Cellular GRK Levels Regulate Sensitivity of the α_{2B}-Adrenergic Receptor to Undergo Agonist-induced Down-regulation.

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Running title: GRK level regulates agonist EC_{50} concentration for α_{2B}-AR down-regulation

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Abbreviations: G-protein coupled receptor kinase (GRK), Norepinephrine (NE), Epinephrine (EPI), adrenoceptor (AR), cyclic adenosine monophosphate (cAMP), neuroblastoma/glioma hydrid cells, NG108-15 (NG108), Glyceraldehyde phosphate dehydrogenase (GAPDH), prostaglandin E1 (PGE1), Polyvinylidene Difluoride (PVDF), G-protein coupled receptor (GPCR), metabotropic glutamate receptor 5 (mGluR5), Delta opioid receptor (δOR), Mitogen activated protein kinase (MAPK), Human embryonic kidney 293 cells (HEK293 cells), Opossum kidney cells (OK cells), $(1,4\text{-}[6,7(n)\text{-}^3H]$benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride ($[^3H]$RX821002) .

ABSTRACT

Chronic co-activation of α_{2B}- and β_2-ARs was recently reported to down-regulate the α_{2B}-AR at a lower threshold EPI concentration compared to when the α_{2B}-AR alone is activated. This is the result of a modest β_2-AR dependent up-regulation of GRK3. In the present study we determined that increasing GRK2 or GRK3 levels, independent of β_2-AR activation, decreases the EC_{50} concentration for agonist-induced down-regulation of the α_{2B}-AR using NG108 cells with or without over-expression (2-10 fold) of GRK2 or GRK3. In parental NG108 cells, the EC_{50} concentration of EPI required for down-regulation of the α_{2B}-AR is 30 µM. A 2-3-fold over-expression of GRK3 in NG108 cells however reduces the EC_{50} to 0.2 µM (a 150-fold decrease) while a comparable over-expression of GRK2 reduces it to 1 µM (a 30-fold decrease). However, when GRK3 or GRK2 in NG108 cells are overexpressed 8-10-fold, the EC_{50} concentration (0.02 µM EPI) for α_{2B}-AR down-regulation is reduced 1000-fold. These data clearly suggest that a modest (2-3 fold) up-regulation of GRK3 is more effective at enhancing the sensitivity of α_{2B}-AR to down-regulation after exposure to EPI than a modest up-regulation of GRK2, but that both GRK2 and GRK3 are equally effective at inducing α_{2B}-AR down-regulation when upregulated 8-10 fold. To our knowledge, this is the first report to systematically demonstrate that GRKs, particularly GRK3, play a pivotal role in modulating the agonist EC_{50} concentration that down-regulates the α_{2B}-AR and thus adds a new dimension to an already intricate signaling network.

INTRODUCTION

The α_{2B}-AR belongs to the superfamily of G-protein coupled receptors (GPCRs) and is activated by the catecholamines NE and EPI. Like most GPCRs, prolonged exposure of the α_{2B}-AR to agonists results in decreased responsiveness, primarily due to down-regulation of the receptors (Thomas and Hoffman, 1986, Convents et al., 1989, Heck and Bylund, 1997). Down-regulation of α_2-AR can result from either a decrease in receptor synthesis (Schaak et al., 2000) or increase in degradation of receptor protein (Heck and Bylund, 1997; Cayla et al., 1999). The commonly accepted model of GPCR regulation following agonist exposure is based on studies with the β_2-AR: Upon agonist binding the receptor is phosphorylated by G protein-coupled receptor kinases (GRK; Benovic et al., 1986), binds β arrestins (Benovic et al., 1987) and is internalized mainly via the clathrin-dependent pathway (Goodman et al., 1996). Following internalization, the receptor is either de-phosphorylated and recycled back to the plasma membrane (Krueger et al., 1997) or targeted to the lysosomes for degradation (Gagnon et al., 1998). Nevertheless, our understanding of factors modulating GPCR down-regulation is very limited.

There is significant ambiguity about the role of GRKs in agonist-induced down-regulation of the α_2-AR. In one heterologous expression system, agonist-induced down-regulation of the α_{2B}-AR (Jewell-Motz and Liggett, 1995) and other α_2-AR subtypes was found not to be dependent on GRK-mediated phosphorylation of these receptors (Jewell-Motz et al., 1997) but instead, dependent on palmitoylation state (Eason et al., 1994). In contrast, mutation of potential GRK phosphorylation sites in the α_{2C}-AR blocks the agonist-induced down-regulation of these receptors expressed in OK cells (Deupree et al., 2002). Moreover, in vivo studies also suggest an association between increases in GRK2 levels and down-regulation of the α_{2A}-AR (Ansonoff and

Etgen, 2001). Thus, there is no clear consensus on the role of GRK in modulating down-regulation of the α_2-AR.

Studies conducted in our lab have added another level of complexity to the intricate role of GRK in modulating down-regulation of the α_2-AR. We have shown that GRK is not only required for α_{2A}- and α_{2B}- AR down-regulation, but that the sensitivity of the α_2-AR to undergo down-regulation is increased if the level of GRK is increased (Bawa et al., 2003; Desai et al., 2004). This observation is interesting for two reasons. First, previous studies suggest that the α_2-AR requires exposure to supra-physiological agonist concentrations to undergo desensitization or down-regulation compared to other adrenoceptors, especially the β-AR (Atkinson and Minneman, 1992; Pleus et al., 1993). Second, there are a number of pathophysiological conditions that are associated with an increase in levels of GRK and abnormalities in GPCR signaling. In a mouse model of cardiac hypertrophy, a marked desensitization of the β-AR was associated with a 3-fold increase in the activity of GRK2 (Choi et al., 1997). In cystic fibrosis lung, a decrease in airway β-AR density is associated with an increase in GRK2 and GRK5 protein levels (Mak et al., 2002). Age-related desensitization of the β-AR is associated with increased expression of GRK2 and GRK3 (Schutzer et al., 2001). Desensitization and down-regulation of the μ opioid receptor during tolerance is associated with up-regulation of GRK2, GRK3 and β-arrestin2 (Hurlé, 2001). In addition, recent studies performed in cultured cells suggest a link between increased GRK levels and the sensitivity of GPCRs for desensitization and down regulation. Muscarinic receptors M_2 (Tsuga et al., 1998) and M_4 (Holroyd et al., 1999) and adenosine A_{2A} receptors (Mundell et al., 1998) are rendered more sensitive to desensitization, internalization or down-regulation when the cellular levels of GRK are increased. Further, prolonged activation of μ- or ORL1- opioid receptors resulted in

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desensitization of these receptors due to agonist-induced up-regulation of GRK2 and GRK3 (Thakker and Standifer 2002). Despite mounting evidence for a role of GRKs in down-regulation of GPCRs in general, and the α_2-AR in particular, current understanding of the effect of changing levels of GRK on the sensitivity of the α_2-AR to undergo desensitization and down-regulation is limited.

To address this issue we utilized NG108 cells, a model in which the requirement of supra-maximal concentration of agonist for α_2-AR desensitization and down-regulation has been demonstrated (Thomas and Hoffman, 1986; Convents et al., 1989). NG108 cells were transfected to express different levels of GRK2 or GRK3 and the EC_{50} concentration of EPI required to desensitize and down-regulate the α_{2B}-AR was determined. Information generated from this study helps to discern the significance of changes in GRK levels in α_{2B}-AR regulation.

MATERIAL AND METHODS

Material: The following drugs were purchased from the indicated sources:

(-) Epinephrine bitartrate (EPI), phenylmethylsulphonylflouride (PMSF), phentolamine, adenosine 3', 5' monophosphate (cAMP), prostaglandin E1 (PGE$_1$), Dulbecco's Modified Eagles medium (DMEM), adrenal cortex extract, hydroxyapatite, HAT (0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) supplement, sodium orthovanadate, sodium pyrophosphate, pepstatin, leupeptin, aprotinin, isobutylmethylxanthine (IBMX), sodium metabisulphite, theophylline, (N-[2-Hydroxyethyl]piperazine-N`-[2-ethanesulfonic acid]) (HEPES), bovine serum albumin and poly-l-lysine hydrobromide, (Sigma Chemical Co., St.Louis, MO); (-) norepinephrine (NE) (RBI, Natick, MA); [^{3}H]cAMP and [^{3}H]RX821002 ((1,4-[6,7(n)-^{3}H]benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride) (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, England); G418 sulphate (Geneticin) (Calbiochem, La Jolla, CA), fetal bovine serum and penicillin-streptomycin (Atlanta Biologicals, Norcross, GA); TEMED and ammonium persulphate (BioRad); anti GRK3 rabbit IgG (cat # sc-563), anti GRK2 rabbit IgG (cat # sc-562) goat antirabbit IgG HRP (cat # sc-2301), goat antimouse IgG HRP (cat # sc-2302) and Enhanced chemiluminiscence reagent (Santa Cruz Biotechnology, CA); mouse antirabbit GAPDH IgG (cat # RDI-TRK4G4C5) (Research Diagnostic Inc, NJ). NG108 cells were obtained from Dr. Graeme Milligan, University of Glasgow, Glasgow, Scotland, UK. GRK2 and GRK3 plasmids (pcDNA3.1 carrying the neomycin resistance cassette) were obtained from Dr. Brian Knoll, University of Houston, Houston, Texas.

Transfection of NG108 cells with GRK2 or GRK3: Stable transfection of GRK2 or GRK3 in NG108 cells was carried out using FUGENE 6 transfection reagent. Cells in 100mm tissue

culture plates (at 30-40% confluence) were incubated at 37°C with a transfection mixture composed of serum-free DMEM-H (HEPES-buffered DMEM) containing 11 µg of DNA/plate and 17 µl of FUGENE. After 48 h, the cells were split (1:12, 1:24 or 1:50) into 100mm tissue culture plates and the medium was supplemented with G418. Surviving colonies were isolated and expanded into cell lines. Whole cell lysates were assayed for the expression of GRK2 or GRK3 protein by Western blotting. NG108 cells transfected to over-express either GRK2 or GRK3 are described as K2/ # or K3/ # respectively, where # is the fold over-expression of GRK2 or GRK3 in the cells.

Cell Culture: NG108 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum, penicillin, streptomycin and HAT supplement. NG108 cells transfected to express either GRK2 or GRK3 were maintained similarly except that the media contained G418 (0.4 mg/ml) to retain selection pressure. All the cell lines were grown either in $75cm^2$ flasks or $150cm^2$ plates. Flasks or plates of cells that were more than 80% confluent were used throughout the study.

Pretreatment: NG108 cells and NG108 cells transfected to over-express either GRK2 or GRK3 were pretreated with vehicle (medium containing 0.1 mM Ascorbate and 1 µM Sodium metabisulphite), or vehicle containing 0.003 - 200 µM EPI for 24 h.

Alpha$_2$-AR agonist concentration response curves: After EPI pretreatment, media containing the drugs was aspirated and the cells were harvested by pipetting fresh drug-free medium against the cells. Intact cells were harvested and assayed for cAMP accumulation as described (Desai et

al., 2004). Briefly, intact cells were first incubated for 10 min at 37°C in HBSS buffer. Then PGE$_1$ (10 nM), NE and cells were added to assay tubes and the tubes were incubated for an additional 10 min at 37°C. All assays were performed in duplicate in a total volume of 0.5 ml. The assay was terminated by removing the tubes to a boiling water bath for 5 min. After boiling, samples were centrifuged for 5 min at 14,000Xg, and cAMP levels in the supernatant fractions were determined in a [^{3}H]cAMP (0.8 pmol) binding assay as previously described (Standifer et al., 1994). Preferential α_2-AR agonists were not used in this study because NG108 cells express imidazoline receptors, the activation of which inhibits cAMP accumulation (Greney et al., 2000). Since all preferential α_2-AR agonists would activate both the α_2- and imidazoline-receptors in the NG108 cells, this would significantly complicate data interpretation.

Membrane preparation for receptor binding: To prepare membranes for receptor binding, the cells were first washed 3 times with PBS (pH-7.4) and then harvested by gentle scraping. The cells were sedimented by centrifugation at 3000Xg for 10 minutes. The cell pellet was then suspended in 10 volumes of Tris-HCl buffer (50 mM, pH 7.7) containing NaCl (100 mM), Na$_2$EDTA (10 mM) and PMSF (0.1 mM) and homogenized with a polytron homogenizer (setting 5, 10 sec). The membranes were incubated for 15 min at 25°C and sedimented by centrifugation (20,000 rpm) for 30 minutes at 4°C. The membranes were immediately used for binding assay.

Radioligand binding assay to determine receptor number: In order to determine the number of α_{2B}-ARs, binding was performed using the α_2-AR antagonist, [^{3}H]RX821002. The membranes (0.25 - 0.30 mg protein/ml) were incubated with [^{3}H]RX821002 (30 nM), in potassium phosphate buffer (25 mM, pH 7.4) at 23°C for 30 min. Assays were performed in

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triplicate and non-specific binding was defined by using 100 µM phentolamine. At the end of the incubation period, the reaction was terminated by adding Tris-HCl buffer (50 mM, pH 8.0 at 4°C) and filtration over Whatman GF/B paper (Brandel). The filter paper was washed thrice with 3-4 ml of the filtration buffer (50 mM Tris-HCl pH 8.0). The amount of radioactivity in the filter paper was determined by scintillation spectroscopy in a Beckman LS6000 liquid scintillation counter.

Western blot analysis: Some of the cells collected either for α_{2B}-AR response assay or receptor binding were used to prepare samples for western blot analysis. Cell pellets were washed once with 1X PBS buffer (pH 7.4), lysed immediately in 100-150 µl of lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton-X 100, 0.1% SDS, 0.02% sodium azide, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin), vortexed and incubated for 30 min in an ice-bath. The resultant cell lysate was diluted with 2X Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1 mg/ml bromophenol blue) and resolved on SDS-PAGE (10% gel). The resolved proteins were electrophoretically transferred to a PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) and the GRK2 or GRK3 expression levels were determined by immunoblotting using anti-GRK2 or GRK3 (1:1000; Santa Cruz Biotechnology, CA) in 2.5% non-fat milk as described (Desai et al., 2004). The blots were stripped and reprobed for GAPDH (Glyceraldehyde phosphate dehydrogenase) as a loading control using mouse anti-rabbit GAPDH (1:8000; RDI, Flanders, NJ).

Protein estimation: Protein concentrations were determined by the Lowry's method (Lowry et al., 1951).

Data Analysis: B_{max}, EC_{50} and maximal response to the α_2AR agonist were determined by non-linear regression analysis using GraphPad Prism version 3.0 (GraphPad Software, Inc. San Diego, CA). Prism also was utilized to estimate the EC_{50} concentrations for α_2AR down-regulation. In these estimations of EC_{50} the iterative curve fitting process was constrained by setting the minimum value for receptor down-regulation at 0. For comparison between groups, the values were expressed as mean ± SEM. Between group comparisons were made either by Student's t-test or one way ANOVA followed by Tukey's post-hoc test where appropriate (GraphPad software, Inc. San Diego, CA), and groups were considered significantly different if $p < 0.05$.

RESULTS

In NG108 cells, the EC_{50} for down-regulation of the α_{2B}-AR is 30 μM EPI (**Figure 1A**). We previously showed that in NG108 cells transfected to express human β_2-AR, the α_{2B}-AR down-regulates after 24 h pretreatment with 0.3 μM EPI, a concentration at which the α_{2B}-AR does not down-regulate in the parental NG108 cells. This down-regulation is associated with a modest increase in the cellular levels of GRK3 (Desai et al., 2004). We therefore wanted to determine whether increasing the levels of GRK3, in the absence of β_2-AR activation, would reduce the concentration of EPI required to down-regulate the α_{2B}-AR. Accordingly, NG108 cells were transfected with GRK3 plasmids and clones over-expressing GRK3 at various levels (2-10 fold over basal) were isolated. The degree of over-expression of GRK3 in these clones compared to the parental NG108 cells is shown in **Table 1**. In NG108 cells transfected to over-express GRK3 (2-3-fold, K3/3) the EC_{50} for down-regulation of the α_{2B}-AR is 0.2 μM EPI. To insure that the decrease in EC_{50} for the down-regulation of the α_{2B}-AR in this clone was not the characteristic of a single clone, but the result of GRK3 over-expression, the α_{2B}-AR binding sites in another clone (K3/2.4) with comparable levels of GRK3 over-expression were also measured after 24 h pretreatment with either 0.1 μM (a concentration at which no down-regulation was observed in K3/3) or 0.3 μM EPI (the minimum concentration at which significant down-regulation of the α_{2B}-AR was observed in K3/3) and the results were similar (**data not shown**). On the other hand, in NG108 cells transfected to over-express GRK3 approximately 10-fold (K3/9.8) the EC_{50} for down-regulation of the α_{2B}-AR is 0.02 μM EPI (**Figure 1A**). The cellular level of GRK3 in K3/3, K3/2.4 and K3/9.8 were not altered by pretreatment with EPI (**Table 2**).

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Clearly, increasing the level of GRK3 renders the α_{2B}-AR more sensitive to agonist-induced down-regulation. Previous results indicate that the α_2-AR may be more susceptible to the action of GRK3 than GRK2 (Desai et al., 2004, Bawa et al., 2003, Diverse-Pierluissi et al., 1996). Therefore the sensitivity of the α_{2B}-AR to increasing levels of GRK2 also was determined. To that end, NG108 cells were transfected with GRK2 plasmids and a number of clones over-expressing GRK2 at various levels (2-10 fold over basal) were isolated. The degree of over-expression of GRK2 in these clones compared to the parental NG108 cells is shown in **Table 1**. In NG108 cells transfected to over-express GRK2 (2-3 fold, K2/2.9) the EC_{50} for down-regulation of the α_{2B}-AR is 1 μM EPI (**Figure 1B**). Similar results were obtained in a second clone (K2/2) with comparable GRK2 over-expression levels following EPI pretreatment (**data not shown**). In NG108 cells transfected to over-express GRK2 about 10 fold (K2/9.5) the EC_{50} for down-regulation is 0.02 μM EPI (**Figure 1B**). Again, we measured the α_{2B}-AR binding sites in another clone (K2/8.5) with comparable levels of GRK2 over-expression after pretreatment with 0.01 μM (a concentration at which no down-regulation was observed in K2/9.5) and 0.03 μM EPI (the minimum concentration at which down-regulation of the α_{2B}-AR was observed in K2/9.5) and the results were similar (**data not shown**). The cellular levels of GRK2 in K2/2.9, K2/2, K2/9.5 and K2/8.5 were not altered by pretreatment with EPI (**Table 2**).

In NG108 cells, 24h pretreatment with 20 μM EPI, the minimum concentration at which significant down-regulation of the α_{2B}-AR is observed, decreases the responsiveness of the α_{2B}-AR (**Figure 2**). Pretreatment with 10 μM EPI for 24h, a concentration at which significant down-regulation of the α_{2B}-AR is not observed, does not decrease the responsiveness of the α_{2B}-AR. The basal cAMP accumulation (pmol/mg protein) in NG108 cells is, Veh: 30.5 ± 2.3, 10 μM EPI: 29.6 ± 1.7 and 20 μM EPI: 31.3 ± 2.9. In NG108 and BN17 cells we always have observed

a decrease in responsiveness of the α_{2B}-AR at the minimum concentration of EPI that causes down-regulation of the α_{2B}-AR. Therefore, we determined whether the loss of α_{2B}-AR binding sites corresponds with loss of α_{2B}-AR responsiveness following 24 h pretreatment with EPI in NG108 cells transfected to over-express GRK2 or GRK3. Prior to that, the α_{2B}-AR responsiveness in naïve GRK2 or GRK3 over-expressing cells also was measured. The acute response to α_{2B}-AR activation was not altered in cells over-expressing GRK3 (**Figure 3A**) or GRK2 (**Figure 3B**) either 2-3 fold or 8-10 fold over basal levels. The basal cAMP accumulation (pmol/mg protein, 32.5 ± 4.2) in all the GRK3 or GRK2 over-expressing clones was similar to that seen in the NG108 cells.

Pretreatment of K3/3 and K3/2.4 for 24 h with 0.3 μM EPI desensitizes α_{2B}-AR signaling (**Figure 4A**). Similarly, a 24 h pretreatment with 0.03 μM EPI desensitizes the α_{2B}-AR signaling in K3/9.8 cells (**Figure 4B**). The α_{2B}-AR signaling also is desensitized in both K2/2.9 and K2/2 following 24 h pretreatment with 3 μM EPI (**Figures 5A**). A 24 h pretreatment with 0.03 μM EPI, the minimum concentration at which significant down-regulation of the α_{2B}-AR is observed, desensitizes the α_{2B}-AR signaling in both K2/8.5 and K2/9.5 (**Figure 5B**).

DISCUSSION

This study demonstrates that increasing the cellular levels of GRK3 or GRK2 increases the sensitivity of the α_{2B}-AR to agonist-induced down-regulation. To our knowledge this is the first evidence that levels of GRK3 or GRK2 in a given cell can regulate the EC_{50} concentration of agonist that down-regulates the α_{2B}-AR. Our report also is the first to demonstrate that when over-expressed at modest levels (2-3 fold) GRK3 is more effective than GRK2 in modulating the α_{2B}-AR. However, when over-expressed at high levels (8-10 fold) this difference between GRK3 and GRK2 is eliminated and both are equally effective.

The results of the present study provide evidence indicating that GRK3 is more effective than GRK2 in the long-term regulation of α_2-AR signaling. Both GRK3 and GRK2 previously have been shown to phosphorylate and desensitize the α_{2A}-AR equi-effectively (Jewell-Motz EA and Liggett SB, 1996). However, in that study GRK3 and GRK2 were over-expressed at very high levels (more than 15-fold); our data suggests that the subtle differences observed at lower, more physiological, levels of GRK3 and GRK2 over-expression were not apparent when GRK were expressed at high levels. There is substantial evidence in the literature to suggest that GRK3 may be a more important player than GRK2 in regulating the α_2-AR upon prolonged agonist exposure. Introduction of recombinant, purified GRKs or synthetic blocking peptides into individual embryonic sensory neurons has demonstrated the involvement of a GRK3-like protein in desensitization of the α_2-AR (Diverse-Pierluissi et al., 1996). We also have previously demonstrated that endogenous GRK3 rather than GRK2 plays an important role in modulation of the α_{2A}- and α_{2B}-AR after prolonged exposure to agonist (Bawa et al., 2003, Desai et al., 2004).

The results of the present study provide additional insights into the mechanisms that enable EPI to desensitize and down-regulate the α_{2B}-AR at lower concentrations than NE in cells that

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express α_2- and β_2-AR. We previously reported that the α_{2B}-ARs endogenously expressed in BN17 cells (NG108 cells transfected to express human β_2-AR) desensitize and down-regulate after exposure to low concentration of EPI (0.3 μM) due to an agonist-induced selective 2-fold up-regulation of GRK3 (Desai et al., 2004). This concentration of EPI was the minimum concentration that down-regulated the α_{2B}-AR and up-regulated GRK3. One of the confounding factors of our results in the BN17 cells was that β_2-AR activation was required for both the up-regulation of GRK3 and the down-regulation of the α_{2B}-AR at low EPI concentration. Therefore, we could not separate the roles of the β_2-AR and of GRK3 in the enhanced sensitivity of the α_{2B}-AR to down-regulation by EPI. Increasing the level of GRK in the absence of β_2-AR, as achieved in the present study, allowed us to distinguish between these possibilities. The data from NG108 cells transfected to over-express GRK3 or GRK2 suggests that a modest increase in cellular levels of GRK, by itself, is sufficient to produce a dramatic decrease in the EC_{50} for agonist-induced down-regulation of the α_{2B}-AR.

A critical role for GRKs in down-regulation of the α_2-AR following prolonged agonist exposure suggested by the present study, is in agreement with some studies but not others. Mutation of potential GRK phosphorylation sites in the third intracellular loop prevents agonist-induced down-regulation of the α_{2C}-AR expressed in OK cells (Deupree et al., 2002). In the human neuroblastoma cell line BE(2)-C, endogenous α_{2A}-AR undergo down-regulation in a GRK3-dependent manner (Bawa et al., 2003). In contrast, in Chinese hamster ovary (CHO) cells, a mutant α_{2A}-AR lacking potential GRK phosphorylation sites in the third intracellular loop does not undergo short-term desensitization but is down-regulated by 24 h agonist treatment (Jewell-Motz et al., 1997). One explanation for these conflicting results could be the difference in level of receptor expression between the different studies. In the OK cells, the WT or mutant α_{2C}-AR

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were expressed at about 40 or 300 fmol/mg protein (Deupree et al., 2002) and in BE(2)C cells, the α_{2A}-AR were expressed at approximately 40 fmol/mg protein (Bawa et al., 2003). In CHO cells where GRK was reported to play no role in down-regulation of the α_{2A}-AR, the receptors were expressed at 700 or 2000 fmol/mg protein. Another explanation could be that different cell types have different mechanisms for agonist-induced down-regulation of GPCRs. There are a number of reports in the literature that suggest cell-specific differences in the mechanism of down-regulation of GPCRs. For example, the δOR undergoes down-regulation either via a GRK-dependent mechanism or a MAP kinase- and tyrosine kinase-dependent mechanism depending on the cell type studied (Shapira et al., 2001). Coincidentally, in this report the GRK-dependent mechanism for down-regulating the δOR was dominant in NT18G cells where the receptors are expressed endogenously at low levels (about 130 fmol/mg protein) whereas the MAP kinase and tyrosine kinase mechanism for down-regulation was dominant in HEK293 cells transfected to express the δOR at high levels (17 pmol/mg protein). Overall, these data suggest that regulation of GPCRs by GRKs after prolonged exposure to agonist is a physiologically relevant phenomenon and one that warrants further investigation.

The present results also suggest that down-regulation of the α_{2B}-AR differs significantly from down-regulation of the β_2-AR with regard to the dose-response relationships for receptor signaling vs. down-regulation. For example, the EC_{50} for adenylyl cyclase activation by fenoterol in BEAS-2B cells (endogenous β_2-AR) is 36 nM. In those cells, fenoterol-induced down-regulation of the β_2-AR is proposed to proceed via low- and high- affinity pathways with EC_{50} of 163 nM and 0.53 nM respectively. The EC_{50} for the low-affinity pathway is about 10-fold greater than the EC_{50} for stimulation of adenylyl cylase and similar to the affinity of fenoterol for the β_2-AR (Williams et al., 2000). In contrast, the affinity for EPI at the α_{2B}-AR in

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NG108 cells is reported to be 3-10 nM and the EC_{50} for α_{2B}-AR-induced inhibition of cAMP accumulation in NG108 cells is about 30 nM, whereas the EC_{50} for down-regulation of the α_{2B}-AR is about 30 μM EPI. At the EC_{50} concentration for α_{2B}-AR down-regulation, the receptor is maximally activated and near saturation, very different from what is observed for the β_2-AR. Moreover, as the cellular level of GRK3 or GRK2 is increased, the EC_{50} for down-regulation of the α_{2B}-AR is reduced such that at 10-fold over-expression of either GRK3 or GRK2, the EC_{50} for down-regulation of the α_{2B}-AR approaches the EC_{50} for α_{2B}-AR-induced inhibition of cAMP accumulation. These results are supportive of the concept that in NG108 cells, the endogenous levels of GRK3 and GRK2 are a rate-limiting factor for down-regulation of the α_{2B}-AR. We hypothesize that the GRKs contribute to down-regulation of α_2-AR by phosphorylating the receptors, thereby facilitating the trafficking of the receptor to lysosomes for degradation. Further, we propose that a modest increase in the cellular level of GRK3 increases the rate of phosphorylation of the α_2-AR relative to the rate of its de-phosphorylation, whereas a modest increase in the level of GRK2 is less able to do so. Hence a small increase in GRK3 levels renders the α_2-AR more sensitive to agonist-induced down-regulation than a similar increase in GRK2. At high levels of GRK3 and GRK2 (~10 fold) over-expression, this difference between the two is eliminated because phosphorylation of the α_2-AR by either GRK dominates over its de-phosphorylation. Future studies will attempt to test this hypothesis.

A final important implication of this study relates to the pathophysiology of disease. There are a number of conditions that are associated with a modest change in levels of GRK and abnormalities in GPCR signaling. Hypothyroidism, a condition where β-AR signaling is compromised, has been reported to exhibit about 50% increase in GRK2 in heart and lung tissue (Penela et al., 2001). In a mouse model of cardiac hypertrophy, a marked desensitization of the

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β-AR was associated with 3-fold increase in the activity of GRK2 (Choi et al., 1997). In cystic fibrosis lung, a decrease in airway β-AR density is associated with increases in GRK2 and GRK5 protein levels (Mak et al., 2002). Age-related desensitization of the β-AR is associated with a 2-fold increase in expression of GRK2 and GRK3 (Schutzer et al., 2001). Desensitization and down-regulation of the μ opioid receptor during tolerance is associated with a 2-fold up-regulation of GRK2, GRK3 and β-arrestin2 (Hurlé, 2001). An increase in the levels of α_{2A}-AR and a decrease in the levels of GRK2 was observed in platelets of patients suffering from major depression and their treatment with the α_2-AR antagonist, mirtazapine, resulted in a 30% up-regulation of GRK2 and a 34% down-regulation of the α_{2A}-AR (Garcia-Sevilla et al., 2004). Our data suggest that the sensitivity of the α_2-AR to undergo agonist-induced down-regulation is dramatically increased by modest changes in the level of GRK2 or GRK3.

In summary, the present study suggests an important role of cellular GRK levels in regulating the sensitivity of the α_{2B}-AR to agonist-induced down-regulation. In addition, the results suggest that small increases in GRK3 expression can have profound effects on α_{2B}-AR regulation. However, the mechanisms whereby GRKs modulate the sensitivity of the α_2-AR for down-regulation remain to be determined.

REFERENCES

Ansonoff MA and Etgen AM (2001) Estrogen increases G protein coupled receptor kinase 2 in the cortex of female rats. Brain Res. 898(1): 186-189.

Atkinson BN and Minneman KP (1992) Preferential desensitization of beta- versus alpha2-adrenergic receptors accelerates loss of response to norepinephrine in primary glial cultures. Mol Pharmacol. 41(4): 688-694.

Bawa T, Altememi GF, Eikenburg DC and Standifer KM (2003) Desensitization of alpha (2A)-adrenoceptor signaling by modest levels of adrenaline is facilitated by beta (2)-adrenoceptor-dependent GRK3 up-regulation. Br J Pharmacol. 138(5): 921-931.

Benovic JL, Strasser RH, Caron MG and Lefkowitz RJ (1986) Beta-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. Proc Natl Acad Sci U S A. 83(9): 2797-2801.

Benovic JL, Kuhn H, Weyand I, Codina J, Caron MG and Lefkowitz RJ (1987) Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). Proc Natl Acad Sci U S A. 84(24): 8879-8882.

Choi D-J, Koch WJ, Hunter JJ and Rockman HA (1997) Mechanism of β-adrenergic receptor desensitization in cardiac hypertrophy is increased β-adrenergic receptor kinase. J. Biol. Chem. 272: 17223-17229.

Convents A, De Backer J-P, Andre C and Vauquelin G (1989) Desensitization of α_2- adrenergic receptors in NG108 cells by (-) adrenaline and phorbol 12-myristate 13-acetate. Biochem J. 262: 245-251.

Desai AN, Standifer KM and Eikenburg DC (2004) Simultaneous alpha2B- and beta2-adrenoceptor activation sensitizes the alpha2B-adrenoceptor for agonist-induced down-regulation. J Pharmacol Exp Ther. In press.

Deupree JD, Borgeson CD and Bylund DB (2002) Down-regulation of the alpha-2C adrenergic receptor: involvement of a serine/threonine motif in the third intracellular loop. BMC Pharmacol. 2(9).

Diverse-Pierluissi M, Inglese J, Stoffel RH, Lefkowitz RJ and Dunlap K (1996) G protein-coupled receptor kinase mediates desensitization of norepinephrine-induced Ca2+ channel inhibition. Neuron. 16(3): 579-585.

Eason MG, Jacinto MT, Theiss CT, Liggett SB (1994) The palmitoylated cysteine of the cytoplasmic tail of alpha 2A-adrenergic receptors confers subtype-specific agonist-promoted downregulation. Proc Natl Acad Sci U S A. 91(23): 11178-11182.

Gagnon AW, Kallal L and Benovic JL (1998) Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the β_2-adrenergic receptor. J. Biol. Chem. 273: 6976-6981.

Garcia-Sevilla JA, Ventayol P, Perez V, Rubovszky G, Puigdemont D, Ferrer-Alcon M, Andreoli A, Guimon J and Alvarez E (2004) Regulation of platelet alpha 2A-adrenoceptors, Gi proteins and receptor kinases in major depression: effects of mirtazapine treatment. Neuropsychopharmacology. 29(3): 580-588.

Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. Nature. 383: 447-450.

Greney H, Ronde P, Magnier C, Maranca F, Rascente C, Quaglia W, Giannella M, Pigini M, Brasili L, Lugnier C, Bousquet P, Dontenwill M (2000) Coupling of I (1) imidazoline receptors to the cAMP pathway: studies with a highly selective ligand, benazoline. Mol Pharmacol. 57(6): 1142-1151.

Heck DA and Bylund DB (1997) Mechanisms of down-regulation of α_2 adrenergic receptor subtypes. J Pharmacol Exp Ther. 282: 1219-1227.

Holroyd EW, Szekeres PG, Whittaker RD, Kelly E, Edwardson JM (1999) Effect of G protein-coupled receptor kinase 2 on the sensitivity of M4 muscarinic acetylcholine receptors to agonist-induced internalization and desensitization in NG108-15 cells. J Neurochem. 73(3): 1236-1245.

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Hurlé MA (2001) Changes in the expression of G protein-coupled receptor kinases and β-arrestin 2 in rat brain during opioid tolerance and supersentivity. J Neurochem. 77(2): 486-492.

Jewell-Motz EA and Liggett SB (1995) An acidic motif within the third intracellular loop of the alpha2C2 adrenergic receptor is required for agonist-promoted phosphorylation and desensitization. Biochemistry. 34(37): 11946-11953.

Jewell-Motz EA, Liggett SB (1996) G protein-coupled receptor kinase specificity for phosphorylation and desensitization of alpha2-adrenergic receptor subtypes. J. Biol. Chem. 271(30): 18082-18087.

Jewell-Motz EA, Donnelly ET, Eason MG and Liggett SB (1997) Role of the amino terminus of the third intracellular loop in agonist-promoted downregulation of the alpha2A-adrenergic receptor. Biochemistry. 36(29): 8858-8863.

Krueger KM, Daaka Y, Pitcher JA and Lefkowitz RJ (1997) The role of sequestration in G-protein coupled receptor resensitization. Regulation of β_2-adrenergic receptor dephosphorylation by vesicular acidification. J. Biol. Chem. 272: 5-8.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Mak JC, Chuang TT, Harris CA and Barnes PJ (2002) Increased expression of G protein-coupled receptor kinases in cystic fibrosis lung. Eur J Pharmacol. 436: 165-172.

Mundell SJ, Luty JS, Willets J, Benovic JL and Kelly E (1998) Enhanced expression of G protein coupled receptor kinase 2 selectively increases the sensitivity of A_{2A} adenosine receptors to agonist induced desensitization. Br J Pharmacol. 125(2): 347-356.

Penela P, Barradas M, Alvarez-Dolado M, Munoz A and Mayor F Jr (2001) Effect of hypothyroidism on G protein-coupled receptor kinase 2 expression levels in rat liver, lung, and heart. Endocrinology. 142(3): 987-991.

Schaak S, Cayla C, Lymperopoulos A, Flordellis C, Cussac D, Denis C and Paris H (2000) Transcriptional down-regulation of the human alpha2C-adrenergic receptor by cAMP. Mol Pharmacol. 58(4): 821-827.

Schutzer WE, Reed JF, Bliziotes M and Mader SL (2001) Upregulation of G protein-linked kinases with advancing age in aorta. Am J Physiol Reg Int Comp Physiol. 280: R897-R903.

Shapira M, Keren O, Gafni M and Sarne Y (2001) Diverse pathways mediate delta-opioid receptor down regulation within the same cell. Brain Res Mol Brain Res. 96(1-2): 142-150.

Standifer KM, Cheng J, Brooks AI, Honrado CP, Su W, Visconti LM, Bielder JL and Pasternak GW (1994) Biochemical and pharmacological characterization of Mu, Delta and Kappa3 opioid receptors expressed in BE(2)-C neuroblastoma cells. J Pharmacol Exp Ther. 270: 1246-1255.

Thakker DR and Standifer KM (2002) Induction of G protein-coupled receptor kinases 2 and 3 contributes to the cross talk between mu and ORL1 receptors following prolonged agonist exposure. Neuropharmacology. 43(6): 979-990.

Thomas JM and Hoffman BB (1986) Agonist induced downregulation of muscarinic and α_2-Adrenergic receptors after inactivation of Ni by pertussis toxin. Endocrinology 119: 1305-1314.

Tsuga H, Kameyama K, Haga T, Honmas T, Lameh J and Sadee W (1998) Internalization and down-regulation of human muscarinic acetylcholine receptor m2 subtype. Role of third intracellular m2 loop and G-protein coupled receptor kinase 2. J. Biol. Chem. 273(9): 5323-5330.

Williams BR, Barber R and Clark RB (2000) Kinetic analysis of agonist-induced down-regulation of the β_2-adrenergic receptor in BEAS-2B cells reveal high and low affinity components. Mol. Pharm. 58: 421-430.

FOOTNOTE

LEGENDS

Figure 1: The EC$_{50}$ for down-regulation of the α_{2B}AR in parental NG108 cells and NG108 cells transfected to over-express GRK3 (A) or GRK2 (B). A: Maximal binding of [^{3}H]RX821002 (30 nM) to α_{2B}AR was determined using membranes prepared from cells pretreated with vehicle and 0.003-200 μM EPI for 24 h. The EC$_{50}$ for down-regulation of the α_{2B}AR in NG108 cells is 30 μM EPI, in K3/3 cells is 0.2 μM EPI and in the K3/9.8 cells is 0.02 μM EPI. B: The EC$_{50}$ for down-regulation of the α_{2B}AR in K2/2.9 cells is 1 μM EPI and in the K2/9.5 cells is 0.02 μM EPI. *NG108 cells transfected to over-express either GRK2 or GRK3 are described as K2/ # or K3/ # respectively, where # is the fold over-expression of GRK2 or GRK3 in the cells.*

Fig 2: Chronic (24 h) pretreatment with 20 μM but not with 10 μM EPI desensitizes the α_{2B}AR in NG108 cells. NE-induced inhibition of 10 nM PGE$_1$-stimulated cAMP accumulation was studied in cells pretreated with vehicle, 10 μM or 20 μM EPI for 24 h. The pretreatment did not alter the potency of NE or the basal cAMP levels in the cells . However, the maximal % inhibition in cells pretreated with 20 μM EPI (32.8 $\pm$ 4.2) was significantly different (*) from that in the cells pretreated with either vehicle (50.6 $\pm$ 3.0) or 10 μM EPI (49.5 $\pm$ 3.7), p< 0.05. n=3-6.

Fig 3: Acute α_{2B}AR signaling is not altered in NG108 cells over-expressing either GRK3 (A) or GRK2 (B). NE-induced inhibition of 10 nM PGE$_1$-stimulated cAMP accumulation was studied in NG108 cells transfected to express either GRK2 or GRK3. The basal cAMP accumulation and potency of NE were similar among all the cell lines. The maximal % inhibition

was NG108 cells (54.7 ± 3.6), K3/3 (53 ± 4.6), K3/2.4 (52.6 ± 4.4), K3/9.8 (60.2 ± 3.6), K2/2 (54 ± 3.7), K2/2.9 (51.9 ± 3.1), K2/8.5 (51.9 ± 4.3) and K2/9.5 (46.4 ± 4.6); p>0.05 (n=3).

Fig 4: Chronic (24 h) pretreatment with 0.3 μM EPI desensitizes the α_{2B}AR in K3/3 and K3/2.4 cells (A), whereas chronic pretreatment with 0.03 μM EPI desensitizes the α_{2B}AR in K3/9.8 (B). A: NE-induced inhibition of 10 nM PGE$_1$-stimulated cAMP accumulation was studied in cells pretreated with vehicle or 0.3 μM EPI for 24 h. The pretreatment did not alter the potency of NE or the basal cAMP levels in the cells. However, the maximal % inhibition in K3/3 cells pretreated with 0.3 μM EPI (33.0 ± 2.8) was significantly different (*) from that in the cells pretreated with vehicle (50.0 ± 2.7), p<0.05. The maximal % inhibition in K3/2.4 cells pretreated with 0.3 μM EPI (35.7 ± 3.7) was significantly different (*) from that in the cells pretreated with vehicle (51.2 ± 3.0), p<0.05. n=3. **B:** NE-induced inhibition of 10 nM PGE$_1$-stimulated cAMP accumulation was studied in K3/9.8 cells pretreated with vehicle, 0.03 μM EPI for 24 h. The pretreatment did not alter the potency of NE or the basal cAMP levels in the cells. However, the maximal % inhibition in cells pretreated with 0.03 μM EPI (28.7 ± 2.5) was significantly different (*) from that in the cells pretreated with vehicle (55.2 ± 2.3), p<0.05. n=3.

Fig 5: Chronic (24 h) pretreatment with 3 μM EPI desensitizes the α_{2B}AR in K2/2.9 or K2/2 cells (A), whereas chronic pretreatment with 0.03 μM EPI desensitizes the α_{2B}AR in K2/9.5 cell. A: NE-induced inhibition of 10 nM PGE$_1$-stimulated cAMP accumulation was studied in K2/2.9 or K2/2 cells pretreated with vehicle or 3 μM EPI for 24 h. The pretreatment did not alter the potency of NE or the basal cAMP levels in the cells. However, the maximal % inhibition in

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K2/2.9 cells pretreated with 3 μM EPI (27.4 ± 1.6) was significantly different (*) from that in the cells pretreated with vehicle (44.3 ± 2.1), p<0.05. n=3. The maximal % inhibition in K2/2 cells pretreated with 3 μM EPI (31.0 ± 2.2) was significantly different (*) from that in the cells pretreated with vehicle (48.7 ± 1.7), p<0.05. n=3. **B:** NE-induced inhibition of 10 nM PGE_1-stimulated cAMP accumulation was studied in cells pretreated with vehicle or 0.03 μM EPI for 24 h. The pretreatment did not alter the potency of NE or the basal cAMP levels in the cells. However, the maximal % inhibition in K2/9.5 cells pretreated with 0.03 μM EPI (33.9 ± 2.8) was significantly different (*) from that in the cells pretreated with vehicle (49.0 ± 2.1), p<0.05. n=3. The maximal % inhibition in K2/8.5 cells pretreated with 0.0 3 μM EPI (34.2 ± 2.0) was significantly different (*) from that in the cells pretreated with vehicle (48.3 ± 2.2), p<0.05. n=3.

TABLE 1: Summary of the GRK2, GRK3 levels in parental NG108 cells and NG108 cells over-expressing either GRK2 or GRK3.

Cell line	GRK2/GAPDH	GRK3/GAPDH	Fold over-expression
NG108 cells			
-	0.94 ± 0.09	0.3 ± 0.02	-
NG108 cells transfected to over-express GRK2			
K2/2.9	2.72 ± 0.25		2.9
K2/2	1.94 ± 0.12	-	2.0
K2/9.5	8.96 ± 1.4	-	9.5
K2/8.5	7.98 ± 1.6	-	8.5
NG108 cells transfected to over-express GRK3			
K3/3	-	0.9 ± 0.21	3
K3/2.4	-	0.72 ± 0.03	2.4
K3/9.8	-	2.96 ± 0.65	9.8

[a] NG108 cells transfected to over-express either GRK2 or GRK3 are described as K2/ # or K3/ # respectively, where # is the fold over-expression of GRK2 or GRK3 in the cells.

Table 2: Summary of GRK2 and GRK3 levels in NG108 cells over-expressing either GRK2 or GRK3 after 24 h pretreatment with NE or EPI

NG108 cells transfected to over-express GRK3

Cell line	GRK3/GAPDH (mean ± SEM)			n
	Veh, 24 h	**0.3 μM NE, 24 h**	**0.3 μM EPI, 24 h**	
K3/3	1.03 ± 0.15	0.96 ± 0.22	1.03 ± 0.15	3
K3/2.4	0.75 ±0.04	0.63 ±0.03	0.75 ±0.05	3
	Veh, 24 h	**0.03 μM NE, 24 h**	**0.03 μM EPI, 24 h**	
K3/9.8	2.98 ±0.55	2.79 ±0.42	3.15 ±0.83	4

NG108 cells transfected to over-express GRK2

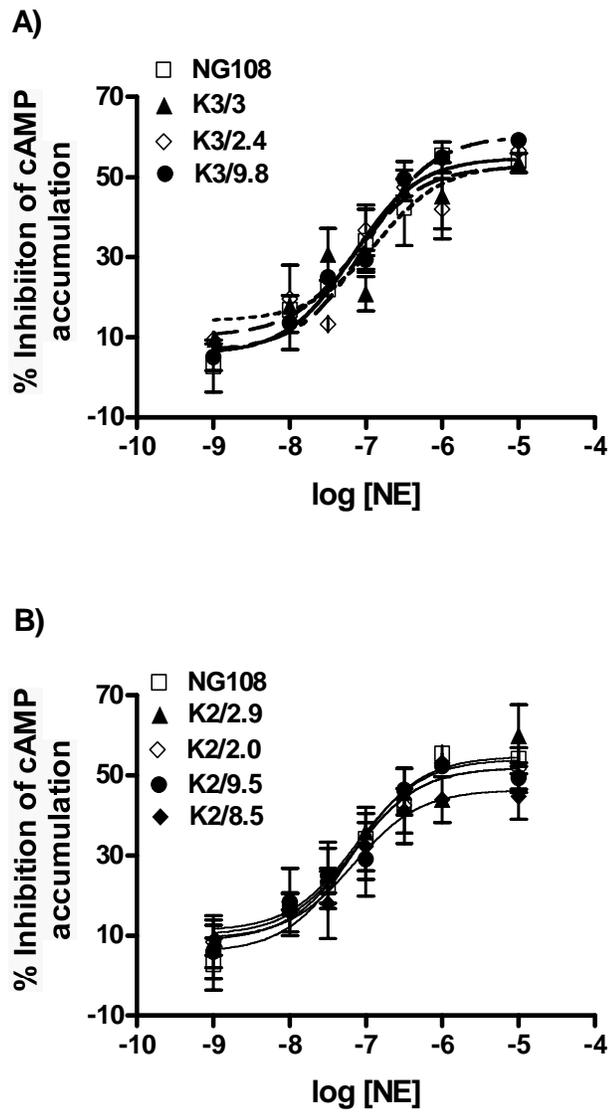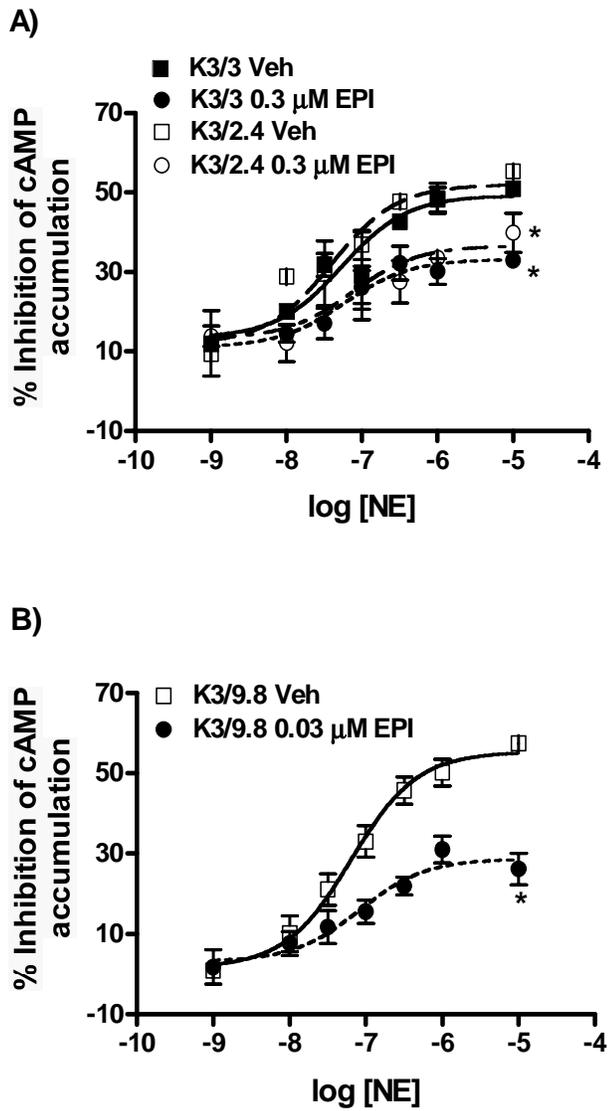| Cell line	GRK2/GAPDH (mean ± SEM)			n
	Veh, 24 h	**3 μM NE, 24 h**	**3 μM EPI, 24 h**	
K2/2.9	2.94 ±0.42	3.17 ±0.06	3.42 ±0.71	3
K2/2	1.84 ±0.08	1.78 ±0.12	1.87 ±0.24	3
	Veh, 24 h	**0.03 μM NE, 24 h**	**0.03 μM EPI, 24 h**	
K2/9.5	8.99 ±1.4	9.16 ± 1.7	9.51 ± 1.8	3
K2/8.5	7.96 ± 1.6	8.98 ± 2.2	9.07 ± 2.3	4

Figure 1
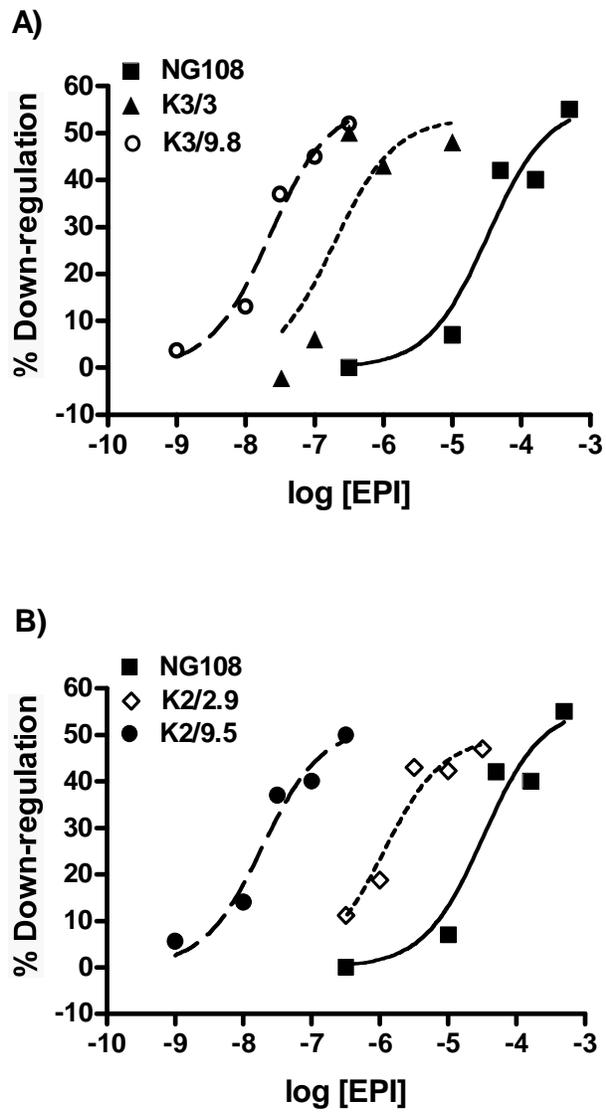
Figure 2

Figure 3

Figure 4

A)

B)

Figure 5

A)

B)