Characterization of ERK1/2 signalling pathways induced by adenosine receptor subtypes in newborn rat cardiomyocytes

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1 Adenosine A_1, A_{2A}, and A_3 receptors (ARs) and extracellular signal-regulated kinase 1/2 (ERK1/2) play a major role in myocardium protection from ischaemic injury. In this study, we have characterized the adenosine receptor subtypes involved in ERK1/2 activation in newborn rat cardiomyocytes.

2 Adenosine (nonselective agonist), CPA (A_1), CGS 21680 (A_{2A}) or Cl-IB-MECA (A_3), all increased ERK1/2 phosphorylation in a time- and dose-dependent manner. The combined maximal response of the selective agonists was similar to adenosine alone. Theophylline (nonselective antagonist) inhibited completely adenosine-mediated ERK1/2 activation, whereas a partial inhibition was obtained with DPCPX (A_1), ZM 241385 (A_{2A}), and MRS 1220 (A_3).

3 PD 98059 (MEK1; ERK kinase inhibitor) abolished all agonist-mediated ERK1/2 phosphorylation. Pertussis toxin (PTX, $G_{i/o}$ blocker) inhibited completely CPA- and partially adenosine- and Cl-IB-MECA-induced ERK1/2 activation. Genistein (tyrosine kinase inhibitor) and Ro 318220 (protein kinase C, PKC inhibitor) partially reduced adenosine, CPA and Cl-IB-MECA responses, without any effect on CGS 21680-induced ERK1/2 phosphorylation. H89 (protein kinase A, PKA inhibitor) abolished completely CGS 21680 and partially adenosine and Cl-IB-MECA responses, without any effect on CPA response.

4 Cl-IB-MECA-mediated increases in cAMP accumulation suggest that A_3AR-induced ERK1/2 phosphorylation involves adenylyl cyclase activation *via* phospholipase C (PLC) and PKC stimulation.

5 In summary, we have shown that ERK1/2 activation by adenosine in cardiomyocytes results from an additive stimulation of A_1, A_{2A}, and A_3ARs, which involves $G_{i/o}$ proteins, PKC, and tyrosine kinase for A_1 and A_3ARs, and Gs and PKA for A_{2A}ARs. Moreover, the A_3AR response also involves a cAMP/PKA pathway *via* PKC activation.

Introduction

Four subtypes of adenosine receptors (ARs) have been characterized and classified into A_1, A_{2A}, A_{2B}, and A_3 (Fredholm *et al.*, 2001). These receptors belong to the G-protein-coupled receptor (GPCR) superfamily. A_1 and A_3ARs activate $G_{i/o}$ protein and inhibit adenylyl cyclase, whereas A_{2A} and A_{2B}ARs signal through G_s and activate adenylyl cyclase. Previous studies have shown that A_1, A_{2A}, and A_3ARs are expressed in rat ventricular cardiomyocytes (Martens *et al.*,

1987; Linden, 1994; Dixon *et al.*, 1996; Xu *et al.*, 1996; Dobson & Fenton, 1997). In the heart, A_1ARs mediate an antiadre-nergic effect as well as a protective effect from ischaemic injury (Mubagwa & Flameng, 2001). Likewise, it has been shown that A_3ARs play a cardioprotective role in rat cardiomyocytes (Safran *et al.*, 2001). A_{2A}ARs mediate a positive inotropic effect (Xu *et al.*, 1996; Dobson & Fenton, 1997) and counteract the antiadrenergic effects of A_1ARs (Norton *et al.*, 1999) in ventricular cardiomyocytes. The role of A_{2A}ARs in cardiopro-tection involves an anti-inflammatory effect rather than a direct effect on cardiomyocytes (Cargnoni *et al.*, 1999).

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Furthermore, it has been reported that $A_{2A}AR$ activation increases the cell death during ischaemia in chick cardiomyocytes (Strickler *et al.*, 1996).

Extracellular signal-regulated kinases 1/2 (ERK1/2) are isoforms of the mitogen-activated protein kinase (MAPK) family, which can be activated by tyrosine kinase receptors and $G_{i/o}$-, G_q-, and G_s-coupled receptors *via* a range of different signalling pathways (Lowes *et al.*, 2002). ERK1/2 are involved in cardiac hypertrophy and can play a protective role in ischaemic myocardium (Michel *et al.*, 2001).

Adenosine has been found to activate ERK1/2 in perfused rat heart (Haq *et al.*, 1998). It is noteworthy that this physiological agonist is released during myocardial hypoxia and ischaemia, and possesses cardioprotective properties (Sommerschild & Kirkeboen, 2000). Although human A_1, A_{2A}, A_{2B}, and A_3ARs transfected in Chinese hamster ovary cells activate ERK1/2 (Schulte & Fredholm, 2000), nothing is known about the regulation of the ERK1/2 signalling pathway by the different subtypes of ARs expressed in the heart, both of which are involved in cardioprotection.

Thus, the aim of the present study was to characterize which AR subtypes activate ERK1/2 and to determine the mechanisms implicated in the ERK1/2 signalling pathway by these receptors in newborn rat cardiomyocytes. We present evidence that ERK1/2 activation by adenosine in newborn rat cardiomyocytes results from an additive stimulation of A_1, A_{2A}, and A_3ARs. ERK1/2 phosphorylation is mainly mediated by A_1 and A_3ARs, and involves $G_{i/o}$ proteins, protein kinase C (PKC), and tyrosine kinase. In addition, the A_3AR response also involves a crosstalk between PLC/PKC and cAMP/ protein kinase A (PKA) signalling pathways. The $A_{2A}AR$ activates ERK1/2 through G_s/PKA pathway.

Methods

Cell culture

Neonatal ventricular myocytes were prepared from 1–4 day-old Wistar rats using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lornes laboratories, Reading, U.K.). The cells were preplated three times for 30 min in a humidified incubator (95% air/5% CO_2 at 37°C) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% (v v^{-1}) foetal calf serum, and penicillin/streptomycin (100 U ml^{-1}), in order to minimize fibroblast contamination. Cardiomyocyte-rich cultures ($>90\%$) were plated onto fibronectin-coated plates at a final density of 1.25×10^5 cells cm^{-2} in supplemented DMEM. For ERK1/2 phosphorylation detected by Western blot analysis, cardiomyocytes were plated onto 12-well plates. For cAMP accumulation assay, the cells were plated onto 24-well plates. After 3 days, confluent and spontaneously beating cells were serum-starved overnight before the experiments.

Western blot analysis

After serum starvation of cardiomyocytes (overnight), assays were carried out in serum-free DMEM in a humidified incubator (95% air/5% CO_2 at 37°C). Agonists, antagonists, and/or inhibitors according to the experiments were added as described in the figure legends. Following stimulation,

cardiomyocytes were washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, 1% (v v^{-1}) IGEPAL CA-630, 0.5% (w v^{-1}) sodium deoxycholate, 0.1% (w v^{-1}) sodium dodecyl sulphate (SDS), 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 0.1 mM phenylmethylsulphonylfluoride (PMSF), 10 μg ml^{-1} aprotinin, and 5 μg ml^{-1} leupeptin). Cell lysates were clarified by centrifugation (5 min; 12, 000 rpm) in an Eppendorf microcentrifuge. A measure of 100 μl of the cell lysate was removed and stored at −20°C until required. Protein concentration was determined using Bio-Rad *DC* Protein assay (Bio-Rad laboratories, Hertfordshire, U.K.) with bovine serum albumin as the standard. Samples in lysis buffer were heated at 95°C in SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer (v v^{-1}). Proteins (20–30 μg) were separated by SDS/PAGE(12% acrylamide gel) using a Bio-Rad Mini-Protean II system (1 h at 200 V). Proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot system (1 h at 100 V in 25 mM Tris, 192 mM glycine, and 20% MeOH). Following transfer, the membranes were washed with tris-buffered saline (TBS) and blocked for 1 h at room temperature in blocking buffer (TBS, 5% (w v^{-1}) skimmed milk powder, 0.1% Tween-20). Blots were then incubated overnight at 4°C with primary antibody against ERK1/2 at 1 : 1000 dilution in blocking buffer. The primary antibody was removed and the blot extensively washed three times for 5 min in TBS/Tween 20. Blots were then incubated for 1 h at room temperature with the goat anti-mouse secondary antibody coupled to horseradish peroxidase (DAKO Ltd, Cambridge, U.K.) at 1 : 1000 dilution in blocking buffer. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech, Little Chalfont, U.K.) and quantified using the programme QuantiScan (BioSoft, Cambridge, U.K.). The uniform transfer of proteins to the nitrocellulose membrane was routinely monitored by transiently staining the membranes with Ponceau S stain (Sigma Chemical Co.) prior to application of the primary antibody. In addition, replicate samples from each experiment were analysed on separate blots using an antibody (1 : 1000) that recognize unphosphorylated (total) ERK1/2. In these experiments, the uniformity of protein loading was confirmed by measuring total ERK1/2 (data omitted from the appropriate figures for clarity).

cAMP accumulation assay

After serum-starvation of cardiomyocytes (overnight), assays were carried out in serum-free DMEM in a humidified incubator (95% air/5% CO_2 at 37°C). Agonists and/or inhibitors according to the experiments were added as described in the figure legends. The cells were incubated for 3 h in a humidified incubator (95% air/5% CO_2 at 37°C) with 500 μl of serum-free DMEM containing [³H]adenine (37 kBq/ well). [³H]adenine-labelled cells were washed twice with Hanks/HEPES buffer and then incubated in 500 μl/well serum-free DMEM containing the cyclic AMP phosphodiesterase inhibitor rolipram (10 μM) for 15 min at 37°C in a humidified incubator. Agonists were added (in 10 μl of medium) 5 min prior to the incubation with 1.5 μM forskolin (10 min). Pathway inhibitors were added 30 min before the

agonist. Incubations were terminated by the addition of 500 μl 5% trichloroacetic acid, after removing the medium. [³H]cyclic AMP was isolated by sequential Dowex-alumina chromatography, as previously described (Dickenson & Hill, 1998). After elution, the levels of [³H]cyclic AMP were determined by liquid scintillation counting.

Statistical analysis

Results are expressed as means ± s.e.m. Dose–response and inhibition response curves were analysed by computer-assisted iteration using the GraphPad Prism (GraphPad software, San Diego, U.S.A.). Statistical significance was determined by analysis of variance (ANOVA) followed by Dunnet's test, and $P < 0.05$ was considered as the limit of statistical significance.

Materials

Bovine serum albumin, DMEM, foetal calf serum, IGEPAL CA-630 ((octylphenoxy)polyethoxyethanol), leupeptin, adenosine (9-β-D-ribofuranosyladenine), forskolin, N^6-cyclopentyladenosine (CPA), 1,3-dipropylcyclopentylxanthine (DPCPX), theophylline, phorbol 12-myristate 13-acetate (PMA), epidermal growth factor (EGF), pertussis toxin (PTX), phospho-specific ERK1/ERK2 (Thr^{202}/Tyr^{204}), and non-phospho-specific

ERK1/2 antibodies were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Trichloroacetic acid, genistein, 3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione (Ro 31-8220), 2′-amino-3′-methoxy-flavone (PD 98059), N-[2-(p-bromocinamylamine)ethyl]-5-iso-quinolisesulphonamide (H-89), rolipram and {1-[6-((17β-3 methoxyestra-1,3,5(10)-trien-17-yl)amoni)hexyl]-1H-pyrrole-2,5-dione} (U-73122) were purchased from Calbiochem (Nottingham, U.K.). 4-[2-[[-6-amino-9-(N-ethyl-β-D-ribofura-nuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid (CGS 21680), 1-[2-chloro-6[[(3-iodophenyl)methyl]ami-no]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide (2-Cl-IB-MECA), 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5ylamino]ethyl)phenol (ZM 241385), and N-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-benzene-acetamide (MRS 1220) were from Tocris (Bristol, U.K.). [8-³H]adenine was obtained from Amersham (Bucks, U.K.).

Results

Adenosine A_1, A_{2A}, and A_3 receptor-induced ERK1/2 activation in rat cardiomyocytes

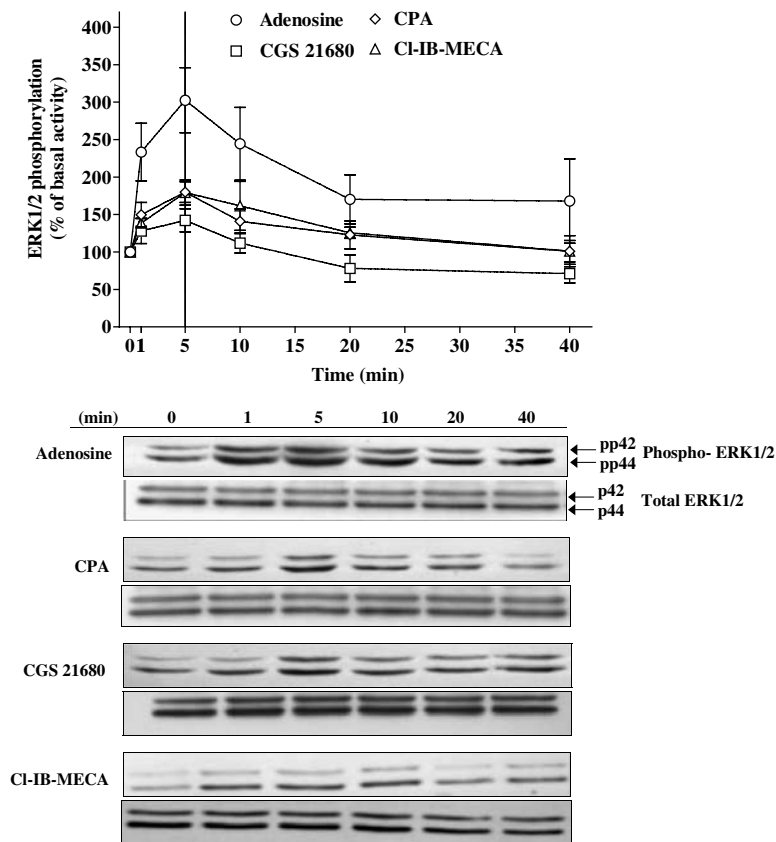In order to investigate which subtype of ARs activates ERK1/2 in newborn rat cardiomyocytes, we used the following

Figure 1 Time course of AR agonist-induced ERK1/2 phosphorylation in newborn rat cardiomyocytes. Serum-starved cardiomyocytes were stimulated with 1 μM adenosine (○), CPA (◇), CGS 21680 (□) or Cl-IB-MECA (△) for the indicated periods of time. Data are expressed as the percentage of the basal level of ERK1/2 phosphorylation (100%). Samples were also analysed on a separate blot, using an antibody that recognizes nonphosphorylated (total) ERK1/2 to confirm equal protein loading. Each point represents the mean ± s.e.m. of 4–6 independent experiments. The immunoblots are representative of the experiments summarized in the graph.

agonists: adenosine (nonselective agonist), CPA (A_1 selective agonist), CGS 21680 (A_{2A} selective agonist) or Cl-IB-MECA (A_3 selective agonist). All agonists induced ERK1/2 phosphorylation in a time- (Figure 1) and dose-dependent (Figure 2a) manner. Although each AR agonist stimulated increases in ERK1 and ERK2 phosphorylation, activation of the ERK1 isoform (p42 MAPK) was dominant. Furthermore, due to the variation in the basal level of ERK2 phosphorylation, all data are presented as the increase in combined ERK1/2 phosphorylation. For each agonist, ERK1/2 activation reached a maximal value at 5 min and decreased back to the basal level after 10 min. The rank order of the maximal stimulation (% over basal activity) was adenosine (203 ± 44, $n = 5$) $\gg$ CPA (78 ± 14, $n = 5$, $P < 0.05$ *versus* adenosine) = Cl-IB-MECA (79 ± 17, $n = 4$, $P < 0.05$ *versus* adenosine) > CGS 21680 (42 ± 15, $n = 4$, $P < 0.01$ *versus* adenosine). When the responses of the A_1, A_{2A}, and A_3 agonists were added, the value (199%) was consistent with the response obtained with adenosine alone (203%). In the same way, from the dose–response curves (Figure 2a), the combined maximal response (E_{max}) of all the three selective agonists (185%; CPA: 65 ± 4, $n = 5$; CGS 21680: 54 ± 19, $n = 4$; Cl-IB-MECA: 66 ± 31, $n = 4$; $P < 0.01$ *versus* adenosine) was similar to the adenosine E_{max} value ($175 \pm 20\%$, $n = 5$). These data suggest that adenosine induces ERK1/2 phosphorylation by stimulating the three adenosine subtypes A_1, A_{2A}, and A_3.

To further characterize the AR subtypes involved in ERK1/2 activation in newborn rat cardiomyocytes, we studied the inhibition of the 0.5 μM adenosine response by theophylline

(nonselective antagonist), DPCPX (A_1 selective antagonist), ZM 241385 (A_{2A} selective antagonist) or MRS 1220 (A_3 selective antagonist) (Figure 2b). Theophylline completely inhibited adenosine-induced ERK1/2 activation ($98 \pm 3\%$, $n = 4$), whereas the inhibition by the selective antagonists was partial (DPCPX: $67 \pm 8\%$, $n = 4$, $P < 0.05$ *versus* theophylline; ZM 241385: $27 \pm 10\%$, $n = 3$, $P < 0.01$ *versus* theophylline; MRS 1220: $54 \pm 12\%$, $n = 3$, $P < 0.01$ *versus* theophylline). The potencies of antagonists were evaluated by their IC_{50} (concentration of antagonist inhibiting 50% of ERK1/2 activation by adenosine) expressed as $-\log_{10} IC_{50}$. They showed that DPCPX ($7.31 \pm 0.79\%$) and MRS 1220 ($7.43 \pm 0.21\%$) were six and nine times more potent, respectively, than theophylline ($6.45 \pm 0.0.35\%$) in inhibiting adenosine-induced ERK1/2 phosphorylation, and both were more efficient than ZM 241385 ($6.95 \pm 0.61\%$). These results suggest that adenosine activates ERK1/2 phosphorylation mainly *via* A_1 and A_3ARs.

Effect of signalling pathway inhibitors on adenosine A_1, A_{2A}, and A_3 receptor-induced ERK1/2 activation in rat cardiomyocytes

$G_{i/o}$-, G_q-, and G_s-PCRs have been shown to activate ERK1/2 *via* a range of signalling pathways involving tyrosine kinase, PKC, and PKA (Kolch *et al.*, 1993; Hawes *et al.*, 1995; Lowes *et al.*, 2002). In order to determine the mechanisms implicated in ERK1/2 activation by the three subtypes of ARs, cardiomyocytes were pretreated with PTX (100 ng ml^{-1}, 16 h)
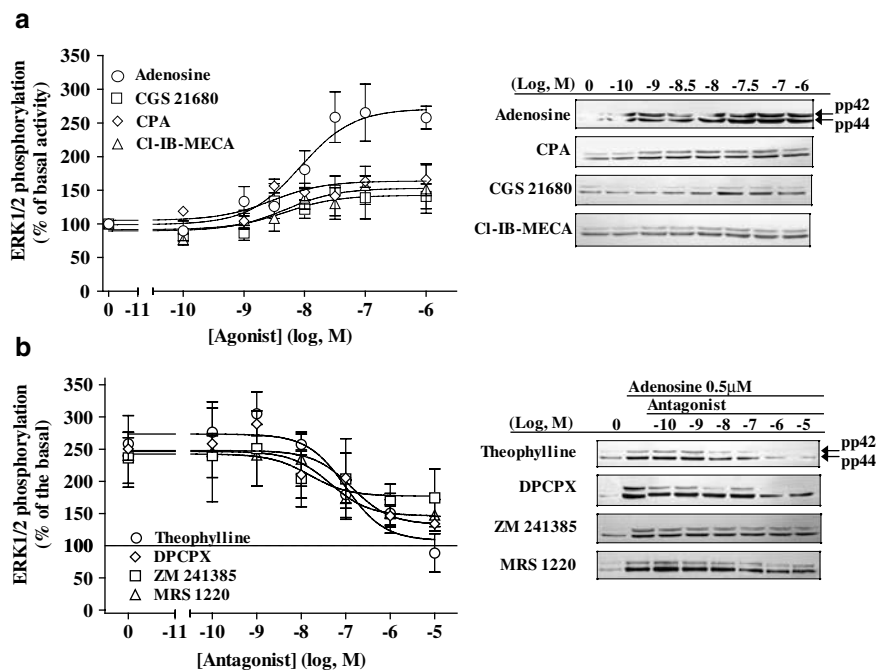

Figure 2 Dose–response curve of ERK1/2 phosphorylation (a) after stimulation for 5 min with adenosine ($\bigcirc$), CPA ($\diamond$), CGS 21680 ($\square$) or Cl-IB-MECA ($\triangle$), and inhibition of 0.5 μM adenosine-induced ERK1/2 phosphorylation (b) by theophylline ($\bigcirc$), DPCPX ($\diamond$), ZM 241385 ($\square$) or MRS 1220 ($\triangle$) in newborn rat cardiomyocytes. From panel (a), agonist potencies were evaluated by their EC_{50} values (concentration of agonist inducing 50% of maximal ERK1/2 phosphorylation) expressed as $-\log_{10} EC_{50}$ (adenosine: 8.23 ± 0.42, $n = 5$; CPA: 8.91 ± 0.15, $n = 5$; CGS 21680 7.98 ± 0.30, $n = 4$; Cl-IB-MECA: 8.61 ± 0.34, $n = 4$). In panel (b), serum-starved cardiomyocytes were preincubated for 30 min with the different antagonists before stimulating was $148 \pm 5\%$. Data are expressed as the percentage of the basal level of ERK1/2 phosphorylation (100%). Each point represents the mean $\pm$ s.e.m. of 4–6 independent experiments. The immunoblots are representative of the experiments summarized in the graph.

to block the $G_{i/o}$ protein, and inhibitors of MEK1 (PD 98059; 50 μM, 30 min), tyrosine kinase (genistein; 100 μM, 30 min), PKC (Ro 318220; 10 μM, 30 min), and PKA (H89; 10 μM, 30 min). Initially, we performed the relevant and essential controls required for the interpretation of data relating to the characterization of ERK1/2 signalling pathways induced by ARs (Figure 3). Indeed, the effects of these inhibitors on the basal level of ERK1/2 phosphorylation were investigated (Figure 3a). Furthermore, we also determined the effects of genistein, Ro 318220, and H89 on ERK1/2 activation induced by EGF (tyrosine kinase-dependent, Figure 3b) and the PKC activator PMA (Figure 3c). As shown in Figure 3b, genistein selectively inhibited the robust increase in ERK1/2 phosphorylation induced by EGF. Similarly, Ro 318220 selectively inhibited PMA-induced increases in ERK1/2 phosphorylation (Figure 3c). Importantly, the PKA inhibitor H89 had no significant effect on EGF or PMA-induced ERK1/2 phosphorylation, indicating that H89 is not inhibiting tyrosine kinase and PKC-dependent pathways of ERK1/2 activation in cardiomyocytes. These observations clearly indicate that genistein and Ro 318220

are selective for tyrosine kinase and PKC-dependent pathways of ERK1/2 activation in rat cardiomyocytes.

The effects of PTX, PD 98059, genistein, Ro 318220, and H89 on agonist-stimulated ERK1/2 phosphorylation are illustrated in Figures 4 and 5. ERK1/2 activation by CPA (A_1 selective agonist) was completely abolished by PTX. PTX partially inhibited ERK1/2 phosphorylation (72%) induced by Cl-IB-MECA (A_3 selective agonist), indicating that ERK1/2 activation by A_3AR stimulation involves PTX-sensitive ($G_{i/o}$) and insensitive (G_q and/or G_s) G protein. PTX was ineffective on CGS 21680 (A_{2A} selective agonist)-induced ERK1/2 phosphorylation. ERK1/2 phosphorylation induced by adenosine was partially inhibited by PTX (66%), indicating that adenosine induces ERK1/2 phosphorylation by stimulating these three subtypes.

As expected, PD 98059 (MEK1 inhibitor) completely abolished the ERK1/2 phosphorylation mediated by all the agonists, showing that MEK1 activation is the common and converging pathway to the three subtypes of ARs.

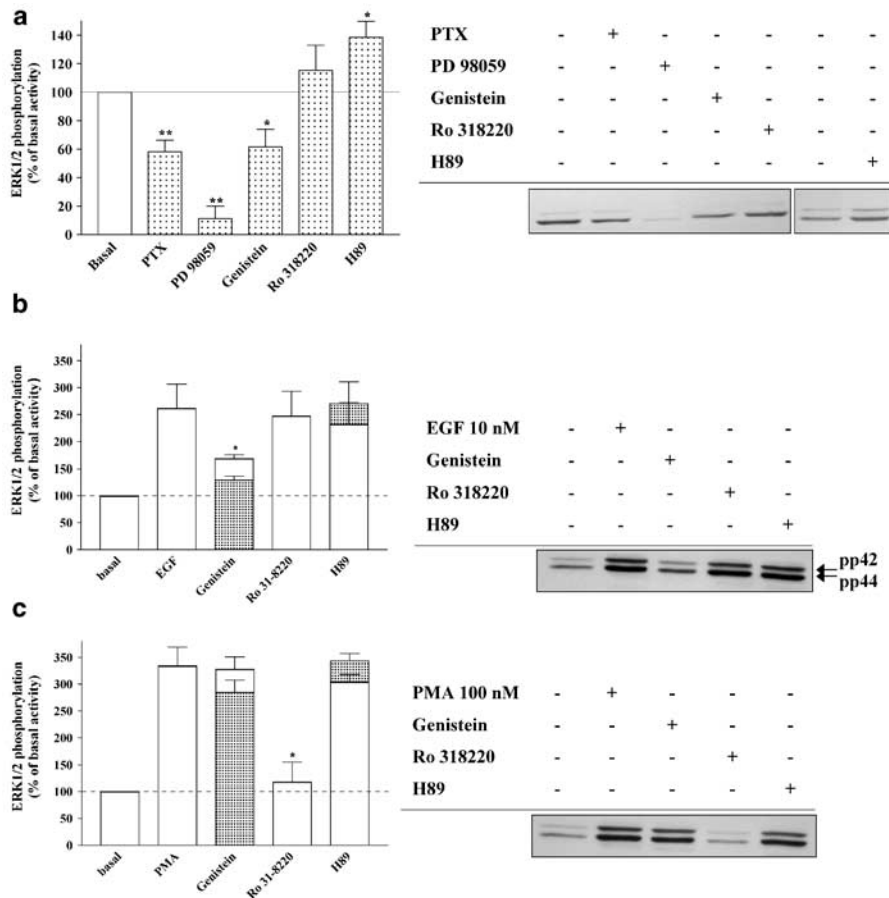Following genistein pretreatment, adenosine, CPA, and Cl-IB-MECA-induced ERK1/2 activation were reduced by 51, 86,

Figure 3 Effect of PTX and protein kinase inhibitors on the basal level of ERK1/2 phosphorylation and ERK1/2 activation triggered by EGF and PMA. Serum-starved cardiomyocytes were pretreated for 16 h with 100 ng ml^{-1} PTX ($G_{i/o}$ protein blocker) and 30 min with 50 μM PD 98059, 100 μM genistein 10 μM Ro 318220 or 10 μM H89. The effects of PTX, PD 98059, genistein, Ro 318220, and H89 on the basal level of ERK1/2 phosphorylation are shown in (a). Panels (b) and (c) show the effect of genistein, Ro 318220, and H89 on 10 nM EGF (b) and 100 nM PMA (c) -induced ERK1/2 phosphorylation (both 5 min stimulation). Data are expressed as the percentage of the basal level ERK1/2 phosphorylation (100%). Each bar represents the mean $\pm$ s.e.m. of four independent experiments. Filled bars indicate the data obtained from the immunoblot, and open bars represent the data after removing the effect of the inhibitors on the basal level of ERK1/2 phosphorylation for each individual experiment and that illustrated in (a). The immunoblots are representative of the experiments summarized in the bar graph. *$P<0.05$ and **$P<0.01$ *versus* basal level of ERK1/2 phosphorylation (panel (a)) and *versus* EGF or PMA-induced ERK1/2 activation (panels (b) and (c)).
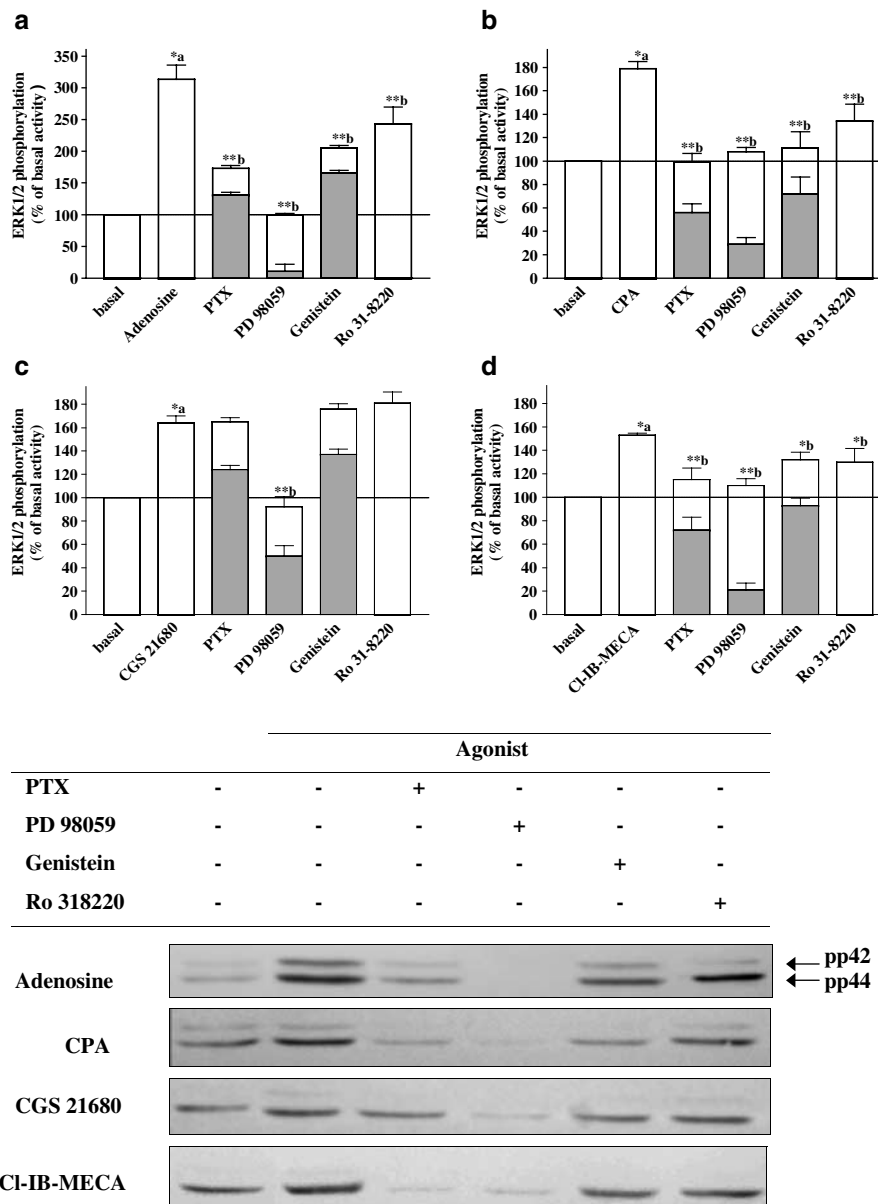
Figure 4 Effect of PTX ($G_{i/o}$ protein blocker), PD 98059 (MEK1 inhibitor), genistein (tyrosine kinase inhibitor), and Ro 318220 (PKC inhibitor) on adenosine (a), CPA (b), CGS 21680 (c) or Cl-IB-MECA (d)-induced ERK1/2 phosphorylation. Serum-starved cardiomyocytes were pretreated for 16 h with 100 ng ml^{-1} PTX and 30 min with 50 μM PD 98059, 100 μM genistein or 10 μM Ro 318220, before stimulating for 5 min with 1 μM adenosine, CPA, CGS 21680 or Cl-IB-MECA. Data are expressed as the percentage of the basal level of ERK1/2 phosphorylation. Each bar represents the mean $\pm$ s.e.m. of 5–6 independent experiments. Filled bars indicate the data obtained from the immunoblot, and open bars represent the data after removing the effect of the inhibitors on the basal level of ERK1/2 phosphorylation for each individual experiment, and illustrated in Figure 3a. The immunoblots are representative of the experiments summarized in the bar graph. *$P < 0.05$ and **$P < 0.01$, (a) *versus* basal activity and (b) *versus* agonist stimulation.

and 40%, respectively. These data suggest that ERK1/2 activation *via* the A_1ARs is mainly tyrosine kinase-dependent, whereas the A_3ARs appear to activate ERK1/2 *via* a tyrosine kinase-dependent and independent pathway. As expected, adenosine showed an intermediate response, taking into account the lack of genistein effect on the CGS 21680 response.

Ro 318220 inhibited the response induced by adenosine, CPA, and Cl-IB-MECA, respectively, by 33, 57, and 43%, without affecting CGS 21680-induced ERK1/2 phosphoryla-

tion. These data indicate that A_1 and A_3ARs stimulate ERK1/2 phosphorylation *via* a pathway that is partially dependent upon PKC.

CGS 21680-induced ERK1/2 phosphorylation was completely inhibited by H89, whereas CPA-stimulated ERK1/2 activation was unaltered. A partial inhibition (about 50%) was achieved with adenosine and Cl-IB-MECA, suggesting that ERK1/2 activation induced by A_3ARs in cardiomyocytes also involves coupling to the G_s/PKA pathway.
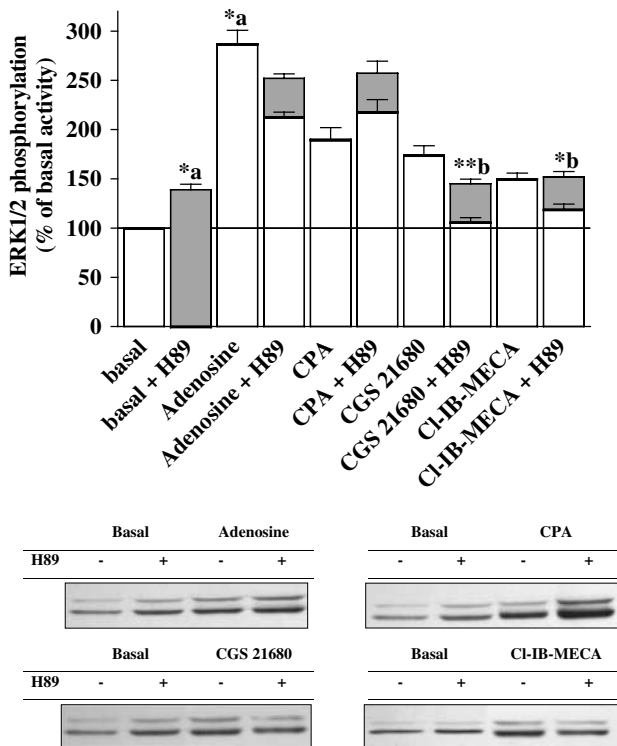
Figure 5 Effect of H89 (PKA inhibitor) on adenosine, CPA, CGS 21680, and Cl-IB-MECA-induced ERK1/2 phosphorylation. Serum-starved cardiomyocytes were pretreated for 30 min with 10 μM H89 before stimulating for 5 min with 1 μM adenosine, CPA, CGS 21680 or Cl-IB-MECA. Data are expressed as the percentage of the basal level of ERK1/2 phosphorylation. H89 alone elicited a small but significant increase in the basal level of ERK1/2 phosphorylation. Each bar represents the mean$\pm$s.e.m. of 4–5 independent experiments. Filled bars indicate the data obtained from the immunoblot and open bars represent the data after removing the effect of the inhibitors on the basal level of ERK1/2 phosphorylation for each individual experiment. The immunoblots are representative of the experiments summarized in the bar graph. $*P < 0.05$ and $**P < 0.01$, (a) *versus* basal activity and (b) *versus* agonist stimulation.

Effect of adenosine A_1, A_{2A}, and A_3 agonists on cAMP accumulation in rat cardiomyocytes

In view of the role of cAMP/PKA in coupling of the A_3ARs to ERK1/2, we investigated the effect of A_3AR activation on cAMP accumulation. A_1 and A_3ARs classically activate $G_{i/o}$ protein and inhibit adenylyl cyclase (Fredholm, 2001). The effects of adenosine (nonselective), CPA (A_1 selective), and Cl-IB-MECA (A_3 selective) on forskolin-stimulated cAMP accumulation are shown in Figures 6a–c, respectively. In addition, we measured cAMP accumulation in response to CGS 21680 (A_{2A} selective; Figure 6d). Adenosine did not inhibit forskolin-induced cAMP accumulation, but increased cAMP formation at concentrations above 100 nM (Figure 6a). The dose–response effects of adenosine presumably reflect A_1, A_{2A}, and A_3AR costimulation, as observed with ERK1/2 activation. As expected, CPA elicited a dose-dependent inhibition of forskolin-stimulated cAMP accumulation by $26 \pm 1\%$, with a potency of 8.8 ± 0.34 (1.5 nM; Figure 6b). The A_{2A}AR agonist CGS 21680 stimulated an increase in cAMP

accumulation of 28% above the basal accumulation, with a pEC$_{50}$ value of 8.50 ± 0.46 (3.18 nM; Figure 6d). Noradrenaline, which was used as a positive control for G_s/PKA pathway activation, stimulated a robust increase in cAMP accumulation ($301 \pm 29\%$ over basal), with a potency of 7.15 ± 0.23 (71 nM). Interestingly, the dose–response curve for Cl-IB-MECA-induced inhibition of forskolin-stimulated cAMP accumulation was biphasic (Figure 7c). Inhibition of forskolin-stimulated cAMP accumulation was observed at lower concentrations of Cl-IB-MECA (maximal inhibition occurring at 3 nM; about 18%), with an approximate pIC$_{50}$ of 9.7 ± 0.8 (0.7 nM). At concentrations above 3 nM, A_3AR stimulation can also induce a reversal of the inhibitory effect on cAMP accumulation. PTX treatment abolished Cl-IB-MECA-mediated inhibition of forskolin-induced cAMP accumulation (observed at low concentrations), and produced an increase in cAMP formation (about 14%) at 1 μM agonist (Figure 6c), indicating that A_3ARs are coupling to the G_i protein and G_s/PKA pathway. PTX treatment also abolished CPA-induced inhibition of forskolin-induced cAMP accumulation, confirming the A_1 AR coupling to $G_{i/o}$ proteins (data not shown). Finally, the inhibition of G_i by PTX produced an increase in adenosine-stimulated cAMP production by 25% in a dose-dependent manner between 0.01 and 30 nM, indicating A_{2A}AR stimulation (Figure 6a). In order to investigate whether the A_3AR directly couples to G_s-protein, PTX-treated cardiomyocytes were stimulated with Cl-IB-MECA in the absence of forskolin. As shown in Figure 7a, 1 μM Cl-IB-MECA (concentration used to determine ERK1/2 signalling pathway activation) did not increase cAMP accumulation, whereas adenosine and CGS 21680 similarly increased cAMP accumulation by 27 ± 3 and $34 \pm 5\%$, respectively.

Since the G_q/PLC/PKC pathway can stimulate cAMP production (Cordeaux & Hill, 2002), and given that A_3ARs activate PLC in cardiomyocytes (Parsons *et al.*, 2000), we determined the role of PKC and PLC in A_3AR-mediated augmentation of forskolin-induced cAMP accumulation in the presence of PTX. Pretreatment with Ro 31822 and U-73122, inhibitors of PKC and PLC, respectively, significantly decreased 1 μM Cl-IB-MECA-mediated cAMP production in the presence of forskolin and PTX (Figure 7b). Overall, these data strongly indicate that A_3ARs are not directly coupled to G_s protein in cardiomyocytes, but rather increase cAMP accumulation *via* PLC/PKC.

Discussion

Adenosine released during myocardial ischaemia plays an important cardioprotective role, *via* the activation of cell surface ARs belonging to the GPCR superfamily (Sommerschild & Kirkeboen, 2000). Previous studies have shown that A_1, A_{2A}, and A_3ARs are functionally expressed in cardiomyocytes (Martens *et al.*, 1987; Linden, 1994; Dixon *et al.*, 1996; Xu *et al.*, 1996; Dobson & Fenton, 1997). Extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, which can be activated by tyrosine kinase receptors and $G_{i/o}$-, G_q-, and G_s-PCRs (Lowes *et al.*, 2002), is involved in cardiac hypertrophy and myocardium protection (Michel *et al.*, 2001). Given the important role of adenosine and ERK1/2 in the heart, the aims of the present study were to characterize the AR subtypes involved in the ERK1/2 activation and to determine the
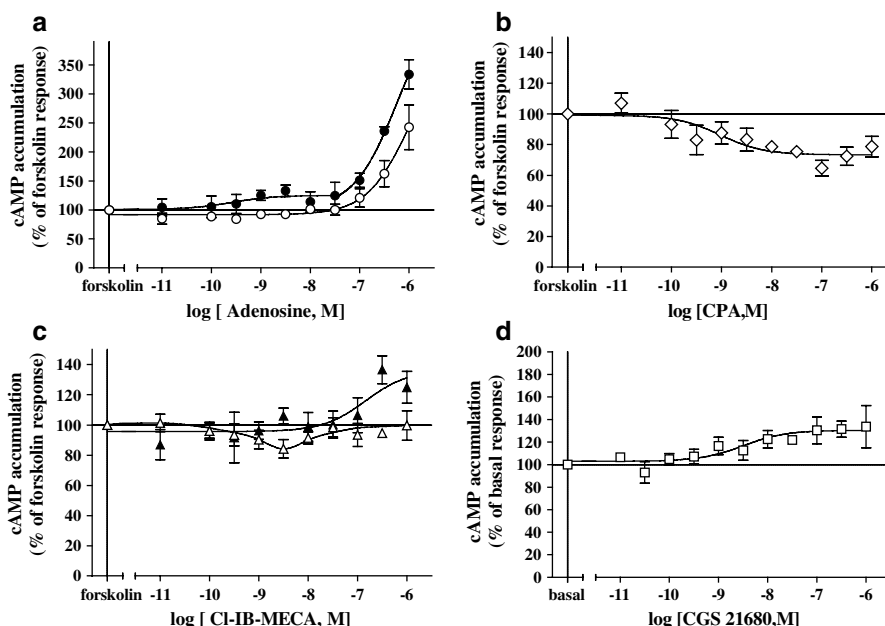
Figure 6 Effect of AR agonists on cAMP accumulation in isolated rat cardiomyocytes. In panels (a)–(c), cells were initially prestimulated for 5 min with the indicated concentrations of adenosine (a), CPA (b), and Cl-IB-MECA (c), prior to stimulation with 1.5 μM forskolin for 10 min in the absence (opened symbols) and presence (filled symbols) of PTX. Cells were pretreated with 100 ng ml^{-1} PTX for 16 h to block G_i/G_o-protein-dependent pathways. Data are expressed as the percentage of the forskolin response (in the absence of agonist = 100%). In panel (d), cells were stimulated with the indicated concentrations of CGS 21680 for 15 min in the absence of forskolin. Data are expressed as the percentage of the basal cAMP accumulation (100%). The results represent mean $\pm$ s.e.m. of 4–6 independent experiments performed in duplicate. Standard error bars not shown are within the symbol.

signalling mechanisms implicated in ERK1/2 activation by these receptors in newborn rat cardiomyocytes.

Our study has shown for the first time that stimulation of A_1, A_{2A}, and A_3ARs activated ERK1/2 in newborn rat cardiomyocytes. Indeed, adenosine (nonselective agonist), CPA (A_1 selective agonist), CGS 21680 (A_{2A} selective agonist), and Cl-IB-MECA (A_3 selective agonist), all induced ERK1/2 phosphorylation in a time- and dose-dependent manner (Figures 1 and 2a). These observations are in agreement with other studies on human A_1, A_{2A}, and A_3ARs in transfected Chinese hamster ovary cells (Dickenson *et al.*, 1998; Schulte & Fredholm, 2000; Graham *et al.*, 2001) and endogenous A_1ARs in DDT$_1$MF-2 cells (Robinson & Dickenson, 2001) and A_{2A}ARs human umbilical venous endothelial cells (Sexl *et al.*, 1997).

It is notable that the combined response of all the three selective agonists was similar to the value obtained with adenosine alone, indicating that this physiological agonist stimulates all the receptor subtypes when it is released in pathological situations or in response to physiological adaptation. This finding is supported by previous studies showing an additive protection against ischaemia induced by a simultaneous activation of A_1 and A_3ARs in cardiomyocytes (Jacobson *et al.*, 2000; Safran *et al.*, 2001). Furthermore, A_1AR-mediated antiadrenergic effects are modulated by costimulation of the A_{2A}AR in rat cardiomyocytes and whole heart (Norton *et al.*, 1999). Adenosine-mediated ERK1/2 activation *via* the three AR receptor subtypes was confirmed by using adenosine-receptor antagonists (Figure 2b). Theophylline (nonselective antagonist) completely inhibited the adenosine-induced ERK1/2 activation, whereas DPCPX

(A_1 selective antagonist), ZM 241385 (A_{2A} selective agonist), and MRS 1220 (A_3 selective agonist) elicited a partial inhibition of this response by 67, 54, and 27%, respectively. The efficiency to inhibit adenosine-mediated ERK1/2 activation was higher using A_1 and A_3 than A_{2A} antagonists. These data suggest that adenosine-induced ERK1/2 phosphorylation in newborn rat cardiomyocytes is highly dependent on A_1 and A_3AR stimulation.

Experiments investigating the signalling pathways associated with ERK1/2 activation revealed that A_1- and A_3AR-mediated ERK1/2 phosphorylation involves a genistein-sensitive tyrosine kinase and MEK1 activation (Figures 4b and d). In addition, A_1AR-mediated ERK1/2 activation was completely abolished by PTX (hence $G_{i/o}$ protein-mediated), whereas the response to Cl-IB-MECA was partially sensitive to PTX (72%). These data are in agreement with previous studies showing that $G_{i/o}$-PCR-mediated ERK1/2 activation, including the A_1AR, involves G protein-derived $\beta\gamma$ subunits and genistein-sensitive *Src*-related tyrosine kinase(s) (Faure *et al.*, 1994; Della Rocca *et al.*, 1997). Also, A_1- and A_3AR-induced increases in ERK1/2 activation were partially sensitive to the PKC inhibitor Ro 318220 (57 and 43% inhibition, respectively), indicating the involvement of a PKC-dependent pathway. There is increasing evidence for a role of PKC in $G_{i/o}$-PCR-mediated ERK1/2 activation (Takeda *et al.*, 1999). The involvement of PKC in A_1AR-induced ERK1/2 activation may reflect the coupling of the A_1AR to PLC/PKC activation in cardiomyocytes (Parsons *et al.*, 2000; Lee *et al.*, 2001). Furthermore, the A_1AR is known to activate PLC *via* $\beta\gamma$ subunits released from PTX-sensitive $G_{i/o}$ proteins (Dickenson & Hill, 1998). Therefore, the PKC-sensitive component of
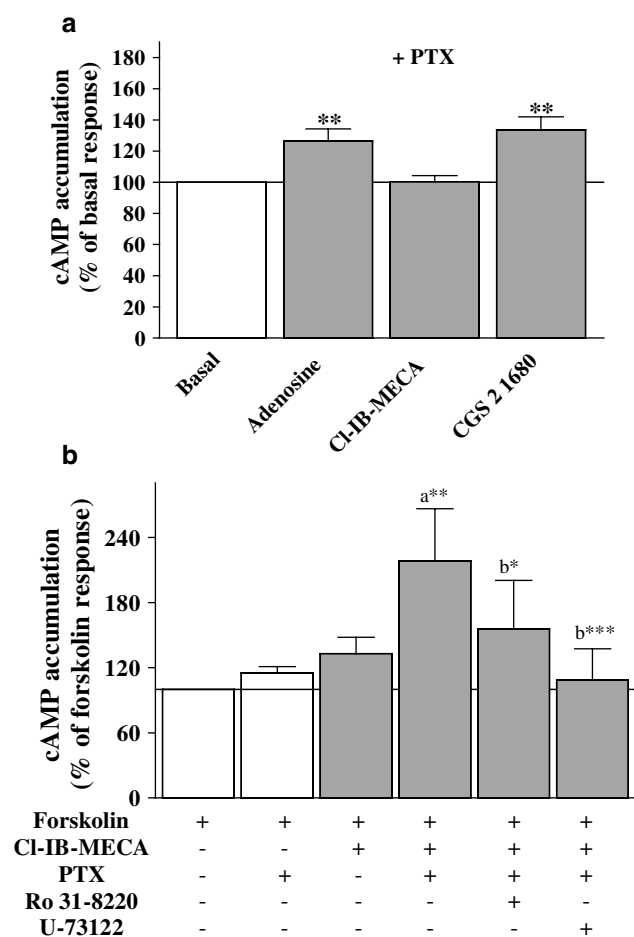
Figure 7 Effect of adenosine, Cl-IB-MECA, and CGS 21680 on basal cAMP accumulation (a), and the effects of Ro 318220 (PKC inhibitor) and U-73122 (PLC inhibitor) on Cl-IB-MECA-mediated augmentation of forskolin-stimulated cAMP accumulation (b). In panel (a), PTX-treated cells ($100 \, \mathrm{ng \, ml^{-1}}$ for 16 h) were stimulated with $1 \, \mu\mathrm{M}$ adenosine, Cl-IB-MECA, and CGS 21680 for 15 min in the absence of forskolin. Data are expressed as the percentage of the basal cAMP accumulation (100%). The results represent mean $\pm$ s.e.m. of 4–5 independent experiments performed in duplicate. $**P < 0.01$ vs basal cAMP accumulation. In panel (b), cells were pretreated with PTX ($100 \, \mathrm{ng \, ml^{-1}}$ for 16 h) to block G_i/G_o-protein-dependent pathways. In addition, the indicated cells were incubated for 30 min with $10 \, \mu\mathrm{M}$ Ro 318220 and $10 \, \mu\mathrm{M}$ U-73122, inhibitors of PKC and PLC, respectively. Cells were then stimulated for 5 min with $1 \, \mu\mathrm{M}$ Cl-IB-MECA prior to stimulation with $1.5 \, \mu\mathrm{M}$ forskolin for 10 min in the continued presence of Cl-IB-MECA. Data are expressed as the percentage of the forskolin response in the absence of Cl-IB-MECA (100%). The results represent the mean $\pm$ s.e.m. of four independent experiments performed in duplicate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, (a) *versus* Cl-IB-MECA/forskolin response and (b) *versus* Cl-IB-MECA/forskolin/PTX response.

A_1AR-induced ERK1/2 activation in cardiomyocytes may involve $\beta\gamma$ subunit-mediated PLC/PKC activation. A similar pathway has been reported for α_{2A} adrenergic receptor-mediated ERK1/2 activation (Della Rocca *et al.*, 1997).

The PKC-dependent pathway associated with A_3AR-induced ERK1/2 activation (PTX-insensitive) may reflect the reported coupling of the A_3AR to G_q and PLC (Palmer *et al.*, 1995; Parsons *et al.*, 2000), since PKC is associated with ERK1/2 activation *via* G_q-PCRs (Hawes *et al.*, 1995; Lowes *et al.*, 2002). We conclude that A_1 and A_3ARs in cardiomyocytes mediate ERK1/2 activation predominantly *via* a $G_{i/o}/$

tyrosine kinase/MEK1 pathway. In addition, A_1AR-activated ERK1/2 phosphorylation also involves a $G_{i/o}/PLC/PKC$-dependent pathway. Furthermore, it is conceivable that the PTX-insensitive component of the A_3AR-mediated ERK1/2 phosphorylation can involve G_q/PLC-mediated PKC activation.

It is notable that A_3AR-mediated ERK1/2 activation was also sensitive to the PKA inhibitor H89, suggesting the involvement of a $G_s/PKA/cAMP$-dependent pathway. Interestingly, A_3ARs have been shown to induce an increase in intracellular calcium and potentiate Ca^{2+} currents *via* PKA activation in A6 renal cells (Reshkin *et al.*, 2000) and hippocampal CA3 pyramidal neuronal cells (Fleming & Mogul, 1997). In addition, the A_3AR stimulates cAMP production in human eosinophils (Ezeamuzie & Philips, 2003). Based on these previous studies and the sensitivity of A_3AR-mediated ERK1/2 activation to H89, we investigated whether the A_3AR couples to $G_s/cAMP$ in cardiomyocytes. Interestingly, the dose–response curve for Cl-IB-MECA-induced inhibition of forskolin-stimulated cAMP accumulation was biphasic (Figure 6c). Inhibition of forskolin-stimulated cAMP accumulation was observed at lower concentrations of Cl-IB-MECA ($< 3 \, \mathrm{nM}$), whereas, at concentrations above $3 \, \mathrm{nM}$, the inhibitory effect was reversed and potentiated in the presence of PTX (Figure 6c), indicating that A_3AR stimulation can also induce an increase in cAMP accumulation. This increase does not involve direct coupling of the A_3AR to Gs or the nonselectivity of Cl-IB-MECA (*via* A_{2A} activation), since this agonist did not induce cAMP accumulation in cells treated with PTX in the absence of forskolin (Figure 7a). Since PKC can directly activate certain isoforms of adenylyl cyclase (Cordeaux & Hill, 2002), and given the evidence for A_3AR coupling to PLC in cardiomyocytes, we determined the role of PLC/PKC in Cl-IB-MECA-mediated augmentation of forskolin-induced cAMP accumulation. Ro 31-8220 (PKC inhibitor) and U-73122 (PLC inhibitor) inhibited the potentiation of forskolin-induced cAMP production by Cl-IB-MECA (Figure 7b). Therefore, these data suggest that the cAMP/PKA-dependent pathway associated with A_3AR-mediated ERK1/2 activation in cardiomyocytes may involve PKC *via* G_q/PLC and not direct coupling to G_s protein. A_3AR coupling to cAMP/PKA may explain the induction of apoptosis in cardiomyocytes after stimulation with high concentration ($\geqslant 10 \, \mu\mathrm{M}$) of Cl-IB-MECA (Shneyvays *et al.*, 2000). The cardioprotective effects of Cl-IB-MECA are only observed using concentrations below $1 \, \mu\mathrm{M}$ (Safran *et al.*, 2001).

$A_{2A}AR$-mediated increases in ERK1/2 phosphorylation in cardiomyocytes appear to involve coupling to G_s protein and, subsequently, PKA activation, since the PKA inhibitor H89 completely blocked the responses to CGS 21680 (Figure 5). These data are strengthened by the activation of cAMP accumulation by CGS 21680, as shown in Figure 6d. In addition, the MEK1 inhibitor PD 98059 blocked A_2AR-mediated ERK1/2 activation. Previous studies have shown that the $A_{2A}AR$ activates ERK1/2 *via* a cascade composed of G_s, adenylyl cyclase, PKA, and MEK1, although additional pathways independent of G_s coupling and PKA activation have also been observed (Sexl *et al.*, 1997; Seidel *et al.*, 1999).

In conclusion, this study has shown for the first time that the activation of ERK1/2 by adenosine in newborn rat cardiomyocytes arises from an additive stimulation of A_1, A_{2A}, and

A_3ARs endogenously expressed in these cells. Adenosine-induced ERK1/2 phosphorylation is mainly mediated by A_1 and A_3ARs, and involves $G_{i/o}$ proteins, PKC, and tyrosine kinase. In addition, the A_3AR response also involves a cAMP/PKA-dependent pathway triggered by A_3AR-induced PKC activation. The $A_{2A}AR$ activates ERK1/2 *via* G_s and PKA. The role of the different ERK1/2 pathways activated by the three AR subtypes and their possible interaction in cardioprotection induced by adenosine remain to be established.

However, we have recently obtained preliminary data which indicate that ERK1/2 are involved in A_1AR- and A_3AR-mediated cardioprotection in isolated rat cardiomyocytes (Germack et al., 2003).

References

CARGNONI, A., CECONI, C., BORASO, A., BERNOCCHI, P., MONOPOLI, A., CURELLO, S. & FERRARI, R. (1999). Role of A2A receptor in the modulation of myocardial reperfusion damage. *J. Cardiovasc. Pharmacol.*, **33**, 883–893.

CORDEAUX, Y. & HILL, S.J. (2002). Mechanisms of cross-talk between G-protein-coupled receptors. *Neurosignals*, **11**, 45–57.

DELLA ROCCA, G.J., VAN BIESEN, T., DAAKA, Y., LUTTRELL, D.K., LUTTRELL, L.M. & LEFKOWITZ, R.J. (1997). Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of G_i- and G_q-mediated pathways on calcium/calmodulin, Pyk 2, and Src kinase. *J. Biol. Chem.*, **272**, 19125–19132.

DICKENSON, J.M., BLANK, J.L. & HILL, S.J. (1998). Human adenosine A_1 receptor and $P2Y_2$-purinoceptor-mediated activation of the mitogen-activated protein kinase cascade in transfected CHO cells. *Br. J. Pharmacol.*, **124**, 1491–1499.

DICKENSON, J.M. & HILL, S.J. (1998). Involvement of G-protein $\beta\gamma$ subunits in coupling the adenosine A_1 receptor to phospholipase C in transfected CHO cells. *Eur. J. Pharmacol.*, **355**, 85–93.

DIXON, A.K., GUBITZ, A.K., SIRINATHSINGHJI, D.J.S., RICHARDSON, P.J. & FREEMAN, T.C. (1996). Tissue distribution of adenosine receptor mRNAs in the rat. *Br. J. Pharmacol.*, **118**, 1461–1468.

DOBSON, J.G. & FENTON, R.A. (1997). Adenosine A2 receptor function in rat ventricular myocytes. *Cardiovasc. Res.*, **34**, 337–347.

EZEAMUZIE, C.I. & PHILIPS, E. (2003). Positive coupling of atypical adenosine A3 receptors on human eosinophils to adenylyl cyclase. *Biochem. Biophys. Res. Commun.*, **300**, 712–718.

FAURE, M., VOYONO-YASENETSKAYA, T.A. & BOURNE, H.R. (1994). cAMP and $\beta\gamma$ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.*, **269**, 7851–7854.

FLEMING, K.M. & MOGUL, D.J. (1997). Adenosine A_3 receptors potentiate hippocampal calcium current by a PKA-dependent/PKC-independent pathway. *Neuropharmacology*, **36**, 353–362.

FREDHOLM, B.B., IJZERIAAN, A.P., JACOBSON, K.A., KLOTZ, K.N. & LINDEN, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.*, **53**, 527–552.

GERMACK, R., MARSHALL, C., GRIFFIN, M. & DICKENSON, J.M. (2003). Role of PKC and ERK1/2 in A_1 and A_3 adenosine receptor-mediated cardioprotection in newborn rat cardiomyocytes. *Eur. J. Biochem.*, **270** (Suppl 1), 180.

GRAHAM, S., COMBES, P., CRUMIERE, M., KLOTZ, K.N. & DICKENSON, J.M. (2001). Regulation of p42/p44 mitogen-activated protein kinase by the human adenosine A_3 receptor in transfected CHO cells. *Eur. J. Pharmacol.*, **420**, 19–26.

HAQ, S.E.A., CLERK, A. & SUGDEN, P.H. (1998). Activation of mitogen-activated protein kinases (p38-MAPKs, SAPKs/JNKs and ERKs) by adenosine in the perfused rat heart. *FEBS Lett.*, **434**, 305–308.

HAWES, B.E., VAN BIESEN, T., KOCH, W.J., LUTTRELL, L.M. & LEFKOWITZ, R.J. (1995). Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. *J. Biol. Chem.*, **270**, 17148–17153.

JACOBSON, K.A., XIC, R., YOUNG, L., CHANG, L. & LIANG, B.T. (2000). A novel pharmacological approach to treating cardiac ischemia. Binary conjugates of A_1 and A_3 adenosine receptor agonists. *J. Biol., Chem.*, **275**, 30272–30279.

KOLCH, W., HEIDECKER, G., KOCHS, G., HUMMEL, R., VAHIDI, H., MISCHAK, H., FINKENZELLER, G., MARME, D. & RAPP, U.R. (1993). Protein kinase C alpha activates RAF-1 by direct phosphorylation. *Nature*, **364**, 249–252.

LEE, J.E., BOKOCH, G. & LIANG, B.T. (2001). A novel cardioprotective role of RhoA: new signalling mechanism for adenosine. *FASAB J.*, **15**, 1886–1894.

LINDEN, J. (1994). Cloned adenosine A_3 receptors: pharmacological properties, species differences and receptor functions. *Trends Pharmacol. Sci.*, **15**, 298–306.

LOWES, V., IP, N. & WONG, Y. (2002). Integration of signals from receptor tyrosine kinases and G protein-coupled receptors. *Neurosignals*, **11**, 5–19.

MARTENS, D., LOHSE, M.J., RAUCH, B. & SCHWABE, U. (1987). Pharmacological characterization of A_1 adenosine receptors in isolated rat ventricular myocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **336**, 342–348.

MICHEL, M.C., LI, Y. & HEUSCHCH, G. (2001). Mitogen-activated protein kinases in the heart. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **363**, 245–266.

MUBAGWA, K. & FLAMENG, W. (2001). Adenosine, adenosine receptors and myocardial protection: an updated overview. *Cardiovasc. Res.*, **52**, 25–39.

NORTON, G.R., WOODIWISS, A.J., McGINN, R.J., LORBAR, M., CHUNG, E.S., HONEYMAN, T.W., FENTON, R.A., DOBSON, J.G. & MEYER, T.E. (1999). Adenosine A_1 receptor-mediated antiadrenergic effects are modulated by A_{2A} receptor activation in rat heart. *Am. J. Physiol.*, **276**, H341–H349.

PALMER, T.M., GETTYS, T.W. & STILES, G.L. (1995). Differential interaction with and regulation of multiple G-proteins by the rat A_3 adenosine receptor. *J. Biol. Chem.*, **270**, 16895–16902.

PARSONS, M., YOUNG, L., LEE, J.E., JACOBSON, K.A. & LIANG, B.T. (2000). Distinct cardioprotective effects of adenosine mediated by differential coupling of receptor subtypes to phospholipases C and D. *FASEB J.*, **14**, 1423–1431.

RESHKIN, S.J., GUERRA, L., BAGORDA, A., DEBELLIS, L., CARDONE, R., LI, A.H., JACOBSON, K.A. & CASAVOLA, V. (2000). Activation of A_3 adenosine receptor induces calcium entry and chloride secretion in A6 cells. *J. Membr. Biol.*, **178**, 103–113.

ROBINSON, A.J. & DICKENSON, J.M. (2001). Regulation of p42/p44 MAPK and p38 MAPK by the adenosine A_1 receptor in DDT$_1$MF-2 cells. *Eur. J. Pharmacol.*, **413**, 151–161.

SAFRAN, N., SHNEYVAYS, V., BALAS, N., JACOBSON, K.A., NAWRATH, H. & SHAINBERG, A. (2001). Cardioprotective effects of adenosine A_1 and A_3 receptor activation during hypoxia in isolated rat cardiac myocytes. *Mol. Cell. Biochem.*, **217**, 143–152.

SCHULTE, G. & FREDHOLM, B.B. (2000). Human adenosine A_1, A_{2A}, A_{2B} and A_3 receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. *Mol. Pharmacol.*, **58**, 477–482.

SEIDEL, M., KLINGER, M., FREISSMUTH, M. & HÖLLER, C. (1999). Activation of mitogen-activated protein kinase by A2A-adenosine receptor *via* a rap1-dependent and *via* a p21rea-dependent pathway. *J. Biol. Chem.*, **274**, 25833–25841.

SEXL, V., MANCUSI, G., HOLLER, C., GLORIA-MAECKER, E., SCHUTZ, W. & FREISSMUTH, M. (1997). Stimulation of mitogen-activated protein kinase *via* the A2A-adenosine receptor in primary human endothelial cells. *J. Biol. Chem.*, **272**, 5792–5799.

SHNEYVAYS, V., JACOBSON, K.A., LI, A.H., NAWRATH, H., ZINMAN, T., ISAAC, A. & SHAINBERG, A. (2000). Induction of apoptosis in rat cardiocytes by A_3 adenosine receptor activation and its suppression by isoproterenol. *Exp. Cell Res.,* **257,** 111–126.

SOMMERSCHILD, H.T. & KIRKEBOEN, K.A. (2000). Adenosine and cardioprotection during ischemia and reperfusion – an overview. *Acta Anaesthesiol. Scand.,* **44,** 1038–1055.

STRICKLER, J., JACOBSON, K.A. & LIANG, B.T. (1996). Direct preconditioning of cultured chick ventricular myocytes novel functions of cardiac adenosine A_{2a} and A_3 receptors. *J. Clin. Invest.,* **98,** 1773–1779.

TAKEDA, H., MATOZAKI, T., TAKADA, T., NOGUCHI, T., YAMAO, T., TSUDA, M., OCHI, F., FUKUNAGA, K., INAGAKI, K. & KASUGA, M. (1999). PI3-kinase γ and protein kinase C-ζ mediate RAS-independent activation of MAP kinase by a G_i protein-coupled receptor. *EMBO J.,* **18,** 386–395.

XU, H., STEIN, B. & LIANG, B. (1996). Characterization of a stimulatory adenosine A_{2A} receptor in adult rat ventricular myocyte. *Am. J. Physiol.,* **270,** H1655–H1661.