in transfected Chinese hamster ovary cells (CHO-A1) cells.

2 In agreement with our previous studies the selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA) stimulated the accumulation of [^{3}H]-IPs in CHO-A1 cells. Pre-treatment with the broad spectrum tyrosine kinase inhibitor genistein (100 μM; 30 min) potentiated the responses elicited by 1 μM (199$\pm$17% of control CPA response) and 10 μM CPA (234$\pm$15%). Similarly, tyrphostin A47 (100 μM) potentiated the accumulation of [^{3}H]-IPs elicited by 1 μM CPA (280$\pm$32%).

3 Genistein (EC_{50} = 13.7$\pm$1.2 μM) and tyrphostin A47 (EC_{50} = 10.4$\pm$3.9 μM) potentiated the [^{3}H]-IP response to 1 μM CPA in a concentration-dependent manner.

4 Pre-incubation with the inactive analogues of genistein and tyrphostin A47, daidzein (100 μM; 30 min) and tyrphostin A1 (100 μM; 30 min), respectively, had no significant effect on the accumulation of [^{3}H]-IPs elicited by 1 μM CPA.

5 Genistein (100 μM) had no significant effect on the accumulation of [^{3}H]-IPs produced by the endogenous thrombin receptor (1 u ml^{-1}; 100$\pm$10% of control response). In contrast, tyrphostin A47 produced a small augmentation of the thrombin [^{3}H]-IP response (148$\pm$13%).

6 Genistein (100 μM) had no effect on the [^{3}H]-IP response produced by activation of the endogenous G_q-protein coupled CCK_A receptor with the sulphated C-terminal octapeptide of cholecystokinin (1 μM CCK-8; 96$\pm$6% of control). In contrast, tyrphostin A47 (100 μM) caused a small but significant increase in the response to 1 μM CCK-8 (113$\pm$3% of control).

7 The phosphatidylinositol 3-kinase inhibitor LY 294002 (30 μM) and the MAP kinase kinase inhibitor PD 98059 (50 μM) had no significant effect on the [^{3}H]-IP responses produced by 1 μM CPA and 1 μM CCK-8.

8 These observations suggest that a tyrosine kinase-dependent pathway may be involved in the regulation of human adenosine A_1 receptor mediated [^{3}H]-IP responses in CHO-A1 cells.

Keywords: Adenosine A_1 receptor; inositol phosphates; tyrosine kinases; genistein; tyrphostin; CHO cells; CCK_A receptors

Introduction

The inositol-lipid specific phospholipase C (PLC) isoenzymes have been classified into PLC-β, PLC-γ and PLC-δ families based on biochemical and molecular biological approaches (Lee & Rhee, 1995). The β isoforms of PLC are regulated by receptors that are coupled to the heterotrimeric family of G-proteins. For example, the Gα subunits belonging to the pertussis toxin-insensitive G_q class of G-proteins (Gα_q, Gα_{11}, Gα_{14}, Gα_{15} and Gα_{16}) activate the β isoforms of PLC (PLC-β1-4; Rhee, 1994; Lee *et al*., 1992). The activation of PLC-β through receptors coupled to the pertussis toxin-sensitive family of G proteins (G_{i1}, G_{i2}, G_{i3} and G_0) is considered to be mediated by the $\beta\gamma$ subunits of these G_i/G_0 proteins (Camps *et al*., 1992; Clapham & Neer, 1993). In marked contrast, the PLC-γ isoforms (PLC-γ1 and PLC-γ2) are activated following tyrosine phosphorylation of specific tyrosine residues (Kim *et al*., 1990; Rhee,1991). For example, PLC-γ1 is activated by the intrinsic protein-tyrosine kinase activity that is associated with receptors for growth factors, e.g. epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Kim *et al*., 1990). In addition, PLC-γ's can be activated by non-receptor tyrosine kinases that are associated with immune system receptors (Carter *et al*., 1991; Park *et al*., 1991a,b).

Recent reports have demonstrated that several members of the G-protein coupled receptor family can activate PLC-γ through tyrosine phosphorylation (Gusovsky *et al*., 1993; Dahr & Shukla, 1994; Marrero *et al*., 1996; Puceat & Vassort, 1996). For example, in cultured rat aortic smooth muscle cells activation of the angiotensin (AT_1) receptor stimulates the tyrosine phosphorylation of PLC-γ1 (Marrero *et al*., 1996). Similar increases in PLC-γ phosphorylation have also been observed following muscarinic M_5 receptor, P_2 purinoceptor and platelet activating factor (PAF) receptor activation (Gusovsky *et al*., 1993; Dahr & Shukla, 1994; Puceat & Vassort, 1996). Furthermore, the non-receptor tyrosine kinase *c-Src* appears to mediate AT_1 and PAF receptor stimulation of PLC-γ1 phosphorylation (Dahr & Shukla, 1994; Marrero *et al*., 1995).

The adenosine A_1 receptor is a member of the seven transmembrane G protein-coupled receptor superfamily (Fredholm *et al*., 1994). Adenosine A_1 receptors couple to the pertussis toxin-sensitive family of inhibitory G proteins (G_{i1}, G_{i2}, G_{i3} and G_0) and as a consequence inhibit the activation of adenylyl cyclase (Olah & Stiles, 1995). In addition, endogenous and transfected adenosine A_1 receptors have also been shown to stimulate pertussis toxin-sensitive increases in inositol

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phospholipid hydrolysis and Ca^{2+} mobilization in a variety of different cell types (Gerwins & Fredholm, 1992; Dickenson & Hill, 1993; Iredale & Hill, 1994; Peakman & Hill, 1995). The sensitivity to pertussis toxin suggests that G_i/G_0 proteins are involved in adenosine A_1 receptor coupling to phospholipase C. At present it is not known whether G_i/G_0 protein derived $\beta\gamma$ subunits are responsible for adenosine A_1 receptor stimulation of phospholipase C (Dickenson & Hill, 1996). However, in view of the increasing number of reports describing G protein-coupled receptor activation of PLC-γ isoforms, it is conceivable that adenosine A_1 receptor-mediated inositol phospholipid hydrolysis occurs through the stimulation of PLC-γ. Furthermore, several members of the G_i/G_0 protein-coupled receptor family have recently been shown to activate the *Src* family of tyrosine kinases through a pertussis toxin-sensitive mechanism (Chen *et al*., 1994; Ishida *et al*., 1995; Dikic *et al*., 1996). Therefore, an alternative mechanism (and still pertussis toxin-sensitive) for adenosine A_1 receptor-induced inositol phospholipid hydrolysis would involve tyrosine kinase phosphorylation of PLC-γ. In the present study we have investigated whether adenosine A_1 receptor stimulation of inositol phospholipid hydrolysis in CHO-A1 cells is sensitive to tyrosine kinase inhibitors.

Methods

Cell culture

Chinese hamster ovary cells (CHO-K1) transfected with the human brain adenosine A_1 receptor sequence (CHO-A1) were a generous gift from Dr Andrea Townsend-Nicholson and Professor John Shine, Garvan Institute, Sydney, Australia. CHO-A1 cells were cultured in 75 cm^2 flasks (Costar) in Dulbeco's modified Eagles Medium/Nutrient F12 (1 : 1) supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum. Cells were maintained at 37°C in a humidified 10% CO_2 atmosphere until confluency and were subcultured (1 : 5 split ratio) using trypsin (0.05% w/v)/EDTA (0.02% w/v). Cells for [^{3}H]-inositol phosphate determinations were grown in 24 well cluster dishes (Costar).

Inositol phospholipid hydrolysis

When confluent cell monolayers were loaded for 24 h with [^{3}H]-*myo*-inositol (37 kBq/well) in 24 well cluster dishes in inositol-free DMEM containing 0.1% 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 the presence of 20 mM LiCl (290 μl $well^{-1}$). Where appropriate tyrosine kinase inhibitors were added at the beginning of this incubation period. Agonists were then added in 10 μl of medium and the incubation continued for 40 min (unless otherwise stated) at 37°C. Incubations were terminated by aspiration of the incubation medium and the addition of 900 μl cold (−20°C) methanol/0.12 M HCl (1 : 1 vv^{-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. 800 μl aliquots of the supernatant were neutralized 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 (X8, 100–200 mesh, chloride form). [^{3}H]-inositol and [^{3}H]-glycerophosphoinositol were removed with 20 ml of distilled water and 10 ml 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. Radioactivity was quantified by scintillation counting in the gel phase (scintillator plus, Packard).

Data analysis

EC_{50} values (concentrations of drug producing 50% of the maximal stimulation) were obtained by computer assisted curve fitting by use of the computer programme InPlot (GraphPAD, California, U.S.A.). Statistical significance was determined by Student's unpaired *t*-test ($P<0.05$ was considered statistically significant). All data are presented as means ± s.e.mean. The *n* in the text refers to the number of separate experiments.

Chemicals

[2-^{3}H]-*myo*-inositol was from New England Nuclear. The sulphated C-terminal octapeptide of cholecystokinin (CCK-8), genistein, daidzein, tyrphostin A1, tyrphostin A47, Ro 31-8220, PD 98059 and LY 294002 were from Calbiochem/Novobiochem (Nottingham, U.K.). Thrombin and N^6-cyclopentyladenosine were obtained from Boehringer and Sigma Chemical Co. (Poole, Dorset, U.K.) respectively. Dulbeco's modified Eagles Medium/Nutrient Mix F-12 (1 : 1) and foetal calf serum were from Sigma Chemical Co. (Poole, Dorset, U.K.). All other chemicals were of analytical grade.

Results

The effects of the tyrosine kinase inhibitors, genistein and tyrphostin A47, on adenosine A_1 receptor, CCK_A-receptor and thrombin receptor-mediated inositol phospholipid hydrolysis have been examined in Chinese hamster ovary cells. We have previously shown that the endogenously expressed thrombin receptor and CCK_A receptor stimulate pertussis toxin-insensitive increases in [^{3}H]-IP accumulation in CHO cells, suggesting coupling to G_q/G_{11}-proteins (Dickenson & Hill, 1996, 1997). In contrast, the transfected G_i/G_0 protein coupled human adenosine A_1 receptor stimulates increases in [^{3}H]-IPs which are sensitive to pertussis toxin (Dickenson & Hill, 1996). The two tyrosine kinase inhibitors employed in this study have different modes of action. Genistein is a potent broad range tyrosine kinase inhibitor and exerts its effects by binding to the ATP binding site (Akiyama *et al*., 1987), whereas tyrphostin A47, structurally resembles tyrosine and inhibits protein tyrosine kinases by blocking the substrate binding site (Gazit *et al*., 1989; Levitzki & Gazit, 1995).

Effect of tyrosine kinase inhibitors on adenosine A_1 receptor-mediated [^{3}H]-inositol phosphate accumulation

The results of our initial experiments investigating the effects of genistein and tyrphostin A47 on adenosine A_1 receptor-mediated [^{3}H]-IP production are shown in Figure 1A. Genistein and tyrphostin A47 (100 μM; 30 min) clearly augmented the accumulation of [^{3}H]-IPs elicited by the selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA; 1 μM). The [^{3}H]-IP response to CPA (after subtracting basal [^{3}H]-IP accumulation) in genistein and tyrphostin A47 treated cells increased significantly to 202 ± 25% ($n=3$; $P<0.05$) and 275 ± 31% ($n=3$; $P<0.05$)

respectively, of the control CPA response (100%). Both tyrosine kinase inhibitors increased the basal accumulation of [^{3}H]-IPs (3316±710 d.p.m. (n=3) and 2875±565 d.p.m. (n=3) in genistein and tyrphostin A47 treated cells compared to 2163±314 d.p.m. (n=3) in control cells). Daidzein, the inactive analogue of genistein, had no significant effect on the accumulation of [^{3}H]-IPs produced by 1 μM CPA (116±9% of control CPA response; n=3). To eliminate any effects the tyrosine kinase inhibitors may be having on protein kinase C we investigated the effects of the selective protein kinase C (PKC) inhibitor Ro 31-8220, and the PKC activator, phorbol 12-myristate 13-acetate (PMA) on adenosine A_1 receptor-mediated [^{3}H]-IP responses. As shown in Figure 1A, Ro 31-8220 (10 μM; 30 min) had no significant effect on the accumulation of [^{3}H]-IPs elicited by 1 μM CPA (109±11% of control CPA response; n=3). However, Ro 31-8220 (10 μM) did reverse the inhibitory effects of PMA (1 μM; 10 min) on CPA stimulated increases in [^{3}H]-IP accumulation (Figure 1B). In marked contrast, genistein (100 μM), tyrphostin A47 (100 μM) and daidzein (100 μM) were unable to reverse the inhibitory effects of PMA (Figure 1B). These data would seem to suggest that the concentrations of genistein and tyrphostin A47 employed in this study (up to 100 μM) are not inhibiting phorbol ester-sensitive PKC isoforms.

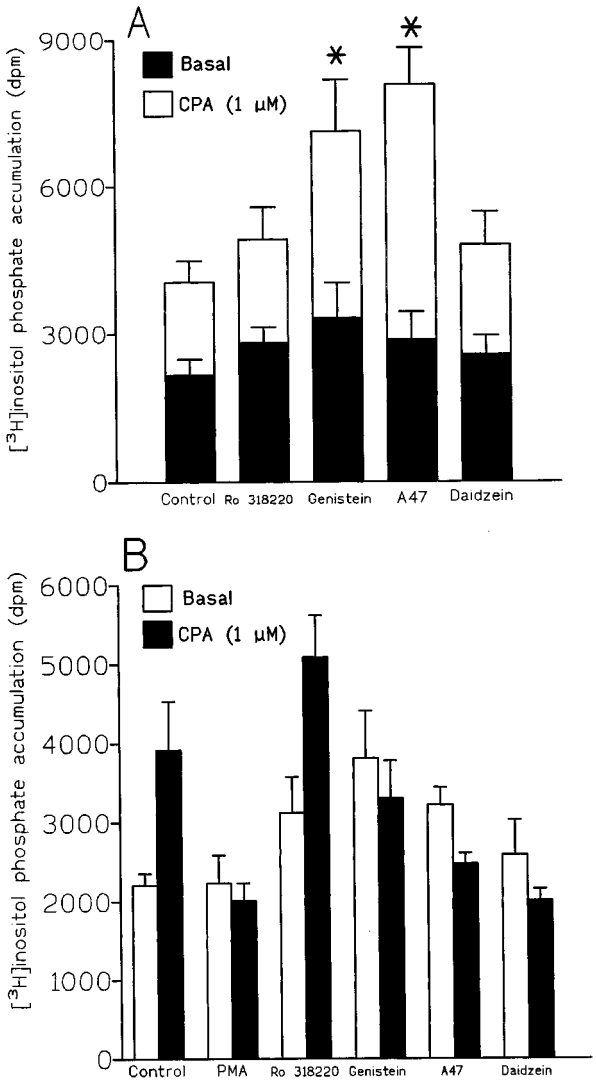

Figure 1 Effect of the tyrosine kinase inhibitors, genistein and tyrphostin A47, and the protein kinase C inhibitor, Ro 31-8220, on adenosine A_1 receptor mediated [^{3}H]-IP accumulation in CHO-A1 cells. (A) Cells were pre-incubated for 30 min with Ro 318220 (10 μM), genistein (100 μM), tyrphostin A47 (100 μM) and daidzein (100 μM). Cells were then stimulated with 1 μM N^6-cyclopentyladenosine (CPA). Values represent the means±s.e.mean from three independent experiments each measured in duplicate. *Statistically significant (P<0.05) from the control CPA response (after subtracting basal accumulation). (B) In these experiments cells were initially pre-incubated for 30 min with Ro 31-8220 (10 μM), genistein (100 μM), tyrphostin A47 (100 μM) and daidzein (100 μM) or 0.1% dimethyl sulphoxide (control and PMA alone treated cells). Cells (except control) were then stimulated with 1 μM PMA for 10 min before stimulating with 1 μM CPA. Values represent the means ±s.e.mean from three independent experiments each measured in duplicate.

Figure 2 shows the effect of genistein pre-treatment (100 μM; 30 min) on the concentration response curve for adenosine A_1 receptor-mediated [^{3}H]-IP production in CHO-A1 cells. Genistein potentiated the [^{3}H]-IP responses produced by 1 μM and 10 μM CPA. Furthermore, the EC_{50} value for CPA stimulated [^{3}H]-IP accumulation in control cells (51±17 nM; n=4; Hill coefficient 1.19±0.23) was significantly different from genistein-treated cells (287±46 nM; n=4; Hill coefficient 0.79±0.04). Interestingly, Okajima *et al.* (1994) reported that genistein is a competitive antagonist of A_1 and A_2 adenosine receptors in FRTL-5 cells. Hence, the ability of genistein to potentiate CPA mediated [^{3}H]-IP accumulation may be compromised by the competitive antagonist properties of genistein (certainly at concentrations of CPA≤100 nM). Finally, genistein potentiated the accumulation of [^{3}H]-IPs elicited by CPA (1 μM) in a concentration-dependent manner (EC_{50}=13.7±1.2 μM; n=3; Figure 3).

We also determined the effect of tyrphostin A47 pre-treatment (100 μM; 30 min) on the concentration response curve for adenosine A_1 receptor-stimulated [^{3}H]-IP accumulation in CHO-A1 cells (Figure 4). Tyrphostin A47 potentiated the [^{3}H]-IP responses elicited by 100 nM (200±15% of control response; n=6), 1 μM (292±43%; n=6) and 10 μM

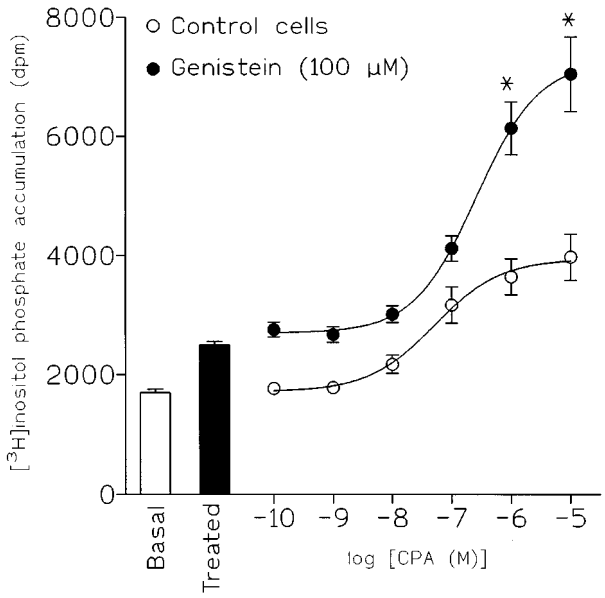

Figure 2 Effect of the tyrosine kinase inhibitor genistein on the concentration-response curve for adenosine A_1 receptor-stimulated [^{3}H]-IP accumulation in CHO-A1 cells. Concentration-response curves for N^6-cyclopentyladenosine (CPA) stimulated [^{3}H]-IP accumulation in absence and presence of genistein (100 μM; 30 min). *Statistically significant (P<0.05) from the response to CPA obtained in control cells. Data are the means±s.e.mean of four independent experiments each measured in triplicate.

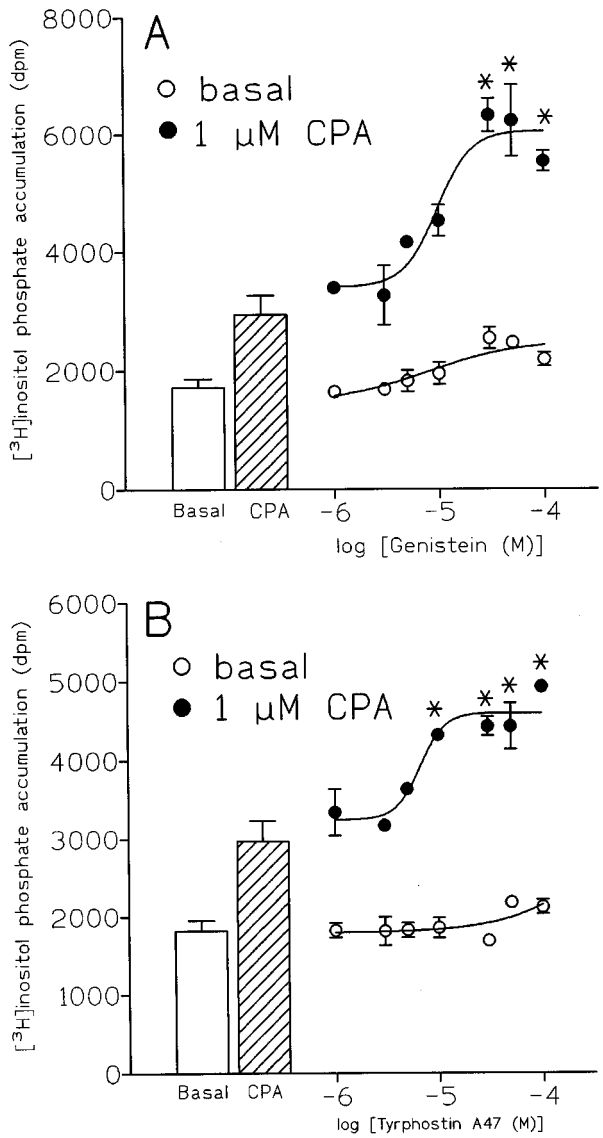

Figure 3 Concentration-response curves for genistein and tyrphostin A47 on the CPA-mediated [^{3}H]-IP response. The effect of increasing concentrations of genistein (A) and tyrphostin A47 (B) on basal and 1 μM N^6-cyclopentyladenosine stimulated [^{3}H]-IP accumulation in CHO-A1 cells (30 pre-incubation). Control responses to CPA (1 μM) are shown in the hatched columns. (A) Data are the means$\pm$s.e.mean of three independent experiments each measured in duplicate. (B) Due to experimental variation in the absolute data obtained the values shown are the means$\pm$range of duplicate determinations obtained in a single experiment. Similar effects were observed in three further experiments. *Statistically significant ($P<0.05$) from the control response to CPA.

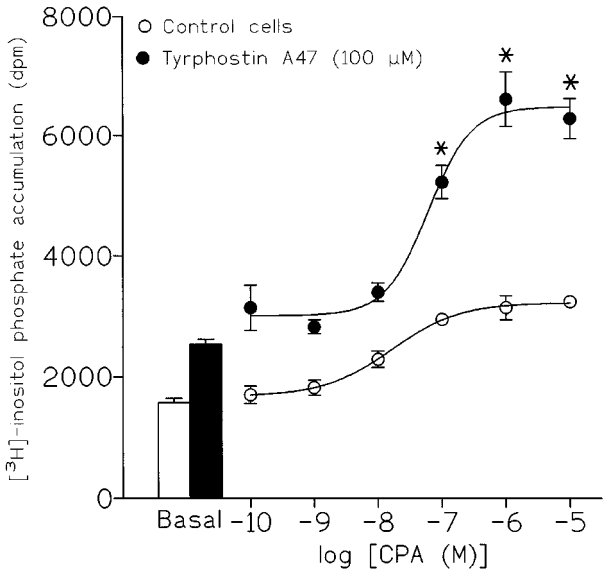

Figure 4 Effect of the tyrosine kinase inhibitor tyrphostin A47 on the concentration-response curve for adenosine A_1 receptor-stimulated [^{3}H]-IP accumulation in CHO-A1 cells. Concentration-response curves for N^6-cyclopentyladenosine (CPA) stimulated [^{3}H]-IP accumulation in absence and presence of tyrphostin A47 (100 μM; 30 min). *Statistically significant ($P<0.05$) from the response to CPA obtained in control cells. Data are the means$\pm$s.e.mean of four independent experiments each measured in triplicate.

(235$\pm$17%; $n=6$) CPA. Furthermore, tyrphostin A47 had no significant effect on the EC_{50} value for CPA stimulated [^{3}H]-IP production (58$\pm$27 nM ($n=6$) in control cells and 71$\pm$15 nM ($n=6$) in tyrphostin A47 treated cells). The potentiation of CPA (1 μM) mediated [^{3}H]-IP responses by tyrphostin A47 was concentration-dependent ($EC_{50}=10.4\pm3.9$ μM; $n=4$; Figure 3). Finally, tyrphostin A1, the negative control for tyrphostin A47, had no significant effect on the accumulation of [^{3}H]-IPs produced by 1 μM CPA (93$\pm$4% of control CPA response; $n=3$).

These data suggest a potential role for a tyrosine kinase-dependent pathway in regulating the adenosine A_1 receptor-mediated [^{3}H]-IP responses in CHO-A1 cells.

Effect of tyrosine kinase inhibitors on thrombin receptor-mediated [^{3}H]-inositol phosphate accumulation

Genistein (100 μM; 30 min) had no significant effect on the accumulation of [^{3}H]-IPs elicited by the maximally effective concentration of thrombin (1 μm l^{-1}; Figure 5A) (Dickenson & Hill, 1997). The response to thrombin in genistein-treated cells (after subtracting basal [^{3}H]-IP accumulation) was 100$\pm$10% ($n=3$) of the control response. In contrast, tyrphostin A47 pre-treatment (100 μM; 30 min) augmented the accumulation of [^{3}H]-IPs elicited by 1 u ml^{-1} thrombin (148$\pm$14% of control response; $n=4$). As shown in Figure 5A, the PKC inhibitor Ro 31-8220 (10 μM; 30 min) markedly increased the [^{3}H]-IP response elicited by 1 u ml^{-1} thrombin (329$\pm$22% of control response; $n=3$; $P<0.05$). Furthermore, Ro 31-8220 reversed the inhibitory effects of PMA (1 μM; 10 min) on thrombin-stimulated [^{3}H]-IP accumulation (Figure 5B). Genistein (100 μM), tyrphostin A47 (100 μM) and daidzein (100 μM) were unable to reverse the inhibitory effects of PMA (Figure 5B). These data further confirm that the concentrations of genistein and tyrphostin A47 employed in this study (up to 100 μM) are not inhibiting phorbol ester-sensitive PKC isoforms.

Effect of tyrosine kinase inhibitors on CCK_A receptor-mediated [^{3}H]-inositol phosphate accumulation

We also determined the effects of genistein and tyrphostin A47 on the accumulation of [^{3}H]-IPs produced by the endogenous G_q-protein coupled CCK_A receptor in CHO-A1 cells (Dickenson & Hill, 1996). Genistein had no effect on the response produced by the sulphated C-terminal octapeptide of cholecystokinin (CCK-8; 1 μM; 99$\pm$6% of control; $n=7$). The effect of genistein (100 μM; 30 min) on the concentration-response curve produced by CCK-8 is shown in Figure 6. There was no significant effect on the EC_{50} value for CCK-8 stimulated [^{3}H]-IP production (22$\pm$11 nM ($n=3$) in control

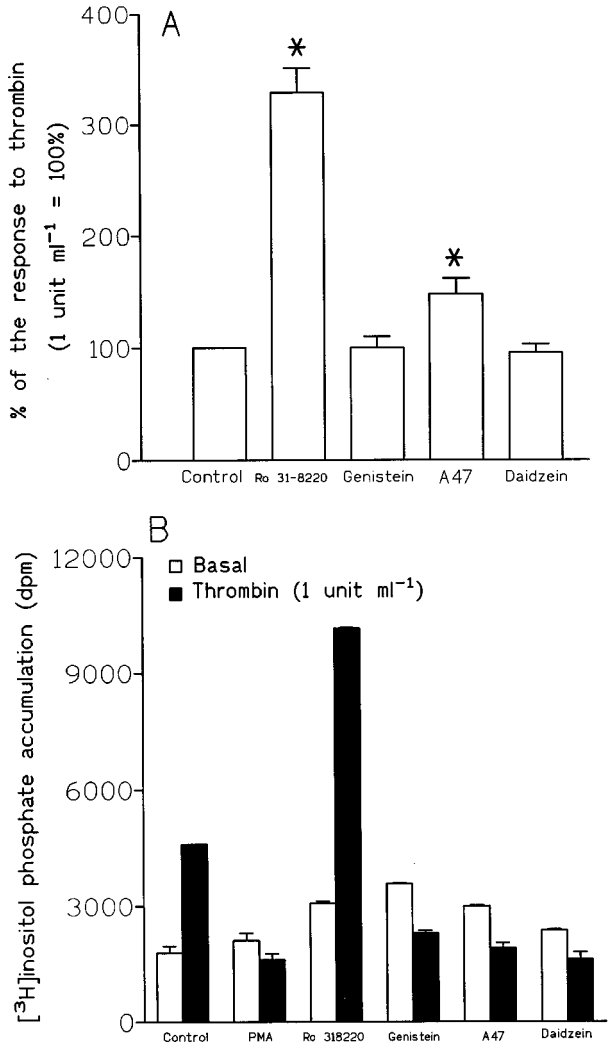

Figure 5 Effect of the tyrosine kinase inhibitors, genistein and tyrphostin A47, and the protein kinase C inhibitor, Ro 31-8220, on thrombin-stimulated [^{3}H]-IP accumulation in CHO-A1 cells. (A) Cells were pre-incubated for 30 min with Ro 31-8220 (10 μM), genistein (100 μM), tyrphostin A47 (100 μM) and daidzein (100 μM). Cells were then stimulated with 1 u ml^{-1} thrombin for 40 min. Values represent the means$\pm$s.e.mean of 3–4 (tyrphostin A47) independent experiments each measured in duplicate. *Statistically significant ($P<0.05$) from the control thrombin response (=100% and after subtracting basal [^{3}H]-IP accumulation). (B) In these experiments cells were initially pre-incubated for 30 min with Ro 31-8220 (10 μM), genistein (100 μM), tyrphostin A47 (100 μM) and daidzein (100 μM) or 0.1% dimethyl sulphoxide (control and PMA alone treated cells). Cells (except control) were then stimulated with 1 μM PMA for 10 min before stimulating with 1 u ml^{-1} thrombin. Values represent the means$\pm$range of duplicate determinations measured in a single experiment. Similar results were obtained in two further experiments.

cells and 23$\pm$10 nM ($n=3$) in genistein treated cells). For comparison the response to 10 μM CPA in these experiments increased significantly (237$\pm$3% of control; $n=3$). Tyrphostin A47 pre-treatment caused a small but significant increase in the [^{3}H]-IP response produced by 1 μM CCK-8 (112.8$\pm$3.5% of control responses; $n=11$; $P<0.05$). Figure 7 shows the effect of tyrphostin A47 (100 μM; 30 min) pre-treatment on the concentration-response curve elicited by CCK-8. In these experiments, tyrphostin A47 had no significant effect on the EC_{50} value for CCK-8 mediated [^{3}H]-IP accumulation (22.5$\pm$7.5 nM ($n=4$) in control cells and 22.5$\pm$4.5 nM ($n=4$) in treated cells).

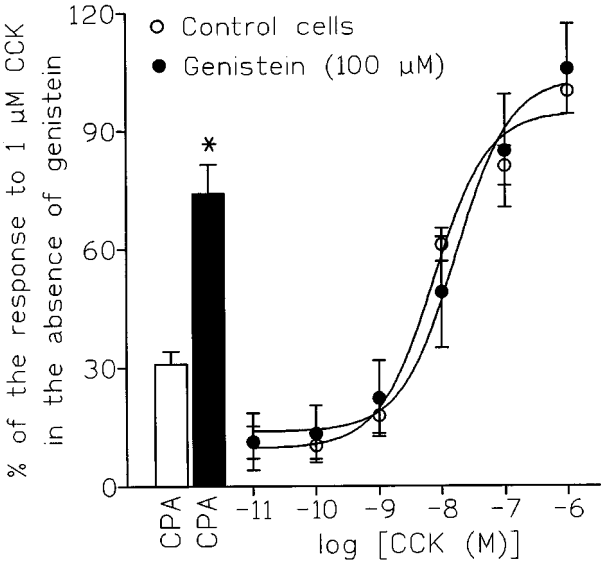

Figure 6 Effect of the tyrosine kinase inhibitor genistein on the concentration-response curve for CCK_A receptor-stimulated [^{3}H]-IP accumulation in CHO-A1 cells. Concentration-response curves for CCK_A receptor-stimulated [^{3}H]-IP accumulation in the absence and presence of genistein (100 μM; 30 min). For comparison the effect of genistein on CPA (10 μM) stimulated [^{3}H]-IP accumulation was included in these experiments (represented by histograms). Values represent the means$\pm$s.e.mean of three independent experiments each measured in triplicate. Data are presented as the percentage of the maximal (100%) [^{3}H]-IP response obtained with 1 μM CCK in control cells (and after subtracting basal [^{3}H]-IP accumulation). Basal [^{3}H]-IP accumulation was 1713$\pm$138 d.p.m ($n=3$) and 2802$\pm$135 d.p.m. ($n=3$) in control and genistein treated cells, respectively. *Statistically significant ($P<0.05$) from the control CPA response.

Effect of phosphatidylinositol-3-kinase and MAP kinase kinase inhibitors on adenosine A_1 receptor-mediated [^{3}H]-inositol phosphate accumulation

Recent evidence has shown that stimulation of the mitogen-activated protein kinase (MAP kinase) pathway by G_i/G_0-protein coupled receptors involves *c-Src* kinase tyrosine activation (Luttrell *et al.*, 1996). Furthermore, several reports have implicated that phosphatidylinositol-3-kinase (PI-3K) may be involved upstream in the activation of tyrosine kinase(s) by G_i/G_0-protein coupled receptors (Hawes *et al.*, 1996; Garnovskaya *et al.*, 1996). Therefore, we investigated whether pre-treatment of CHO-A1 cells with the selective PI-3K inhibitor LY 294002 (Vlahos *et al.*, 1994) would also potentiate adenosine A_1 receptor-mediated [^{3}H]-IP accumulation. However, as shown in Figure 8A, LY 294002 (30 μM) had no significant effect on the adenosine A_1 receptor-mediated [^{3}H]-IP response (Figure 8A). Finally, inhibition of the MAP kinase signalling pathway downstream of tyrosine kinase activation was investigated using the selective MAP kinase kinase inhibitor PD 98059 (Dudley *et al.*, 1995). As shown in Figure 8B, PD 98059 (50 μM; 30 min) had no significant effect on the accumulation of [^{3}H]-IPs produced by CPA or CCK-8.

Discussion

In our previous studies we have shown that the human transfected G_i/G_0 protein-coupled adenosine A_1 receptor stimulates a pertussis toxin-sensitive increase in [^{3}H]-IP accumulation in CHO-A1 cells (Megson *et al.*, 1995;

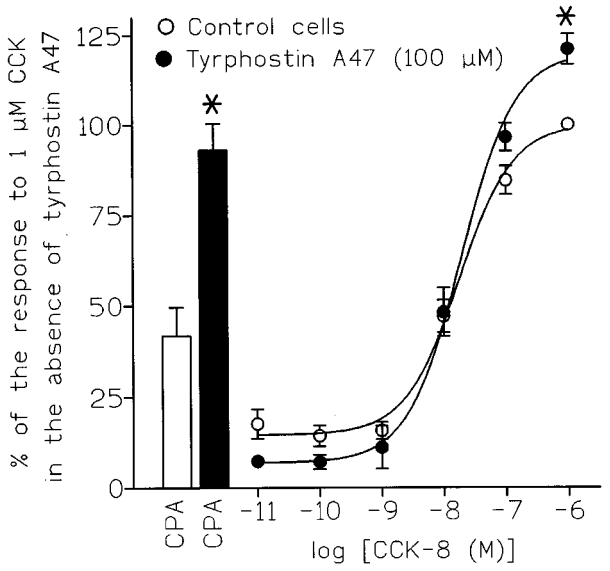

Figure 7 Effect of the tyrosine kinase inhibitor tyrphostin A47 on the concentration-response curve for CCK_A receptor-stimulated [^{3}H]-IP accumulation in CHO-A1 cells. Concentration-response curves for CCK_A receptor-stimulated [^{3}H]-IP accumulation in the absence and presence of tyrphostin A47 (100 μM; 30 min). For comparison the effect of tyrphostin A47 on CPA (10 μM) stimulated [^{3}H]-IP accumulation was routinely measured in these experiments (represented by histograms). Values represent the means$\pm$s.e.mean of four independent experiments each measured in triplicate. Data are presented as the percentage of the maximal (100%) [^{3}H]-IP response obtained with 1 μM CCK in control cells (after subtracting basal [^{3}H]-IP accumulation). Basal [^{3}H]-IP accumulation was 1697$\pm$145 d.p.m. (n=4) and 2445$\pm$180 d.p.m. (n=4) in control and tyrphostin A47-treated cells, respectively. *Statistically significant ($P<0.05$) from the control responses.

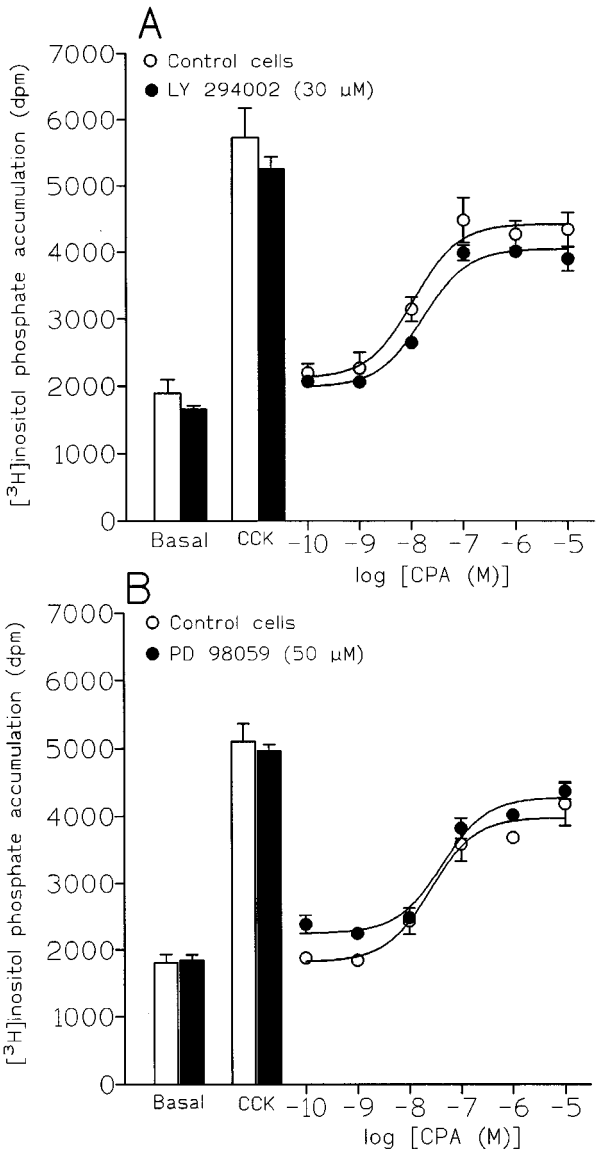

Figure 8 Effect of the phosphatidylinositol-3-kinase inhibitor LY 294002 and the MAP kinase kinase inhibitor PD 98059 on the concentration-response curve for adenosine A_1 receptor-stimulated [^{3}H]-IP accumulation in CHO-A1 cells. (A) Concentration-response curves for adenosine A_1 receptor stimulated [^{3}H]-IP accumulation in absence and presence of LY 294002 (30 μM; 30 min). Basal and CCK-8 (1 μM) stimulated [^{3}H]-IP accumulation in control and LY 294002 treated cells are represented by histograms. Values represent the means$\pm$s.e.mean of three independent experiments each measured in triplicate. (B) As in (A) except that experiments were performed in the absence and presence of PD 98059 (50 μM; 30 min). Values represent the means$\pm$s.e.mean of three independent experiments each measured in triplicate.

Dickenson & Hill, 1996). The initial aim of the present study was to investigate whether adenosine A_1 receptor-mediated [^{3}H]-IP accumulation in CHO-A1 cells is sensitive to blockade by protein tyrosine kinase inhibitors. The rationale for performing these experiments was to determine whether tyrosine phosphorylation of PLC-γ is involved in adenosine A_1 receptor-mediated inositol phospholipid hydrolysis in CHO cells. However, and to our surprise, the findings presented in this study demonstrate that adenosine A_1 receptor-mediated [^{3}H]-IP accumulation in CHO-A1 cells can be potentiated by pre-treating cells with the tyrosine kinase inhibitors, genistein and tyrphostin A47. To our knowledge this is the first report describing the augmentation of adenosine A_1 receptor-stimulated [^{3}H]-IP responses by tyrosine kinase inhibitors. In marked contrast, the [^{3}H]-IP responses produced by the endogenous G_q/G_{11} protein-coupled CCK_A receptor were insensitive to pre-treatment with genistein. Similarly, genistein pre-treatment did not potentiate thrombin receptor-mediated inositol phospholipid hydrolysis. Our previous studies have shown that thrombin-stimulated [^{3}H]-IP accumulation in CHO cells is also pertussis toxin-insensitive suggesting the involvement of G_q/G_{11} proteins (Dickenson & Hill, 1997). Hence, the effects of genistein appear to be specific for the G_i/G_0 protein-coupled adenosine A_1-receptor. However, tyrphostin A47 pre-treatment did augment CCK_A receptor and thrombin receptor-mediated [^{3}H]-IP responses at maximally-effective concentrations of CCK-8 (1 μM; 113% of control) and thrombin (1 u ml^{-1}; 148% of control) respectively. Interestingly, both genistein and tyrphostin A47 increased the basal accumulation of [^{3}H]-IPs (independent of agonist activation). These data would seem to suggest that the basal level of IP turnover/PLC activation is also sensitive to the degree of tyrosine phosphorylation within the cell.

Genistein and tyrphostin A47 have been reported to cause inhibition of protein kinase C (O'Dell *et al.*, 1991). We addressed this potential problem in a series of experiments using the selective PKC activator, PMA, and the selective PKC inhibitor Ro 31-8220 (Davis *et al.*, 1989). Pre-treatment with PMA abolished both adenosine A_1 receptor and thrombin receptor-mediated [^{3}H]-IP responses. As expected, pre-treatment with Ro 31-8220, reversed the inhibition of adenosine A_1 and thrombin receptor [^{3}H]-IP responses induced by PMA. However, genistein and tyrphostin A47 pre-treatment, did not

reverse PMA-mediated inhibition of adenosine A_1 receptor or thrombin receptor-mediated [^{3}H]-IP responses. These data would seem to suggest that the potentiating effects of genistein and tyrphostin A47 are not a consequence of PKC inhibition. Interestingly, we observed no marked potentiation of the CPA-induced [^{3}H]-IP response in CHO cells pre-treated with the PKC inhibitor Ro 31-8220. In contrast, the thrombin-induced [^{3}H]-IP response was significantly potentiated by Ro 31-8220. These data suggest that PKC exerts a tonic inhibition of the thrombin-induced inositol phospholipid hydrolysis pathway (and not the adenosine A_1 receptor-mediated pathway) in CHO-A1 cells and therefore may be involved in the desensitization of the thrombin receptor following agonist stimulation.

The precise mechanism(s) underlying the desensitization of the adenosine A_1 receptor are not fully understood (Olah & Stiles, 1995). It is known, however, that adenosine A_1 receptor phosphorylation increases 3–4 fold in DDT_1MF-2 cells treated with the A_1 receptor agonist R-PIA (Ramkumar *et al*., 1991). Whether the increase in receptor phosphorylation represents phosphoserine, phosphothreonine or phosphotyrosine is not known. Furthermore, Ramkumar *et al*. (1993) have demonstrated that the bovine adenosine A_1 receptor can be phosphorylated *in vitro* by the G-protein receptor kinase-2 (GRK-2). The findings of this study (i.e. genistein and tyrphostin A47 potentiating adenosine A_1 receptor-mediated [^{3}H]-IP responses) would seem to suggest that a tyrosine kinase-dependent signalling pathway may also be involved in regulating (desensitizing) the adenosine A_1 receptor in CHO-A1 cells.

The adenosine A_1 receptor couples to the pertussis toxin-sensitive family of inhibitory G proteins (G_{i1}, G_{i2}, G_{i3} and G_0). Interestingly, there have been several papers describing the tyrosine phosphorylation of G protein α_i/α_0 subunits (Krupinsky *et al*., 1988; Hausdorff *et al*., 1992). For example, the insulin receptor was found to catalyze tyrosine phosphorylation of $G_{i\alpha}$ and $G_{o\alpha}$ (Krupinsky *et al*., 1988). More recently, Hausdorff *et al*. (1992) found that the non-receptor tyrosine kinase $pp60^{c\text{-}src}$ phosphorylated recombinant $G_{s\alpha}$, $G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$ and $G_{o\alpha}$ subunits. The functional consequence of G-protein α subunit tyrosine phosphorylation is unclear. However, in A431 human epidermoid carcinoma cells tyrosine phosphorylation of $G_{s\alpha}$ by the EGF receptor inhibited bradykinin-induced stimulation of cyclic AMP accumulation (Liebmann *et al*., 1996). These observations raise a number of interesting questions which are relevant to the present study: (i) are the potentiating effects of genistein and tyrphostin A47 on adenosine A_1 receptor-mediated [^{3}H]-IP responses related to the level of $G_{i\alpha}$ subunit tyrosine phosphorylation?; (ii) is adenosine A_1 receptor-mediated tyrosine phosphorylation of $G_{i\alpha}$ subunits occurring in CHO cells? and (iii) if so, what effect would $G_{i\alpha}$ subunit tyrosine phosphorylation have on adenosine A_1 receptor signalling? Clearly, further studies are required to address these interesting questions.

There have been several recent reports describing tyrosine phosphorylation of G protein-coupled receptors. For example, the β_2-adrenergic receptor is a substrate for the insulin receptor tyrosine kinase and in the presence of insulin is phosphorylated on tyrosine residues 350 and 354 (Baltensperger *et al*., 1996). Furthermore, in CHO cells transfected with the β_2-adrenergic receptor, insulin pre-treatment inhibits isoprenaline-stimulated cyclic AMP accumulation suggesting that tyrosine phosphorylation of the β_2-adrenergic receptor inhibits coupling to G_s (Karoor *et al*., 1996). In addition, the angiotensin II AT_1 receptor has been reported to be a substrate for the *Src* family of tyrosine kinases (Paxton *et al*., 1994). Examination of the amino acid sequence of the human adenosine A_1 receptor reveals that there are two potential tyrosine phosphorylation sites within the second intracellular loop (Tyr-106 and Tyr-116) and three within the third intracellular loop (Tyr-205, Tyr-225 and Tyr-226). Future studies using site-directed mutagenesis of these potential tyrosine phosphorylation sites should enable the possible role of tyrosine phosphorylation in adenosine A_1 receptor signalling and desensitization to be determined. Interestingly, during the preparation of this manuscript, Ciruela *et al*. (1997) reported agonist-stimulated tyrosine phosphorylation of the adenosine A_1 receptor in DDT_1MF-2 cells.

The observations described in this paper would indicate that tyrosine kinases are involved in the regulation of adenosine A_1 receptor second messenger responses. Recent studies have reported that stimulation of the MAP kinase signalling pathway by G_i/G_0 protein-coupled receptors involves *c-Src* tyrosine kinase activation (Luttrell *et al*., 1996). Indeed, numerous G_i/G_0 and G_q/G_{11} protein-coupled receptor have been shown to stimulate increases in *Src* family tyrosine kinase activity (Chen *et al*., 1994; Ishida *et al*., 1995; Dikic *et al*., 1996; Rodriguez-Fernandez & Rozengurt, 1996). Whether the adenosine A_1 receptor activates a member of the *c-Src* family of non-receptor tyrosine kinases remains to be determined. Several studies have also implicated that PI-3K is involved upstream in the activation of tyrosine kinase(s) by G_i/G_0-protein coupled receptors (Hawes *et al*., 1996; Garnovskaya *et al*., 1996). Based on these findings one would predict that the selective PI-3K inhibitor LY 294002 (Vlahos *et al*., 1994) may also potentiate adenosine A_1 receptor-mediated [^{3}H]-IP accumulation (by inhibiting tyrosine kinase activation). However, LY 294002 pre-treatment had no significant effect on adenosine A_1 receptor-mediated [^{3}H]-IP responses. The G_i/G_0 protein-coupled receptor stimulation of the MAP kinase signalling pathway (downstream of tyrosine kinase activation) is thought to involve activation of the Ras → Raf-1 → MAP kinase kinase (or MEK) → cascade (Malarkey *et al*., 1995). Hence, it is conceivable that kinases downstream of tyrosine kinase activation (i.e. Raf-1, MEK or MAP kinase) may be able to regulate the adenosine A_1 receptor. In this study we employed the MEK inhibitor PD 98059 (Dudley *et al*., 1995) to block the MAP kinase pathway downstream of Raf. However, PD 98059 had no significant effect on adenosine A_1 receptor-mediated [^{3}H]-IP accumulation. These observations suggest that MEK and MAP kinase are not involved in regulating the adenosine A_1 receptor. Finally, there have been several interesting reports describing the 'trans-activation' of EGF and PDGF receptors by G protein-coupled receptors, e.g. lysophosphatidic acid and thrombin (Daub *et al*., 1996; Luttrell *et al*., 1997). Hence, it is possible that receptor tyrosine kinases (EGF and PDGF receptors) may be involved in regulating adenosine A_1 receptor signalling.

In summary, these observations provide the first evidence for a possible role of a tyrosine kinase-dependent pathway (and hence tyrosine phosphorylation) in regulating adenosine A_1 receptor-mediated inositol phospholipid hydrolysis in CHO-A1 cells. However, further investigation is needed to determine whether the human adenosine A_1 receptor becomes tyrosine phosphorylated during agonist stimulation of the receptor.

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