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Enhanced expression of G protein-coupled receptor kinase 2 selectively increases the sensitivity of A_{2A} adenosine receptors to agonist-induced desensitization

[1]Stuart J. Mundell, [1]Jason S. Luty, [1]Jon Willets, [2]Jeffrey L. Benovic & [1,3]Eamonn Kelly

[1]Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K. and [2]Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Philadelphia 19107, U.S.A.

1 G protein-coupled receptor kinases (GRKs) are thought to be important in mediating the agonist-induced phosphorylation and consequent desensitization of G protein-coupled receptor (GPCR) responses. We have previously shown that stable expression of a dominant negative mutant G protein-coupled receptor kinase 2 (GRK2) construct in NG108-15 mouse neuroblastoma x rat glioma cells suppresses the agonist-induced desensitization of A_{2A} and A_{2B} adenosine receptor-stimulated adenylyl cyclase activity (Mundell *et al*., 1997). To further determine the role of GRK2 in agonist-induced desensitization of these adenosine receptors, we stably overexpressed wild type GRK2 in NG108-15 cells.

2 In homogenates prepared from cells overexpressing GRK2, the acute stimulation of adenylyl cyclase by activation of A_{2A} and A_{2B} adenosine receptors was markedly reduced, but could be reversed by pretreating the cells with AD (adenosine deaminase), to remove extracellular adenosine from the medium. On the other hand, acute stimulation of adenylyl cyclase by secretin, iloprost, NaF and forskolin was the same in GRK2 overexpressing cells and plasmid-transfected control cells.

3 Cells overexpressing GRK2 were more sensitive to adenosine receptor agonist-induced desensitization than plasmid-transfected control cells. This effect was selective since the agonist sensitivity of desensitization for secretin and IP-prostanoid receptor-stimulated adenylyl cyclase activity was not affected by GRK2 overexpression.

4 These results further implicate GRK2 as the likely mechanism by which A_2 adenosine receptors undergo short-term desensitization in NG108-15 cells, and indicate that even when overexpressed, GRK2 retains its substrate specificity for native receptors in intact cells. Furthermore, the susceptibility of GPCRs to desensitization appears to depend on the level of GRK expression, such that in cells that express high levels of GRK2, low agonist concentrations may be sufficient to trigger GRK-mediated desensitization.

Keywords: G protein-coupled receptor; G protein-coupled receptor kinase; desensitization; A_2 adenosine receptor; NG108-15 cells

Introduction

The desensitization of GPCR responses following prolonged agonist treatment has been widely reported, and increasingly receptor phosphorylation is considered to play a major role in this phenomenon (Hausdorff *et al*., 1990). Agonist-induced phosphorylation of GPCRs is thought to occur as the result of activation of second messenger-regulated kinases such as cyclic AMP-dependent protein kinase (Clark *et al*., 1989), or by activation of a specific family of kinases, known as GRKs, which phosphorylate only the agonist-occupied form of the receptor and hence lead to homologous desensitization (Benovic *et al*., 1986). Once phosphorylated by GRKs, GPCRs become uncoupled from G-proteins, a process effected by another class of proteins called arrestins, which bind to the phosphorylated receptor and prevent coupling to the G-protein (Lohse *et al*., 1990). At present six members of the GRK family have been identified, termed GRK1 to GRK6 (reviewed in Haga *et al*., 1994; Premont *et al*., 1995; Sterne-Marr & Benovic, 1995; Ferguson *et al*., 1996; Chuang *et al*., 1996).

The NG108-15 mouse neuroblastoma x rat glioma hybrid cell line expresses both GRK2 and lower levels of GRK3 (Mundell *et al*., 1997). In the latter study we reported that when NG108-15 cells are stably transfected with a dominant negative mutant form of GRK2 (Kong *et al*., 1994), there is slower agonist-induced desensitization of A_{2A} and A_{2B} adenosine receptor-stimulated adenylyl cyclase activity. This suggests that the short term agonist-induced homologous desensitization of A_{2A} and A_{2B} adenosine receptor responsiveness is mediated by GRK2 or a closely related receptor kinase. Interestingly in the same study, the agonist-induced desensitization of secretin and IP-prostanoid receptor-stimulated adenylyl cyclase activity was unaffected in cells expressing dominant negative mutant GRK2. Thus in intact cells, the selectivity of GRKs for agonist-occupied receptor substrates may be greater than initially suggested by reconstitution experiments. In the present study we have further characterized A_2 adenosine receptor desensitization, this time in NG108-15 cells that overexpress wild-type GRK2. We find that increased GRK2 expression enhances the sensitivity of A_2 adenosine receptors to desensitization. Furthermore, selectivity of GRK2 action is retained at high levels of GRK2 expression, since the desensitization of other G_S-coupled responses is unaffected.

Methods

Cell culture and transfection with the GRK2 construct

NG108-15 neuroblastoma x glioma hybrid cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing

[3] Author for correspondence.

6% fetal calf serum, 100 units ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin, and supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. For culture of NG108-15 cells stably transfected with pMEP4 vector or pMEP4 containing the coding sequence for bovine GRK2, the above medium was supplemented with 200 $\mu g\ ml^{-1}$ hygromycin. For culture of NG108-15 cells stably transfected with pCMVneo vector or pCMVneo containing the dominant negative mutant form of GRK2 (Kong *et al*., 1994), the above medium was supplemented with 200 $\mu g\ ml^{-1}$ geneticin.

For stable overexpression of GRK2 in NG108-15 cells, the coding sequence of bovine GRK2 (Benovic *et al*., 1986) was expressed in the mammalian expression vector pMEP4 using standard techniques (Sambrook *et al*., 1989). Briefly, bovine GRK2 was excised from pBluescript using *Nhe*I and ligated into the *Nhe*I site of pMEP4 (Groger *et al*., 1989). The ligation mix was incubated overnight at 16°C and used to transfect XL1-blue *E. coli* by electroporation using a Biorad Gene Pulser according to the manufacturer's instructions. Bacterial colonies transfected with pMEP4 were then grown in the presence of 50 $\mu g\ ml^{-1}$ carbenecillin, and selected colonies expanded and the pMEP4 extracted and digested with *Nhe*I to confirm the presence of a $\approx$2 kbp insert. For one plasmid preparation, the 5′ and 3′ flanks of the GRK2 insert in pMEP4 were sequenced with a Genesis 2000 automatic sequencer to confirm the insertion of the GRK2 insert in the correct orientation. This was then used to transform NG108-15 cells using Lipofectamine. For transfection, the bovine GRK2 construct in pMEP4, or pMEP4 itself was linearized before mixing with Lipofectamine and incubated with plated cells according to the manufacturer's instructions (20 μg of DNA was incubated with 20 units *Cla*I and the linearized DNA mixed with DMEM and Lipofectamine before addition to cells in monolayer). After 5 h, 6% fetal calf serum was added to the medium and the cells left overnight. Next day this medium was replaced with normal complete medium and after a further day the medium was supplemented with 200 $\mu g\ ml^{-1}$ hygromycin. Surviving colonies were isolated and expanded into cell lines.

Western blotting

Cells were lysed by addition of 200–500 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.4, 200 mM NaCl, 10 mM EDTA, 1% Triton-X 100, 0.2 mg ml^{-1} benzamidine, 0.1 mg ml^{-1} leupeptin and 0.5 mM phenylmethylsulphonylfluoride) to cell monolayers. Insoluble cell fractions were then pelleted by centrifugation in a microcentrifuge at 13,000 r.p.m. and 4°C for 3 min. Aliquots of the supernatant were then snap-frozen in liquid nitrogen and stored at − 70°C. When required, 40 μg of cell lysate was added to sodium dodecyl sulphate (SDS) sample buffer (final loading concentration 63 mM Tris, pH 6.5, 100 mM dithiothreitol, 1% SDS, 11.6% glycerol and 0.02% bromophenol blue) and resolved by SDS–PAGE according to the method of Laemmli (1970). Recombinant GRK2 (Kim *et al*., 1993) was used as a standard. Protein was then transferred to nitrocellulose and incubated first with a monoclonal antibody that recognizes an epitope within residues 500–531 of the carboxy terminus of bovine GRK2 (Loudon *et al*., 1996), and then with a sheep anti-mouse Ig horseradish peroxidase linked secondary antibody, followed by enhanced chemiluminescence (ECL) detection according to the manufacturer's instructions.

Rhodopsin phosphorylation

Bovine rod outer segments were prepared and rhodopsin phosphorylation carried out exactly as previously described (Gagnon & Kelly, 1997). Briefly, for rhodopsin phosphorylation by cell lysate preparations, confluent cells from 75 cm^2 culture flasks were harvested and washed with 10 ml of ice-cold phosphate buffered saline (PBS). To the resultant cell pellet 0.5–1 ml of ice-cold lysis buffer was added (200 mM NaCl, 20 mM HEPES, pH 7.5, 10 mM EDTA, 1 mM dithiothreitol, 0.02% Triton X-100, 0.2 mg ml^{-1} benzamidine, 0.02 mg ml^{-1} leupeptin and 0.5 mM phenylmethylsulphonylfluoride) and the cells disrupted by homogenization with a polytron twice for 20 s pulses at maximum setting. The homogenates were then centrifuged in eppendorf tubes at 13,000 r.plm. at 4°C for 5 min, and the resulting supernatnat transferred to fresh tubes and placed on ice. Following protein assay, 2–3 μl of lysate was added to a solution containing (final assay concentrations) 20 mM Tris, pH 7.5, 2 mM EDTA, 5 mM Mg^{2+}, 1 mM ATP and 1 μCi [λ-^{32}P]-ATP. Then, under safe light illumination, rhodopsin was added to each tube to give a final rhodopsin concentration of 5 μM and a total incubation volume of 20 μl. Following incubation at 30°C under normal laboratory illumination (some tubes were incubated in the dark), the reaction was stopped by addition of 10 μl of 3 × SDS sample buffer (8% SDS, 50 mM Tris, pH 6.5, 10% glycerol, 5% mercaptoethanol, and 0.005% bromophenol blue) and the samples incubated for at least 30 additional min to denature the rhodopsin. The latter was then separated from other proteins by SDS–PAGE and phosphorylation of rhodopsin assessed by autoradiography of the dried gel.

Adenylyl cyclase assay

Where required, drugs were added directly to the culture medium for varying times. Cells were harvested in 10 ml of ice-cold PBS and pelleted by centrifugation at 200 × *g* for 1 min. The resulting pellets were washed twice in 10 ml of ice-cold PBS and frozen at −70°C until required. Adenylyl cyclase activity was measured by a protein binding assay (Brown *et al*., 1971). Cell pellets were thawed and homogenized in a glass dounce homogenizer containing ice-cold homogenization buffer (0.3 M sucrose, 25 mM Tris, pH 7.4). A 40 μl sample of homogenate was then added to 30 μl premix buffer (final assay concentration 50 mM Tris, pH 7.5, 5 mM Mg^{2+}, 1 mM ATP, 1 μM GTP, 250 μM 4-(3-Butoxy-4-methoxybenzyl) imidazolidin-2-one (Ro201724) as phosphodiesterase inhibitor, 20 mM creatine phosphate and 130 U ml^{-1} creatine phosphokinase) and 30 μl of drug at the relevant concentration. The tubes were incubated at 37°C for 10 min and the reaction terminated by the addition of 20 μl of 100% trichloroacetic acid and the tubes placed on ice for 10 min. Precipitated protein was pelleted by centrifugation at 2900 × *g* for 20 min at 4°C and 50 μl of the resulting supernatant added to 50 μl of 1 M NaOH and 200 μl of 50 mM Tris, pH 7.4, 4 mM EDTA (TE buffer); 50 μl of this solution was then added to fresh tubes containing 100 μl TE buffer, 100 μl [^{3}H]-cyclic AMP in TE buffer (about 20,000 c.p.m.) and 100 μl of binding protein in TE buffer (to give final concentration of $\sim$750 μg protein ml^{-1}; prepared from bovine adrenal cortex). Tubes containing 100 μl of standard concentrations of cyclic AMP (0.125–20 pmol) were used to construct a standard curve. After 2 h incubation at 4°C, 200 μl of TE buffer containing charcoal (Norit GSX; 50 mg ml^{-1} final concentration) and bovine serum albumin (2 mg ml^{-1} final concentration) were added and 15 min later the tubes were centrifuged at 2900 × *g* for 20 min at 4°C. The resulting supernatant was transferred into vials for liquid scintillation counting. Standard curve data were fitted to a logistic expression (Graphpad) and

the unknowns read off. Protein content of homogenates was determined (Bradford, 1976) and adenylyl cyclase activity expressed as pmol cyclic AMP min^{-1} mg^{-1} protein.

Whole cell cyclic AMP accumulation

NG108-15 cells were seeded in 12- or 24-well plates. On the experimental day, the cell culture medium was replaced with 0.5 ml of fresh culture medium 1–2 h before the experiment. 30 min before agonist addition, 250 μM Ro 201724 was added to each well. At time 0 agonist or vehicle was added to each well and the plates replaced in the cell incubator. At various time points after this 20 μl of 100% trichloroacetic acid was added to stop the reaction. Following completion of the experiment, cells were scraped from each well and the resulting mixture centrifuged at $2900 \times g$ for 20 min at 4°C. 50 μl of the resulting supernatant was then added to 50 μl of 1 M NaOH and 200–1000 μl of TE buffer. 50 μl of this solution was then assayed for cyclic AMP content as described in the previous section. Results from whole cell cyclic AMP assays were expressed as fold-increase over basal.

Experimental design and statistics

Where appropriate, concentration-effect curves or time courses of desensitization were analysed by the iterative fitting programme GraphPad Prism (GraphPad Software). Log concentration-effect curves were fitted to logistic expressions for single site analysis. Values of $t_{0.5}$ for agonist-induced desensitization were obtained by fitting data to a single exponential curve. Where appropriate, statistical significance of different values was assessed by Student's *t*-test or by 2-way ANOVA using the GraphPad InStat or Prism programmes.

Materials

[8-^{3}H]-cyclic AMP (925 GBq $mmol^{-1}$), [γ-^{32}P]-ATP (185 TBq $mmol^{-1}$), Hybond ECL nitrocellulose membrane, ECL detection kit, sheep anti-mouse Ig horseradish peroxidase linked secondary antibody and Hyperfilm-ECL luminescence detection film were all obtained from Amersham International plc. Cell growth medium, Lipofectamine, geneticin and restriction enzymes were from GIBCO BRL, hygromycin from Boehringer Mannheim Ltd., and 2-(*p*-carboxyethyl)phenylamino-5′N-carboxamidoadenosine (CGS-21680) and 8-(3-chlorostyrl)caffeine (CSC) from Research Biochemicals International. The protein assay reagent was from Pierce and Warriner. All other reagents and drugs were obtained from the Sigma Chemical Co.

Results

G protein-coupled receptor kinase 2 (GRK2) overexpression in NG108-15 cells

NG108-15 cells were transfected with plasmid vector alone (pMEP4), or with plasmid vector containing GRK2. After culture in medium containing 200 μg ml^{-1} hygromycin, surviving clones were isolated and expanded into cell lines. Two clones were identified (B4 and B7) that expressed high levels of GRK2 (respective levels of GRK2 around 20-fold and 30-fold that seen in plasmid-transfected cells; Figure 1a). Inclusion of 2.5 μM cadmium chloride in the growth medium for 24 h moderately enhanced GRK2 expression in B4 and B7 (data not shown), but since constitutive expression was already high, it was decided to use cells without cadmium chloride pretreatment for most of the subsequent experiments. In addition, one clone was identified (B5) that had moderately enhanced levels of GRK2 (around 3 fold over plasmid-transfected); in this clone cadmium chloride (2.5 μM, 24 h) increased GRK2 expression to between 5 and 10 fold that seen in plasmid-transfected controls controls (data not shown). Plasmid control transfected cells (named P1 and P5) expressed GRK2 levels similar to wild-type NG108-15 cells (Figure 1a). The ability of whole cell lysates to enhance phosphorylation of bovine rhodopsin was next examined. A small amount of light-dependent rhodopsin phosphorylation was observed using lysates from non-transfected or plasmid transfected cells. In contrast, lysates prepared from B7 cells exhibited much greater light-dependent rhodopsin phosphorylation (Figure 1b).

Acute activation of adenylyl cyclase activity in stably transfected cells

Initially, agonist-stimulated adenylyl cyclase activity was measured in homogenates from non-pretreated GRK2-over-expressing and pMEP4 cells. This indicated that there was no difference in basal or secretin, iloprost, NaF or forskolin-stimulated adenylyl cyclase activity between P1, P5, B4 and B7 cells (Figure 2). Acute A_{2A} and A_{2B} adenosine receptor-stimulated adenylyl cyclase activity was then assessed, using 3 μM 2-(*p*-carboxyethyl)phenylamino-5′N-carboxamidoadenosine (CGS-21680) for selective A_{2A} adenosine receptor activation, and 100 μM 5′-(N-ethylcarboxamido)-adenosine (NECA) in the presence of 1 μM 8-(3-chlorostyryl)caffeine (CSC) for selective A_{2B} adenosine receptor activation. We have shown previously (Mundell & Kelly, 1998), along with others

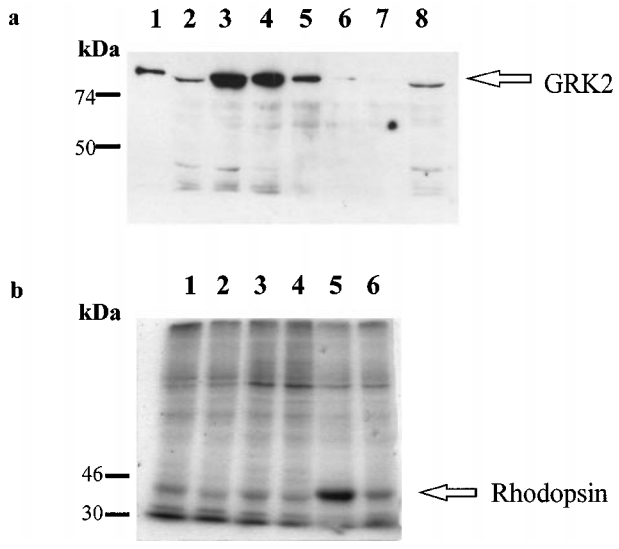

Figure 1 GRK2 overexpression in NG108-15 cells. (a) Whole cell lysates were subjected to SDS–PAGE followed by Western transfer and immunoblotting with a monoclonal antibody that recognizes GRK2. Lane 1, 20 ng of purified GRK2; Lane 2, 40 μg P1 plasmid control lysate; Lane 3, 40 μg B7 overexpressing cell lysate; Lane 4, 10 μg B7 overexpressing cell lysate; Lane 5, 2.5 μg B7 overexpressing cell lysate; Lane 6, 0.62 μg B7 overexpressing cell lysate; Lane 7, 0.31 μg B7 overexpressing cell lysate; Lane 8, 40 μg wild-type NG108-15 cell lysate. (b) Lysate preprations (30 μg) of NG108-15 cells were incubated with [λ-^{32}P]-ATP and bovine rhodopsin before being subjected to SDS–PAGE followed by autoradiography of the dried gel. Lane 1, wild type lysate (light); Lane 2, wild type lysate (dark); Lane 3, P5 lysate (light); Lane 4, P5 lysate (dark); Lane 5, B7 lysate (light); Lane 6, B7 lysate (dark).

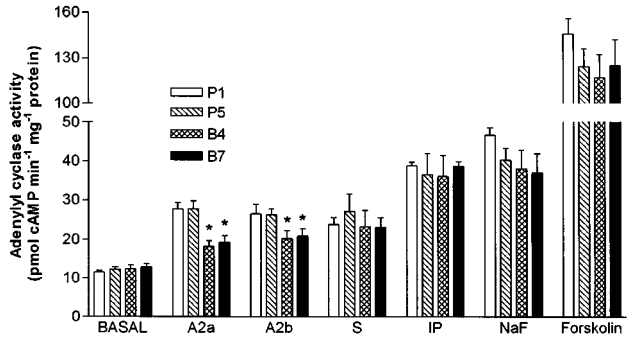

Figure 2 Acute adenylyl cyclase activity in pMEP4 plasmid transfected cells (P1 and P5) and GRK2-overexpressing (B4 and B7) NG108-15 cells. Whole cell homogenates from non-pretreated cells were incubated with or without (BASAL) agonist for 10 min. A_{2A} receptor-stimulated activity was assessed in the presence of 3 μM CGS-21680, A_{2B} in the presence of 100 μM NECA + 1 μM CSC. Other drug concentrations were 0.1 μM secretin (S), 1 μM iloprost (IP), 10 mM Sodium fluoride (NaF) and 10 μM forskolin. Values represent means ± s.e.mean from four separate experiments, each performed in triplicate. *$P<0.05$, Student's t-test, indicates values for A_{2A} and A_{2B} adenosine receptor-stimulated activity for B4 and B7 homogenates significantly lower than P5 and P1 cells.

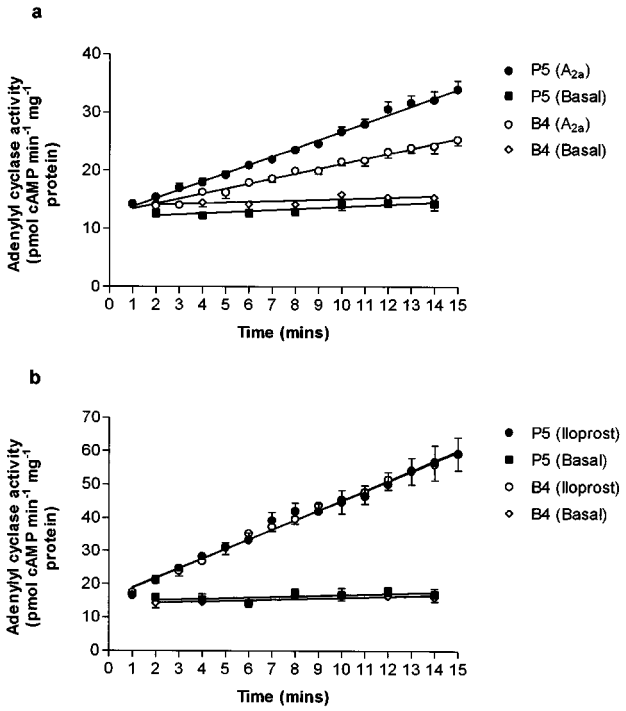

Figure 3 Time-dependent cyclic AMP accumulation in homogenates from GRK2-overexpressing (B4) and plasmid-transfected (P5) NG108-15 cells for (a) the A_{2A} adenosine receptor (3 μM CGS-21680) and (b) the IP-prostanoid receptor (1 μM Iloprost). In (a) and (b) values are means ± s.e.mean from five separate experiments. In (a) CGS-21680-stimulated cyclic AMP accumulation over time is significantly higher in plasmid control (P5) versus GRK2 over-expressing (B4) cells (2-way ANOVA, $P<0.05$).

(Alexander *et al*., 1996; Mirabet *et al*., 1997), that 3 μM CGS-21680 will activate A_{2A} and not A_{2B} adenosine receptors. A_{2B} adenosine receptor activity can be assessed using a combination of 100 μM NECA and 1 μM of the selective A_{2A} adenosine receptor antagonist CSC (Mundell & Kelly, 1998), since this concentration of CSC does not inhibit A_{2B} adenosine receptor mediated responses (Peters *et al*., 1998). Acute A_{2A} and A_{2B} adenosine receptor-stimulated adenylyl cyclase was markedly reduced in B4 and B7 cells when compared to P1 and P5 cells. For example, when compared to P1 cells, the percentage reduction in A_{2A} and A_{2B} adenosine receptor responsiveness in B7 cells was 64.5 ± 7.0 and 49.0 ± 12.5 respectively, whilst when compared to P5 cells, the percentage reduction in A_{2A} and A_{2B} adenosine receptor responsiveness in B4 cells was 59.5 ± 8.4 and 47.0 ± 9.4, respectively. To further characterize this effect, time courses of adenylyl cyclase activation in P5 and B4 cells were constructed (Figure 3). This indicated that A_{2A} adenosine receptor-stimulated adenylyl cyclase activity was linear up to 15 min in both P5 and B4 cells, but that A_{2A} adenosine receptor-stimulated activity was lower in B4 cells. No such difference was observed between P5 and B4 when iloprost-stimulated adenylyl cyclase activity was assessed (Figure 3b). Therefore, the A_{2A} adenosine receptors are probably already desensitized before agonist addition. Since these cells release adenosine into the medium (Nagy *et al*., 1989; Williams *et al*., 1993), it is possible that this is sufficient to cause A_{2A} adenosine receptor desensitization in GRK2-overexpressing cells. Therefore, P5 and B4 cells were preincubated with medium in the presence or absence of 0.5 units ml^{-1} adenosine deaminase (AD) for 24 h, to remove extracellular adenosine (this concentration of AD is sufficient to completely inhibit 100 μM adenosine-stimulated cyclic AMP accumulation in intact non-transfected NG108-15 cells; in non-pretreated cells, incubation with 100 μM adenosine for 30 min causes a 14.9 ± 1.1-fold increase in cyclic AMP accumulation over basal, whereas in cells pretreated with 0.5 units ml^{-1} AD for 5 min, incubation with 100 μM adenosine for 30 min did not increase cyclic AMP accumulation; 1.0 ± 0.1 fold; $n=4$). Following AD, concentration-response curves to CGS-21680 were constructed in cell homogenates prepared from these cells (Figure 4). AD pretreatment had no effect on CGS-21680-stimulated adenylyl cyclase activity in P5 cells, but markedly increased agonist-stimulated activity in B4 cells, almost to levels observed in P5 cells. This suggests that adenosine released into the medium from cells is sufficient to desensitize A_{2A} adenosine receptor responsiveness in GRK2-overexpressing but not pMEP4-transfected cells. Therefore, in order to be able to assess the rate and extent of A_{2A} adenosine receptor desensitization in GRK2-overexpressing cells, all further experiments were undertaken after pretreatment with AD for 24 h, unless otherwise indicated.

Agonist-induced desensitization of A_{2A} adenosine receptor-stimulated adenylyl cyclase in GRK2 overexpressing cells

Initially, P1 and B7 cells were incubated in the presence or absence of 10 μM NECA for up to 1 h in order to assess the rate of desensitization of A_{2A} adenosine receptor responsiveness (Figure 5a). This indicated that using this concentration of NECA, desensitization was moderately faster in B7 than P1 cells ($t_{0.5}$ for desensitization in B7 cells was 5 ± 4 min and for P1 cells 11 ± 5 min, the latter showing rates of desensitization similar to wild type cells; Mundell & Kelly, 1998). However, when a lower concentration of NECA was employed (0.1 μM), a marked difference in the rate of desensitization was observed between P1 and B7 cells (Figure 5b). Under these conditions the $t_{0.5}$ for desensitization of P1 cells was 58 ± 12 min, but that for B7 cells markedly faster at 16 ± 7 min. The maximum extent of desensitization, however, was the same irrespective of the presence of GRK2 overexpression and agonist pretreat-

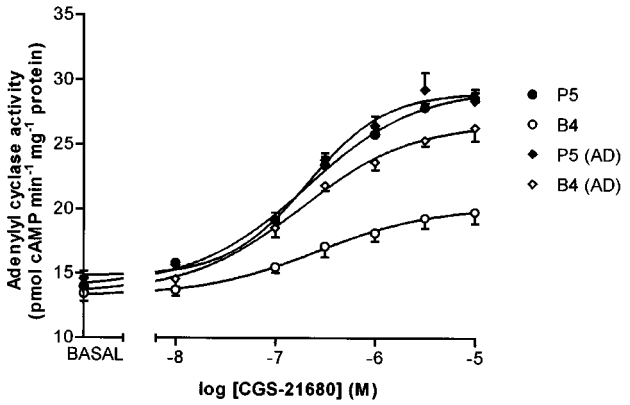

Figure 4 Increase following AD pretreatment (0.5 units ml^{-1}; 24 h) of acute A_{2A} adenosine receptor-stimulated (0.01–10 μM CGS-21680) adenylyl cyclase activity in GRK2-overexpressing (B4) but not plasmid transfected control cells (P5). Values are means ± s.e.mean from four separate experiments. CGS-21680-stimulated adenylyl cyclase activity was significantly higher in AD-pretreated B4 cells compared to B4 cells given no AD pretreatment (2-way ANOVA, $P<0.05$). EC_{50} values were as follows: P5 no AD 0.23 μM (0.12–0.32), P5 with AD 0.21 μM (0.14–0.31), B4 no AD 0.27 μM (0.07–0.93) and B4 with AD, 0.18 μM (0.10–0.31). Values in parentheses represent 95% confidence intervals.

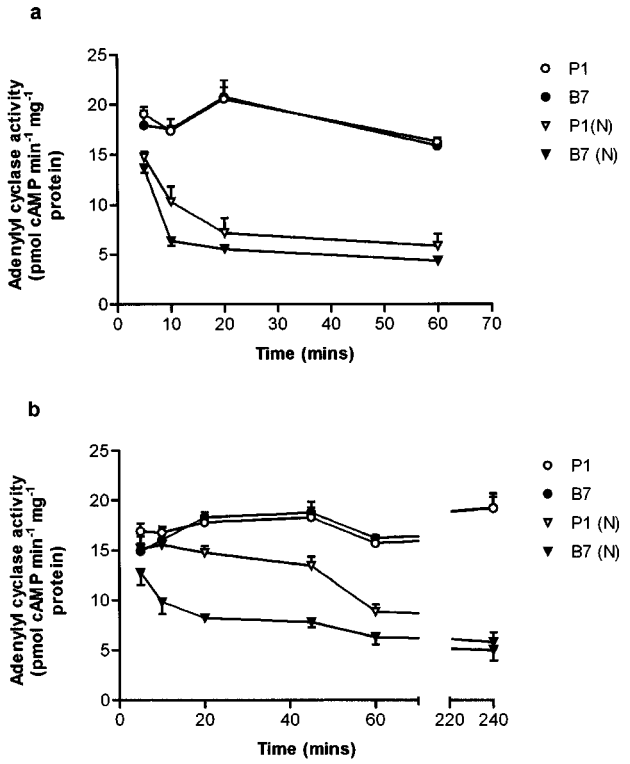

Figure 5 Time-course of desensitization of A_{2A} adenosine receptor-stimulated adenylyl cyclase activity in AD-pretreated (0.5 units ml^{-1}; 24 h) GRK2-overexpressing (B7) and plasmid transfected control (P1) cells. Cells were either given no NECA pretreatment or pretreated with (a) 10 μM or (b) 0.1 μM NECA for varying lengths of time. Subsequently A_{2A} (3 μM CGS-21680) receptor-stimulated adenylyl cyclase activity was measured. Values in (a) and (b) represent means ± s.e.mean from five separate experiments. The $t_{0.5}$ values for desensitization in 10 μM NECA-pretreated cells were 11 ± 5 min in P1 cells and 5 ± 4 min in B7 cells, and for 0.1 μM NECA-pretreated cells were 58 ± 12 min in P1 cells and 16 ± 7 min in B7 cells. In (b) desensitization was significantly greater for A_{2A} adenosine receptors in B7 compared to P1 cells pretreated with 0.1 μM NECA (2-way ANOVA, $P<0.05$).

ment concentration. Thus GRK2 overexpression markedly enhances the sensitivity of A_{2A} adenosine receptors to agonist-induced desensitization. To further quantify this effect, P1 and B7 cells were incubated with different concentrations of NECA for 20 min, and percentage desensitization assessed (Figure 6a). The EC_{50} for NECA-induced desensitization in P1 cells (1.1 μM) was similar to the EC_{50} of NECA for A_{2A} adenosine receptor-stimulated adenylyl cyclase activity in wild type cells (0.7 μM; Mundell & Kelly, 1998). However, the concentration-desensitization curve for NECA was 18 fold more potent in B7 cells. Interestingly, in a similar experiment, the concentration-desensitization curve for NECA was shifted 7 fold to the right in GRK2 dominant negative mutant expressing cells (D16) when compared to a plasmid control (P7; Figure 6b).

Relationship between level of GRK2 expression and extent of A_{2A} adenosine receptor desensitization

In order to determine the level of GRK2 overexpression that is necessary to alter the sensitivity of A_{2A} adenosine receptors to desensitization, experiments were performed using B5 cells, which overexpress GRK2 to a more moderate extent than B7 (around 3 fold as opposed to around 30 fold, respectively). In the absence of AD pretreatment, acute A_{2A} adenosine receptor-stimulated activity in B5 cells was not significantly reduced compared to P1 cells (Figure 7a), however activity was reduced when B5 cells were pretreated with cadmium chloride (2.5 μM; 24 h), which increases GRK2 overexpression in B5 cells to at least 5 fold that seen in P1 or wild type cells. Cadmium chloride pretreatment did not affect adenylyl cyclase activity in P1 cells (Figure 7a). AD pretreatment (0.5 units ml^{-1}; 24 h) completely reversed the reduction in acute A_{2A} adenosine receptor-stimulated adenylyl cyclase activity seen in B7 cells, or B5 cells pretreated with cadmium (compare Figure 7a, b). Finally, the relative degree of agonist-induced desensitization was assessed in P1, B7 and B5 cells. Cells were pretreated with AD (0.5 units ml^{-1}; 24 h) before being further incubated with or without 1 μM NECA for 20 min. In subsequent homogenates, 3 μM-CGS21680-stimulated adenylyl cyclase activity was assessed. Some A_{2A} adenosine receptor desensitization was observed in P1 cells (Figure 7c), but the level of desensitization was not greater in B5 cells, unlike B7 cells. However, cadmium chloride pretreatment of B5 cells led to a significantly greater desensitization of the A_{2A} adenosine receptor response in B5 than P1 cells (Figure 7c).

Desensitization of cyclic AMP accumulation in intact NG108-15 cells

In addition to adenylyl cyclase activity in cell homogenates, desensitization of G_S-coupled receptor responses was also assessed in intact GRK2-overexpressing cells. Cells grown in multi-well plates were incubated with agonist for varying lengths of time before cyclic AMP content was assessed. In response to 100 μM NECA, reflecting activation of both A_{2A} and A_{2B} adenosine receptors, cyclic AMP accumulation in P5 cells increased rapidly before levelling off after 10–20 min (Figure 8a). Preincubation with AD did not affect NECA-stimulated cyclic AMP accumulation in P5 cells. On the other hand, NECA-stimulated cyclic AMP accumulation in B5 cells was markedly less than in P5 cells, whether or not the cells were preincubated with AD. However, cyclic AMP accumulation was higher in B4 cells with AD pretreatment when compared to B4 cells not pretreated with this agent. Thus the difference between P5 and B4 curves following AD probably

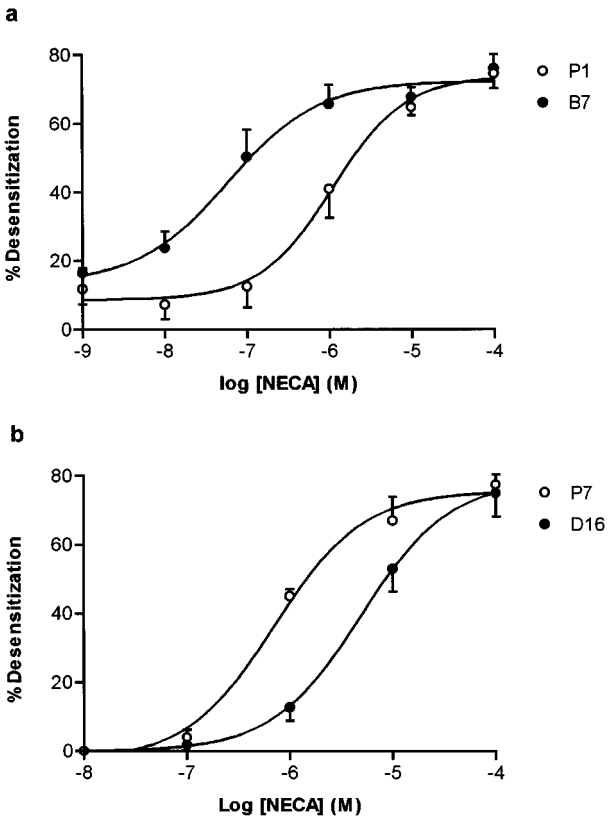

Figure 6 Concentration-dependency of agonist-mediated desensitization of adenosine A_{2A} receptor-stimulated adenylyl cyclase activity in AD pretreated (0.5 units ml^{-1}; 24 h) NG108-15 cells. (a) P1 or B7 cells were pretreated with various concentrations of NECA for 20 min before harvesting and washing. Subsequent cell homogenates were challenged with 3 μM CGS-21680 and adenylyl cyclase activity assessed. (b) P7 or D16 cells (pCMVneo or GRK2 dominant negative mutant, respectively) were pretreated as above and 3 μM CGS-21680-stimulated adenylyl cyclase activity measured. Values represent means $\pm$ s.e.mean from five separate experiments in each case. EC_{50} values for desensitization with NECA for 20 min were for B7 cells, 0.06 μM (0.01–0.11), P1 cells 1.1 μM (0.7–1.5), P7 cells 0.7 μM (0.4–1.2) and for D16 cells 4.9 μM (2.5–9.7). Values in parentheses represent 95% confidence intervals. Percentage desensitization of adenylyl cyclase activity was calculated as: [(stimulated activity in non-desensitized cells – stimulated activity in desensitized cells)/stimulated activity in non-desensitized cells] $\times$ 100.

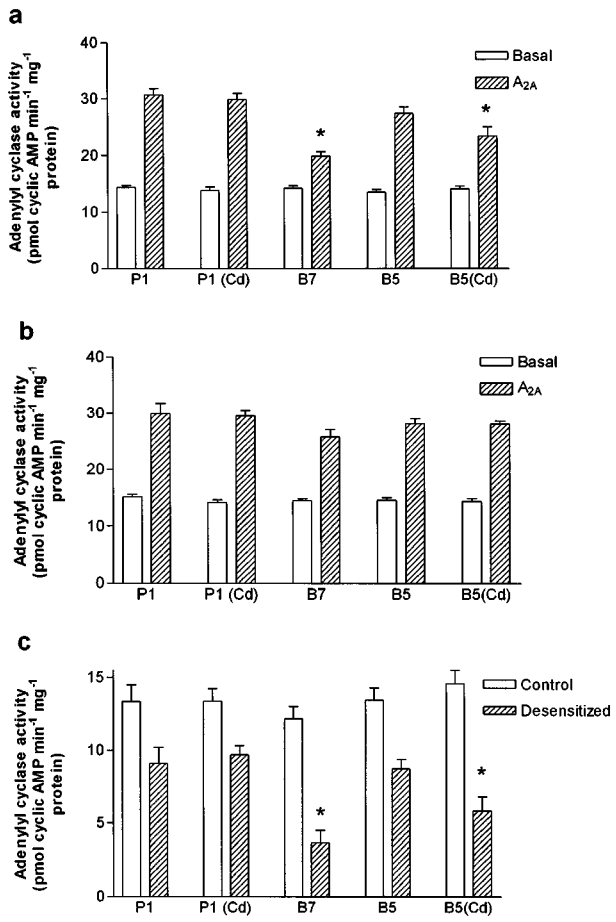

Figure 7 Sensitivity of A_{2A} adenosine receptors to desensitization is related to the level of GRK2 expression. (a) Whole cell homogenates of P1, B7 and B5 cells were incubated with (A_{2A}) or without (basal) 3 μM CGS-21680 for 10 min. In some cases, cells were pretreated with 2.5 μM cadmium chloride for 24 h (Cd). Values are means $\pm$ s.e.mean from five experiments. *$P<0.05$, Student's *t*-test, indicates 3 μM CGS-21680-stimulated values significantly lower than value for P1. (b) Whole cell homogenates of P1, B7 and B5 cells pretreated with AD (0.5 units ml^{-1}; 24 h) were incubated with (A_{2A}) or without (basal) 3 μM CGS-21680 for 10 min. In some cases, cells were pretreated with 2.5 μM cadmium chloride for 24 h (Cd). Values are means $\pm$ s.e.mean from five experiments. (c) Desensitization of A_{2A} adenosine receptor-stimulated adenylyl cyclase activity in P1, B7 and B5 cells. All cells were pretreated with AD (0.5 units ml^{-1}; 24 h), and then with (desensitized) or without (control) 1 μM NECA for 20 min. Subsequently 3 μM CGS-21680-stimulated adenylyl cyclase activity was measured in cell homogenates. Values are means $\pm$ s.e.mean from four experiments, and represent agonist-stimulated activity (ie. basal adenylyl, cyclase activity has been subtracted in each case). *$P<0.05$, Student's *t*-test, indicates 3 μM CGS-21680-stimulated values significantly lower than CGS-21680-stimulated value for desensitized P1.

gives a true reflection of enhanced desensitization of the combined A_2 receptor response due to GRK2 overexpression. Iloprost-stimulated cyclic AMP accumulation was also assessed in P5 and B4 cells, but no difference in accumulation was observed (Figure 8b).

Effect of GRK2 overexpression on desensitization of other G_S-coupled receptors

NG108-15 cells also express secretin and IP-prostanoid receptors coupled in a stimulatory fashion to adenylyl cyclase. It was thus of interest to see if GRK2 overexpression also affects agonist-induced desensitization of these receptor responses. P1 and B7 cells were pretreated with various concentrations of secretin or iloprost, and then homogenates prepared and challenged with secretin or iloprost respectively to assess desensitization. However, in marked contrast to A_{2A} adenosine receptor responsiveness, the loss of responsiveness to secretin (Figure 9a) or iloprost (Figure 9b) was the same in P1 and B7 cells. Thus the ability of GRK2 overexpression to enhance the sensitivity of A_{2A} adenosine receptors to desensitization is highly selective, since other G_S-coupled responses are not affected.

Discussion

This study provides further evidence to indicate that in intact cells, GRK2 represents an important mechanism whereby natively expressed A_{2A} adenosine receptors undergo desensitization. Recent studies show that canine A_{2A} adenosine

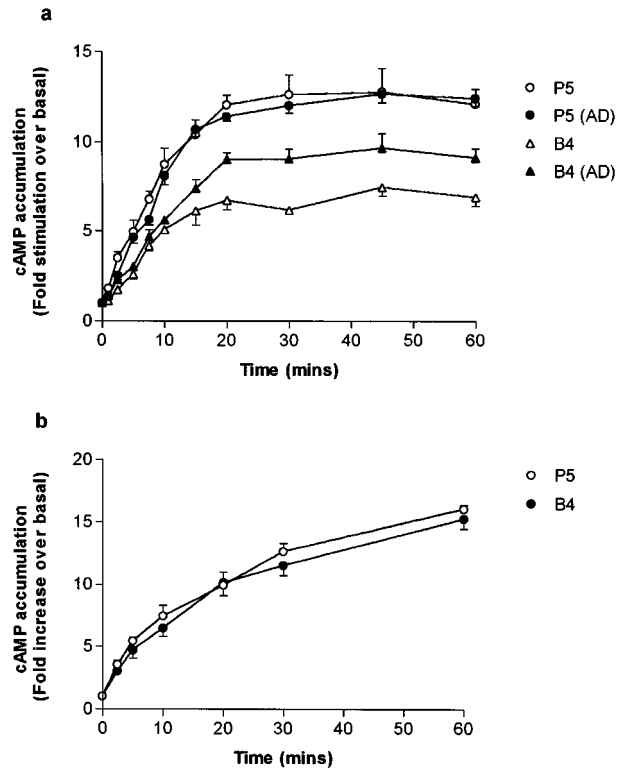

Figure 8 Cyclic AMP accumulation in intact P5 and B4 cells following addition of (a) 100 μM NECA, or (b) 1 μM iloprost. In (a), cells were pretreated in either the absence (open symbols) or presence (filled symbols) of AD (0.5 units ml^{-1}; 24 h). Cyclic AMP accumulation was lower in B4 than P5 cells whether or not B4 cells were pretreated with AD ($P<0.05$, 2-way ANOVA). Values represent means$\pm$s.e.mean from four to five separate experiments. Basal cyclic AMP values were as follows: (a) P5 cells no AD 31.6$\pm$6.8, P5 cells with AD 28.5$\pm$6.2, B4 cells no AD 35.6$\pm$5.8 and B4 cells with AD 32.8$\pm$4.3 pmol cyclic AMP mg^{-1} protein, and for (b) P5 cells 31.1$\pm$6.2 and B4 29.5$\pm$7.1 pmol cyclic AMP mg^{-1} protein.

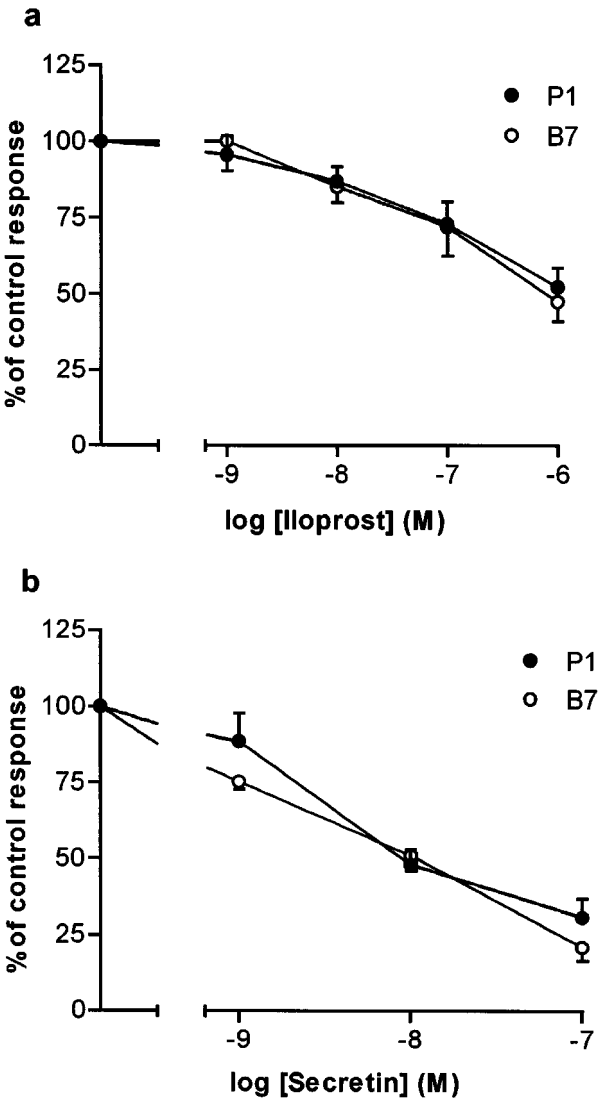

Figure 9 Lack of effect of GRK2 overexpression on agonist-mediated desensitization of (a) IP-prostanoid and (b) secretin receptor-stimulated adenylyl cyclase activity. P1 and B7 cells were pretreated with (a) iloprost (0.001 – 1.0 μM) for 1 h or (b) secretin (0.001 – 0.1 μM) for 5 min, before being harvested and washed. Subsequent cell homogenates were challenged with (a) 1 μM iloprost or (b) 0.1 μM secretin. The control response was that to 1 μM iloprost or 0.1 μM secretin in homogenates from P1 or B7 cells not pretreated with agonist. Control adenylyl cyclase activity in the presence of 1 μM iloprost was 162.8$\pm$43.7 and 170.1$\pm$27.0 pmol cAMP min^{-1} mg^{-1} protein for P1 and B7 cells respectively, and for 0.1 μM secretin was 31.6$\pm$2.5 and 32.9$\pm$2.0 pmol cAMP min^{-1} mg^{-1} protein for P1 and B7 cells respectively. Values in (a) and (b) represent means$\pm$s.e.mean from five or four separate experiments, respectively.

receptors stably expressed in Chinese Hamster Ovary cells undergo agonist-dependent phosphorylation and desensitization (Palmer *et al.*, 1994; Palmer & Stiles, 1997). Furthermore, since phosphorylation of the canine A_{2A} adenosine receptor is not induced by activation of the second messenger-dependent kinases PKA and PKC, then it seems likely that a GRK-like kinase mediates this effect (Palmer *et al.*, 1994). The present study complements previous work (Mundell *et al.*, 1997) and further supports a role for GRK2 in the desensitization of A_{2A} adenosine receptors.

We were initially surpised to find that in GRK2-overexpressing cells, acute stimulation of adenylyl cyclase by activation of A_{2A} and A_{2B} adenosine receptors was markedly reduced, whilst stimulation due to a range of other receptor and non-receptor agonists was unchanged. The time course of adenylyl cyclase activation by the selective A_{2A} adenosine receptor agonist CGS-21680 revealed that activation was linear for both P5 and B4 cells, suggesting that the A_{2A} adenosine receptors are already desensitized before addition of the agonist. This could be due to GRK-mediated phosphorylation of unoccupied A_{2A} adenosine receptors in overexpressing cells, or to agonist present in the growth medium occupying A_2 adenosine receptors and promoting phosphorylation in the GRK2-overexpressing cells. The former is possible since a number of GPCRs have been shown to undergo limited agonist-independent phosphorylation in the presence of high levels of GRKs (Diviani *et al.*, 1996). However, we (Williams *et al.*, 1993) and others (Nagy *et al.*, 1989) have shown that these cells release adenosine into the growth medium, which could be sufficient to promote A_2 adenosine receptor desensitization in GRK2-overexpressing cells. We have previously employed AD to remove extracellular adenosine in cultures of NG108-15 (Williams *et al.*, 1993), and hence pretreated cells with this agent to determine if adenosine was responsible for the lowered A_2 adenosine receptor-stimulated adenylyl cyclase activity in GRK2-overexpressing cells. The results clearly showed that removal of extracellular adenosine reversed the

loss of acute A_{2A} adenosine receptor function in GRK2 overexpressing cells, but had no effect on plasmid-transfected controls. Thus the NG108-15 cell growth medium contains sufficient adenosine to desensitize A_2 adenosine receptors when GRK2 expression is high, whereupon the A_2 adenosine receptors are more sensitive to agonist-induced desensitization. Given the lower sensitivity of A_{2B} adenosine receptors to agonist stimulation (Mundell & Kelly, 1998), it seems surprising that this subtype is desensitized in response to endogenous adenosine, but in the presence of GRK2 overexpression the concentration of extracellular adenosine is presumably high enough, and present for long enough, to promote desensitization of both A_2 adenosine receptor subtypes.

To determine the rate of desensitization of A_{2A} adenosine receptor responsiveness in GRK2-overexpressing cells and plasmid-transfected controls, cells were pretreated with AD for 24 h and then exposed to the adenosine receptor agonist NECA for varying lengths of time. When using a pretreatment concentration of NECA that would maximally activate A_{2A} adenosine receptors (Mundell & Kelly, 1998), the rate of desensitization of A_{2A} adenosine receptor-stimulated adenylyl cyclase activity was moderately higher in B7 than P5 cells. Given our findings with extracellular adenosine, it was reasoned that A_{2A} adenosine receptors may be much more sensitive to lower agonist concentrations, so accordingly cells were pretreated with 0.1 μM NECA, a concentration that will submaximally activate A_{2A} adenosine receptors in these cells (Mundell & Kelly, 1998). Under these conditions, desensitization was much more rapid in GRK2-overexpressing cells than plasmid-transfected controls, confirming the finding that overexpression of GRK2 lowers the agonist concentration requirement for desensitization. This was further confirmed by pretreating GRK2-overexpressing and plasmid control cells with different concentrations of NECA for a fixed period of time (20 min). Under these conditions the concentration of agonist pretreatment versus desensitization curve was shifted to the left in GRK2 overexpressing cells compared to control cells, and, interestingly, similar treatment of dominant negative mutant GRK2 cells revealed a shift of the curve to the right, indicating that these cells are less sensitive to A_{2A} adenosine receptor desensitization than controls. Experiments using the B5 clone, which overexresses GRK2 around 3 fold and between 5 and 10 fold in the absence and presence of cadmium chloride respectively, indicates that in NG108-15 cells at least a 5 fold increase in GRK2 is necessary in order to detect changes in the sensitivity of A_{2A} adenosine receptors to agonist-induced desensitization. Thus for a natively expressed GPCR, sensitivity to agonist-induced desensitization is highly dependent on the level of GRK2 expression and function. This is important since, for example, if GRKs are concentrated at synapses as has been reported (Arriza *et al*., 1992), then desensitization of receptor responses at synapses may occur at significantly lower concentrations of agonist than at other sites where GRKs are less well expressed. In addition, different cell lines express different levels of GRK2, which differentially affects β_2-adrenoceptor sequestrian (Ménard *et al*., 1997). Furthermore, increased expression of GRKs, as has been observed for example in the heart during cardiac failure (Ungerer *et al*., 1994; Choi *et al*., 1997), may have important effects on cardiac function, not least enhanced sensitivity of β-adrenoceptors to desensitization.

A question remains as to the exact nature of A_2 adenosine desensitization, since in the absence of ligand binding and receptor phosphorylation studies we cannot definitively conclude that the loss of responsiveness is due to receptor phosphorylation rather than the disappearance of A_{2A} adenosine receptors from the cell surface, or indeed that plasma membrane surface A_{2A} receptor expression is the same in GRK2 overexpressing and control cells. Thus the enhanced desensitization in GRK2-overexpressing cells may be due to increased loss of receptors from the cell surface compared to plasmid transfected controls. As discussed previously (Mundell *et al*., 1997; Mundell & Kelly, 1998), ligand binding to adenosine A_2 adenosine receptors is extremely difficult due to the presence of a high capacity non-receptor binding site for these ligands in many cell types (Hutchison *et al*., 1990). Attempts to label the A_{2A} adenosine receptor in NG108-15 cells with the agonist ligands [^{3}H]-NECA (Keen *et al*., 1989) and [^{3}H]-CGS-21680 (S.P.H. Alexander, personal communication) have proven unsuccessful. At present A_{2A} adenosine receptor binding studies are only realistic in cells expressing high levels of endogenous receptor, or cells transfected with high levels of receptor (Palmer & Stiles, 1997). However, it is very unlikely that receptor internalization accounts for the loss of responsiveness, since we (Mundell & Kelly, 1997) and others (Palmer *et al*., 1994) find that the rapid desensitization of A_{2A} adenosine receptor responsiveness is completely unaffected by inhibitors of internalization, whereas resensitization is totally blocked by these agents. Furthermore, down regulation of A_{2A} adenosine receptors only appears to occur after many hours of agonist treatment (Palmer *et al*., 1994).

A number of studies have now demonstrated that co-expression of GPCRs and GRKs leads to marked agonist-dependent phosphorylation of the receptor and desensitization of the receptor response (Diviani *et al*., 1996; Oppermann *et al*., 1996). On the other hand there have been few studies where the effects of manipulating GRK levels on desensitization of natively expressed receptors has been investigated. This is an important issue since it cannot be assumed that receptors expressed at a very high level in a particular cellular environment will faithfully reflect the behaviour of a natively-expressed receptor. Transgenic mice with cardiac-specific overexpression of GRK2 (Koch *et al*., 1995) or GRK5 (Rockman *et al*., 1996) display reduced basal and isoprenaline-stimulated adenylyl cyclase activity in cardiac tissue compared to control, presumably due to desensitization of receptors by endogenous noradrenaline and adrenaline in the presence of increased GRK expression. These results reflect our findings to some extent, although we observed no change in basal adenylyl cyclase activity in NG108-15 cells overexpressing GRK2, but this may reflect the high expression of β-$_1$-adrenoceptors in cardiac tissue (and hence significant receptor-G_S coupling in the presence of low agonist concentrations). In an earlier study to assess the effects of GRK2 overexpression, Chinese Hamster Ovary cells were stably transfected to express both β_2-adrenoceptors and GRK2 (Pippig *et al*., 1993). This work reported a modest increase in the desensitization of β_2-adrenoceptor-stimulated adenylyl cyclase activity following GRK2 overexpression, but the cells were only pretreated with a high concentration of isoprenaline (10 μM; maximal for adenylyl cyclase activation). It is possible that a more pronounced effect on desensitization would have been evident if lower agonist concentrations had been used in pretreatment, as we discovered in the present work.

Apart from our own studies in NG108-15 cells (Keen *et al*., 1989; Mundell *et al*., 1997; Mundell & Kelly, 1998), A_2 adenosine receptor desensitization has been characterized in a number of other cell types, including rat aortic vascular smooth cells (Anand-Srivastava *et al*., 1989), bovine aortic endothelial cells (Luty *et al*., 1989), rat phaeochromocytoma PC-12 cells (Chern *et al*., 1995) and Chinese Hamster Ovary

cells (Palmer & Stiles, 1997). In intact tissues functional A_2 adenosine receptor desensitization has been reported in rat nucleus tractus solitarius (Barracco *et al*., 1996) and porcine coronary artery (Makujina & Mustafa, 1993). In most of these cases the mechanisms underlying desensitization were not investigated, but it will be interesting in the future to assess the involvement of GRKs in A_2 adenosine receptor desensitization in these cell types and tissues.

When NG108-15 cells express dominant negative mutant GRK2, there is a selective reduction in A_2 adenosine receptor desensitization, that due to prolonged activation of secretin and IP-prostanoid receptors being unaffected (Mundell *et al*., 1997). In the same way overexpression of GRK2 selectively enhanced desensitization of A_2 adenosine receptor responsiveness without affecting that to secretin or the IP-prostanoid receptor agonist iloprost. This is somewhat surprising since we expected the selectivity of GRK2 to be reduced by overexpression. This result therefore suggests that either secretin and IP-prostanoid receptors are not substrates for GRK2, or that the cellular environment of NG108-15 cells prevents such an interaction, or that these G_S-coupled receptors are phosphorylated but that this does not affect desensitization. As far as we are aware, these two receptors have not been tested as substrates for GRK phosphorylation, although the human IP-prostanoid receptor is a substrate for protein kinase C-mediated phosphorylation (Smyth *et al*., 1996) and the secretin receptor is phosphorylation by unknown kinases in an agonist-dependent manner (Ozcelebi *et al*., 1995). However, we and others have shown that agonist-induced desensitization of IP-prostanoid responsiveness in NG108-15 cells is mediated by concurrent loss of IP-prostanoid receptor and $G_{S\alpha}$ (Williams & Kelly 1994; Adie *et al*., 1992) whereas secretin receptor desensitization in Chinese Hamster Ovary cells is dependent mainly upon phosphorylation-independent internalization of the receptor (Holtmann *et al*., 1996). Interestingly, in the transgenic studies discussed above (Koch *et al*., 1995; Rockman *et al*., 1996), cardiac-specific overexpression of GRK2 inhibited β-adrenoceptor and angiotensin II responses, whereas overexpression of GRK5 reduced only the response to β-adrenoceptor activation. Also Kong *et al*. (1994) showed that expression of dominant negative mutant GRK2 in human bronchial epithelial cells selectively reduced agonist-induced desensitization of β_2-adrenoceptor-stimulated but not prostaglandin E receptor-stimulated cyclic AMP accumulation. Thus in relation to native receptors in intact cells, GRKs appear to exert high levels of substrate selectivity.

In conclusion, this study demonstrates that overexpression of GRK2 selectively enhances the sensitivity of natively expressed adenosine A_2 adenosine receptors to agonist-induced desensitization. It will be of interest in future to overexpress other GRKs and respective dominant negative mutants in NG108-15 cells in order to assess changes in sensitivity of adenosine A_2 adenosine receptors and other GPCRs to desensitization. In addition, it will be important to determine whether GRK2-mediated phosphorylation is a universal mechanism of homologous A_2 adenosine receptor desensitization, or whether the desensitization of A_2 adenosine receptor responses in different cell types is subject to variations in cellular expression of GRKs or other components of desensitization.

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