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A Dominant Negative Mutant of the G Protein-Coupled Receptor Kinase 2 Selectively Attenuates Adenosine A_2 Receptor Desensitization

STUART J. MUNDELL, JEFFREY L. BENOVIC, and EAMONN KELLY

Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK (S.J.M., E.K.), and Department of Biochemistry and Molecular Pharmacology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 (J.L.B.).

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SUMMARY

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 responses. NG108–15 mouse neuroblastoma X rat glioma cells express a wide range of G protein-coupled receptors and significant levels of GRK2. Therefore, to determine the role of GRK2 in agonist-induced desensitization of various G_s-coupled receptors in NG108–15 cells, we stably transfected cells with a dominant negative mutant GRK2 construct (Lys220Arg). In homogenates prepared from cells overexpressing the dominant negative mutant GRK2, the acute stimulation of adenylyl cyclase by various receptor and nonreceptor agonists was the same as in control cells stably transfected with plasmid only. NG108–15 cells express both A_{2a} and A_{2b} adenosine receptors, which mediate activation of adenylyl cyclase, with both of these responses being subject to agonist-induced desensitization with a $t_{1/2}$ of 15–20 min. In dominant negative mutant GRK2 cells, the rates of desensitization of A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase were markedly slower than in plasmid transfected controls, with the latter being similar to wild-type cells. After a 20-min treatment with an adenosine agonist, the desensitization of A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase in dominant negative mutant GRK2 cells was less than half that seen in plasmid transfected control cells. On the other hand, the agonist-induced desensitization of secretin and IP-prostanoid receptor-stimulated adenylyl cyclase was the same in dominant negative mutant GRK2 cells as in plasmid transfected control cells. These results indicate that in intact cells, GRK2 may mediate the desensitization of adenosine A_2 receptors. Furthermore, there seems to be selectivity of GRK2 action between G_s-coupled receptors because the agonist-induced desensitization of secretin and IP-prostanoid receptor-stimulated adenylyl cyclase was not affected by dominant negative mutant GRK2 overexpression.

Agonist-induced phosphorylation is thought to play a major role in the desensitization of G protein-coupled receptor responses (1). Receptor phosphorylation generally occurs as the result of activation of second messenger-regulated kinases such as cAMP-dependent protein kinase (2) or through activation of a specific family of kinases, known as GRKs, that phosphorylate only the agonist-occupied form of the receptor and hence lead to homologous desensitization (3). Once phosphorylated by GRKs, G protein-coupled receptors become uncoupled from G proteins, a process that is effected by another class of proteins called arrestins, which bind to the phosphorylated receptor and prevent coupling to the G protein (4). At present, six members of the GRK family have been identified: GRK1 through GRK6 (for a review, see Refs. 5–7). GRK1, also known as rhodopsin kinase, is mainly confined to retinal cells (8), whereas GRK2, also known as β-adrenergic receptor kinase-1, is widely distributed. The other GRKs also seem to be widely distributed, except GRK4, whose expression is mainly confined to the testes (9).

An important question relates to the specificity of action of these kinases. In reconstitution studies, these kinases are generally able to phosphorylate a range of agonist-occupied G protein-coupled receptors (10, 11), but much less is known about the selectivity of GRKs for native receptors in intact cells. In a recent study, this was addressed by stably expressing a dominant negative mutant form of GRK2 in human bronchial epithelial cells (12). The mutant GRK2 contained a Lys220Arg mutation in the catalytic region of the enzyme that blocks its kinase activity and in a reconstitution assay was able to inhibit the ability of wild-type GRK2 to phosphorylate the β_2-adrenoceptor. In the intact cells, the dominant negative mutant GRK2 was found to suppress desensitiza-

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ABBREVIATIONS: GRK, G protein-coupled receptor kinase; DNM GRK2, dominant negative mutant G protein-coupled receptor kinase 2; NECA, 5′-(*N*-ethylcarboxamido)-adenosine; CSC, 8-(3-chlorostyryl)caffeine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TE, Tris/EDTA; SDS, sodium dodecyl sulfate; ECL, enhanced chemiluminescence.

tion of β_2-adrenoceptor-stimulated cAMP formation but not that stimulated by prostaglandin E_1 (12). Thus, in intact cells, the selectivity of GRKs for agonist-occupied receptor substrates may be greater than initially suggested in reconstitution experiments.

NG108–15 mouse neuroblastoma X rat glioma cells express at least four different G_s-coupled receptors, all of which have been shown to desensitize, including the adenosine A_{2a} and A_{2b} receptors (13), secretin receptors (14), and IP-prostanoid receptors (15). These cells therefore represent an interesting model system in which to further explore the involvement of GRKs in desensitization of native receptors in intact cells. Initially, we established that NG108–15 cells express significant levels of GRK2 (16). Then, to assess a possible role for GRK2 in the desensitization of these G_s-coupled receptors, we stably overexpressed dominant negative mutant GRK2 in NG108–15 cells. Our results indicate that in these cells, GRK2 is selectively involved in the agonist-induced desensitization of adenosine A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase but not in the agonist-induced desensitization of secretin or IP-prostanoid receptor-stimulated adenylyl cyclase activity.

Experimental Procedures

Reagents. [8-^{3}H]cAMP (925 GBq/mmol), Hybond ECL nitrocellulose membrane, ECL detection kit, sheep anti-mouse and donkey anti-rabbit immunoglobulin horseradish peroxidase-linked secondary antibodies, and Hyperfilm ECL detection film were obtained from Amersham International (Buckinghamshire, UK). Cell growth medium, Lipofectamine, and *Hin*dIII were from GIBCO BRL (Paisley, UK), and CGS-21680 and CSC were from Research Biochemicals Inc (Natick, MA). The protein assay reagent was from Pierce and Warriner (Rockford, IL). All other reagents and drugs were obtained from Sigma Chemical (Poole, Dorset, UK).

Cell culture and transfection. NG108–15 neuroblastoma X glioma hybrid cells were cultured in Dulbecco's modified Eagle's medium containing 6% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and supplemented with 1 μM aminopterin, 100 μM hypoxanthine, and 16 μM thymidine. For transfection, the dominant negative mutant GRK2 construct in pCMVneo (12) or pCMVneo itself (17) was linearized before being mixed with Lipofectamine and incubated with plated cells according to the manufacturer's instructions. Briefly, 20 μg of DNA was incubated with 20 units of *Hin*dIII, and the linearized DNA was mixed with Dulbecco's modified Eagle's medium and Lipofectamine before addition to cells in monolayer. After 5 hr, 6% fetal calf serum was added to the medium, and the cells were left overnight. On the next day, this medium was replaced with normal complete medium; after an additional day, the medium was supplemented with 200 μg/ml geneticin. Surviving colonies were isolated and expanded into cell lines.

Western blotting. Cells were lysed by the 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 benzamidine, 0.1 mg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride) to cell monolayers. Insoluble cell fractions were then pelleted by centrifugation in a microcentrifuge at 13,000 rpm and 4° for 3 min. Aliquots of the supernatant were then snap-frozen in liquid nitrogen and stored at −70°. When required, 40 μg of cell lysate was added to SDS sample buffer (final loading concentration, 63 mM Tris, pH 6.5, 100 mM dithiothreitol, 1% SDS, 11.6% glycerol, and 0.02% bromphenol blue) and resolved by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (18). Recombinant GRK2 or GRK3 (19) was used as standard. Protein was then transferred to nitrocellulose and incubated first with a monoclonal antibody that recognizes an epitope within residues 500–531 of the carboxyl terminus of bovine GRK2 (20) or a polyclonal antibody that recognizes GRK3 (raised against a fusion protein containing amino acids 648–671 of the carboxyl terminus of bovine GRK3) and then with a sheep anti-mouse or a donkey anti-rabbit immunoglobulin horseradish peroxidase-linked secondary antibody respectively, followed by ECL detection according to the manufacturer's instructions.

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 phosphate-buffered saline and pelleted by centrifugation at 200 × *g* for 1 min. The resulting pellets were washed twice in 10 ml of ice-cold phosphate-buffered saline and frozen at −70° until use. Adenylyl cyclase activity was measured by a protein binding assay (21). 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 of premix buffer (final assay concentration, 50 mM Tris, pH 7.5, 5 mM Mg^{2+}, 1 mM ATP, 1 μM GTP, 250 μM Ro201724 [4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one] as phosphodiesterase inhibitor, 20 mM creatine phosphate, and 130 units/ml creatine phosphokinase) and 30 μl of drug at the relevant concentration. The tubes were incubated at 37° for 10 min, and the reaction terminated by the addition of 20 μl of 100% trichloroacetic acid; the tubes placed on ice for 10 min. Precipitated protein was pelleted by centrifugation at 2900 × *g* for 20 min at 4°, and 50 μl of the resulting supernatant was added to 50 μl of 1 M NaOH and 200 μl of TE buffer (50 mM Tris, 4 mM EDTA, pH 7.4); 50 μl of this solution was then added to fresh tubes containing 100 μl of TE buffer, 100 μl of [^{3}H]cAMP in TE buffer (~20,000 cpm), and 100 μl of binding protein in TE buffer (to give final concentration of ~750 μg of protein/ml; prepared from bovine adrenal cortex). Tubes containing 50 μl of standard concentrations of cAMP (0.125–20 pmol) were used to construct a standard curve. After a 2-hr incubation at 4°, 200 μl of TE buffer containing charcoal (Norit GSX; 50 mg/ml final concentration) and bovine serum albumin (2 mg/ml final concentration) was added; 15 min later, the tubes were centrifuged at 2900 × *g* for 20 min at 4°. The resulting supernatant was transferred into vials for liquid scintillation counting. Standard curve data were fitted to a logistic expression (GraphPAD Software, San Diego, CA), and the unknowns were read off. Protein content of homogenates was determined (22), and adenylyl cyclase activity was expressed as pmol of cAMP/min/mg of protein.

Whole-cell cAMP accumulation. NG108–15 cells were seeded onto 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 hr before the experiment. Thirty minutes before agonist addition, 250 μM Ro201724 was added to each well. At time 0, agonist or vehicle was added to each well; at various time points afterward, 20 μl of 100% trichloroacetic acid was added to stop the reaction. After completion of the experiment, cells were scraped from each well. and the resulting mixture was centrifuged at 2900 × *g* for 20 min at 4°. Fifty microliters of the resulting supernatant was then added to 50 μl of 1 M NaOH and 200-1000 μl of TE buffer. Fifty microliters of this solution was then assayed for cAMP content as described above. Results from whole-cell cAMP assays were expressed as fold-increase over basal.

Experimental design and statistics. Where appropriate, concentration-effect curves were analyzed by the iterative fitting program GraphPAD InPlot and GraphPAD Prism (GraphPAD Software). Log concentration-effect curves were fitted to logistic expressions for single-site analysis. Where appropriate, statistical significance of different values was assessed by Student's *t* test or by two-way ANOVA using the GraphPAD InStat or Prism program.

Results

Dominant negative mutant GRK2 (DNM GRK2) overexpression in NG108–15 cells. In whole-cell lysate preparations of NG108–15 cells, GRK2 was present at ~50 ng/mg

whole-cell protein (Fig. 1A). Cells were transfected with plasmid vector alone (pCMVneo) or with plasmid vector containing DNM GRK2 (Lys220Arg). After culture in medium containing geneticin (200 μg/ml), surviving clones were isolated and expanded into cell lines. Of 25 clones screened for mutant GRK2 overexpression, 3 were used (named D16, D19, and D28) that expressed high levels of the mutant GRK2 (Fig. 1A). Mutant GRK2 had the same electrophoretic mobility as wild-type GRK2. From titration experiments (data not shown), we estimate that DNM GRK2 expression was >60-fold that of wild-type GRK2 in D16 and D28 cells and ~30-fold greater in D19 cells. The relatively low levels of GRK3 in NG108–15 cells (~10 ng/mg of whole-cell protein) were not altered by DNM GRK2 overexpression (Fig. 1B). Plasmid control transfected cells (named P7 and P10) expressed GRK2 levels similarly to wild-type NG108–15 cells (Fig. 1A). Initially, agonist-stimulated adenylyl cyclase activity was measured in homogenates from nonpretreated DNM GRK2 and pCMVneo cells. This indicated that there was no difference in basal or acute agonist-stimulated adenylyl cyclase activity between DNM GRK2 and pCMVneo cells (Fig. 2).

Agonist-induced desensitization of A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase in DNM GRK2 cells. NG108–15 cells express adenosine A_{2a} and A_{2b} receptors, both of which desensitize in response to agonist with a $t_{1/2}$ of 15–20 min (13); we therefore investigated whether the

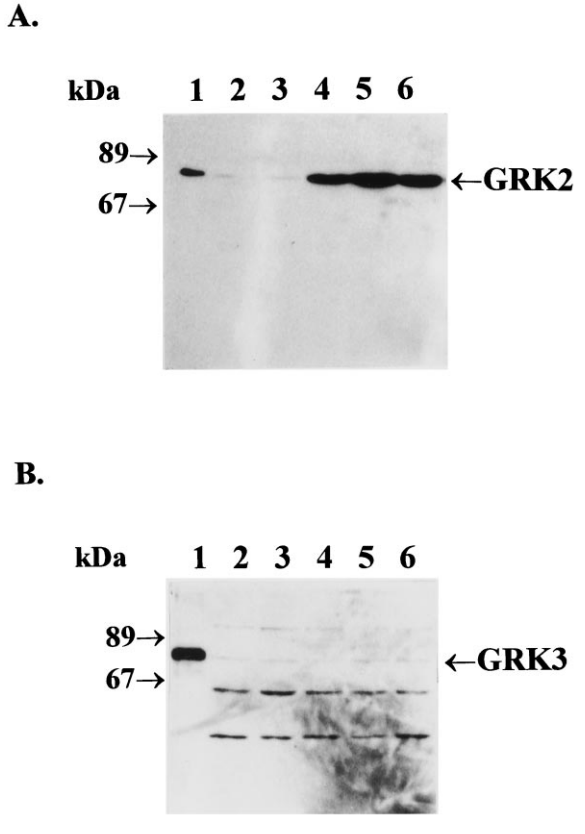

Fig. 1. DNM GRK2 overexpression in NG108–15 cells. Whole-cell lysates (40 μg) were subjected to SDS-polyacrylamide gel electrophoresis followed by Western transfer and immunoblotting with a monoclonal antibody that recognizes GRK2 and DNM GRK2 (A) or a polyclonal antibody that recognizes GRK3 (B). *Lanes 1*, 10 ng of purified GRK2 (A) or 3 ng of GRK3 (B). *Lanes 2*, wild-type NG108–15 lysate. *Lanes 3*, P7. *Lanes 4*, D19. *Lanes 5*, D16. *Lanes 6*, D28.

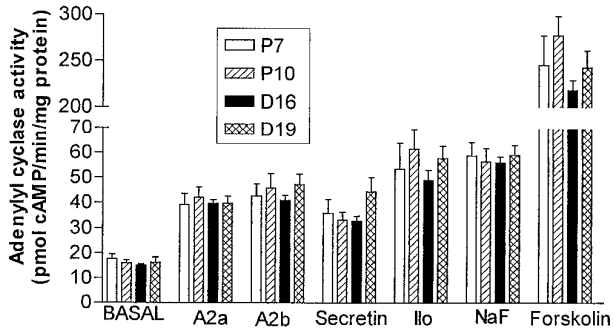

Fig. 2. Acute adenylyl cyclase activity in DNM GRK2 (D16 and D19) and pCMVneo (P7 and P10) NG108–15 cells. Whole-cell homogenates from nonpretreated cells were incubated with or without (*BASAL*) agonist for 10 min. A_{2a} receptor-stimulated activity was assessed in the presence of 3 μM CGS21680, and A_{2b} receptor-stimulated activity was assessed in the presence of 100 μM NECA plus 1 μM CSC. Other drug concentrations were 0.1 μM secretin, 1 μM iloprost (*Ilo*), 10 mM sodium fluoride (*NaF*), and 10 μM forskolin. Values represent mean ± standard error from four separate experiments, each performed in triplicate.

desensitization of these responses was altered in DNM GRK2 cells. Accordingly, DNM GRK2 and pCMVneo cells were pretreated with the A_{2a}/A_{2b} adenosine receptor agonist NECA for 30 min, and subsequent adenylyl cyclase assays performed on cell homogenates. For desensitization of A_{2a} and A_{2b} receptor responsiveness, cells were pretreated with 10 or 100 μM NECA, respectively, because we have previously shown these concentrations to produce maximal activation of each subtype (13). A_{2a} adenosine receptor-stimulated adenylyl cyclase activity was measured in the presence of 3 μM CGS21680 [2-(*p*-carboxyethyl)phenylamino-5′-*N*-carboxyamidoadenosine]. The acute stimulation of adenylyl cyclase by this concentration of CGS21680 is abolished by 1 μM concentration of the selective A_{2a} receptor antagonist CSC (stimulated activity with 3 μM CGS21680 alone was 16.6 ± 0.6 pmol of cAMP/min/mg of protein and with 3 μM CGS21680 in the presence of 1 μM CSC was 1.4 ± 0.5 pmol of cAMP/min/mg of protein, six experiments). Adenosine A_{2b} receptor-stimulated adenylyl cyclase activity was measured in the presence of 100 μM NECA plus 1 μM concentration of the selective A_{2a} receptor antagonist CSC. Pretreatment with 10 μM NECA for 30 min reduced A_{2a} receptor-stimulated adenylyl cyclase by 64 ± 3% and 57 ± 4% in P7 and P10 cells, respectively, but by only 41 ± 3% and 29 ± 4% in D16 and D19 cells (Fig. 3A; four experiments for each). Pretreatment with 100 μM NECA for 30 min reduced A_{2b} receptor-stimulated adenylyl cyclase by 60 ± 4% and 66 ± 5% in P7 and P10 cells but by only 39 ± 5% and 37 ± 5% in D16 and D19 cells (Fig. 3B; four experiments for each). In further experiments, clone D28 was compared with wild-type cells: A_{2a} desensitization was 55 ± 16% in wild-type cells but only 24 ± 9% in D28, and A_{2b} desensitization was 63 ± 15% in wild-type cells and 34 ± 9% in D28 (four experiments).

The time course of desensitization of A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase in response to NECA was next determined for P7 and D16 cells (Fig. 4). The time courses for desensitization of both responses in P7 were very similar to those we previously reported in wild-type cells ($t_{1/2}$ = 15–20 min; Ref. 13); however, the rate of desensitization of A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase in D16 cells (now >30 min) was much slower than that in P7 cells. For example, after a 20-min treatment with NECA, there was marked

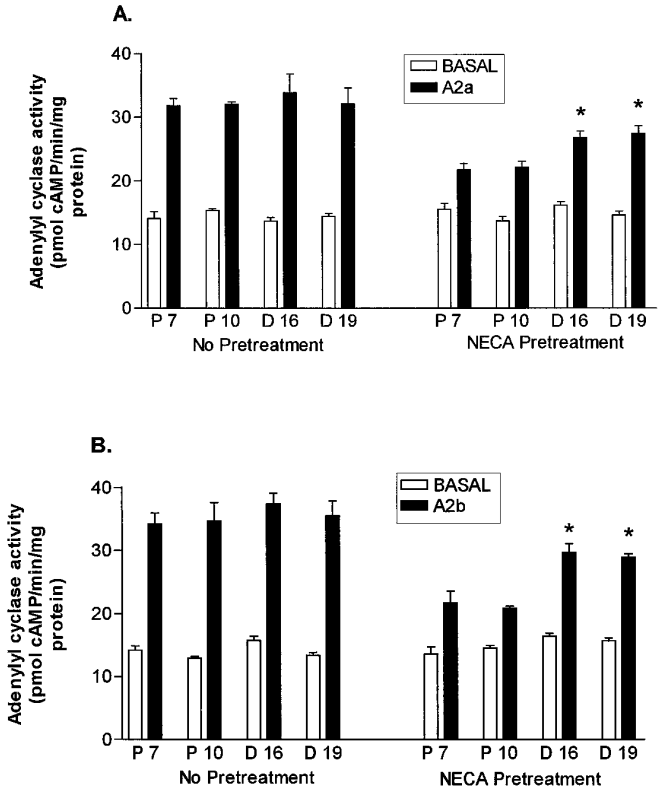

Fig. 3. Reduced desensitization of (A) A_{2a} and (B) A_{2b} receptor-stimulated adenylyl cyclase activity in DNM GRK2 NG108–15 cells. DNM GRK2 (D16 and D19) and pCMVneo (P7 and P10) cells received no pretreatment or were pretreated for 30 min with 10 μM (A) or 100 μM (B) NECA. Subsequent adenylyl cyclase assays were performed with no agonist addition (*BASAL*) or the addition of 3 μM CGS21680 for A_{2a} receptor stimulation (A) and 100 μM NECA plus 1 μM CSC for A_{2b} receptor stimulation (B). Values represent mean ± standard error from five separate experiments, each performed in triplicate. *, $p < 0.05$, Student's *t* test, values for D16 and D19 cells significantly different from those for P7 and P10 cells, respectively.

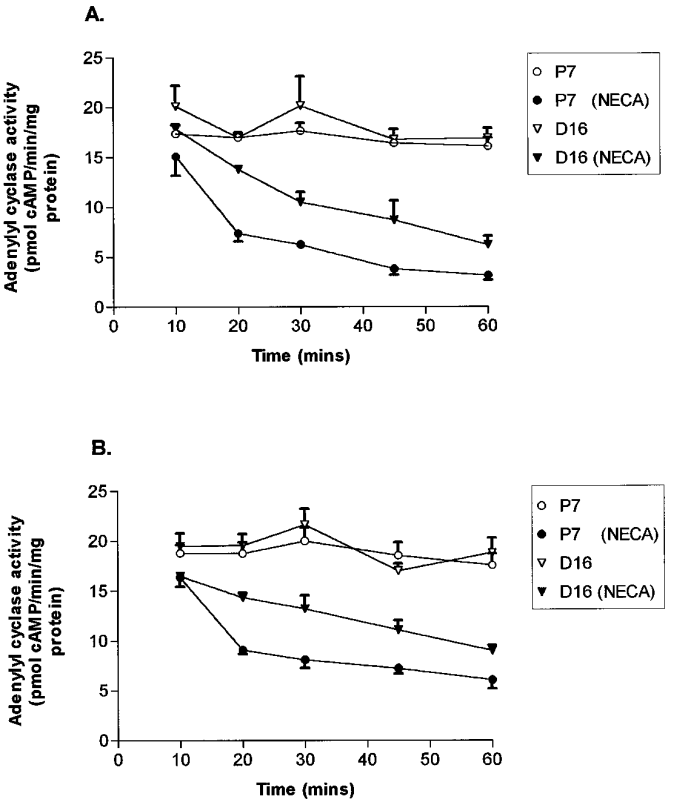

Fig. 4. Time course of desensitization of adenosine A_{2a} (A) and A_{2b} (B) receptor-stimulated adenylyl cyclase activity in P7 and D16 cells. Cells received no pretreatment or were pretreated with 10 μM (A_{2a}) or 100 μM (A_{2b}) NECA for varying lengths of time. Subsequently A_{2a} (3 μM CGS21680) and A_{2b} (100 μM NECA plus 1 μM CSC) receptor- stimulated adenylyl cyclase activity was measured. Values represent mean ± standard error from five separate experiments, each performed in triplicate. Desensitization was significantly less for both adenosine receptors in D16 compared with P7 cells (two-way ANOVA, $p < 0.05$).

desensitization of both A_{2a} and A_{2b} responses in P7 cells, whereas there was very little desensitization in D16. On the other hand, at 1 hr after NECA, there was marked desensitization of A_{2a} and A_{2b} responses in both P7 and D16, but desensitization was still greater in the former.

Agonist-induced desensitization of other G_s-coupled receptor responses in DNM GRK2 cells. To determine the selectivity of GRK2-induced desensitization, we examined the desensitization of IP-prostanoid and secretin receptor-stimulated adenylyl cyclase in pCMVneo and DNM GRK2 cells. The IP-prostanoid response desensitizes slowly (Fig. 5), whereas the secretin response desensitizes very rapidly (Fig. 6). However, unlike the adenosine A_2 responses, the desensitization of secretin and IP-prostanoid receptor-stimulated adenylyl cyclase was the same in pCMVneo and DNM GRK2 cells (Figs. 5 and 6A). Because the secretin response desensitizes extremely rapidly, we wanted to ensure that a possible effect of DNM GRK2 was not missed at very short time points. Accordingly, we exposed cells to secretin for ~5 sec before washing, but even under these conditions, there was marked desensitization, which was the same in P10 and D19 cells (Fig. 6B).

Desensitization of cAMP accumulation in intact NG108–15 cells. In addition to adenylyl cyclase activity in

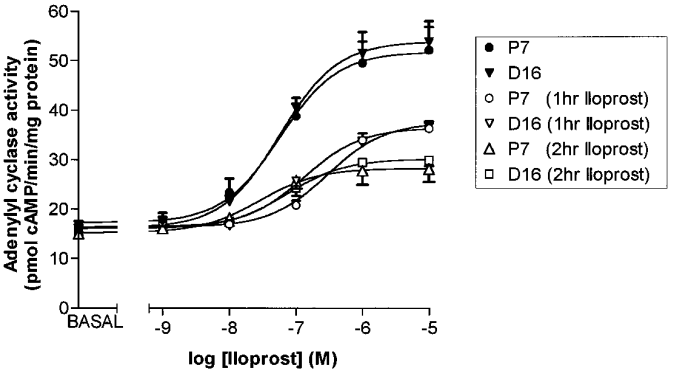

Fig. 5. Lack of effect of DNM GRK2 overexpression on the agonist-induced desensitization of IP-prostanoid receptor-stimulated adenylyl cyclase activity. P7 and D16 cells received no pretreatment or were pretreated with 1 μM iloprost for 1 or 2 hr. Values represent mean ± standard error from five separate experiments, each performed in triplicate.

cell homogenates, desensitization of G_s-coupled receptor responses was assessed in intact GRK2 DNM cells. Cells grown in multiwell plates were incubated with agonist for varying lengths of time before cAMP content was assessed. These experiments indicated that in response to 100 μM NECA, A2 (A_{2a} + A_{2b}) receptor-stimulated cAMP accumulation was greater in D16 than in P7 cells (Fig. 7A). In contrast, 33 μM

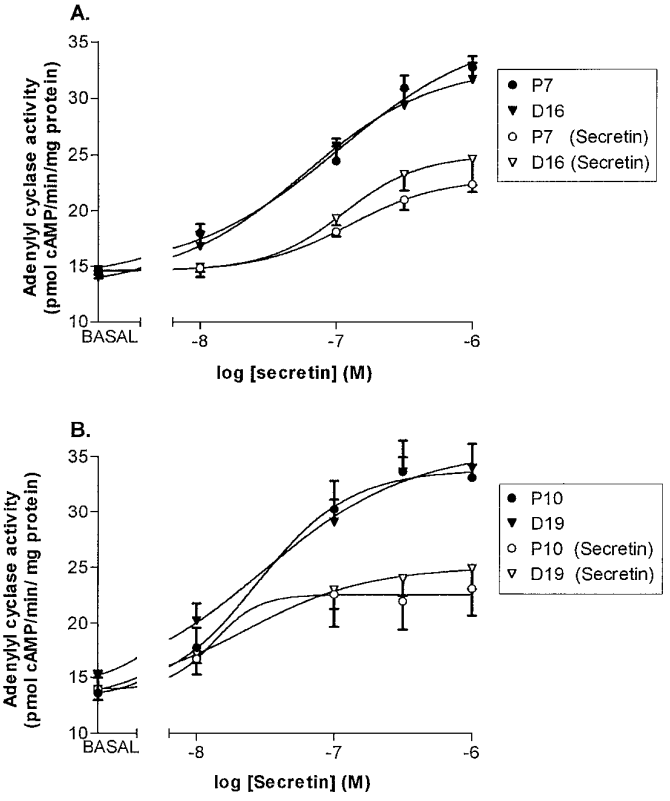

Fig. 6. Lack of effect of DNM GRK2 overexpression on the agonist-induced desensitization of secretin receptor-stimulated adenylyl cyclase activity. A, P7 and D16 cells received no pretreatment or were pretreated with 0.1 μM secretin for 1 min. B, P10 and D19 cells received no pretreatment or were pretreated with 0.1 μM secretin for 5 sec. Values represent mean $\pm$ standard error from four separate experiments, each performed in triplicate.

forskolin produced equivalent increases in cAMP in D16 and P7 cells (Fig. 7B). Very similar data were obtained in D19 and P10 cells (data not shown). Furthermore, 1 μM iloprost and 0.1 μM secretin produced similar increases in cAMP accumulation in D16 and P7 cells (Fig. 7, C and D).

Discussion

This study provides the first evidence to suggest that in intact cells, the agonist-induced desensitization of natively expressed adenosine A_2 receptors involves GRK2 and/or other kinases with similar regulatory mechanisms. In addition, this study underlines the selectivity of GRK-mediated processes in intact cells because neither the agonist-induced desensitization of secretin nor IP-prostanoid receptor-stimulated adenylyl cyclase activity was affected by DNM GRK2 overexpression. A recent study (23) indicates that canine A_{2a} adenosine receptors expressed in Chinese hamster ovary cells undergo rapid agonist-dependent phosphorylation that correlates with loss of agonist-stimulated adenylyl cyclase activity. Agonist-dependent phosphorylation of the A_{2b} receptor has not yet been demonstrated. We have shown previously that the short term agonist-dependent homologous desensitization of A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase in NG108–15 cells is not mediated by cAMP-dependent protein kinase (24). Thus, if phosphorylation of these two A_2 receptor subtypes underlies their desensitization in NG108–15 cells, a GRK-mediated process seems likely. In the current study, Western blotting analysis indicated that wild-type NG108–15 cells express significant levels of GRK2 and much lower levels of GRK3. Attempts to identify GRK5 and GRK6 in these cells were, however, equivocal (data not shown). We therefore decided to overexpress DNM GRK2 in the cells, and analysis of overexpression indicated that some of the DNM cells used in this study expressed a >60-fold excess of DNM GRK2 compared with wild-type GRK2 in wild-type or plasmid transfected control cells. An excess of 10–20-fold DNM GRK2 was previously shown to be effective in inhibiting β_2-adrenoceptor phosphorylation by wild-type GRK2 in a reconstitution assay (12).

Recent studies indicate that cellular overexpression of GRKs or DNM GRK2 can interfere with signaling of phosphoinositidase C-linked receptors, possibly by inhibiting the coupling between receptor and G protein or by inhibiting the availability of phosphatidylinositol bisphosphate (25, 26). However, in the current study, the overexpression of DNM GRK2 did not affect acute agonist-stimulated adenylyl cyclase activity in NG108–15 cell homogenates, indicating that the mutant protein does not have an unexpected interaction with this signal transduction process. In cells pretreated with the adenosine A_2 receptor agonist NECA, subsequent A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase activity in cell homogenates was greater in DNM than in plasmid-transfected control cells. This indicates that there was less A_2 receptor desensitization in the DNM cells and, furthermore, that endogenous GRK2 may mediate, at least in part, the desensitization of A_{2a} and A_{2b} responses. The time course for NECA-mediated desensitization of A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase in DNM GRK2 cells revealed that mutant overexpression slowed down rather than stopped desensitization. This could mean either that wild-type GRK2 still phosphorylates the A_2 receptors but at a slower rate in the presence of DNM GRK2 or that the GRK2 pathway is blocked but mechanisms other than GRK2-mediated phosphorylation are involved. For example, in PC12 cells, activation of the A_{2a} receptor leads to a rapid heterologous desensitization involving phosphorylation of type VI adenylyl cyclase (27). However, this cannot be the case in NG108–15 cells because A_2 desensitization is strictly homologous (28). Another possibility is that down-regulation of A_2 receptors occurs, but the available evidence suggests that much longer periods of agonist exposure (>5 hr) are required to observe A_{2a} receptor down-regulation (23). Unfortunately, it is very difficult for us to assess A_{2a} or A_{2b} receptor binding in NG108–15 cells because we have previously demonstrated that receptor binding is masked by the presence of a low affinity, high capacity binding site for adenosine and related ligands in these cells (28), which is almost certainly a stress protein (29). Therefore, we cannot formally rule out the possibility that adenosine receptor expression has changed in DNM GRK2 cells and that this underlies the altered desensitization in these cells. However, the fact that acute A_2 receptor-stimulated adenylyl cyclase activity is the same in DNM GRK2 and plasmid-transfected cells suggests that there are no overt differences in A_2 receptor density.

The conclusion that GRK2 is likely involved in desensitization of the A_2 responses in NG108–15 cells is further supported by the results of our intact-cell studies, in which cAMP accumulation due to NECA was greater in D16 than in P7 cells. This is similar to the effects of DNM GRK2 overex-

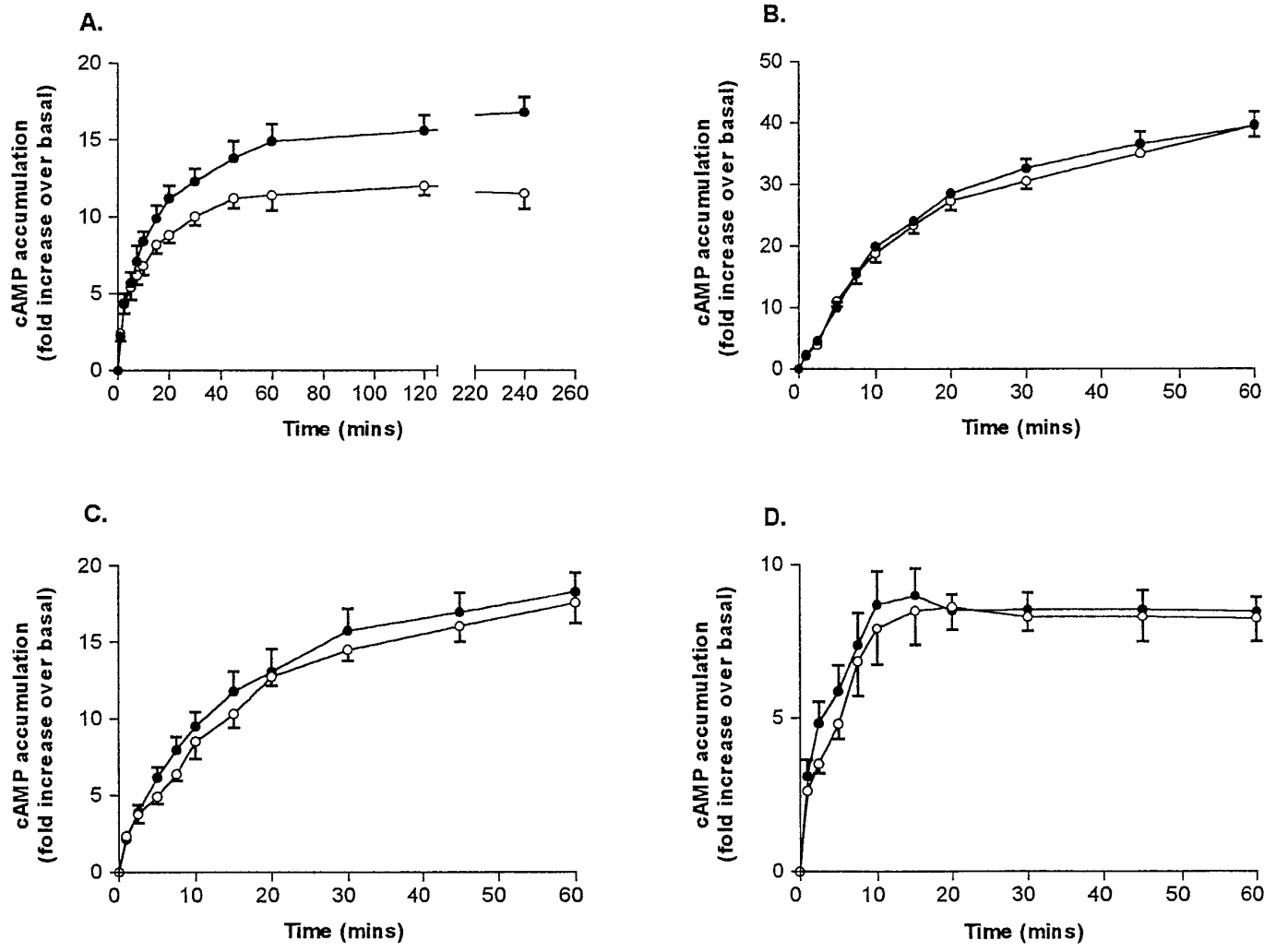

Fig. 7. cAMP accumulation in intact P7 (○) and D16 (●) cells after the addition of 100 μM NECA (A), 33 μM forskolin (B), 1 μM iloprost (C), or 0.1 μM secretin (D). Values represent mean ± standard error from four or five separate experiments. After the addition of NECA, cAMP accumulation was significantly greater in D16 cells than in P7 cells ($p < 0.05$, two-way ANOVA), but there was no difference after the addition of forskolin, iloprost, or secretin. Note different scales for fold-increases in cAMP accumulation.

pression on β_2-adrenoceptor-stimulated cAMP accumulation in human bronchial epithelial cells (12). Importantly, cAMP accumulation triggered by forskolin, iloprost, and secretin was exactly the same in D16 and P7 cells, suggesting the lack of involvement of GRK2 in these responses. Of further interest, cAMP levels after 4-hr incubation with NECA remained substantially higher in D16 than in P7 cells, suggesting that DNM GRK2 has prolonged effects on A_2 receptor desensitization. A similar effect has been observed with desensitization of thyrotropin receptor-stimulated cAMP accumulation in rat thyroid FRTL5 cells transfected with a GRK5 antisense construct (30). In the current study, and somewhat surprisingly, the rate of forskolin- and iloprost-stimulated cAMP accumulation decreased markedly with time, suggesting that other factors modulate the rate of cAMP production in intact cells. Experiments with the cAMP-dependent protein kinase inhibitor H89 (30 μM; data not shown), however, indicate that phosphorylation of adenylyl cyclase is not the cause.

Both the A_{2a} and the A_{2b} receptors contain a number of serine and threonine residues in the third intracellular loop and carboxyl tail (31, 32), which may represent targets for GRK2, as they do in other G protein-coupled receptors such as the α_{2C2}- (33) and β_2- (34) adrenergic receptors. However, currently, we have no direct evidence for GRK phosphorylation of adenosine A_2 receptors, although the A_3 adenosine receptor is a GRK2 substrate (35). It will be important in the future to demonstrate that A_2 receptors become phosphorylated on agonist exposure. Also, we do not know how selective DNM GRK2 is in inhibiting GRK2 action in intact cells. For example, at high levels of expression, DNM GRK2 could block the activity of another GRK. One way to address this in future studies will be to assess A_2 receptor desensitization in cells overexpressing DNM GRKs other than GRK2.

In sharp contrast to the adenosine A_2 receptor subtypes, the desensitization of secretin- and IP-prostanoid receptor-stimulated adenylyl cyclase was unaffected in DNM GRK2 cells. The secretin response desensitizes extremely rapidly in these cells ($t_{1/2}$ = a few seconds), and a recent study of secretin receptors expressed in Chinese hamster ovary cells indicates that agonist-induced receptor phosphorylation, although rapid, plays a relatively minor part in desensitization (36). Instead, phosphorylation-independent receptor internalization seems to be the dominant process in loss of respon-

siveness, which may explain the lack of effect of GRK2 DNM on secretin desensitization in NG108–15 cells. GRK-mediated receptor phosphorylation and arrestins have, in fact, been implicated in the mechanism of β_2-adrenergic receptor internalization (37, 38); however, overexpression of DNM GRK2 in human bronchial epithelial cells inhibited β_2-adrenergic receptor desensitization but did not affect receptor internalization (12).

IP-prostanoid receptors, on the other hand, have not been shown to be a substrate for phosphorylation by GRKs or other kinases. However, we (39) and others (40) have implicated a relatively slow $t_{1/2}$ (~1 hr) and concurrent loss of IP-prostanoid receptors and $G_{s\alpha}$ in agonist-induced desensitization of IP-prostanoid responsiveness in NG108–15 cells, and the data from the current study further suggest that phosphorylation by GRK2, even if it does occur, is not important in the desensitization of this response.

It is unclear what determines the selectivity of GRKs for agonist-occupied receptors in intact cells, but it is clear that it does not simply relate to the number of serine/threonine residues present. In a comparison of the mouse or rat amino acid sequences of the receptors studied here, there are varying numbers of serine/threonine residues in the putative third intracellular loops and carboxyl tails (31, 32, 41, 42). For example, in the carboxyl tail, the A_{2a} receptor contains 18, the A_{2b} receptor contains 9, the secretin receptor contains 9, and the IP-prostanoid receptor contains 15 serine/threonine residues. Other research suggests that acidic amino acid residues (glutamate and aspartate) in close proximity to the relevant serine/threonine residues are crucial for GRK-mediated receptor phosphorylation (33), but sequence analysis of the four receptors studied here does not reveal any obvious differences in acidic amino acid distribution or content in the third intracellular loop or carboxyl tail of these receptors (31, 32, 41, 42). Therefore, as-yet-undetermined factors may regulate the specificity of GRKs for agonist-occupied receptors in intact cells. In summary, the data presented here further indicate that dominant negative mutant GRKs can be used to investigate the involvement of GRKs in agonist-induced desensitization of G protein-coupled receptor responses. In particular, it is apparent that different receptors have differential dependencies on GRKs for agonist-induced desensitization. It will be interesting in future studies to extend our investigation to the role of GRKs in the desensitization of G_i/G_o-coupled receptors in these cells.

References

1. Hausdorff, W. P., M. G. Caron, and R. J. Lefkowitz. Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J.* **4:**2881–2889 (1990).
2. Clark, R. B., J. Friedman, R. A. F. Dixon, and C. D. Strader. Identification of a specific site required for rapid heterologous desensitization of the β-adrenergic receptor by cAMP-dependent protein kinase. *Mol. Pharmacol.* **36:**343–348 (1989).
3. Benovic, J. L., R. H. Strasser, M. G. Caron, and R. J. Lefkowitz. β-Adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. USA* **83:**2797–2801 (1986).
4. Lohse, M. J., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. β-Arrestin: a protein that regulates β-adrenergic receptor function. *Science (Washington D. C.)* **248:**1547–1550.
5. Haga, T., K. Haga, and K. Kameyama. G protein-coupled receptor kinases. *J. Neurochem.* **63:**400–412 (1994).
6. Premont, R. T., J. Inglese, and R. J. Lefkowitz. Protein kinases that phosphorylate activated G protein-coupled receptors. *FASEB J.* **9:**175–182 (1995).
7. Sterne-Marr, R., and J. L. Benovic. Regulation of G protein-coupled receptors by receptor kinases and arrestins. *Vitamins Hormones* **51:**193–234 (1995).
8. Somers, R. L., and D. C. Klein. Rhodopsin kinase activity in the mammalian pineal and other tissues. *Science (Washington D. C.)* **226:**182–184 (1984).
9. Ambrose, C., M. James, G. Barnes, C. Lin, G. Bates, M. Altherr, M. Duyao, N. Groot, D. Church, J. J. Wasmuth, H. Lehrach, D. Housman, A. Buckler, J. F. Gusells, and M. E. MacDonald. A novel G protein-coupled receptor kinase gene cloned from 4p16.3. *Hum. Mol. Genet.* **1:**697–703 (1992).
10. Benovic, J. L., J. W. Regan, H. Matsui, F. Mayor Jr., S. Cotecchia, L. M. F. Leeb- Lundberg, M. G. Caron, and R. J. Lefkowitz. Agonist-dependent phosphorylation of the α_2-adrenergic receptor by the β-adrenergic receptor kinase. *J. Biol. Chem.* **262:**17251–17253 (1987).
11. Kwatra, M. M., J. L. Benovic, M. G. Caron, R. J. Lefkowitz, and M. M. Hosey. Phosphorylation of chick heart muscarinic cholinergic receptors by the β-adrenergic receptor kinase. *Biochemistry* **28:**4543–4547 (1989).
12. Kong, G., R. Penn, and J. L. Benovic. A β-adrenergic receptor kinase dominant negative mutant attenuates desensitization of the β_2-adrenergic receptor. *J. Biol. Chem.* **269:**13084–13087 (1994).
13. Mundell, S. J., S. Smith, and E. Kelly. Evidence for coexistence and desensitization of A_2 adenosine receptor subtypes in NG108–15 cells. *Br. J. Pharmacol.* **118:**P17 (1996).
14. McKenzie, F. R., E. J. Adie, and G. Milligan. Prostanoid-mediated down regulation of G_s in NG108–15 cells. *Biochem. Soc. Trans.* **19:**81S (1991)
15. Kelly, E., M. Keen, P. Nobbs, and J. MacDermot. Segregation of discrete $G_{s\alpha}$ mediated responses that accompany homologous or heterologous desensitization in two related somatic hybrids. *Br. J. Pharmacol.* **99:**309–316 (1990)
16. Kelly, E., and J. L. Benovic. Stable expression of G-protein coupled receptor kinase 2 dominant negative mutant (GRK2 DNM) in NG108–15 cells. *Br. J. Pharmacol.* **118:**P142 (1996).
17. Baker, S. J., S. Markowitz, E. R. Fearon, J. K. V. Willson, and B. Vogelstein. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science (Washington D. C.)* **249:**912–915 (1990)
18. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227:**680–685 (1970).
19. Kim, C. M., Dion, S. B., Onorato, J. J., and J. L. Benovic. Expression and characterization of two β-adrenergic receptor kinase isoforms using the baculovirus expression system. *Receptor* **3:**39–55 (1993).
20. Loudon, R. P., Perussia, B., and J. L. Benovic. Differentially regulated expression of the G-protein-coupled receptor kinases, βARK and GRK6, during myelomonocytic cell development in vitro. *Blood* **88:**4547–4557 (1996).
21. Brown, B. L., J. D. M. Albano, R. P. Ekins, A. M. Sgherzi, and W. Tampion. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* **121:**561–562 (1971).
22. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254 (1976).
23. Palmer, T. M., T. W. Gettys, K. A. Jacobson, and G. L. Stiles. Desensitization of the canine A_{2a} adenosine receptor: delineation of multiple processes. *Mol. Pharmacol.* **45:**1082–1094 (1994).
24. Keen, M., E. Kelly, A. Krane, A. Austin, R. Wiltshire, N. Taylor, K. Docherty, and J. MacDermot. Cyclic AMP produces desensitization of prostacyclin and adenosine A_2 receptors in hybrid cell lines but does not affect G_s function. *Biochim. Biophys. Acta* **1134:**157–163 (1992).
25. Diviani, D., A.-L. Lattion, N. Larbi, P. Kunapuli, A. Pronin, J. L. Benovic, and S. Cotecchia. Effect of different G protein-coupled receptor kinases on phosphorylation and desensitization of the α_{1B}-adrenergic receptor. *J. Biol. Chem.* **271:**5049–5058 (1996).
26. Oppermann, M., N. J. Freedman, R. W. Alexander, and R. J. Lefkowitz. Phosphorylation of the type 1A angiotensin II receptor by G protein-coupled receptor kinases and protein kinase C. *J. Biol. Chem.* **271:**13266–13272 (1996),
27. Chern, Y., J.-Y. Chiou, H.-L. Lai, and M.-H. Tsai. Regulation of adenylyl cyclase type VI activity during desensitization of the A_{2a} adenosine receptor-mediated cyclic AMP response: role for protein phosphatase 2a. *Mol. Pharmacol.* **48:**1–8 (1995).
28. Keen, M., E. Kelly, P. Nobbs, and J. MacDermot. A selective binding site for ^{3}H-NECA that is not an adenosine A_2 receptor. *Biochem. Pharmacol.* **38:**3827–3833 (1989).
29. Hutchison, K. A., B. Nevins, F. Perini, and I. H. Fox. Soluble and membrane-associated human low affinity adenosine binding protein (adenotin): properties and homology with mammalian and avian stress proteins. *Biochemistry* **29:**5138–5144 (1990).
30. Nagayama, Y., K. Tanaka, T. Hara, H. Namba, S. Yamashita, K. Taniyama, and M. Niwa. Involvement of G-protein-coupled receptor kinase-5 in homologous desensitization of the thyrotropin receptor. *J. Biol. Chem.* **271:**10143–10148 (1996).
31. Fink, J. S., D. R. Weaver, S. A. Rivkees, R. A. Peterfreund, A. E. Pollack, E. M. Adler, and S. M. Reppert. Molecular cloning of the rat A_2 adenosine receptor: selective co-expression with D_2 dopamine receptors in rat striatum. *Mol. Brain Res.* **14:**186–195 (1992).
32. Stehle, J. H., S. A. Rivkees, J. J. Lee, D. R. Weaver, J. D. Deeds, and S. M. Reppert, S. M. Molecular cloning and expression of the cDNA for a novel A_2-adenosine receptor subtype. *Mol. Endocrinol.* **6:**384–393 (1992).
33. Jewell-Motz, E. A., and S. B. Liggett. An acidic motif within the 3rd

intracellular loop of the α_{2C2}-adrenergic receptor is required for agonist-promoted phosphorylation and desensitization. *Biochemistry* **34:**11946–11953 (1995).
34. Hausdorff, W. P., M. Bouvier, B. F. O'Dowd, G. P. Irons, M. G. Caron, and R. J. Lefkowitz. Phosphorylation sites on two domains of the β_2-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J. Biol. Chem.* **264:**12657–12665 (1989).
35. Palmer, T. M., J. L. Benovic, and G. L. Stiles. Agonist-dependent phosphorylation and desensitization of the rat A_3 adenosine receptor. *J. Biol. Chem.* **270:**29607–29613 (1995).
36. Holtmann, M. H., B. F. Roettger, D. I. Pinon, and L. Miller. Role of receptor phosphorylation in desensitization and internalization of the secretin receptor. *J. Biol. Chem.* **271:**23566–23571 (1996).
37. Ferguson, S. S. G., W. E. Downey III, A.-M. Colapietro, L. S. Barak, L. Ménard, and M. G. Caron. Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science (Washington D. C.)* **271:**363–366 (1996).
38. Goodman, O. B., Jr., J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen, and J. L. Benovic. β-Arrestin acts as a clathrin adaptor in endocytosis of the β_2-adrenergic receptor. *Nature (Lond.)* **383:**447–450 (1996).
39. Williams, R. J., and E. Kelly. $G_{s\alpha}$-dependent and -independent desensitization of prostanoid IP receptor-activated adenylyl cyclase in NG108–15 cells. *Eur. J. Pharmacol.* **268:**177–186 (1994).
40. Adie, E. J., I. Mullaney, F. R. McKenzie, and G. Milligan. Concurrent down-regulation of IP prostanoid receptors and the α-subunit of the stimulatory guanine-nucleotide-binding protein (G_s) during prolonged exposure of neuroblastoma X glioma cells to prostanoid agonists. *Biochem. J.* **285:**529–536 (1992).
41. Ishihara, T., S. Nakamura, Y. Kaziro, T. Takahashi, K. Takahashi, and S. Nagata. Molecular cloning and expression of a cDNA encoding the secretin receptor. *EMBO J.* **10:**1635–1641 (1991).
42. Namba, T., H. Oida, Y. Sugimoto, A. Kakizuka, M. Negishi, A. Ichikawa, and S. Narumiya. cDNA cloning of a mouse prostacyclin receptor. *J. Biol. Chem.* **269:**9986–9992 (1994).

Send reprint requests to: Dr. Eamonn Kelly, Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, United Kingdom. E-mail: e.kelly@bristol.ac.uk