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G protein-coupled receptor kinase 6 (GRK6) selectively regulates endogenous secretin receptor responsiveness in NG108-15 cells

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1 To determine the role of G protein-coupled receptor kinases (GRKs) in the regulation of endogenous secretin receptor responsiveness, we have transiently overexpressed both wild-type (WT) and dominant negative mutant (DNM) GRKs in NG108-15 mouse neuroblastoma × rat glioma hybrid cells and investigated the effects of this on agonist-stimulated adenylyl cyclase activity.

2 Overexpression of WT GRK6 selectively inhibited secretin-stimulated cyclic AMP accumulation (fold stimulation of cyclic AMP above basal following 15 min incubation with 100 nM secretin was 12.1 ± 2.0 and 6.2 ± 0.8 in control and WT GRK overexpressing cells, respectively) without affecting cyclic AMP responses mediated by the adenosine A_2 receptor agonist 5′-(*N*-ethylcarboxamido) adenosine (NECA) or the prostanoid-IP receptor agonist iloprost, or the direct activator of adenylyl cyclase, forskolin. On the other hand DNM GRK6 ($Lys^{215}Arg$) overexpression produced the opposite effect – a selective increase in the secretin-stimulated cyclic AMP response was observed in cells overexpressing DNM GRK6 compared to plasmid-transfected cells (fold stimulation of cyclic AMP above basal following 15 min incubation with 100 nM secretin was 12.6 ± 2.7 and 29.6 ± 5.6 for control and DNM GRK6-overexpressing cells, respectively).

3 Overexpression of WT GRK5 likewise inhibited the secretin-stimulated cyclic AMP response, however, this effect was not as selective as with GRK6, since adenosine A_2 receptor responsiveness was also suppressed by GRK5 overexpression. Unlike DNM GRK6, overexpression of DNM GRK5 failed to modulate secretin or A_2 adenosine receptor signalling suggesting that endogenous GRK5 is unlikely to regulate desensitization of these receptors in NG108-15 cells.

4 Overexpression of WT GRK2 did not affect secretin-stimulated cyclic AMP accumulation. Instead, GRK2 overexpression selectively inhibited A_2 adenosine receptor responsiveness, confirming our previous findings.

5 Together these results suggest a selective role of endogenous GRK6 in regulating secretin receptor responsiveness in NG108-15 cells. In addition, these data indicate that GRKs exert a surprising degree of selectivity in the regulation of natively expressed GPCR responses.
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Abbreviations: DNM, dominant negative mutant; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; NECA, 5′-(*N*-ethylcarboxamido) adenosine; PKA, protein kinase A; PKC, protein kinase C; GFP, Green Fluorescent Protein

Introduction

G protein-coupled receptors (GPCRs) exhibit a ubiquitous phenomenon known as desensitization, or loss of agonist-induced receptor responsiveness, to protect cells from over-stimulation and enable dynamic responsiveness to new stimuli. The molecular mechanisms underlying desensitization are best characterized for the class I rhodopsin/β-adrenoceptor family of GPCRs, where receptor phosphorylation is considered to play a major role (Hausdorff *et al*., 1990). GPCR phosphorylation generally involves two different families of serine/threonine protein kinases. Firstly, second messenger-dependent protein kinases A and C (PKA and PKC) can phosphorylate both active and unstimulated receptors to impair functional receptor-G protein coupling (heterologous desensitization). Secondly, G protein-coupled receptor kinases (GRKs) have been implicated in the homologous desensitization of only the agonist-occupied form of the receptor. At present, seven GRKs have been identified which have been classified into three sub-families based on structural and regulatory properties: GRK1, also known as rhodopsin kinase, is localised specifically in the cytosol of retinal cells; GRK2 and 3 are localised in the cell cytosol and are widely distributed; GRKs 4, 5 and 6 (GRK4 family) appear to be constitutively associated with the cell membrane, and whilst GRKs 5 and 6 are ubiquitously expressed, GRK4 is mainly expressed in the testis (Krupnick & Benovic, 1998). A common mechanism of homologous desensitization is GRK-mediated receptor phosphorylation which promotes the binding of adaptor proteins known as arrestins to the receptor, which inhibits receptor-G protein coupling, and also facilitates internalization of the receptor (Krupnick & Benovic, 1998). The internalized receptor is then targeted to either recycling or lysosomal (degradation) pathways perpetuating the dynamism of GPCR signalling (Ferguson, 2001).

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The peptide hormone secretin is an important regulator of pancreatic, biliary, and gastrointestinal physiology (Ulrich *et al*., 1998). In addition, secretin has been reported to affect cardiac output and muscle contractility (Sitniewska *et al*., 2002). A neuromodulatory role for secretin and its receptors has also been suggested, supported recently by the demonstration of high affinity secretin binding sites in specific regions of the rat brain (Nozaki *et al*., 2002) and by the use of secretin in clinical trials as a potential treatment for autism (Horvath *et al*., 1998). The known physiological effects of secretin receptor activation are mediated through G_s and G_q-coupled signalling pathways (Ulrich *et al*., 1998).

The secretin receptor itself is the prototypic member of the class II family of G protein-coupled receptors. Other members of the class II family include receptors for pituitary adenylate cyclase-activating polypeptide (PACAP), calcitonin, parathyroid hormone and vasoactive intestinal polypeptide (VIP). These GPCRs share less than 12% sequence homology with members of the larger and more extensively studied class I rhodopsin/β-adrenoceptor family (Segre & Goldring, 1993). However, like the class I GPCRs, receptors of the secretin family clearly exhibit agonist-mediated desensitization in response to prolonged agonist stimulation, but the precise mechanisms involved remain to be clarified (Holtmann *et al*., 1996). We have previously demonstrated that endogenous secretin receptor responsiveness in NG108-15 mouse neuroblastoma × rat glioma hybrid cells can be inhibited by both activation of PKA and PKC (Ghadessy & Kelly, 2002). The downstream activation of PKA arising from secretin-stimulated cyclic AMP signalling is likely to function as a negative feedback control. In contrast, PKC activation (which was achieved by phorbol ester treatment or G_q-coupled receptor cross-talk), appears to heterologously regulate secretin receptor signalling in NG108-15 cells (Ghadessy & Kelly, 2002). Both second messenger-dependent protein kinase and GRK-mediated phosphorylation of the secretin receptor has been demonstrated in various recombinant systems (Shetzline *et al*., 1998). Secretin receptors overexpressed in HEK293 cells were phosphorylated by coexpressed GRK2, 3 or 5, and phosphorylation correlated with enhanced desensitization of the secretin cyclic AMP response. Similar recombinant studies on another class II GPCR, the vasoactive intestinal polypeptide type-1 ($VPAC_1$) receptor, have shown agonist-mediated phosphorylation and desensitization by GRK2, 3, 5 and 6 (Shetzline *et al*., 2002).

The NG108-15 cell line is a convenient system to study the functional desensitization of the secretin receptor and other native G_s-coupled receptors, as these cells also express adenosine A_2 receptors and prostanoid-IP receptors (Hamprecht, 1977). We have previously shown that GRK2 is selectively involved in the agonist-induced desensitization of adenosine A_2 receptors, but not in the regulation of secretin or prostanoid-IP receptor responsiveness (Mundell *et al*., 1997; 1998). As NG108-15 cells express a number of the GRKs, the cells provide an interesting model system in which to further explore the specificity of GRKs in regulating endogenous receptor responsiveness, and in particular the secretin receptor. To assess the involvement of different GRKs in receptor responsiveness, we have overexpressed both wild-type and dominant negative mutant forms of GRK in NG108-15 cells and determined the subsequent effects on agonist-stimulated adenylyl cyclase activity. Our results indicate unexpected GRK specificity between the different natively expressed G_s-coupled receptors in this cell line.

Methods

Materials

[8-^{3}H]-cyclic AMP (925 Gbq mmol^{-1}) was obtained from Amersham International, U.K. Hybond Enhanced Chemiluminescence (ECL) nitrocellulose membrane, ECL detection kit and Hyperfilm-ECL luminescence detection film were also obtained from Amersham International, U.K. Colour Markers for SDS–PAGE (Wide range; C3437) and horseradish peroxidase (HRP)-linked secondary antibodies were from Sigma, U.K. The GRK5 and GFP antibodies were from Santa Cruz Biotechnology, CA, U.S.A. Lipofectamine™ 2000 Reagent was from Invitrogen, U.K. Cell culture medium and supplements were from GIBCO Life Sciences, U.K. Synthetic porcine secretin was obtained from Sigma, U.K. All other reagents and drugs were from Sigma, U.K.

Cell culture and transfections

NG108-15 mouse neuroblastoma × rat glioma hybrid cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 6% foetal calf serum, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The culture medium was supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. Cells were maintained at 37°C in humidified conditions under 5% CO_2.

For transfection, cells were seeded in 100 mm petri dishes and transfected with Lipofectamine™ 2000 Reagent according to the manufacturer's instructions. Transfections were performed using 5 μg of DNA construct with a 1 : 3 ratio of DNA to Lipofectamine™ 2000 Reagent. Cells were transfected with empty vector ($pcDNA_3$ or pEGFP-C2), human WT GRK5 in $pcDNA_3$, human WT GRK6 in $pcDNA_3$, bovine WT GRK2 in pEGFP-C2 (Willets *et al*., 2002), bovine WT β-arrestin-1 in pEGFP-N1 (Mundell *et al*., 2002), DNM GRK5 in $pcDNA_3$ (Tiruppathi *et al*., 2000), or DNM GRK6 in $pcDNA_3$ (Milcent *et al*., 1999). The latter two DNM constructs contain a single point mutation at $Lys^{215}Arg$. The next day, transfected cells were seeded into poly-L-lysine (0.1 mg ml^{-1}) coated 6 or 24-well microtiter plates for Western Blotting and cyclic AMP accumulation studies, respectively. The generation of NG108-15 cell lines stably expressing vector alone ($pcDNA_3$) or human WT GRK6 (in $pcDNA_3$) has been previously described (Willets & Kelly, 2001).

Whole cell cyclic AMP accumulation

Whole cell cyclic AMP accumulation assays were carried out as previously described (Ghadessy & Kelly, 2002). Briefly, NG108-15 cells were seeded onto 24-well microtiter 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. Fifteen minutes before agonist addition, the phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one (Ro201724; 250 μM) was added directly to each well to prevent degradation of the cyclic AMP

generated during the incubation period. Unless drug concentrations are otherwise stated, secretin (100 nM), forskolin (10 μM), 5′-(*N*-ethylcarboxamido) adenosine (NECA; 10 μM), iloprost (a prostacyclin analogue; 1 μM) or vehicle was added to each well at time point 0 and the cell plate returned to the incubator. At various time points thereafter, 20 μl of 100% trichloroacetic acid was added to terminate the signalling reaction. Cyclic AMP concentrations were measured in a competition assay using [^{3}H]-cyclic AMP as previously described (Mundell *et al*., 1997). The protein content of cell monolayers was determined as described previously (Bradford, 1976) and cyclic AMP accumulation expressed as pmol cyclic AMP mg^{-1} protein, or as fold-increase over basal.

Western blotting

The level of protein overexpression in transfected NG108-15 cells was determined using SDS–PAGE and Western blotting. Cells seeded in poly-L-lysine-treated 6-well plates were subjected to cell lysis 48 h after transfection, by addition of 100 μl of ice-cold lysis buffer (HEPES 20 mM, pH 7.4, NaCl 200 mM, EDTA 10 mM, 1% Triton X-100, 0.2 mg ml^{-1} benzamidine, 0.01 mg ml^{-1} leupeptin, 0.5 mM phenylmethylsulfonyl fluoride and 0.002 mg ml^{-1} aprotinin). The cell lysate was then centrifuged at 14,000 r.p.m. and 4°C for 3 min to pellet the insoluble cell fractions. Aliquots of the supernatant were snap-frozen in liquid nitrogen and stored at −70°C. When required, 40μg of the cell lysate protein was added to sodium dodecyl sulphate (SDS) loading buffer (final loading concentration of 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). In each gel a positive control was included using either a lysate sample from a cell line stably overexpressing GRK6 or a sample of recombinant protein (GRK2 and GRK5). Another lane containing pre-stained SDS–PAGE colour molecular weight markers was used as standards. Resolved proteins were transferred to Hybond-ECL nitrocellulose membranes and incubated first with GRK-specific antibodies: GRK2, mouse-monoclonal antibody that recognizes an epitope within residues 500–531 of the carboxyl-terminus of bovine GRK2 (Loudon *et al*., 1996); GRK5, rabbit-polyclonal antibody (Santa Cruz Biotechnology, CA, U.S.A.); GRK6, rabbit-polyclonal antibody that recognizes an epitope of residues 98–136 of human GRK6 (Loudon *et al*., 1996); GFP, rabbit-polyclonal antibody (Santa Cruz Biotechnology, CA, U.S.A.). Membranes were then incubated with appropriate horseradish peroxidase conjugated secondary antibodies (1 : 1000) to enable protein detection by enhanced chemiluminescence (ECL) according to the manufacturer's instructions.

Experimental design and statistics

Standard curve data and concentration-effect curves were fitted to logistic expressions (non-linear regression was used to fit a sigmoidal curve of variable slope), for single-site analysis using GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.). Time-course assays were constructed using point to point measurements. Results were expressed as pmol cyclic AMP min^{-1} mg^{-1} protein or as fold-stimulation over basal cyclic AMP. Where appropriate, statistical significance of different values was assessed by a paired *t*-test (two-tailed) or two-way ANOVA using GraphPad Prism Software, statistically significant differences being assumed where $P<0.05$.

Results

Effect of WT GRK6 and DNM GRK6 overexpression on agonist-stimulated cyclic AMP accumulation

Initial studies were performed using NG108-15 cells stably overexpressing WT GRK6 (Willets & Kelly, 2001). In whole cell cyclic AMP accumulation studies, secretin-stimulated cyclic AMP accumulation was markedly inhibited in GRK6-expressing cells compared to plasmid-transfected cells over a 30 min time-period, whereas that due to iloprost, NECA and forskolin addition was unchanged (data not shown). However, due to problems with clonal variation in agonist-stimulated cyclic AMP accumulation, coupled with our desire to investigate a range of GRK constructs, it was decided to employ a transient expression approach.

NG108-15 cells were transiently transfected with either WT GRK6, dominant negative mutant (DNM) GRK6 (Lys^{215}Arg) or empty vector (pcDNA$_3$). As judged by Western blotting (Figure 1A), both WT GRK6 and DNM GRK6 were overexpressed in the cells, demonstrated by immuno-reactive bands at approximately 66 kDa with similar electrophoretic mobilities to the immunoreactive band in non-transfected NG108-15 cells (presumably endogenous GRK6). Densitometric analysis of the immunoblots revealed that WT GRK6 and DNM GRK6 were expressed at approximately 20-fold over endogenous GRK6. The functional kinase activity of these constructs had been previously determined in [γ-^{32}P]-ATP phosphorylation studies using bovine (α-casein as a substrate; whereas WT GRK6 readily phosphorylated the substrate, DNM GRK6 did not (Willets *et al*., 2002).

The ability of WT GRK6 to regulate secretin receptor signalling was examined in a time-course assay of secretin-stimulated cyclic AMP accumulation. Over the 1 h time period investigated, the desensitization profile of secretin (100 nM)-stimulated cyclic AMP accumulation in pcDNA$_3$-transfected (control) cells was similar to that we have previously observed in non-transfected NG108-15 cells (Ghadessy & Kelly, 2002), with cyclic AMP generation levelling off after 15–30 min of agonist stimulation (Figure 2A). However, in cells overexpressing WT GRK6, secretin-stimulated cyclic AMP accumulation was significantly decreased as compared to control-transfected cells over the same time-period. To further examine the effect of WT GRK6 overexpression on secretin receptor responsiveness, concentration-effect curves to secretin were produced after 15 min of agonist stimulation. WT GRK6 overexpression inhibited secretin-stimulated cyclic AMP accumulation over the range of secretin concentrations tested (Figure 3), but there was no difference in the EC_{50} values for secretin-stimulated cyclic AMP accumulation between control and WT GRK6-expressing cells (15.5 ± 7.1 and 9.7 ± 4.9 nM, respectively).

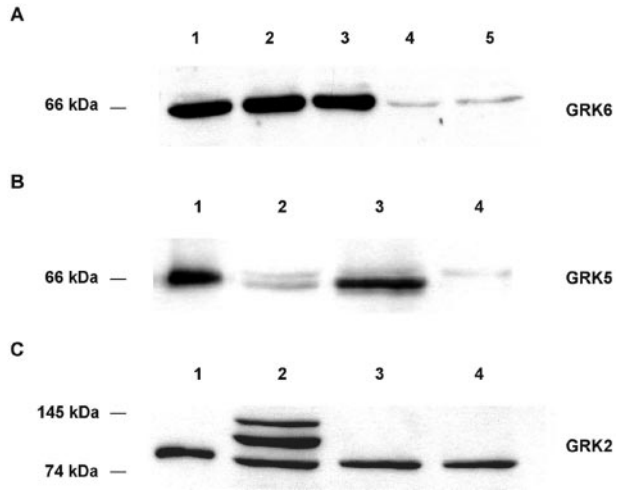

Figure 1 GRK overexpression in NG108-15 cells. Cells were transfected with 5 μg DNA containing empty vector, wild-type (WT) GRK or DNM GRK constructs. Whole cell lysates were subjected to SDS–PAGE followed by Western transfer and immunoblotting with GRK-specific primary antibodies as detailed in the Methods. (A) GRK6 overexpression in NG108-15 cells. Lane 1, lysate from NG108-15 cells stably expressing WT GRK6. In lanes 2–4 lysate preparations were from transiently transfected cells. Lane 2, lysate from cells transiently expressing WT GRK6; Lane 3, lysate from cells transiently expressing DNM GRK6; Lane 4, lysate from cells transiently expressing the vector pcDNA$_3$; Lane 5, lysate from non-transfected NG108-15 cells. (B) GRK5 overexpression in NG108-15 cells. Lane 1, 8 ng of purified GRK5; Lane 2, lysate from cells transiently expressing WT GRK5; Lane 3, lysate from cells transiently expressing DNM GRK5; Lane 4, lysate from cells transiently expressing the vector pcDNA$_3$. (C) GRK2 overexpression in NG108-15 cells. Lane 1, 8 ng of purified GRK2; Lane 2, lysate from cells transiently expressing WT GRK2; Lane 3, lysate from cells transiently expressing the vector pEGFP-C2; Lane 4, lysate from non-transfected NG108-15.

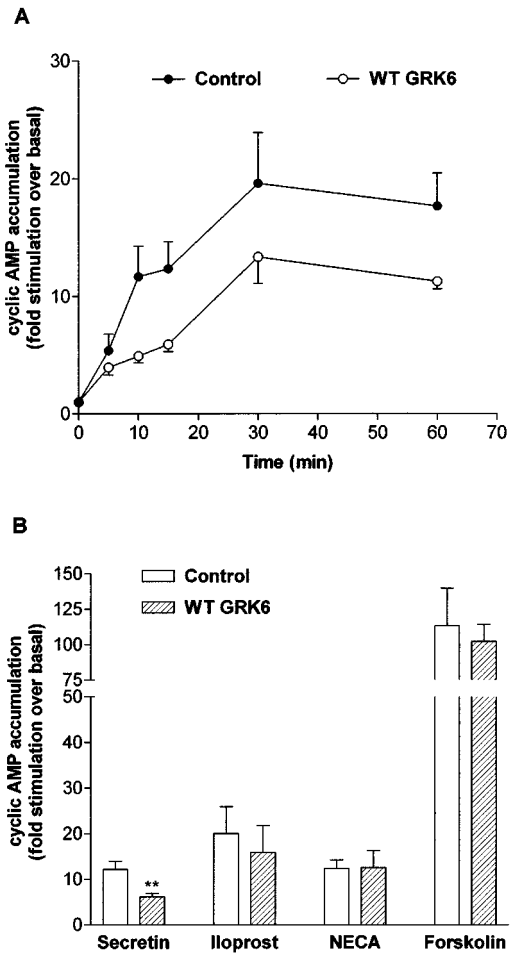

Figure 2 WT GRK6 overexpression selectively inhibits secretin-stimulated cyclic AMP accumulation. (A) WT GRK6-overexpressing and plasmid-transfected control cells were incubated with secretin (100 nM) for the times indicated. Secretin-stimulated cyclic AMP accumulation was significantly decreased in cells overexpressing WT GRK6 versus control ($P=0.008$, two-way ANOVA; $n=5$). (B) WT GRK6-overexpressing and plasmid-transfected control cells were incubated with either secretin (100 nM), iloprost (1 μM), NECA (10 μM) or forskolin (10 μM) for 15 min. Cyclic AMP accumulation is expressed as fold-stimulation over basal and data are mean$\pm$ s.e.mean of values from at least five independent experiments with each point performed in quadruplicate ($P=0.002$ compared to respective control, paired t-test).

The specificity of WT GRK6 in attenuating secretin receptor responsiveness was next investigated by comparing basal and acute agonist-stimulated cyclic AMP responses in WT GRK6 overexpressing cells. In all experiments, prostanoid-IP and adenosine A_2 receptor responsiveness was measured using iloprost (1 μM) and NECA (10 μM), respectively. In addition, the direct activation of adenylyl cyclase activity by forskolin (10 μM) was measured. Using a 15 min time-point of agonist challenge, secretin (100 nM)-stimulated cyclic AMP accumulation was significantly decreased from 12.1 ± 2.0 to 6.2 ± 0.8 fold stimulation over basal in control and WT GRK6-transfected cells, respectively (Figure 2B). In contrast, cyclic AMP responses to iloprost, NECA and forskolin were unaffected by WT GRK6 overexpression (over the same time-period, basal cyclic AMP levels were also unchanged in control and WT GRK6-transfected cells, being 202.5 ± 57.5 and 218.3 ± 43.0 pmol cyclic AMP mg^{-1} protein, respectively; $n=6$).

To investigate whether suppression of endogenous GRK6 activity can alter receptor responsiveness, DNM GRK6 was transiently overexpressed in NG108-15 cells. As shown in Figure 4A, secretin-stimulated cyclic AMP accumulation was significantly increased in DNM-expressing cells as compared to control-transfected cells over time. Indeed DNM GRK6 overexpression potentiated cyclic AMP accumulation over the range of secretin concentrations tested (Figure 3), but without changing secretin's EC_{50} (values for control-transfected and DNM GRK6-expressing cells were 15.5 ± 7.1 and $19.1\pm$ 6.9 nM, respectively). The selectivity of DNM GRK6 in enhancing secretin receptor responsiveness was investigated again by comparison with basal and other acute agonist-stimulated cyclic AMP responses in DNM GRK6-overexpressing cells. After 15 min, basal cyclic AMP levels in control and DNM GRK6-transfected cells were similar at 132.5 ± 46.0 and 148.3 ± 32.3 pmol cyclic AMP mg^{-1} protein, respectively, $n=5$. Furthermore, DNM GRK6 overexpression was selective for the secretin-mediated cyclic AMP response with an increase from 12.6 ± 2.7 to 29.6 ± 5.6 fold stimulation over basal in control and DNM GRK6-transfected cells, respectively, but no effect on cyclic

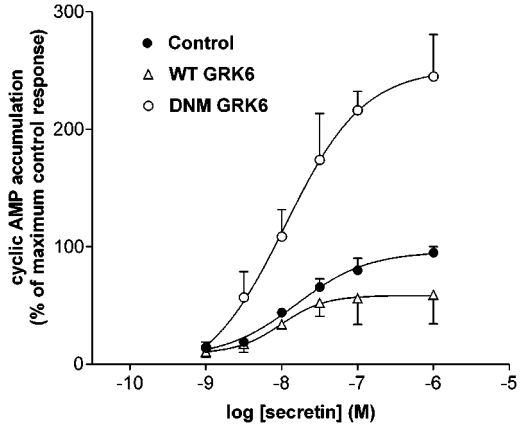

Figure 3 Effect of WT GRK6 or DNM GRK6 overexpression on concentration-effect curves for secretin-stimulated cyclic AMP accumulation. Cells transiently expressing $pcDNA_3$ (control), WT GRK6 or DNM GRK6 were incubated with secretin at the concentrations indicated for 15 min. Values for cyclic AMP accumulation have been expressed as a per cent of the maximum level of cyclic AMP obtained in control cells. Data points represent the mean ± s.e.mean of values from three independent experiments with each point performed in quadruplicate.

AMP accumulation due to the other agonists (Figure 4B). Thus, overexpression of GRK6 or inhibition of endogenous GRK6 activity using a DNM construct, selectively regulates secretin receptor responsiveness in NG108-15 cells.

Effect of GRK5 or DNM GRK5 overexpression on agonist-stimulated cyclic AMP accumulation

GRK5, like GRK6, is a member of the GRK4 subfamily of GRKs, and GRK5 and 6 share sequence and functional similarities. In order to investigate whether GRK5 can regulate secretin receptor signalling in a similar manner to GRK6, NG108-15 cells were transiently transfected with either WT GRK5, DNM ($Lys^{215}Arg$) GRK5 or empty vector ($pcDNA_3$). The levels of protein overexpression were assessed by Western blot (Figure 1B). Overexpression of WT GRK5 and DNM GRK5 (lanes 2 and 3 respectively) was demonstrated by immunoreactive bands at approximately 66 kDa, a similar electrophoretic molecular weight as purified GRK5 (lane 1). Using densitometric analysis, WT and DNM GRK5 were overexpressed approximately 5 and 25 times over the endogenous level of GRK5 expression. Closer examination of the immunoblots shows a different pattern of electrophoretic mobility between the WT and DNM GRK5 proteins. The bands are likely to represent different autophorylation states of the kinase, and similar observations have been made in related studies (Pronin & Benovic, 1997; Milcent *et al*., 1999).

In a similar manner to WT GRK6 overexpression, secretin-stimulated cyclic AMP accumulation was decreased over time in WT GRK5-expressing cells when compared to control-transfected cells (Figure 5A). Again, the specificity of WT GRK5 in attenuating secretin receptor responsiveness was examined by comparing basal and agonist-stimulated cyclic AMP responses in WT GRK5 overexpressing cells at a 15 min time-point of agonist challenge. Basal cyclic AMP levels were similar in control and WT GRK5-transfected cells, being 117.9 ± 21.9 and 130.6 ± 35.1 pmol cyclic AMP mg^{-1} protein, respectively, $n = 5$. Secretin-stimulated cyclic AMP accumulation was decreased from 11.0 ± 0.8 to 6.5 ± 1.3 fold stimulation over basal in control and WT GRK5-transfected cells, respectively (Figure 5B). Interestingly, the cyclic AMP response to NECA was also decreased by WT GRK5 overexpression, from 49.3 ± 7.9 to 27.8 ± 5.7 fold stimulation over basal in control and WT GRK5-transfected cells, respectively. In contrast, iloprost and forskolin-stimulated cyclic AMP accumulation was not affected by WT GRK5 overexpression.

In contrast to the results obtained with DNM GRK6, secretin-stimulated cyclic AMP accumulation was not affected by inhibition of endogenous GRK5 activity with DNM GRK5 overexpression (Figure 6). This seems unlikely to be due to insufficient DNM GRK5 expression, since the mutant was expressed at a higher level than WT GRK5, and in fact was expressed at approximately the same level as DNM GRK6, which did enhance secretin responsiveness. In addition, DNM GRK5 overexpression failed to affect any agonist-stimulated cyclic AMP responses, including that mediated by NECA, which like secretin was inhibited by WT GRK5 overexpression (Figure 6B; after 15 min basal cyclic AMP levels were similar in control and DNM GRK5-transfected cells and were 106.0 ± 26.7 and 115.2 ± 39.6 pmol cyclic AMP mg^{-1} protein, respectively, $n = 4$). These results suggest that although secretin and NECA-stimulated cyclic AMP responses can be selectively inhibited by WT GRK5 overexpression, endogenous GRK5 does not appear to regulate agonist responsiveness.

Effect of GRK2 overexpression on agonist-stimulated cyclic AMP accumulation

Having established that secretin receptor responsiveness can be regulated by GRK5 and 6, the involvement of a different GRK subtype, GRK2, in secretin receptor signalling was investigated. NG108-15 cells were transiently transfected with WT GRK2 or empty vector (pEGFP-C2). The pEGFP-C2 vector which contained the WT GRK2 gene provided a convenient assessment of the efficiency of transfection obtained when using Lipofectamine™ 2000 Reagent in NG108-15 cells. Using confocal microscopy, we estimated 50–75% of the transfected cells functionally expressed the pEGFP-C2 vector. Relative expression levels for the WT GRK2-overexpressing cells compared with vector (pEGFP-C2)-transfected cells and endogenous GRK2 expression are shown in Figure 1C. Immunoreactive bands at approximately 70–75 kDa were observed in all lysate samples; these bands have a slightly faster electrophoretic mobility than that seen for purified GRK2 which resolves as a band of approximately 80 kDa (lane 1), as we have previously noted (Mundell *et al*., 1998). In the lysate sample from WT GRK2-transfected cells (lane 2), two other prominent immunoreactive bands were observed between 100 and 145 kDa. The middle band at approximately 110 kDa is likely to represent overexpressed GFP-tagged GRK2 protein since GFP has a molecular weight of 29–30 kDa. However, at present we do not know the identity of the higher molecular weight band.

In order to investigate the effect of WT GRK2 over-expression on agonist-stimulated cyclic AMP accumulation in

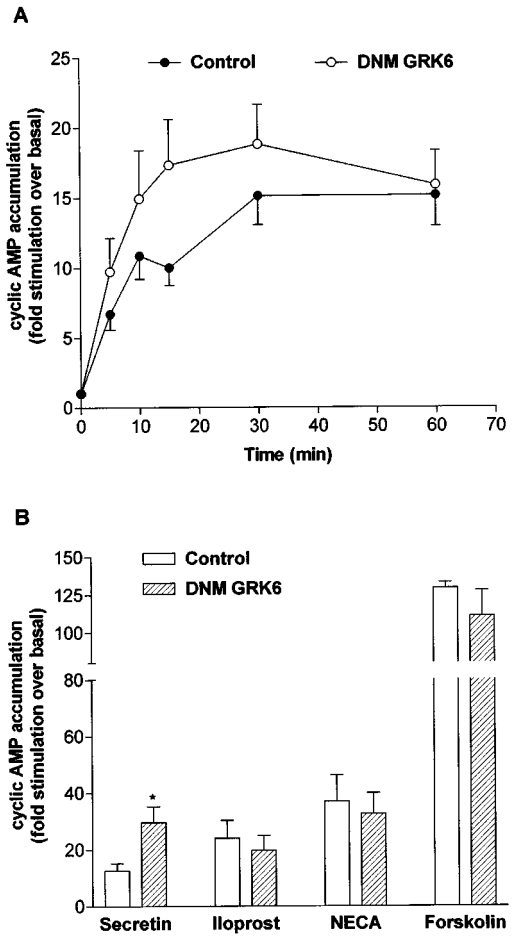

Figure 4 DNM GRK6 overexpression selectively enhances secretin-stimulated cyclic AMP accumulation. (A) DNM GRK6-overexpressing and plasmid-transfected control cells were incubated with secretin (100 nM) for the times indicated. Secretin-stimulated cyclic AMP accumulation was significantly increased in cells overexpressing DNM GRK6 versus control ($P=0.03$, two-way ANOVA, $n=5$). (B) DNM GRK6-overexpressing and plasmid-transfected control cells were challenged with either secretin (100 nM), iloprost (1 μM), NECA (10 μM) or forskolin (10 μM) for 15 min. Cyclic AMP accumulation is expressed as fold-stimulation over basal and data are mean$\pm$ s.e.mean of values from at least five independent experiments with each point performed in quadruplicate ($P<0.05$ compared to respective control, paired t-test).

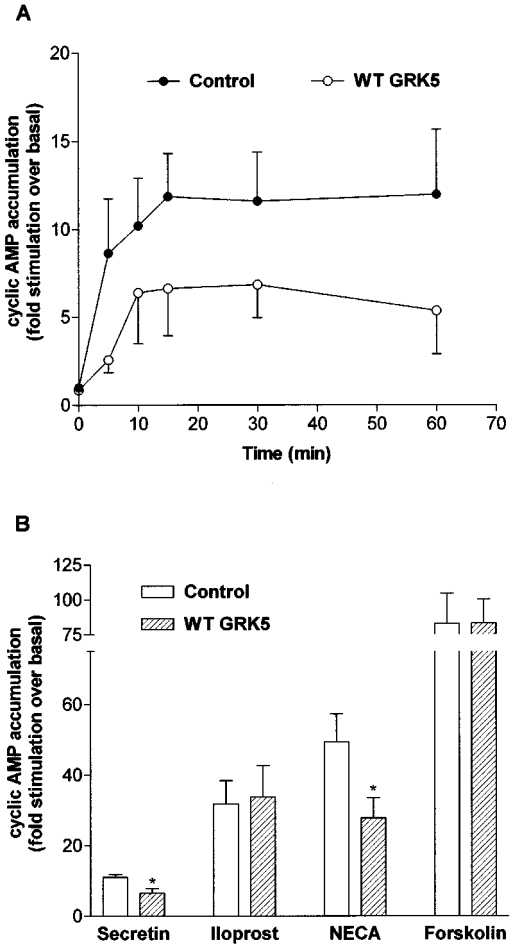

Figure 5 WT GRK5 overexpression inhibits secretin-stimulated cyclic AMP accumulation. (A) WT GRK5-overexpressing and plasmid-transfected control cells were challenged with secretin (100 nM) for the times indicated. Secretin-stimulated cyclic AMP accumulation was significantly reduced in cells overexpressing WT GRK5 versus control ($P=0.0038$, two-way ANOVA, $n=4$). (B) WT GRK5-overexpressing and plasmid-transfected control cells were challenged with either secretin (100 nM), iloprost (1 μM), NECA (10 μM) or forskolin (10 μM) for 15 min. Cyclic AMP accumulation is expressed as fold-stimulation over basal and data are mean$\pm$ s.e.mean of values from at least five independent experiments with each point performed in quadruplicate. Both secretin and NECA-stimulated cyclic AMP accumulation were reduced by WT GRK5 overexpression as compared to control values ($P=0.0338$ and $P=0.0101$, respectively, paired t-test).

NG108-15 cells, cells were challenged as before with either secretin, forskolin, iloprost or NECA and the resulting level of cyclic AMP measured after 15 min of agonist stimulation. Basal cyclic AMP levels were similar in control and WT GRK2-transfected cells, after 15 min being 131.8 ± 33.0 and 122.2 ± 16.8 pmol cyclic AMP mg^{-1} protein, respectively; $n=5$. In contrast to the results obtained with WT GRK6 overexpression, a selective inhibition of NECA-stimulated cyclic AMP accumulation was observed in WT GRK2-overexpressing cells (Figure 7). NECA-stimulated cyclic AMP accumulation was decreased from 23.5 ± 2.2 to 16.0 ± 1.6 fold stimulation over basal in control and WT GRK2-transfected cells, respectively. Cyclic AMP accumulation in response to secretin, iloprost and forskolin was unchanged following WT GRK2-transfection (Figure 7B). These data with a transient transfection approach confirm that GRK2 can selectively regulate A_2 adenosine receptor-stimulated cyclic AMP accumulation in NG108-15 cells.

Effect of β-arrestin-1 overexpression on agonist-stimulated cyclic AMP accumulation

The effect of β-arrestin-1 overexpression (Figure 8A) on agonist-stimulated cyclic AMP accumulation was also investigated. Compared to plasmid-transfected controls, β-arrestin-1 overexpression reduced both secretin and adenosine A_2 receptor-stimulated cyclic AMP accumulation (Figure 8B,C). Interestingly, this effect was selective for these two responses, since iloprost and forskolin-stimulated cyclic AMP

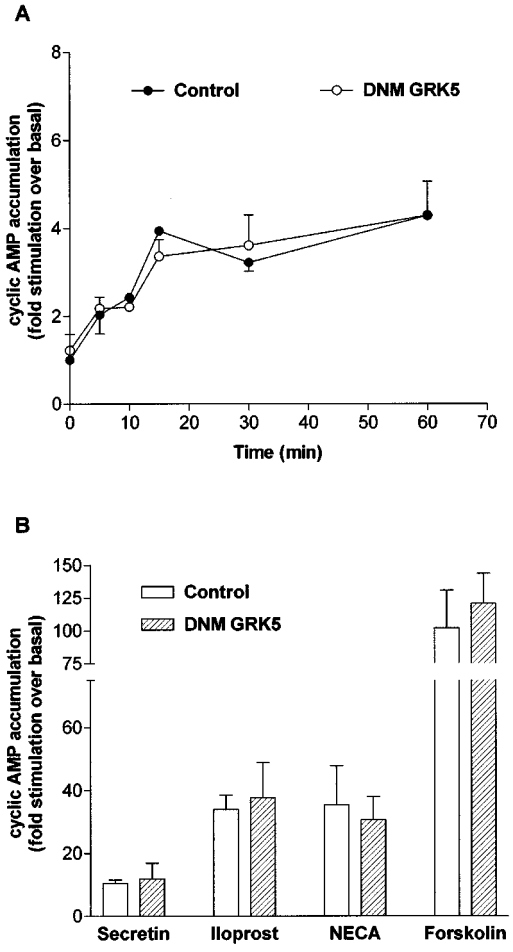

Figure 6 Effect of DNM GRK5 overexpression on agonist-stimulated cyclic AMP accumulation. (A) DNM GRK5-overexpressing and plasmid-transfected control cells were challenged with secretin (100 nM) for the times indicated. Secretin-stimulated cyclic AMP accumulation was not significantly affected by DNM GRK5 overexpression compared to control ($P>0.05$, two-way ANOVA, $n=3$). (B) DNM GRK5-overexpressing and plasmid-transfected (control) cells were challenged with either secretin (100 nM), iloprost (1 μM), NECA (10 μM) or forskolin (10 μM) for 15 min. There was no difference in agonist-stimulated cyclic AMP accumulation between DNM GRK5-overexpressing and control cells ($n=4$). Data are mean$\pm$s.e.mean of values from at least three independent experiments with each point performed in quadruplicate.

accumulation was not affected by β-arrestin-1 overexpression (Figure 8D).

Discussion

In this study, we have examined the potential roles of GRK2, 5 and 6 in the regulation of endogenous secretin receptor responsiveness in NG108-15 cells. Overexpression of WT GRK6 selectively inhibited secretin-stimulated cyclic AMP accumulation, without affecting adenosine A_2 or prostanoid-IP receptor responsiveness, or cyclic AMP accumulation stimulated by the direct activator of adenylyl cyclase, forskolin. Together these results suggest that the suppression of secretin receptor responsiveness occurs at the level of the receptor. However, these effects do not prove that GRK6 is involved in the physiological regulation of secretin receptor responsiveness, even though we could detect endogenous GRK6 in these cells. Therefore to address this issue, we overexpressed a dominant negative mutant (DNM) form of GRK6 ($Lys^{215}Arg$) in NG108-15 cells, to inhibit endogenous GRK6 activity. This construct has previously been used to block endogenous GRK6 activity in SH-SY5Y cells, implicating GRK6 in the desensitization of endogenous M_3 mACh receptors (Willets *et al*., 2002). The authors reported that DNM GRK6 overexpression inhibited agonist-stimulated M_3 mACh receptor phosphorylation and desensitization by around 50%. In the present study, secretin receptor-stimulated cyclic AMP accumulation was increased in DNM GRK6-expressing cells compared to plasmid-transfected controls, whereas that due to activation of adenosine A_2 or prostanoid-IP receptors, or to direct activation of adenylyl cyclase with forskolin, was not. This strongly suggests that endogenous GRK6 regulates secretin receptor responsiveness in these cells, and also that the kinase activity of GRK6 is crucial for this effect, since DNM GRK6 produced the opposite effect. Nevertheless, desensitization of secretin-stimulated cyclic AMP accumulation was still observed in DNM GRK6-expressing cells. This could be because the DNM GRK6 is not fully effective as a WT GRK6 inhibitor, or that the transient expression protocol did not lead to construct expression in all cells (e.g. 50–75% transfection efficiency with GRK2-GFP), or that other mechanisms such as PKA-mediated feedback inhibition (Ghadessy & Kelly, 2002) are also effective. Indeed, a combination of these factors seems likely.

GRK6 is a member of the GRK4 subfamily of GRKs which also comprises GRK4 and 5. Based on sequence and functional similarities, we decided to investigate whether GRK5 can also regulate secretin receptor signalling. Both GRK5 and 6 contain autophosphorylation sites within the carboxyl-terminus region of the kinase sequence and autophosphorylation is a prerequisite for functional kinase activity (reviewed in Penn *et al*., 2000). In the present study, the immunoblots revealed a different pattern of electrophoretic mobility between the WT and DNM GRK5 proteins (Figure 1B). WT GRK5 appeared to migrate as a doublet, however only the lower molecular weight species was evident for the DNM GRK5 protein. The loss of the higher molecular weight band in DNM GRK5-expressing lysate (which most likely represents autophosphorylated GRK5) suggests that the point mutation ($Lys^{215}Arg$) impairs the ability of GRK5 to autophosphorylate and consequently results in a kinase-dead protein. Similar electrophoretic migration bands have been observed for the different versions of GRK5 and 6 in related studies (Milcent *et al*., 1999; Pronin & Benovic, 1997). The overexpression of WT GRK5 inhibited the secretin-stimulated cyclic AMP response to a similar extent as that observed with WT GRK6 overexpression. On the other hand this inhibitory effect of GRK5 on secretin receptor responsiveness was not as selective as with GRK6, as the adenosine A_2 receptor-stimulated cyclic AMP response was likewise inhibited by GRK5 overexpression. Since DNM GRK5 overexpression did not appreciably modulate receptor signalling, then endogenous GRK5 activity seems unlikely to mediate desensitization of the secretin and adenosine A_2 receptors.

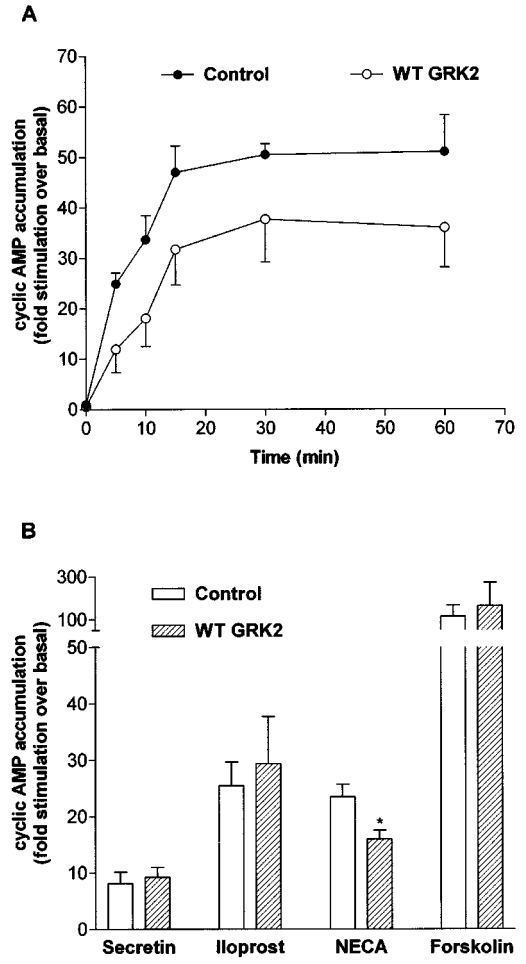

Figure 7 WT GRK2 overexpression selectively inhibits NECA-stimulated cyclic AMP accumulation. (A) WT GRK2-overexpressing and plasmid-transfected control cells were challenged with NECA (10 μM) for the times indicated. NECA-stimulated cyclic AMP accumulation was decreased in cells overexpressing WT GRK2 versus control ($P=0.0012$, two-way ANOVA, $n=5$). (B) WT GRK2-overexpressing and plasmid-transfected control cells were challenged with either secretin (100 nM), iloprost (1 μM), NECA (10 μM) or forskolin (10 μM) for 15 min. Data are mean$\pm$s.e.mean of values from five independent experiments with each point performed in quadruplicate. GRK2 overexpression selectively inhibited NECA-stimulated cyclic AMP accumulation compared to control ($P=0.02$, paired t-test).

However there could be other reasons for the lack of effect of DNM GRK5 in this cell system; for example the mutant kinase may have reduced affinity for receptor substrates, or the kinase activity of the endogenous GRK5 present in the cells could be constitutively inhibited due to calmodulin-stimulated autophosphorylation of GRK5 or PKC-mediated phosphorylation of the kinase (reviewed in Penn *et al*., 2000).

Using a combination of approaches involving stable transfection of NG108-15 cells with WT GRK2 (Mundell *et al*., 1998), DNM GRK2 (Mundell *et al*., 1997), or antisense GRK2 constructs (Willets *et al*., 1999), we have previously shown that this kinase selectively regulates agonist-induced desensitization of adenosine A_2 receptors. In these earlier studies, secretin and prostanoid-IP receptor responsiveness was not affected by changes in the level of GRK2 activity or expression. In the present study, the specificity of GRK2 in adenosine A_2 receptor regulation was confirmed following transient expression of WT GRK2 in these cells. However the overexpression of GRK2 (and also GRK5 and 6) failed to modulate prostanoid-IP receptor responsiveness in NG108-15 cells. These data are consistent with previous studies where desensitization of IP-prostanoid receptor responsiveness was shown to be due to down-regulation of the receptor protein and $G_{s\alpha}$ (Williams & Kelly, 1994), and to be independent of GRK (Smyth *et al*., 2000).

The inability of GRK2 to modulate endogenous secretin receptor responsiveness differs from findings with the recombinant secretin receptor (Shetzline *et al*., 1998). In the latter study, rat secretin receptors transiently expressed in HEK293 cells were phosphorylated and desensitized by co-expression of GRK2, 3 or 5, but interestingly not GRK6. On the other hand, when expressed in Chinese Hamster Ovary cells, a carboxyl-terminal truncated secretin receptor, which does not undergo agonist-induced phosphorylation, still displays marked desensitization, apparently by receptor internalization (Holtmann *et al*., 1996). Thus the mechanism of secretin receptor desensitization may be cell context-dependent. Another difference between the present study and that by Shetzline *et al*. (1998) is that levels of secretin receptor following transient expression in HEK293 cells are likely to be far higher than in NG108-15 cells (the latter is reported to be very low at approximately 25 fmol mg protein^{-1}; Gossen *et al*., 1990). It is possible that high levels of receptor expression lead to protein–protein interactions not seen at physiological levels of receptor expression, but this does not explain the marked difference in the action of GRK6 on secretin receptor responsiveness in NG108-15 versus HEK293 cells. Another possibility is that endogenous secretin receptors and secretin receptors heterologously expressed under the control of an artificial promoter are processed and expressed in the cell differently, which may lead to differential regulation. It is interesting to note that the IP-prostanoid receptor also endogenously expressed in NG108-15 cells (Williams & Kelly, 1994; Mundell *et al*., 1998) or other cell types (Nilius *et al*., 2000) desensitizes slowly in a GRK- and PKC-independent manner, but IP-prostanoid receptors heterologously expressed in HEK293 cells undergo very rapid agonist-induced phosphorylation and desensitization (Smyth *et al*., 1998).

The role of GRKs in internalization of the secretin receptor has also been investigated in recombinant studies (Walker *et al*., 1999). In HEK293 cells co-expressing the secretin receptor and GRK2 or 5, no effect of overexpressed GRKs on receptor sequestration was observed. Instead, PKA-mediated phosphorylation of the receptor was suggested to promote receptor internalization in these cells. We have previously shown PKA to regulate endogenous secretin receptor responsiveness after longer time periods of agonist stimulation in NG108-15 cells, suggesting a delayed effect of PKA in regulating desensitization and/or internalization (Ghadessy & Kelly, 2002). In the present study, the effects of GRK5 and 6 overexpression on inhibiting secretin receptor responsiveness were observed within 5 min of agonist challenge, suggesting that GRKs are involved in acute mechanisms of secretin receptor desensitization. However, the possibility that overexpression of GRKs results in down-regulation of the secretin receptors from the cell surface cannot at present be discounted, since a constitutive loss of

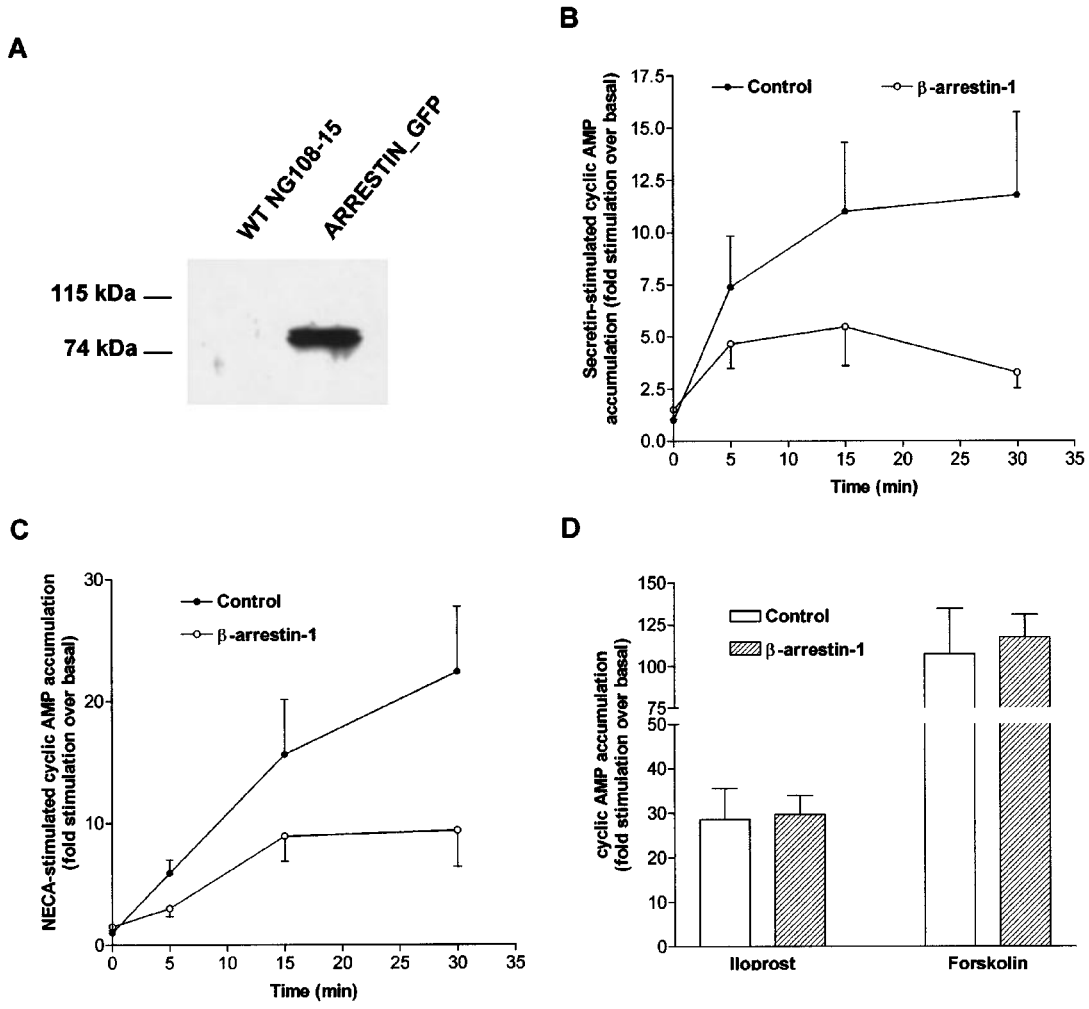

Figure 8 WT β-arrestin-1 overexpression inhibits secretin and NECA-stimulated cyclic AMP accumulation. (A) β-arrestin-1 overexpression in NG108-15 cells. Cells were transfected with WT β-arrestin-1-GFP. Whole cell lysates were subjected to SDS–PAGE followed by Western transfer and immunoblotting with an anti-GFP antibody as detailed in the Methods. A strong band of the expected molecular weight ($\sim$70 kDa) was observed following β-arrestin-1-GFP transfection. β-arrestin-1-overexpressing and plasmid-transfected control cells were incubated with (B) secretin (100 nM) or (C) NECA (10 μM) for the times indicated. Both secretin and NECA-stimulated cyclic AMP accumulation was significantly decreased in cells overexpressing β-arrestin-1 versus control ($P=0.0239$ and $P=0.0258$, respectively; two-way ANOVA). (D) Overexpression of β-arrestin-1 did not affect iloprost or forskolin-stimulated cyclic AMP accumulation at a 15 min time-point of agonist challenge. Cyclic AMP accumulation is expressed as fold stimulation over basal and data are mean $\pm$ s.e.mean of values from four independent experiments with each point performed in quadruplicate.

functional secretin receptors would also be manifested by an apparent decrease in receptor responsiveness. For example, a recent study reported that overexpression of DNM GRK2 enhances endogenous calcitonin receptor signalling in Chinese Hamster Ovary cells by increasing calcitonin receptor number (Horie & Insel, 2000). Binding studies at the secretin receptor would therefore be useful to assess whether the effects of GRK overexpression reflect enhanced short-term desensitization, or instead longer term changes in secretin receptor expression. However, the very low level of secretin receptors in NG108-15 cells (Gossen *et al*., 1990) makes this approach difficult.

In addition to the secretin receptor, GRK6 is also implicated in the agonist-induced desensitization of other class II GPCRs, including the vasoactive intestinal polypeptide type-1 ($VPAC_1$ receptor; Shetzline *et al*., 2002), and the calcitonin gene-related peptide (CGRP) receptor (Aiyar *et al*., 2000). The regulation of class II GPCRs by GRK6 does not however appear to be a common theme as desensitization of the parathyroid hormone and pituitary adenylate cyclase-activating polypeptide (PACAP) receptors has been shown to involve GRKs other than GRK6 (Dautzenberg & Hauger, 2001; Flannery & Spurney, 2001). In terms of the selectivity of GRK6 in regulating secretin receptor responsiveness in NG108-15 cells, this may arise from different affinities of the GRKs for activated receptor substrates, or alternatively to compartmentalization of signalling components such as the co-localization of the receptor with GRK6 at the plasma membrane in NG108-15 cells. Using confocal microscopy, we have observed co-localization of HA-epitope tagged β_2-adrenoceptors and GFP-tagged GRK6 at the plasma membrane in recombinant NG108-15 cells (data not shown).

A similar cellular distribution of GRK6 and the secretin receptor may exist. The idea of compartmentalization of GPCR signalling components is becoming increasingly accepted, and there is evidence that cellular compartmentalization may function to fine-tune cell signalling (Cordeaux & Hill, 2002). We have already suggested that compartmentalization may exist at the level of receptor-adenylyl cyclase coupling specificity in NG108-15 cells (Ghadessy & Kelly, 2002), and the same phenomenon may contribute to receptor-GRK specificity in homologous desensitization.

Finally, we examined the ability of β-arrestin-1 overexpression to modify secretin receptor responsiveness. Overexpression of this arrestin subtype inhibited secretin and adenosine A_2 receptor-stimulated cyclic AMP formation. One scenario is that GRK6 phosphorylates the secretin receptor, thus promoting the association of the arrestins with the receptor. The effect of β-arrestin-1 on adenosine A_2 responsiveness is not unexpected, since we have previously shown GRK2 to mediate adenosine A_2 receptor desensitization in NG108-15 cells (Mundell *et al*., 1997). Interestingly, as with GRK manipulation, β-arrestin-1 overexpression led to selective effects, since IP-prostanoid and forskolin-stimulated cyclic AMP formation were unaffected. Arrestins may therefore be involved in endogenous secretin receptor desensitization, and indeed secretin receptor stimulation promotes β-arrestin-1-GFP translocation from cytosol to membrane (Walker *et al*., 1999). However, since arrestin manipulation was reported not to affect secretin receptor internalization (Walker *et al*., 1999), it is possible that β-arrestin-1 interaction with the secretin receptor mediates agonist-induced desensitization but not internalization.

In conclusion, we show that secretin receptor responsiveness can be selectively regulated by endogenous GRK6 in NG108-15 cells. Thus the natively expressed secretin receptor in NG108-15 cells appears to be regulated by GRK6, as well as by PKA, and also heterologously following activation of PKC by other GPCRs (Ghadessy & Kelly, 2002). An important aim of future studies will be to identify the factors that determine the selectivity of different GRKs for GPCRs.

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