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

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Running title: GRK level regulates agonist $EC_{50}$ concentration for $\alpha_{2B}$-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-[6,7(n)-$^3$H]benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride ([$^3$H]RX821002) .

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**ABSTRACT**

Chronic co-activation of $\alpha_{2B}$- and $\beta_2$-ARs was recently reported to down-regulate the $\alpha_{2B}$-AR at a lower threshold EPI concentration compared to when the $\alpha_{2B}$-AR alone is activated. This is the result of a modest $\beta_2$-AR dependent up-regulation of GRK3. In the present study we determined that increasing GRK2 or GRK3 levels, independent of $\beta_2$-AR activation, decreases the $EC_{50}$ concentration for agonist-induced down-regulation of the $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-AR and thus adds a new dimension to an already intricate signaling network.

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## INTRODUCTION

The $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_2$-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 $\beta_2$-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 $\alpha_2$-AR. In one heterologous expression system, agonist-induced down-regulation of the $\alpha_{2B}$-AR (Jewell-Motz and Liggett, 1995) and other $\alpha_2$-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 $\alpha_{2C}$-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 $\alpha_{2A}$-AR (Ansonoff and



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Etgen, 2001). Thus, there is no clear consensus on the role of GRK in modulating down-regulation of the $\alpha_2$-AR.

Studies conducted in our lab have added another level of complexity to the intricate role of GRK in modulating down-regulation of the $\alpha_2$-AR. We have shown that GRK is not only required for $\alpha_{2A}$- and $\alpha_{2B}$- AR down-regulation, but that the sensitivity of the $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-AR in particular, current understanding of the effect of changing levels of GRK on the sensitivity of the $\alpha_2$-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 $\alpha_2$-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 $\alpha_{2B}$-AR was determined. Information generated from this study helps to discern the significance of changes in GRK levels in $\alpha_{2B}$-AR regulation.

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## 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



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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



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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 14,000X*g*, 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 $\alpha_2$-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 $\alpha_2$-AR agonists would activate both the $\alpha_2$- 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), $Na_2EDTA$ (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 $\alpha_{2B}$-ARs, binding was performed using the $\alpha_2$-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 $\alpha_{2B}$-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).

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**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 $\alpha_2$AR 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 $\alpha_2$AR 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 $p < 0.05$.

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**RESULTS**

In NG108 cells, the $EC_{50}$ for down-regulation of the $\alpha_{2B}$-AR is 30 μM EPI (**Figure 1A**). We previously showed that in NG108 cells transfected to express human $\beta_2$-AR, the $\alpha_{2B}$-AR down-regulates after 24 h pretreatment with 0.3 μM EPI, a concentration at which the $\alpha_{2B}$-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 $\beta_2$-AR activation, would reduce the concentration of EPI required to down-regulate the $\alpha_{2B}$-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 $\alpha_{2B}$-AR is 0.2 μM EPI. To insure that the decrease in $EC_{50}$ for the down-regulation of the $\alpha_{2B}$-AR in this clone was not the characteristic of a single clone, but the result of GRK3 over-expression, the $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-AR more sensitive to agonist-induced down-regulation. Previous results indicate that the $\alpha_2$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-AR is observed, decreases the responsiveness of the $\alpha_{2B}$-AR (**Figure 2**). Pretreatment with 10 μM EPI for 24h, a concentration at which significant down-regulation of the $\alpha_{2B}$-AR is not observed, does not decrease the responsiveness of the $\alpha_{2B}$-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



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a decrease in responsiveness of the $\alpha_{2B}$-AR at the minimum concentration of EPI that causes down-regulation of the $\alpha_{2B}$-AR. Therefore, we determined whether the loss of $\alpha_{2B}$-AR binding sites corresponds with loss of $\alpha_{2B}$-AR responsiveness following 24 h pretreatment with EPI in NG108 cells transfected to over-express GRK2 or GRK3. Prior to that, the $\alpha_{2B}$-AR responsiveness in naïve GRK2 or GRK3 over-expressing cells also was measured. The acute response to $\alpha_{2B}$-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 $\alpha_{2B}$-AR signaling (**Figure 4A**). Similarly, a 24 h pretreatment with 0.03 μM EPI desensitizes the $\alpha_{2B}$-AR signaling in K3/9.8 cells (**Figure 4B**). The $\alpha_{2B}$-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 $\alpha_{2B}$-AR is observed, desensitizes the $\alpha_{2B}$-AR signaling in both K2/8.5 and K2/9.5 (**Figure 5B**).

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## DISCUSSION

This study demonstrates that increasing the cellular levels of GRK3 or GRK2 increases the sensitivity of the $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_{2B}$-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 $\alpha_2$-AR signaling. Both GRK3 and GRK2 previously have been shown to phosphorylate and desensitize the $\alpha_{2A}$-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 $\alpha_2$-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 $\alpha_2$-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 $\alpha_{2A}$- and $\alpha_{2B}$-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 $\alpha_{2B}$-AR at lower concentrations than NE in cells that



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express $\alpha_2$- and $\beta_2$-AR. We previously reported that the $\alpha_{2B}$-ARs endogenously expressed in BN17 cells (NG108 cells transfected to express human $\beta_2$-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 $\alpha_{2B}$-AR and up-regulated GRK3. One of the confounding factors of our results in the BN17 cells was that $\beta_2$-AR activation was required for both the up-regulation of GRK3 and the down-regulation of the $\alpha_{2B}$-AR at low EPI concentration. Therefore, we could not separate the roles of the $\beta_2$-AR and of GRK3 in the enhanced sensitivity of the $\alpha_{2B}$-AR to down-regulation by EPI. Increasing the level of GRK in the absence of $\beta_2$-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 $\alpha_{2B}$-AR.

A critical role for GRKs in down-regulation of the $\alpha_2$-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 $\alpha_{2C}$-AR expressed in OK cells (Deupree et al., 2002). In the human neuroblastoma cell line BE(2)-C, endogenous $\alpha_{2A}$-AR undergo down-regulation in a GRK3-dependent manner (Bawa et al., 2003). In contrast, in Chinese hamster ovary (CHO) cells, a mutant $\alpha_{2A}$-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 $\alpha_{2C}$-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 $\alpha_{2A}$-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 $\alpha_{2A}$-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 $\alpha_{2B}$-AR differs significantly from down-regulation of the $\beta_2$-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 $\beta_2$-AR) is 36 nM. In those cells, fenoterol-induced down-regulation of the $\beta_2$-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 $\beta_2$-AR (Williams et al., 2000). In contrast, the affinity for EPI at the $\alpha_{2B}$-AR in



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NG108 cells is reported to be 3-10 nM and the $EC_{50}$ for $\alpha_{2B}$-AR-induced inhibition of cAMP accumulation in NG108 cells is about 30 nM, whereas the $EC_{50}$ for down-regulation of the $\alpha_{2B}$-AR is about 30 μM EPI. At the $EC_{50}$ concentration for $\alpha_{2B}$-AR down-regulation, the receptor is maximally activated and near saturation, very different from what is observed for the $\beta_2$-AR. Moreover, as the cellular level of GRK3 or GRK2 is increased, the $EC_{50}$ for down-regulation of the $\alpha_{2B}$-AR is reduced such that at 10-fold over-expression of either GRK3 or GRK2, the $EC_{50}$ for down-regulation of the $\alpha_{2B}$-AR approaches the $EC_{50}$ for $\alpha_{2B}$-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 $\alpha_{2B}$-AR. We hypothesize that the GRKs contribute to down-regulation of $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-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 $\alpha_{2A}$-AR and a decrease in the levels of GRK2 was observed in platelets of patients suffering from major depression and their treatment with the $\alpha_2$-AR antagonist, mirtazapine, resulted in a 30% up-regulation of GRK2 and a 34% down-regulation of the $\alpha_{2A}$-AR (Garcia-Sevilla et al., 2004). Our data suggest that the sensitivity of the $\alpha_2$-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 $\alpha_{2B}$-AR to agonist-induced down-regulation. In addition, the results suggest that small increases in GRK3 expression can have profound effects on $\alpha_{2B}$-AR regulation. However, the mechanisms whereby GRKs modulate the sensitivity of the $\alpha_2$-AR for down-regulation remain to be determined.

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**FOOTNOTE**

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**LEGENDS**

**Figure 1: The $EC_{50}$ for down-regulation of the $\alpha_{2B}$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 $\alpha_{2B}$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 $\alpha_{2B}$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 $\alpha_{2B}$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 $\alpha_{2B}$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 ± 4.2) was significantly different (*) from that in the cells pretreated with either vehicle (50.6 ± 3.0) or 10 μM EPI (49.5 ± 3.7), $p< 0.05$. n=3-6.

**Fig 3: Acute $\alpha_{2B}$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



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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 $\alpha_{2B}$AR in K3/3 and K3/2.4 cells (A), whereas chronic pretreatment with 0.03 μM EPI desensitizes the $\alpha_{2B}$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 $\alpha_{2B}$AR in K2/2.9 or K2/2 cells (A), whereas chronic pretreatment with 0.03 μM EPI desensitizes the $\alpha_{2B}$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.

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**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.

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**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 |

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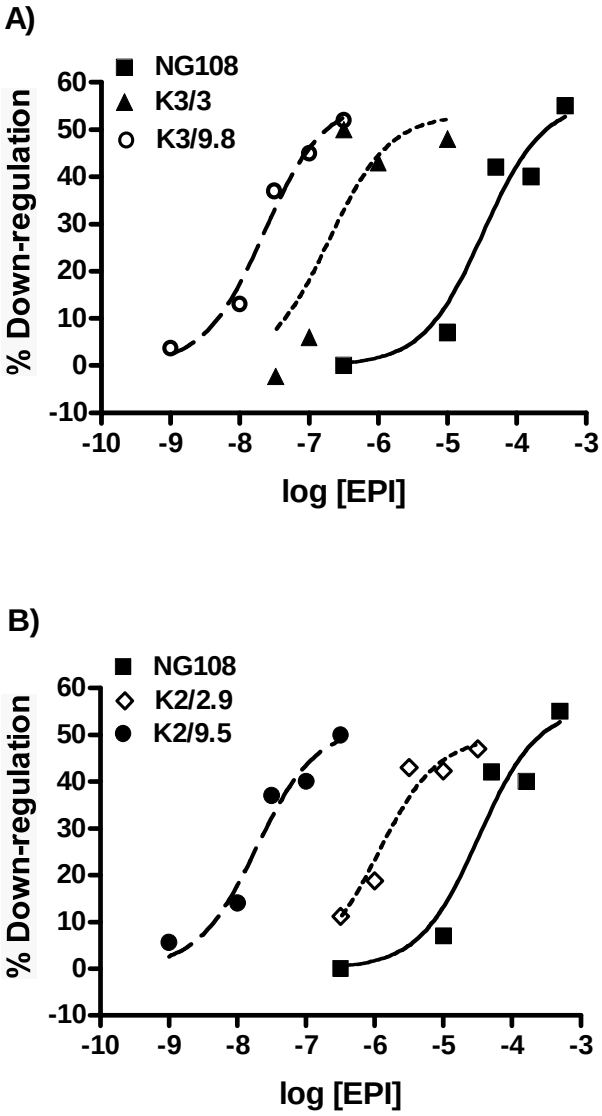
# Figure 1

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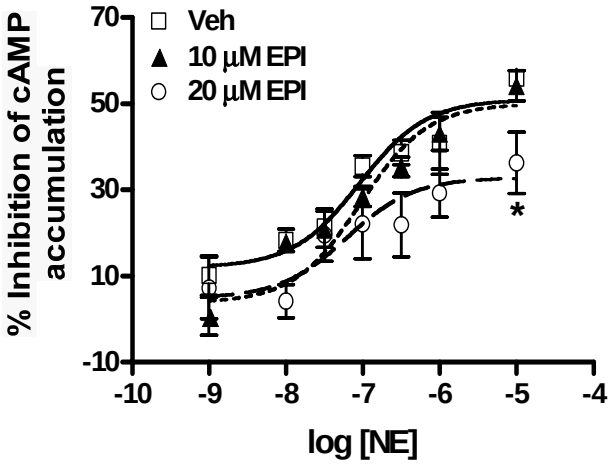
# Figure 2

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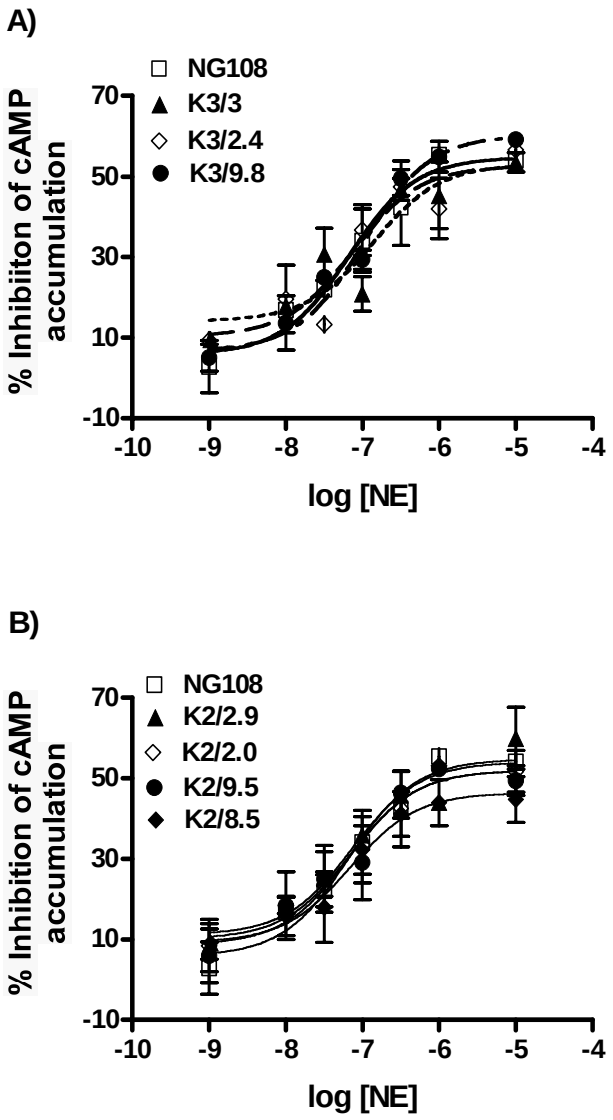
# Figure 3

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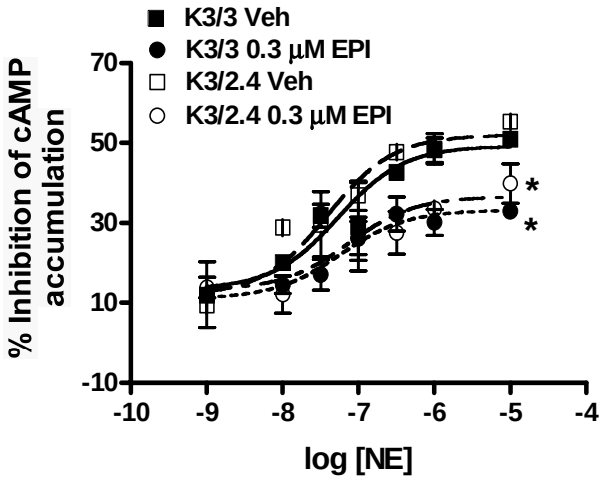
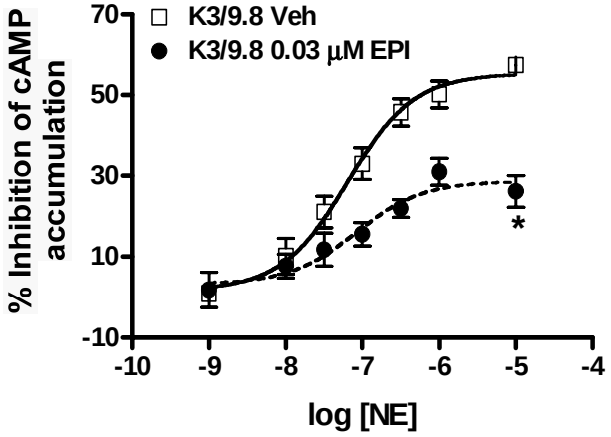
# Figure 4

A)




B)

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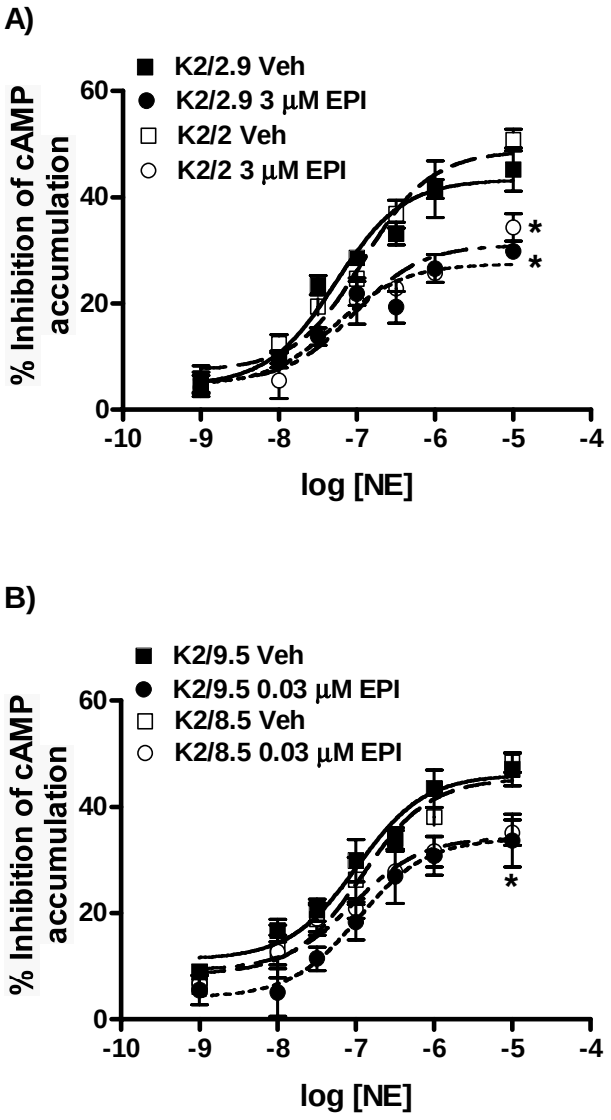
# Figure 5

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