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A role of protein kinase Cμ in signalling from the human adenosine A_1 receptor to the nucleus

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1 Stimulation of adenosine A_1 receptors produced a stimulation of c-fos promoter-regulated gene transcription in Chinese hamster ovary (CHO)-A1 cells expressing the human A_1 receptor. Gene transcription was monitored using a luciferase-based reporter gene (pGL3).

2 This response to the A_1 receptor agonist N^6-cyclopentyladenosine (CPA) was sensitive to inhibition by pertussis toxin, the MEK-1 inhibitor PD 98059 and by the phosphatidylinositol-3-kinase inhibitors wortmannin and LY 294002. The response was also completely abolished by the protein kinase C (PKC) inhibitor Ro-31-8220.

3 Several isoforms of PKC can be detected in CHO-A1 cells (α, δ, ε, μ, ι, ζ), but only PKCα, PKCδ and PKC were downregulated by prolonged treatment with phorbol esters. The c-fos-regulated luciferase response to A_1 agonists was not, however, inhibited by 24 h pretreatment with the phorbol ester phorbol 12,13-dibutyrate (PDBu). This observation, together with the fact that a significant attenuation (40%) of the c-fos-luciferase response to PDBu and A_1 agonist was produced by low concentrations of the PKC inhibitor Gö 6976 suggests a role for PKCμ.

4 Stimulation of CHO-A1 cells with CPA stimulated the activation of endogenous PKCμ as measured by autophosphorylation. This was rapid, occurred within 1 – 2 min, but returned to basal levels after 30 min. Furthermore, transient expression of a constitutively active form of PKCμ resulted in a significant increase in c-fos-regulated gene expression.

5 Taken together, these data suggest that PKCμ plays an important role in the ability of the adenosine A_1 receptor to signal to the nucleus.
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Keywords: PKCμ; adenosine A_1 receptors; reporter genes; c-fos; protein kinase C; MAP kinase; gene transcription

Abbreviations: AP-1, activator protein 1; CHO, Chinese hamster ovary; CPA, cyclopentyladenosine; CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; ERK, extracellular regulated kinase; MAP kinase, mitogen-activated protein kinase; PDBu, phorbol 12,13-dibutyrate; PI3 kinase, phosphatidylinositol-3-kinase; PKC, protein kinase C; SIE, sis-inducible element; SRE, serum response element; SRF, serum response factor

Introduction

Expression of the immediate early gene c-fos is rapidly induced in response to a wide range of extracellular stimuli including growth factors, cytokines, serum, G-protein-coupled receptors and phorbol esters (Karin & Smeal, 1992; Edwards, 1994; Hill & Treisman, 1995). Like the products of many other immediate early genes, c-fos acts as a transcription factor to regulate expression of other genes and is involved ultimately in the control of cell growth and proliferation. The c-fos promoter region is known to contain several regulatory elements including a serum response element (SRE), a sis-inducible element (SIE), a site for activator protein 1 (AP-1) and a cyclic AMP response element (CRE) (Hill & Treisman, 1995). Transcription factors such as cyclic AMP binding protein (CREB), fos, jun and serum response factor (SRF) activate transcription by binding to these regulatory elements (Hill & Treisman, 1995). At the SRE, a ternary complex forms between SRF and a ternary complex factor such as Elk-1 to mediate responses to growth factors and mitogens via activation of mitogen-activated protein (MAP) kinases such as the extracellular regulated kinases ERK-1 and ERK-2 (Hill & Treisman, 1995; Price *et al*., 1996).

MAP kinase pathways can be activated by a range of different G-protein-coupled receptors (Hawes *et al*., 1995; Robinson & Cobb, 1997; Selbie & Hill, 1998). The human adenosine A_1 receptor is an example of a $G_{i/o}$-coupled receptor that can stimulate the MAP kinase pathway (Dickenson *et al*., 1998). Stimulation of ERK-1/2 activity by A_1 receptor agonists is sensitive to inhibition by pertussis toxin and the phosphatidylinositol-3-kinase (PI3 kinase) inhibitors wortmannin and LY 294002 (Dickenson *et al*., 1998). These findings are consistent with the pathway proposed for $G_{i/o}$-coupled receptors, which requires G-protein $\beta\gamma$ subunits and activation of PI3 kinase leading to a Ras-dependent MAP kinase activation (Hawes *et al*., 1995; Van Biesen *et al*., 1996). In contrast, G_q-coupled receptors appear to activate the MAP kinase pathway *via* activation of protein kinase C (PKC) and a Ras-independent pathway (Hawes *et al*., 1995). In keeping with this hypothesis, we have recently reported that signalling from the $G_{q/11}$-coupled histamine H_1 receptor (Hill

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et al., 1997) to the c-fos promoter is mediated via protein kinase Cα after activation of phospholipase Cβ (Megson *et al*., 2001).

The PKC serine/threonine protein kinase family is made up of at least 12 different isoforms (Mellor & Parker, 1998). Recent studies have provided some evidence for a role of specific PKC isoforms in the activation of MAP kinases by different mitogens (Ueda *et al*., 1996; Mackenzie *et al*., 1997; Kim *et al*., 1999). Since adenosine A_1-receptor activation can lead to stimulation of phospholipase Cβ activity *via* $G_{i/o}$-$\beta\gamma$ subunits (Dickenson & Hill, 1998; Megson *et al*., 1995), we have investigated whether specific PKC isoforms are involved in signalling from the adenosine A_1 receptor to the human c-fos promoter.

Methods

Materials

pcDNA3PKC$\mu\Delta$PH was a gift from Dr F.J. Johannes (University of Stuttgart, Germany), pcDNA3 was purchased from Invitrogen. PGL3-fosluc3 and pCMV-SPAP were generous gifts from Professor P.E. Shaw (University of Nottingham, U.K.) and Dr S. Rees (GlaxoSmithKline, Stevanage, U.K.), respectively. Cell culture flasks and 24-well cluster dishes were from Costar Corning, High Wycombe, Bucks. Dulbecco's modified Eagles medium (DMEM)/nutrient mix F12 HAM, L-glutamine, foetal calf serum (FCS), forskolin, Hank's balanced salt solution, HEPES, phorbol-12-myristate-13-acetate, phorbol 12,13-dibutyrate, N^6-cyclopentyladenosine (CPA) and pertussis toxin were supplied by Sigma (Poole, Dorset). [^{3}H]-8-cyclopentyl-1,3,-dipropylxanthine (DPCPX) was obtained from NEN DuPont (Stevenage, U.K.) and adenosine 5′-[γ-^{32}P] triphosphate from Amersham Pharmacia Biotech U.K. Ltd (Little Chalfont, Bucks, U.K.). Ro-31-8220, PD 98059, Gö 6976 wortmannin, LY 294002 and Gö 6983, were purchased from Calbiochem (Nottingham, U.K.) and adenosine deaminase was obtained from Roche Diagnostics (East Sussex, U.K.). Antibodies raised against dual-phosphorylated forms of ERK1/2 (E10 monoclonal), p38 (polyclonal) and JNK (polyclonal) and antibodies recognising ERK1/2, p38 and JNK were from New England Biolabs (Herts, U.K.). Antibodies against PKC isoforms α, β, δ, ε, τ/λ and ζ were from BD Transduction Laboratories (Kentucky, U.S.A.). Antibody to PKCμ (D-20) was obtained from Santa Cruz Biotechnology (California, U.S.A.). All other chemicals were of analytical grade.

Expression of recombinant human adenosine A_1 receptors in Chinese hamster ovary cells

The pSVL plasmid containing the human adenosine A_1-receptor cDNA was obtained from ATCC. The adenosine A_1-receptor cDNA was subcloned into the *Not*I/*Apa*I sites of the eukaryotic expression vector pcDNA3. Chinese hamster ovary (CHO)-K1 cells (European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K.) were transfected with pcDNA3A_1R using transfectam (Promega) according to the manufacturer's instructions. Stably transfected CHO-K1 cells were selected using 400 μg ml^{-1} geneticin (G418, Gibco) for 2 weeks. A single clonal line was then isolated (CHO-A1) from CHO-K1 cells resistant to G418 by dilution cloning. CHO-A1 cells were grown at 37°C in a humidified air/CO_2 atmosphere (95 : 5) in 75 cm^2 flasks. For measurement of c-fos promoter activity, CHO-A1 cells were secondarily transfected with a reporter vector encoding firefly luciferase (pGL3-fosluc3) under the control of the full c-fos promoter (−711 to +1 bases; Shaw *et al*., 1989), together with a zeocin selectable vector pZeoSV (Invitrogen). Cells (CHO-A1fos) were subsequently selected with 200 μg ml^{-1} zeocin. The cells were grown in DMEM/nutrient mix F12 HAM (1 : 1) supplemented with 2 mM L-glutamine and 10% foetal calf serum. Cells for measurement of luciferase activity were grown in 24-well cluster dishes. Cells for Western blot analysis were grown in 100 mm dishes or 162 cm^2 flasks. All experiments were performed on confluent monolayers.

Measurement of A_1-receptor-stimulated luciferase activity

Confluent CHO-A1fos cell monolayers, in 24-well cluster dishes, were incubated at 37°C in a humidified air/CO_2 atmosphere (95 : 5) for 24 h in 1 ml serum-free DMEM/F12 media immediately prior to agonist administration. The medium was aspirated and replaced with 1 ml fresh serum-free DMEM/F12 medium. Agonists (10 μl) or foetal calf serum (FCS) (100 μl; total volume 1 ml) were then added and the incubation continued for 6 h. Where appropriate, antagonists were added 30 min prior to agonist administration. Luciferase activity in cell lysates was then monitored using the Promega luciferase assay system according to the manufacturer's instructions.

Imaging of luciferase expression in living CHO-A1 cells

CHO-A1fos cells were grown in 60 mm dishes and, once confluent, the medium was replaced with serum-free DMEM/F12 for 24 h. Cells were washed once with 2 ml Hanks' balanced salt solution containing 20 mM HEPES, pH 7.4 and then incubated in 4.5 ml medium containing 2 mM luciferin at 37°C on the heated stage of a Zeiss Axiovert S100TV microscope. Cells were stimulated by the addition of 500 μl medium containing CPA (0.1 μM). Cells were imaged under $\times$10 magnification and approximately 100 – 200 cells were visible in the field of view. Light emitted from the cells was detected with a Photek ICCD325 camera and recorded over a 6 h period. Images were obtained by integrating the signal obtained from each 1 h period.

Cell extracts for PKC detection following prolonged treatment with phorbol ester

CHO-A1 cells, grown to confluency in T162 flasks, were treated for a further 24 h with phorbol ester phorbol 12,13-dibutyrate (PDBu, 1 μM) or vehicle control, in DMEM/nutrient mix F12 HAM (1 : 1) supplemented with 2 mM L-glutamine. After this period, cell monolayers were washed twice with ice-cold phosphate-buffered saline (PBS: 138 mM NaCl, 2.7 mM KCl, 12.9 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4, pH 7.4) and then harvested from the culture flasks using a cell scraper. The detached cells were collected by centrifugation at 1500 $\times$ *g* for 5 min. The cell pellet was then resuspended in 500 μl ice-cold lysis buffer containing protease inhibitors (20 mM Tris-HCl, 10 mM EGTA, 1 mM EDTA,

1 mM dithiothreitol, 0.1 mM leupeptin, 0.1 mM phenylmethylsulphonylfluoride, 1 μg ml^{-1} soybean trypsin inhibitor, 1 mM benzamidine, pH 7.4). The cell suspension was then lysed by sonication on ice. Lysates were subsequently centrifuged (5 min, 300 × *g*) to remove intact cells and cell debris.

The protein content of cell lysates was determined by the method of Bradford (1976), using bovine serum albumin as a standard. Protein-matched samples (40 μg) were heated at 95°C for 5 min in SDS/PAGE sample buffer (0.05 M Tris-HCl, 20% (v v^{-1}) glycerol, 4% (w v^{-1}) sodium dodecyl sulphate, 10% 2-mercaptoethanol, trace brilliant blue dye pH 6.8), and then subjected to Western blot analysis.

Western blot analysis

Protein samples were separated by SDS/PAGE (7.5% acrylamide gel) using the Bio-Rad Mini-Protean II system. Following transfer of proteins to nitrocellulose membranes, the membranes were blocked overnight in blocking buffer (5% (w v^{-1}) low-fat dried milk in PBS/0.1% (v v^{-1}) Tween 20), at 4°C. The blots were then incubated with primary anti-PKC antibodies (Transduction Laboratories, distributed by Affiniti Research Products Ltd, Exeter, U.K.) for 2 h at room temperature in blocking buffer. The blots were washed briefly in washing buffer (PBS/0.1% (v v^{-1}) Tween 20) and were then incubated with secondary antibody (horseradish-peroxidase conjugated goat anti-mouse IgG, Fc specific, affinity isolated antibody, Sigma, Poole, Dorset), in blocking buffer, for 1 h at room temperature. The secondary antibody was removed, the blots washed twice briefly with washing buffer before developing the blots using the Enhanced Chemiluminescence detection system (Amersham). For PKCμ, the antibody (D-20) was obtained from Santa Cruz and the secondary antibody was swine anti-rabbit HRP-conjugated antibody (Dako).

MAP kinase activation

MAP kinase activation was determined by Western blotting using antiserum specific for phosphorylated forms of 42 and 44 kDa MAP kinase. CHO-A1 cells were grown to confluence in 100 mm dishes and then incubated in serum-free medium for 24 h. Cells were then incubated for 30 min at 37°C in 5 ml Hank's HEPES buffer (containing inhibitors where appropriate). Agonists (plus inhibitors where appropriate) were then added in a total volume of 5 ml. Incubations were terminated by washing the cells twice with ice-cold PBS and cell lysates were then prepared as described above for the PKC down-regulation experiments. Protein was determined by the method of Bradford (1976). Anti-phospho-P38 and anti-phospho-JNK were used to probe Western blots for P38 and JNK activation. Sorbitol (0.5 M) was used as positive control for P38 and JNK activation. Protein samples (40 μg) were separated by SDS/PAGE (10% acrylamide gels) essentially as described above with the exception that phosphatase inhibitors (2 mM sodium orthovanadate, 50 mM sodium fluoride and 1 mM β-glycerophosphate) were included in the lysis buffer.

Immunoprecipitation and in vitro *kinase activity of PKC*μ

CHO-A1fos cells were grown to confluence in 100 mm dishes and then the medium replaced with serum-free DMEM/F12 media for 24 h. Cells were washed with Hank's balanced salt solution containing 20 mM HEPES (pH 7.4) and then incubated for 30 min in 5 ml Hank's/HEPES buffer. Agonists were added in a further 5 ml Hank's/HEPES medium for the indicated time at 37°C. Whole-cell lysates were prepared from confluent 100 mm diameter dishes of CHO-A1fos cells, which had been grown in serum-free DMEM/F12 for 24 h. The cell monolayer was washed twice with 10 ml ice-cold PBS and cells were removed using a cell scraper. The cell suspension was centrifuged at 1500 × *g* for 5 min and the pellet then resuspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% v v^{-1} Nonidet P-40, 0.1% w v^{-1} SDS, 0.5% w v^{-1} sodium deoxycholate, pH 7.4) containing phosphatase inhibitors (2 mM sodium orthovanadate, 1 mM β-glycerophosphate, 50 mM sodium fluoride) and one tablet of complete protease inhibitors (EDTA-free, Boehringer Mannheim) per 10 ml and transferred to an Eppendorf tube. The cell suspension was passed rapidly through a 21G needle to promote lysis. The cell lysate was then placed on a rotator for 2 h at 4°C. Lysates were then centrifuged at 13,400 × *g* for 10 min. Protein content was determined by the method of Lowry *et al.* (1951). Samples (1 mg protein) were then made up to 1 ml in RIPA buffer containing phosphatase and protease inhibitors. Anti-PKCμ antibody (5 μg) (Santa Cruz) was added to each sample and the incubation continued for 4 h at 4°C on a rotator. PKCμ was then precipitated with protein A/Sepharose beads in Tris-buffered saline containing Tween-20 0.1% (TBS/T). After a further 2 h, samples were centrifuged (13,400 × *g*, 10 min, 4°C) and the supernatant discarded. The pellet was washed twice with 1 ml RIPA buffer, and then twice with TBS/T. Finally, the pellet was washed with 1 ml ice-cold kinase assay buffer (50 mM Tris HCl, 10 mM $MgCl_2$ and 2 mM dithiothreitol, pH 7.4) and 30 μl kinase buffer added to each protein A pellet. [γ-^{32}P]-ATP (2 μCi) was then added to each sample in 20 μl kinase assay buffer and incubated at 30°C for 10 min. Samples were then centrifuged at 10,500 × *g* for 2 min. The supernatant was removed and 20 μl 2 × sample treatment buffer (20% v v^{-1} glycerol, 4% w v^{-1} SDS, 0.05 M Tris HCl, 10% v v^{-1} β-mercaptoethanol, trace brilliant blue dye, pH 6.8) added to each sample and heated at 95°C for 1 min. Samples were then centrifuged at 10,500 × *g* for 2 min and the supernatant subjected to SDS/PAGE on 10% polyacrylamide gels. Proteins were subsequently transferred to nitrocellulose and γ-^{32}P detected by autoradiography and exposure to film at −70°C.

Transient transfection of a constitutively active PKCμ

The cDNA for a constitutively active form of PKCμ (pcDNA3-PKC$\mu\Delta$PH) which has a deletion of the entire PH domain of PKCμ (K417-G553; Hausser *et al.*, 2001) was transiently transfected into CHO-A1 cells using lipofectamine. CHO-A1 cells (in 75 cm^2 flasks) were cotransfected with pcDNA3-PKC$\mu\Delta$PH or control vector (pcDNA3) (4 μg), together with pGL3-fosluc3 (1 μg) and pCMV-SPAP (1 μg). At 18 h after transfection, cells were passaged and used to seed 24-well plates (for luciferase assays) or 75 cm^2 flasks (for Western blotting). Once confluent, cells were serum-starved for 24 h prior to performing luciferase assay as described above. At the end of the serum-free period, 500 μl of medium was also removed from each well and assayed for the presence of human secreted placental alkaline phosphatase (SPAP) to

control for transfection efficiency. SPAP was measured as described previously (Selbie *et al*., 1997).

^{3}H-DPCPX binding to CHO-A1fos membranes

CHO-A1fos cells from two confluent 162 cm^2 flasks were detached using Dulbecco's PBS solution containing 5 mM EDTA at 37°C for 5 min. After centrifugation (150 × *g* for 5 min), membranes were prepared by resuspending the cells in 10 ml of ice-cold Tris-EDTA buffer (50 mM; 1 mM; pH 7.4) followed by homogenisation using a glass homogeniser (20 strokes) and centrifugation at 20,000 × *g* for 15 min. The resulting pellet was resuspended in 600 μl of Tris-EDTA buffer and kept on ice. CHO-A1fos cell membranes (10 μl) were incubated with increasing concentrations of [^{3}H]-DPCPX in Tris-EDTA buffer containing adenosine deaminase (1 U ml^{-1}) and Triton X-100 (0.01%) in a total volume of 200 μl. Nonspecific binding was determined in the presence of 5 mM theophylline. After 90 min at room temperature, the incubation was stopped by rapid filtration through Whatman GF/B filters (presoaked in 0.3% polyethylenimine) using a Brandel MR24 cell harvester. Filters were washed three times with 10 ml of ice-cold Tris-EDTA buffer and the tritium content determined by liquid scintillation counting.

[^{3}H]DPCPX binding in intact cells

CHO-A1fos cells were seeded into 24-well plates and once confluent transferred to serum-free DMEM/F12 medium containing 2 mM glutamine for 24 h. The medium was then removed and replaced with 250 μl per well of Hank's balanced salt solution containing 0.1% w v^{-1} bovine serum albumin, 2 U ml^{-1} adenosine deaminase, 0.001% v v^{-1} Triton X-100, 20 mM HEPES (pH 7.4). Cells were then incubated for 30 min at 37°C in the presence or absence of PKC inhibitors before addition of [^{3}H]DPCPX (in 50 μl medium) to give a final concentration of 3 nM. Nonspecific binding was determined with 10 μM xanthine amine congener. The incubation was then continued for a further 60 min. The medium was then removed and each well washed twice with 1 ml ice-cold Hank's balanced salt solution. NaOH (250 μl, 0.5 M) was then added to each well and the plate heated for 15 min at 60°C. HCl (50 μl, 1 M) was then added to each well and the entire contents transferred to a scintillation vial. Tritium content was then determined by liquid scintillation counting.

Data analysis

Agonist concentration – response curves were fitted to a logistic equation using the nonlinear regression programme Prism (GraphPad Software, San Diego, CA, U.S.A.). The equation fitted was

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

where E_{MAX} is the maximal agonist response, A is the agonist concentration and n is the Hill coefficient.

Results

Adenosine A_1-receptor-stimulated gene expression

Specific binding of [^{3}H]DPCPX to CHO-A1 cell membranes yielded values of 277 ± 68 fmol mg^{-1} protein and 3.5 ± 0.7 nM (n = 6) for the B_{MAX} and K_D. CHO-A1 cells, expressing a luciferase reporter gene under the transcriptional control of the human c-fos promoter, responded to FCS (5.86 ± 0.24-fold over basal levels, n = 23), CPA (2.14 ± 0.08-fold, n = 17) and PDBu (5.84 ± 0.60-fold, n = 7) (Figure 1a). Responses to agonists (ATP and CCK) of endogenously expressed $G_{q/11}$-coupled P_{2Y2} and CCK receptors were much smaller (Figure 1a). Significant (P < 0.05; two-way ANOVA) induction of the c-fos-regulated luciferase reporter was detectable at 1 h in response to CPA (10 μM) and was maximal between 4 and 6 h (Figure 1b; n = 3). This time course is also apparent in real-time imaging studies of CPA (1 μM)-stimulated luciferase expression in CHO-A1 cells incubated with 2 mM luciferin (Figure 2). In contrast, levels of luciferase expression remained unchanged in untreated cells for up to 6 h (Figures 1b and 2).

CPA produced a concentration-dependent increase in luciferase expression ($\log EC_{50}$ – 7.62 ± 0.07, n = 19) which was shifted to higher agonist concentrations by the competitive adenosine A_1-receptor antagonist dipropylcyclopentylxanthine (DPCPX; apparent K_D = 2.7 ± 0.7 nM, n = 3; Figure 3a; $\log K_D$ = −8.6 ± 0.1). This response to CPA was completely

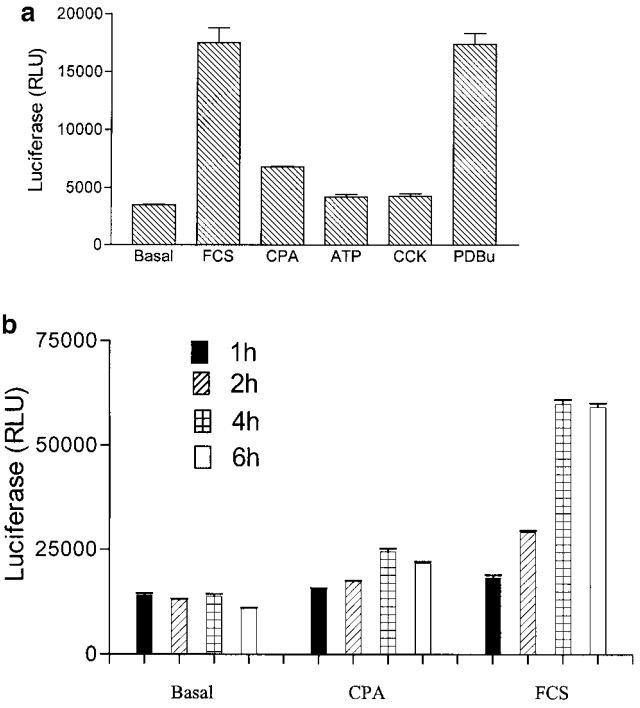

Figure 1 Influence of CPA and FCS on human c-fos promoter-regulated luciferase expression in CHO-K1 cells expressing the human adenosine A_1-receptor. After 24 h in serum-free medium, agonist incubation was for (a) 6 h or (b) 1 – 6 h. (a) Responses to CPA (10 μM), FCS (10%), ATP (100 μM), cholecystokinin (CCK; 1 μM) and PDBu (1 μM). (b) Responses to 10% FCS and 10 μM CPA. Data represent mean ± s.e.m. of triplicate determinations in a representative experiment. Luciferase activity is expressed as relative light units (RLU). Similar data were obtained in four separate experiments.

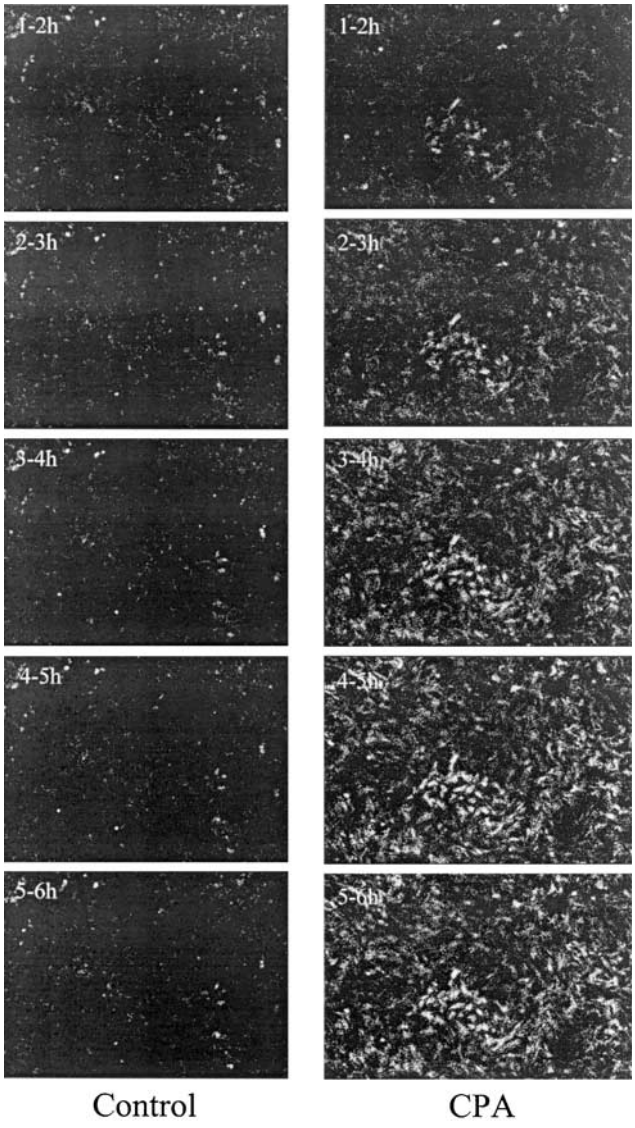

Figure 2 Real-time imaging of CPA-induced luciferase expression in CHO-A1 cells. CHO-A1 cells were grown in serum-free DMEM-F12 for 24 h prior to assay. Cells were then treated with CPA (1 μM) and luciferase expression was monitored over a 6 h period in the presence of 2 mM luciferin. Light emitted from the cells was integrated over sequential 1 h periods after the start of the assay. Images are at $\times 10$ magnification. Similar results were obtained in two other separate experiments.

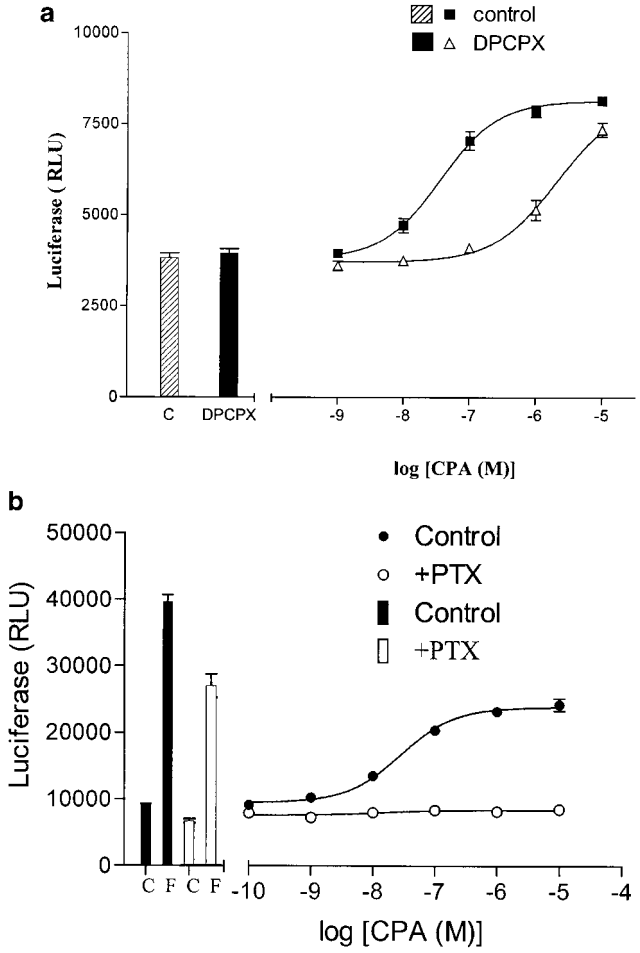

Figure 3 Inhibition of adenosine-stimulated c-fos luciferase expression in CHO-A1 cells by (a) the selective adenosine A_1-receptor antagonist DPCPX or (b) pertussis toxin (PTX). Cells were serum-starved for 24 h before the addition of agonists as described under Methods. (a) Cells were incubated for 30 min in the presence or absence of DPCPX (100 nM) before stimulation with CPA for 5.5 h. In (b) this serum-free period was in the presence of 100 ng ml^{-1} pertussis toxin and the agonist incubation period was for 6 h. Cells were then lysed and luciferase activity measured. RLU = relative light units. Values represent mean $\pm$ s.e.m. of triplicate determinations in a single representative experiment. Bars show the basal (C) responses obtained in the absence or presence of 100 nM DPCPX and the responses to 10% FCS (F) in control cells and those that have been treated with PTX. Similar results were obtained from two other experiments.

attenuated by 24 h pretreatment with pertussis toxin (100 ng ml^{-1}; $n=6$; Figure 3b) indicating an involvement of $G_{i/o}$-proteins. The c-fos luciferase response to CPA (10 μM) was also attenuated by the PI3-kinase inhibitors (PI3K) wortmannin (by 83.9 $\pm$ 3.1%, $n=3$, $P<0.01$; Figure 4a) and LY 294002 (by 74.9 $\pm$ 8.7%, $n=4$, $P<0.01$; Figure 4b). These are two structurally unrelated compounds that inhibit PI3K activity by different mechanisms (Yano *et al*., 1993; Vlahos *et al*., 1994; Wymann *et al*., 1996).

Role of ERK-1/2 in A_1 receptor-stimulated gene expression

The sensitivity of the A_1-receptor-mediated gene transcription response to inhibitors of PI3 kinase is consistent with a role for ERK-1/2 in signalling to the c-fos promoter. In keeping with this hypothesis, the MEK-1 inhibitor PD 098059 (50 μM; Dudley *et al*., 1995) almost completely abolished the luciferase response to 10 μM CPA (90.5 $\pm$ 3.9% inhibition, $P<0.01$; $n=3$; Figure 5a). Stimulation of CHO-A1 cells with CPA (1 μM) increased the levels of phosphorylated ERK-1 and ERK-2, with maximal levels of phosphorylation occuring between 2 and 5 min of agonist stimulation (Figure 5b; $n=3$). This response to CPA was concentration-dependent with phosphorylation of ERK-1 and ERK-2 being detected at 10 nM CPA, and maximal levels of A_1-receptor-stimulated ERK phosphorylation occuring at 100 nM CPA (Figure 5c). These data are similar to those reported by us previously in a different A_1-receptor-expressing line (Dickenson *et al*., 1998). In contrast, CPA (1 μM; 2 min – 6 h) had no effect on the

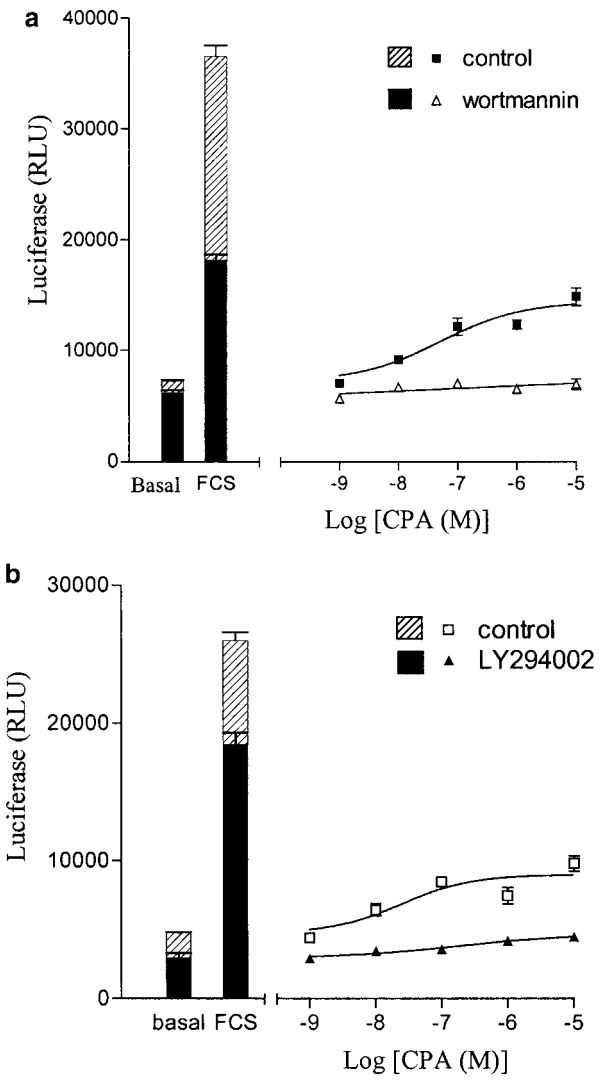

Figure 4 Effect of the PI-3 kinase inhibitors (a) wortmannin and (b) LY294002 on CPA-mediated luciferase response in CHO-A1fos cells. Quiescent CHO-A1fos cells were pretreated with (a) wortmannin (100 nM) or vehicle control (0.1% dimethyl sulphoxide) or (b) LY294002 (30 μM) or vehicle control (0.1% dimethyl sulphoxide) for 30 min before stimulating with CPA or 10% FCS for 5.5 h. Cells were then lysed and luciferase activity measured. RLU = relative light units. Bars show the basal response and that obtained to 10% FCS in the presence or absence of inhibitors. The results shown are a representative experiment. Values represent mean ± s.e.m. of triplicate determinations. Similar results were obtained from two other experiments.

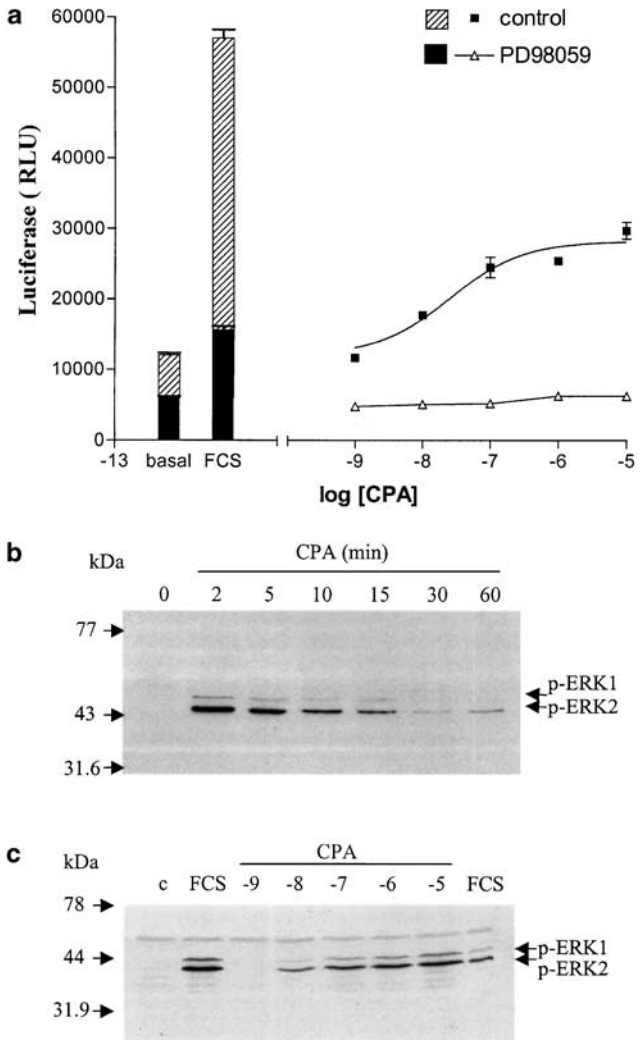

Figure 5 Role of ERK-1/2 in the c-fos promoter-regulated luciferase response to CPA. (a) Effect of the MEK1/2 inhibitor PD98059 on CPA-mediated luciferase response in CHO-A1fos cells. Quiescent CHO-A1fos cells were pretreated with PD98059 (50 μM) or vehicle control (0.1% dimethyl sulphoxide) for 30 min before stimulating with CPA or 10% FCS for 5.5 h. Cells were then lysed and luciferase activity measured. RLU = relative light units. The results shown are a representative experiment. Values represent mean ± s.e.m. of triplicate determinations. Similar results were obtained from two other experiments. (b) Western blot analysis of ERK1/2 phosphorylation in CHO-A1 cells treated with CPA (0 – 60 min). Quiescent CHO-A1 cells were treated with CPA 1 μM, for the indicated time. Whole-cell lysates were resolved by SDS – PAGE (10%) and transferred to nitrocellulose. Membranes were then probed with antisera specific for the phosphorylated ERK1/2. Similar results were obtained from two other independent experiments. (c) Effect of CPA concentration on ERK1/2 phosphorylation. Quiescent CHO-A1 cells were treated with different concentrations of CPA, 10% FCS or vehicle control (c), for 5 min. Whole-cell lysates were resolved by SDS – PAGE (10%) and transferred to nitrocellulose and membranes then probed with antisera specific for the phosphorylated ERK1/2.

phosphorylation of p38 or JNK isoforms in CHO-A1 cells ($n = 3$; data not shown; 0.5 M sorbitol was used as positive control).

Role for PKCμ in adenosine A_1-receptor-mediated c-fos promoter activation

The involvement of PKC isoforms in the response to CPA was investigated initially using the PKC inhibitor Ro-31-8220, which is active against classical, novel and atypical isoforms of PKC (Wilkinson *et al*., 1993; Standaert *et al*., 1997). Ro-31-8220 (10 μM) produced a complete inhibition of CPA (10 μM) stimulated luciferase expression (96.4 ± 3.2% inhibition; $P < 0.01$, $n = 3$; Figures 6 and 8a). The PKC activator PDBu was also able to stimulate the c-fos promoter (Figures 1 and 6), and this effect was also abolished by Ro-31-8220 (Figures 6 and 8a). Expression of the classical PKC isoform α, the novel

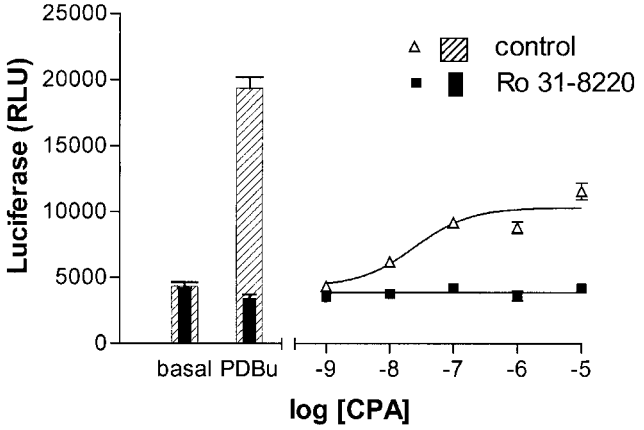

Figure 6 Influence of the PKC inhibitor Ro-31-8220 on adenosine A_1-receptor-stimulated c-fos-luciferase activity. Cells were serum starved for 24 h, the media were then replaced with fresh-serum-free media containing 10 μM Ro-31-8220 or vehicle control and the incubation continued for 30 min before agonist addition (in 10 μl medium) for a further 5.5 h. Values represent mean $\pm$ s.e.m. of triplicate determinations in a single experiment. Similar data were obtained in two further experiments.

isoforms δ and ε, the atypical isoforms i/λ and ζ and PKC μ were detected in CHO-A1 cells by Western blotting of whole-cell lysates with isoform-specific antibodies (Figure 7a). PKCβ and PKCθ were not detectable in these cells. Treatment of CHO-A1 cells with PDBu for 24 h (1 μM; $n=3$) completely downregulated PKCα, PKCδ and PKCε (Figure 7a). In contrast, levels of the other PKC isoforms were unaffected by this treatment (Figure 7a). Pretreatment of CHO-A1 cells with PDBu (1 μM, 24 h, $n=3$) abolished the c-fos luciferase response to PDBu (1 μM), but was without significant effect on the response to CPA (Figure 7b). These data suggest that the A_1 receptor does not activate the c-fos promoter via PKCα, PKCδ or PKCε.

To investigate further the involvement of PKC isoforms in the response to CPA, two compounds Gö 6983 and Gö 6976 that can discriminate between different PKC isoforms were investigated (Martiny-Baron *et al*., 1993; Gschwendt *et al*., 1996). Gö 6983 inhibits the activity of recombinant PKCα, PKCβ, PKCγ, PKCδ and PKCζ with IC_{50} values of 7 – 60 nM, but requires concentration above 10 μM to begin to inhibit PKCμ (Gschwendt *et al*., 1996). In contrast, Gö 6976 is a potent inhibitor of PKCα, PKCβ and PKCμ (also known as PKD) (Martiny-Baron *et al*., 1993; Gschwendt *et al*., 1996). Gö 6983 (10 μM) was able to completely inhibit the luciferase response to PDBu, but was much less effective as an inhibitor of CPA-stimulated responses (Figure 8b). Concentrations of Gö 6983 greater than 100 nM were required to achieve any significant inhibition of the CPA response, whereas the response to PDBu was inhibited by 30.7 $\pm$ 7.2% ($n=4$) by 100 nM Gö 6983 (Figure 8b). In contrast, 100 nM Gö 6976 inhibited luciferase expression in response to stimulation with PDBu and CPA by 30.1 $\pm$ 3.5 and 38.1 $\pm$ 3.4%, respectively ($n=5$; Figure 8c). The maximal level of inhibition achieved with this inhibitor (10 μM Gö 6976) was, however, only *circa* 50% the response to each agonist (47.9 $\pm$ 6.0% PDBu; 52.5 $\pm$ 9.3% CPA; $n=5$; Figure 8c). None of the inhibitors studied had a marked effect on the binding of [^{3}H]DPCPX to intact CHO-A1 cells (Figure 9). These data, taken together with the results from the PDBu-induced downregulation studies, point to a role for PKCμ in the luciferase response to CPA.

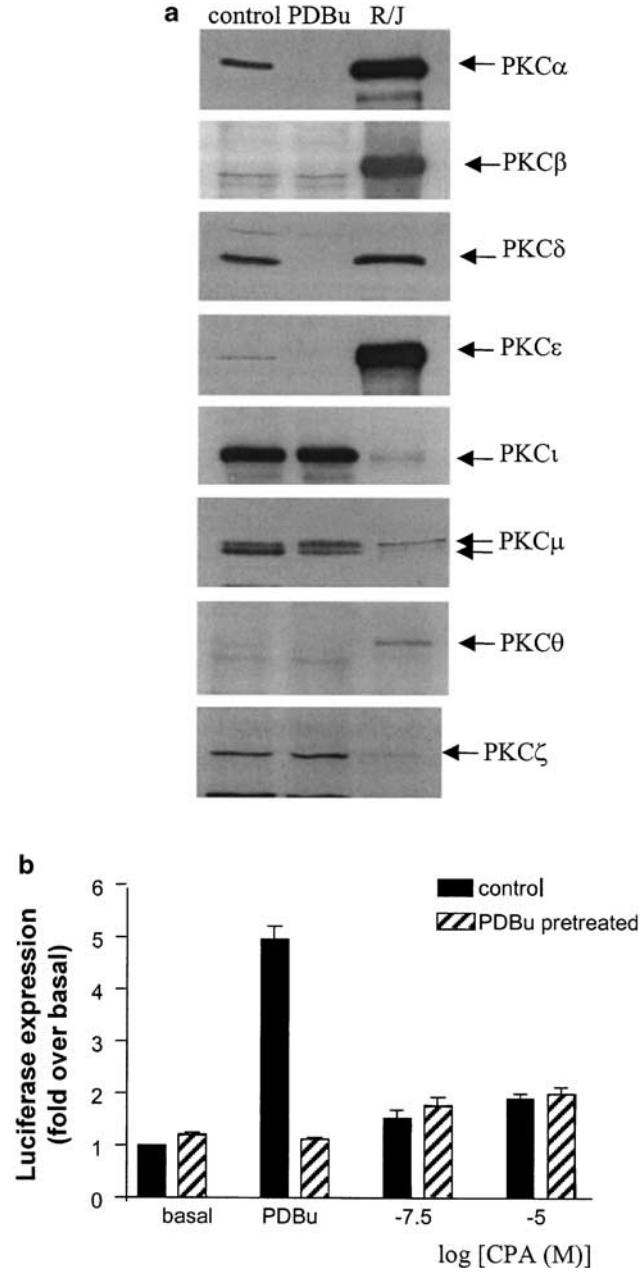

Figure 7 PKC isoforms in CHO-A1 cells. (a) Downregulation of specific isoforms by pretreatment with PDBu. Cells were preincubated for 24 h with 1 μM PDBu or vehicle and then whole-cell lysates prepared. Isoforms were detected by Western blotting with isoform-specific antibodies. Commercially prepared lysates were used as positive controls to confirm isoform identity. Lysate prepared from Jurkat cells (J) was used as positive controls for PKCθ and PKCμ. Lysate prepared from rat brain (R) was used as positive controls for the remaining PKC isoforms. Data shown are from a representative experiment. Similar results were obtained in two other independent experiments. (b) Effect of 24 h PDBu pretreatment on CPA- and PDBu-mediated c-fos-promoter-driven luciferase expression. Confluent CHO-A1 cells were treated with PDBu (1 μM) in serum-free DMEM/F12 medium for 24 h. Cells were then treated with CPA, PDBu (1 μM) or vehicle control for 5.5 h. Values represent the mean $\pm$ s.e.m. of three independent experiments each measured in triplicate.

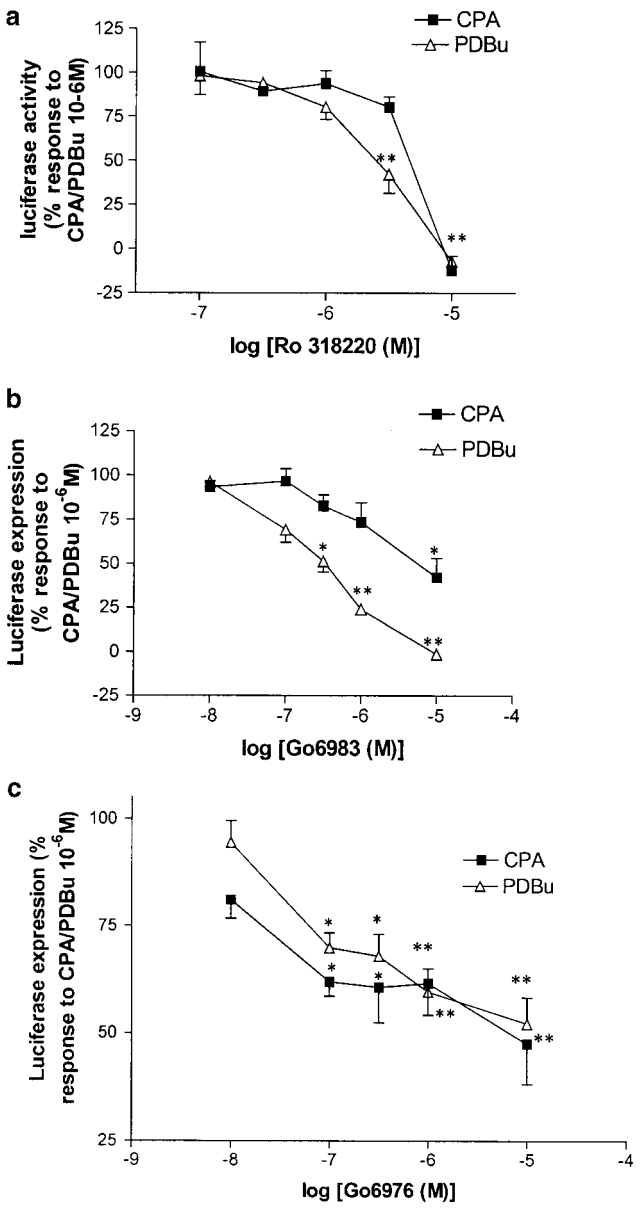

Figure 8 Influence of the PKC inhibitors (a) Ro-31-8220, (b) Go 6983 and (c) Go 6976 on c-fos-promoter-regulated luciferase expression in CHO-A1 cells in response to CPA (1 μM) or PDBu (1 μM). Cells were pretreated for 30 min with indicated concentrations of PKC inhibitor and then stimulated with either CPA (1 μM) or PDBu (1 μM) for 5.5 h. Cells were then lysed and assayed for luciferase expression. Data are expressed as a percentage of the control response to CPA or PDBu in the absence of inhibitor. Values represent the mean $\pm$ s.e.m. of three (a), four (b) or five (c) independent experiments, each measured in triplicate. $^{*}P<0.05$, $^{**}P<0.01$, for cells treated with PKC inhibitor compared to untreated cells.

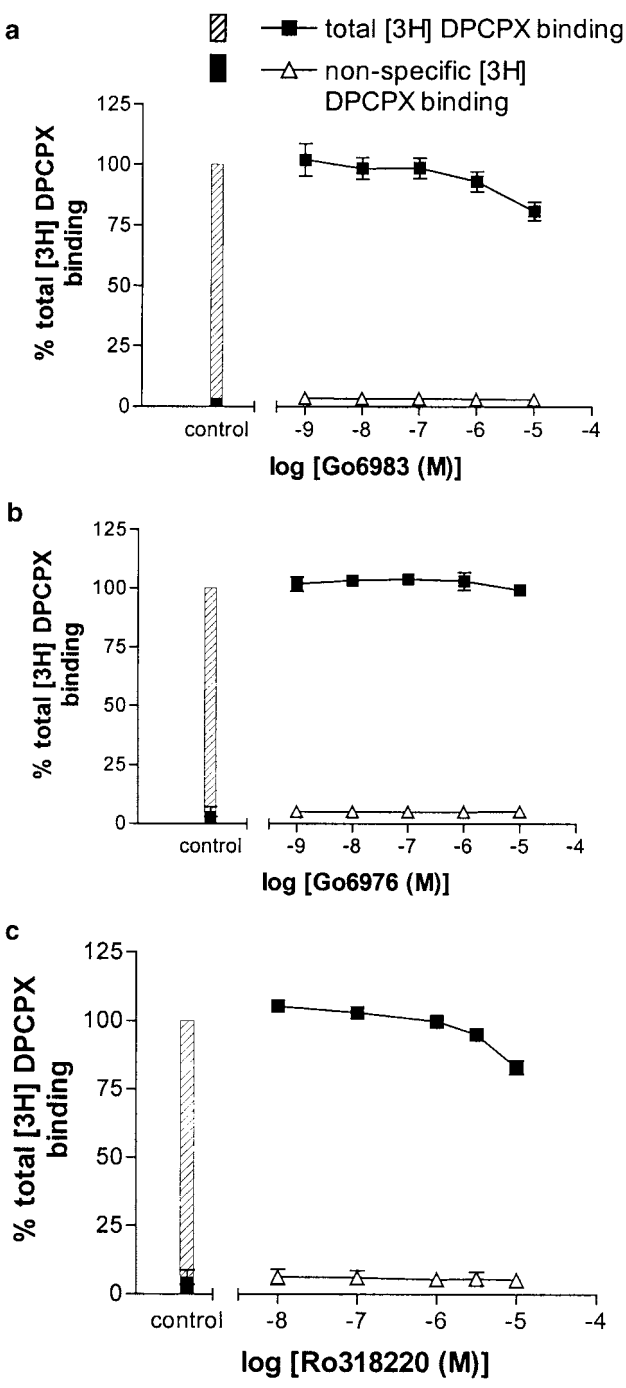

Figure 9 Effect of (a) Gö 6983, (b) Gö 6976 and (c) Ro-31-8220 on [^{3}H]DPCPX binding in CHO-A1 cells. Quiescent CHO-A1fos cells were incubated with the indicated concentrations of PKC inhibitor, 3 nM [^{3}H]DPCPX and where indicated 10 μM XAC (to define nonspecific binding) for 1 h, 37°C. Cells were then lysed by heating with alkali and bound [^{3}H]DPCPX determined. Data were normalised to [^{3}H]DPCPX binding in the absence of 10 μM XAC. Values represent the mean $\pm$ s.e.m. of three independent experiments, each measured in duplicate.

In vitro kinase assays showed that treatment of CHO-A1 cells with PDBu (1 μM, 10 min) stimulated the activation of endogenous PKCμ as measured by autophosphorylation ($n=7$; Figure 10). Stimulation of CHO-A1 cells with CPA (1 μM; $n=4$) also resulted in activation of PKCμ (Figure 10). This was rapid, occurred within 1 – 2 min of CPA addition, but returned towards basal levels after approximately 30 min (Figure 10a, b). Transient coexpression of a constitutively active form of PKCμ (in the vector pcDNA3) together with the pGL3fosluc3 reporter vector into CHO-A1 cells (Figure 11) resulted in a significant increase in c-fos-regulated luciferase expression (1.9 $\pm$ 0.3-fold over basal levels; $n=4$; data corrected for transfection efficiency), when compared to the data

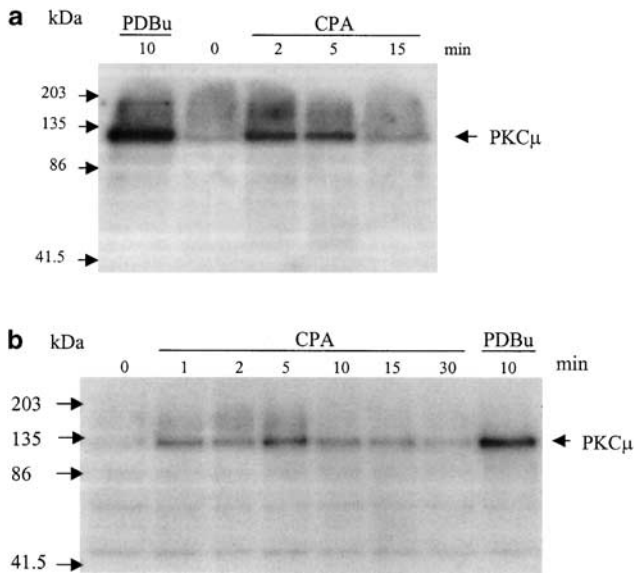

Figure 10 Time course of endogenous PKCμ phosphorylation following adenosine-A_1 receptor activation in CHO-A1 cells. Quiescent cells were treated with CPA (1 μM) or PDBu (1 μM) for the indicated times (a, b) and then lysed. Lysates were immunoprecipitated with antisera specific for PKCμ, and PKCμ phosphorylation determined by an *in vitro* kinase assay followed by SDS–PAGE separation (10% gel), transfer to nitrocellulose and autoradiography. Equal loading was confirmed by reprobing with antisera specific for PKCμ and detection by a colorimetric-based HRP method (not shown).

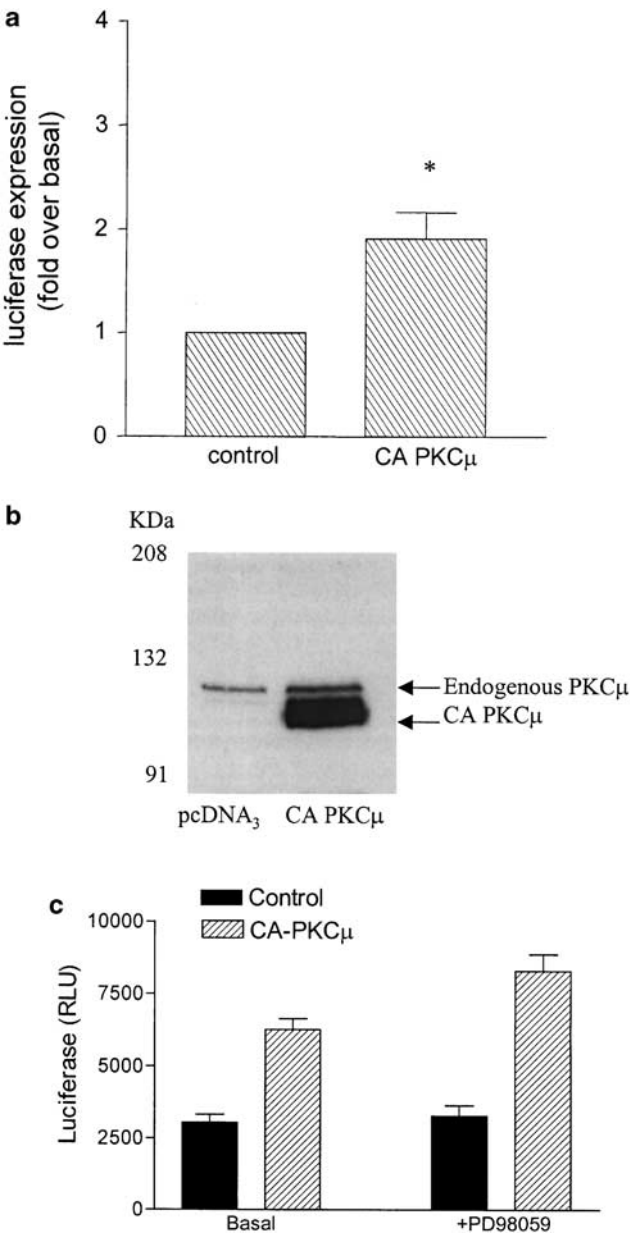

Figure 11 Effect of constitutively active PKCμ (CA PKCμ) on the c-fos promoter-regulated expression of luciferase in CHO-A1 cells. CHO-A1 cells were transiently cotransfected with pGL3fosluc3, pCMV-SPAP and $pcDNA_3$ (control) or $pcDNA_3$PKC$\mu\Delta$PH. At 48 h post-transfection, cells were grown in serum-free DMEM/F12 for 24 h prior assay. (a) Luciferase expression ($n=4$). Data are expressed relative to basal levels following correction for transfection efficiency using the secretion of SPAP. *$P<0.001$; two-way ANOVA. (b) Representative Western blot showing expression of endogenous PKCμ and constitutively active PKCμ which runs as a smaller band because of the deletion of the plekstrin homology domain. Whole-cell lysates were separated by SDS–PAGE (7.5%) and transferred to nitrocellulose. The blot was then probed with antisera specific for PKCμ. Similar results were obtained from three other experiments. (c) Effect of PD 98059 (50 μM) on the c-fos luciferase response to constitutively active PKCμ. Transient transfection was performed as described in (a). Data are from a single experiment. Similar results were obtained in two further experiments.

obtained with the equivalent amount of the control pcDNA3 vector (Figure 11). Interestingly, the effect of the constitutively active form of PKCμ on c-fos-regulated gene expression was not attenuated by the MEK-1 inhibitor PD 98059 (50 μM; Figure 11c). Expression of constitutively active PKCμ did not, however, stimulate phosphorylation of ERK-1 or ERK-2 (Figure 12).

Discussion

Distinct pathways have been shown to mediate the effects of G_i- and G_q-coupled receptors on MAP kinase activation (Hawes *et al*., 1995). These studies have shown that G_i-coupled receptors stimulate the MAP kinase pathway *via* G$\beta\gamma$-subunits and Ras, while G_q-coupled receptors stimulate MAP kinase and cell proliferation *via* PKC and activation of Raf-1 (Hawes *et al*., 1995). In keeping with this hypothesis, we have recently shown that G_q-coupled histamine H_1 receptors signal to the c-fos promoter via the activation of PKCα (Megson *et al*., 2001). We have also shown that the $G_{i/o}$-coupled adenosine A_1 receptor can activate ERK-1/2 in a manner which is insensitive to the PKC inhibitor Ro 31-8220 (Dickenson *et al*., 1998). In the present study, we have provided evidence that stimulation of c-fos promoter activity by the human adenosine A_1 receptor is mediated by pertussis toxin-sensitive $G_{i/o}$-proteins and can be almost completely attenuated by inhibitors of MEK-1 and PI3K, which both appear to act upstream of ERK-1/2 (Dickenson *et al*., 1998). Thus, we have previously shown that ERK-1/2 activity is inhibited by 80–90% by inhibitors of PI3K and 89% by the MEK-1 inhibitor PD 98059 (Dickenson *et al*., 1998). ERK-1/2 are the predominant MAP kinases activated by adenosine A_1-receptor stimulation in CHO-A1 cells, since we were unable to detect activation of either P38 or JNK by the A_1-agonist CPA. However, surprisingly, the c-fos

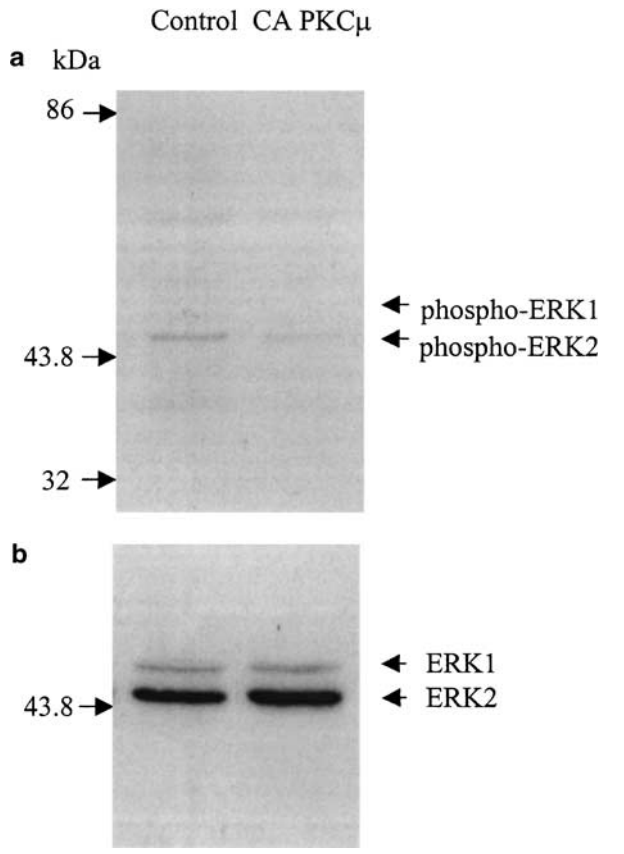

Figure 12 Effect on ERK-1/2 phosphorylation of the overexpression of constitutively active PKCμ (PKC$\mu\Delta$PH) in CHO-A1 cells. CHO-A1 cells were transiently cotransfected with pGL3fosluc3, pCMV-SPAP and pcDNA$_3$ (control) or pcDNA$_3$PKC$\mu\Delta$PH (ΔPH). At 48 h post-transfection, cells were transferred to serum-free DMEM/F12 for 24 h prior. Whole-cell lysates from these quiescent cells were then prepared and separated by SDS – PAGE on 10% gel. Blots were probed with (a) anti-phospho ERK1/2 antibody or (b) anti-ERK1/2 antibody. Results from a single experiment are shown. Similar results were obtained in one other experiment.

promoter-regulated luciferase response to adenosine A_1-receptor stimulation could be completely inhibited by the PKC inhibitor Ro-31-8220.

The c-fos response to adenosine A_1-receptor activation was also sensitive to inhibition by two other PKC inhibitors. These are compounds (Gö 6983 and Gö 6976) that can discriminate between different isoforms of PKC (Johannes *et al*., 1994; Newton, 1995; Mellor & Parker, 1998). Thus, Gö 6983 is a potent inhibitor of PKCα, PKCβ, PKCγ, PKCδ and PKCζ, while Gö 6976 inhibits PKCα, PKCβ and PKCμ (Martiny-Baron *et al*., 1993; Gschwendt *et al*., 1996; Way *et al*., 2000). The partial sensitivity (*circa* 40%) of the responses to both PDBu and CPA to low concentrations of Gö 6976 suggests an involvement of PKCα, PKCβ or PKCμ. However, PKCβ was not detectable in CHO-A1 cell lysates and an involvement of PKCα in the response to adenosine A_1-receptor stimulation is unlikely since 24 h pretreatment with PDBu downregulated PKCα, PKCδ, and PKCε, but had no effect on the CPA-induced luciferase response. The inhibitory effect of low concentrations of Gö 6976, however, would tend to rule out major roles for PKCδ, and PKCε. Taken together, these data suggest that *circa* 40% of the c-fos luciferase response to CPA is mediated by PKCμ.

The partial inhibition of the c-fos response to the phorbol ester PDBu by Gö 6976 could also be because of the inhibition of PKCμ. However, since the c-fos promoter-regulated luciferase response to PDBu can be completely attenuated by downregulation of PKCα, PKCδ and PKCε, it is likely that any role for PKCμ in the response to PDBu is secondary to activation of one of these three other PKC isoforms. In keeping with this hypothesis, it has been suggested that PKCμ activation and phosphorylation can be downstream of other PKC isoforms, particularly novel PKC isoforms such as PKCε (Zugaza *et al*., 1996). Thus, if the activation of PKCμ by PDBu is indirect via other PKC isoforms that are susceptible to downregulation by PDBu treatment (e.g. PKCα, PKCδ and PKCε), then such treatment would inhibit subsequent responses mediated via PKCμ.

Several studies have demonstrated that phorbol esters can activate the c-fos promoter or one of the transcriptional elements contained within it (Whitmarsh *et al*., 1995; Arai & Escobedo, 1996; Megson *et al*., 2001). Soh *et al*. (1999) have also demonstrated that PKCα and PKCε mediate phorbol ester-induced activation of the c-fos SRE via activation of raf/MEK1/ERK/Elk-1 pathway. However, if PKCα was the primary isoform responsible for PDBu-induced luciferase activity, then Gö 6976 should have completely inhibited the response. The fact that there is a distinct plateau (at *circa* 40% inhibition) in the concentration – effect curve for the inhibition produced by Gö 6976, strongly suggests that a novel PKC isoform (PKCδ or PKCε) in addition to PKCμ or PKCα is involved in the response to PDBu itself.

Comparison of the concentration – effect curve obtained for Gö 6983 suggests that the response to CPA is much less sensitive to this inhibitor than that for PDBu, and significant inhibition of the CPA response is only observed at the highest concentrations used. It is therefore likely that PKCμ is the only PKC isoform involved in the response to A_1-receptor stimulation. The inhibitory effect of the higher concentrations of Gö 6983 on the CPA response would be compatible with its weaker ability to inhibit PKCμ. Binding studies with ^{3}H-DPCPX confirmed that Gö 6976 had no significant effect at the level of the A_1 receptor, although a small inhibition was observed at the highest concentrations of Gö 6983 and Ro-31-8220 used, which might contribute to a small extent to their inhibitory effect on luciferase responses. Ro-31-8220 has also been reported to inhibit the activity of rsk2 and p70S6K *in vitro* at similar concentrations to those found to inhibit PKC (Alessi, 1997). These effects may also contribute to the complete attenuation by Ro-31-8220 of the CPA-induced signal to the c-fos promoter at the highest concentration used in the present study (10 μM).

Adenosine A_1-receptor stimulation and PDBu were both able to increase PKCμ activation in CHO-A1 cells. The response to CPA was rapid and transient, producing a peak response between 2 and 5 min after agonist addition. This was very similar to the time course for stimulation of MAP kinase phosphorylation in the same cells. Similar kinetics of PKCμ autophosphorylation have been observed following activation of other G-protein-coupled receptors (Zugaza *et al*., 1997; Paolucci *et al*., 2000). In keeping with a role for PKCμ in the stimulation of c-fos-regulated luciferase expression by both CPA and PDBu, a constitutively active mutant of PKCμ

(PKC$\mu\Delta$PH) was able to stimulate luciferase expression. PKCμ has previously been reported to activate the SRE within the thymidine kinase promoter (Hausser *et al*., 2001). The c-fos promoter contains a similar SRE transcriptional element; so this is the likely target of the PKCμ pathway. Hausser *et al*. (2001) found that raf-1 and ERK1/2 were located downstream of PKCμ in pathways to activate the SRE in HEK 293 cells. However, in the present study, constitutively active PKCμ did not alter the phosphorylation of ERK-1 or ERK-2 in CHO-A1 cells. Given the similarities in the time course CPA-stimulated ERK1/2 and PKCμ phosphorylation, and the insensitivity of the ERK response to Ro-31-8820 (Dickenson *et al*., 1998), it is likely that PKCμ is acting downstream of ERK1/2. Consistent with this hypothesis, the effect of constitutively active PKCμ on c-fos-regulated luciferase expression was not attenuated by the MEK-1 inhibitor PD 98059. It remains to be established, however, whether the PKCμ pathway represents a separate and parallel pathway to the normal $G_{i/o}$-coupled receptor pathway involving G$\beta\gamma$ subunits and the ras/raf-1/MEK-1/ERK/Elk-1 system or a point of divergence immediately downstream of ERK1/2. The complete sensitivity to the MEK-1 inhibitor PD 98059 of the c-fos luciferase response produced by adenosine A_1-receptor stimulation, however, suggests a divergence of signalling downstream of ERK1/2.

In summary, these studies have shown that PKCμ plays a role in the ability of the human adenosine A_1 receptor to signal to the nucleus. The data obtained are consistent with a pathway involving PI3 kinase, MEK-1 and ERK1/2 leading to stimulation of the c-fos-promoter SRE after activation of PKCμ. The fact that only a component (*circa* 50%) of the adenosine A-receptor response is sensitive to the PKCμ inhibitor Gö 6976 suggests that there may be a divergence of the signalling cascade to the c-fos promoter downstream of ERK1/2.

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