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RESEARCH PAPER

Chronic adrenaline treatment fails to down-regulate the $Del_{301–303}$-α_{2B}-adrenoceptor in neuronal cells

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Background and purpose: A polymorphism of the human α_{2B}-adrenoceptor ($Del_{301–303}$-α_{2B}-adrenoceptor) has been described, and this receptor exhibits reduced G-protein-coupled receptor kinase (GRK) phosphorylation and impaired short-term desensitization. Expression of the $Del_{301–303}$-α_{2B}-adrenoceptor also is associated with an increased risk for myocardial infarction in humans. Recent evidence from our laboratory suggests a quantitative relationship between cellular GRK3 expression levels and the sensitivity of the α_{2B}-adrenoceptor to agonist-induced down-regulation. Therefore, the present study was undertaken to study agonist-induced down-regulation of the wild-type (WT)- and $Del_{301–303}$-α_{2B}-adrenoceptor in a neuronal cell model.
Experimental approach: Haemagglutinin (HA) epitope-tagged WT- and $Del_{301–303}$-α_{2B}-adrenoceptor containing plasmids were constructed and the receptors were stably or transiently transfected in neuroblastoma/glioma hybrid NG108 cells. The expression levels in stable transfects were ~50 fmol·mg^{-1}. These cells were used to examine agonist-induced down-regulation and phosphorylation of the WT- and $Del_{301–303}$-α_{2B}-adrenoceptor.
Key results: The $Del_{301–303}$-α_{2B}-adrenoceptor, compared with the WT-α_{2B}-adrenoceptor, displayed reduced adrenaline-stimulated (20 μM) phosphorylation and did not down-regulate in response to adrenaline (20–1000 μM). Using immunofluorescence labelling, we observed that transiently transfected WT-α_{2B}-adrenoceptors internalized upon adrenaline treatment whereas the $Del_{301–303}$-α_{2B}-adrenoceptor did not. Finally, we determined the effect of adrenaline on the $Del_{301–303}$-α_{2B}-adrenoceptor in cells stably over-expressing GRK3 3-fold. In spite of the GRK3 over-expression, 20–1000 μM ADR failed to down-regulate or to increase phosphorylation of the $Del_{301–303}$-α_{2B}-adrenoceptor in these cells.
Conclusions and implications: The results suggest that the 301–303 deletion mutation of the α_{2B}-adrenoceptor eliminates agonist-induced down-regulation, an effect that cannot be overcome by increasing agonist concentration or by modest GRK3 over-expression.
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Keywords: BE(2)-C cells; down-regulation; receptor binding; α_2-adrenoceptor; human neuroblastoma; polymorphism; phosphorylation

Abbreviations: GPCRs, G protein-coupled receptors; GRK, G-protein coupled receptor kinases; NG108, neuroblastoma/glioma hybrid cells, NG108-15; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride

Introduction

α_2-Adrenoceptors modulate many important functions including sympathetic and parasympathetic tone, the release of noradrenaline from sympathetic nerve terminals, vascular tone, lipolysis and insulin release (Westfall and Westfall, 2006; receptor nomenclature follows Alexander *et al.*, 2008). Alterations in α_2-adrenoceptor function reportedly contribute to the pathogenesis of hypertension, heart failure and myocardial infarction (Leenen, 1999; Aggarwal *et al.*, 2001; Brunner-La Rocca *et al.*, 2001; Francis, 2001). Therefore, understanding the mechanisms that govern the long-term regulation of these receptors has significant physiological and pathophysiological importance. However, most of the information currently available regarding the regulation of α_2-adrenoceptor signalling focuses on short-term rather than long-term regulation.

α_2-Adrenoceptors are phosphorylated by G protein-coupled receptor kinases (GRKs). In general, phosphorylation of G protein-coupled receptors (GPCRs) by GRKs is the proximal

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event in short-term GPCR desensitization, and mechanisms that impair receptor phosphorylation impair desensitization (Lefkowitz *et al.*, 1998; Clark and Knoll, 2002; Premont, 2005). While long-term desensitization of the α_2-adrenoceptors most relevant to chronic disease appears to depend largely on the loss of receptor protein, or down-regulation, the relationship between receptor phosphorylation and receptor down-regulation is unclear (Innamorati *et al.*, 1999; Tsao *et al.* 2001; Von Zastrow, 2001; 2003). Moreover, the role for GRKs in α_2-adrenoceptor down-regulation is particularly controversial. For example, the α_{2A}-adrenoceptor undergoes short-term agonist-induced desensitization that is associated with an increase in receptor phosphorylation. Specifically, four serines in the third intracellular loop of α_{2A}-adrenoceptors are the primary sites of receptor phosphorylation by GRKs. Deletion of some or all of these serines results in proportionally reduced agonist-induced receptor phosphorylation and eliminates short-term desensitization (Eason and Liggett, 1992; Eason *et al.*, 1995; Jewell-Motz *et al.*, 1997). However, deletion of these four serines does not affect agonist-induced down-regulation of the α_{2A}-adrenoceptor. Similarly, dissociation between the proximal step in receptor regulation, namely phosphorylation, and long-term receptor regulation has been reported for the α_{2B}-adrenoceptor. The site(s) of phosphorylation by GRKs in the α_{2B}-adrenoceptor are not known, but insights into the relationship between receptor phosphorylation, desensitization and down-regulation have been gained from the study of a polymorphic form of the human α_{2B}-adrenoceptor. Specifically, this polymorphism of the human α_{2B}-adrenoceptor, the $Del_{301–303}$-α_{2B}-adrenoceptor results from the deletion of three glutamic acid residues in its third intracellular loop. The $Del_{301–303}$-α_{2B}-adrenoceptors exhibit reduced agonist-induced receptor phosphorylation, impaired short-term α_{2B}-adrenoceptor desensitization and is associated with increased risk for myocardial infarction (Eason and Liggett, 1992; Jewell-Motz and Liggett, 1995; Small *et al.*, 2001; Snapir *et al.*, 2001). Long-term regulation of the $Del_{301–303}$-α_{2B}-adrenoceptor was not studied. However, in a previous study, deletion of amino acids 294–309 from α_{2B}-adrenoceptor reduced agonist-stimulated phosphorylation but had no effect on down-regulation, suggesting a lack of correlation between α_{2B}-adrenoceptor phosphorylation and down-regulation.

The above results are of interest because we have recently reported several lines of evidence that argue for a direct influence of GRKs, particularly GRK3, in the process of agonist-induced α_2-adrenoceptor down-regulation. First, in neuronal cell lines that express α_{2A}- or α_{2B}-adrenoceptors and β_2-adrenoceptors, treatment with adrenaline produced down-regulation of the α_2-adrenoceptor at concentrations 70-fold lower than those required for down-regulation by noradrenaline (Bawa *et al.*, 2003; Desai *et al.*, 2004). This difference was caused by twofold increase in GRK3 levels by adrenaline, but not noradrenaline, in these neuronal cells. Second, in neuronal NG108 cells, the EC_{50} for α_{2B}-adrenoceptor down-regulation by adrenaline is ~20 μM. However, when GRK3 expression is increased two to threefold by transfection, this results in a dramatic (70–100-fold) decrease in the adrenaline EC_{50} for down-regulation of the α_{2B}-adrenoceptor. Finally, a five to sixfold over-expression of the dominant negative C-terminal portion of GRK3, $GRK3_{ct}$, prevents long-term adrenaline-induced α_{2B}-adrenoceptor desensitization and down-regulation, as well as recruitment of GRK3 to the membrane (Desai *et al.*, 2005). Collectively, these data suggest that GRK3-mediated receptor phosphorylation may be the determining factor that modulates the sensitivity of the α_{2B}-adrenoceptor to down-regulation. Therefore, the present study was undertaken to examine agonist-induced phosphorylation and down-regulation of wild-type (WT)- and $Del_{301–303}$-α_{2B}-adrenoceptors in our neuronal cell model. The results suggest that the human $Del_{301–303}$-α_{2B}-adrenoceptor is deficient in agonist-induced receptor phosphorylation and down-regulation and these deficiencies are not overcome by higher agonist concentrations or increased GRK3 expression in our neuronal model.

Methods

Cell culture

The neuroblastoma/glioma hybrid NG108 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, streptomycin and HAT® supplement in a humidified atmosphere (5% CO2:95% air). All cells were grown until 70–80% confluent prior to adrenaline (ADR)-treatment.

Generation of stable clones

Non-tagged human wild type (WT) and $Del_{301–303}$-α_{2B}-adrenoceptor DNA were kindly provided by Dr Stephen Liggett, University of Maryland. The WT- and $Del_{301–303}$-α_{2B}-adrenoceptor DNA were subcloned from a PBC12BI vector into a pcDNA3.1 (zeo+) vector with an N-terminal haemagglutinin (HA) tag epitope (YPY DVP DYA). The HA-tagged WT- and $Del_{301–303}$-α_{2B}-adrenoceptor plasmids were generated by polymerase chain reaction (PCR) utilizing the restriction enzyme sites for Kpn 1 at the 5′end and Xho 1 at the 3′ end. The primers used were: forward primer, 5′-AGCTTTGTTTAAACATGTACCCAT-3′, and reverse primer, 5′-CCGGTACCTGTGTATACAGG-3′. The sequences of HA-WT- and $Del_{301–303}$-α_{2B}-adrenoceptor DNA constructs were verified by nucleotide sequencing. Stable transfection of HA-tagged human WT- and $Del_{301–303}$-α_{2B}-adrenoceptors in NG108 cells was carried out using Effectene transfection reagent. Cells in 100 mm tissue culture plates (at ~60% confluence) were incubated at 37°C with a transfection mixture composed of serum-free HEPES-buffered DMEM (DMEM-H) containing 2.5 μg of DNA/plate and 30 μL of Effectene reagent. After 48 h, the cells were split (1:2, 1:6 or 1:12) into 100 mm tissue culture plates and the medium was supplemented with zeocin (0.4 $mg{\cdot}mL^{-1}$). Surviving colonies were isolated and expanded into cell lines. Whole cell lysates were checked for the expression of HA-WT- and $Del_{301–303}$-α_{2B}-adrenoceptor protein by Western blotting and α_2-adrenoceptor radioligand binding with ^{3}H-rauwolscine.

Transient transfection of NG108 cells

NG108 cells (~70% confluent) were transfected with HA-WT- and $Del_{301–303}$-α_{2B}-adrenoceptors separately in 10 cm tissue

culture dishes using FuGENE 6 transfection reagent (2 μg DNA:6 μL FuGENE 6) in a total volume of 8 mL of serum-free NG108 media as per manufacturer's instructions. After 8 h incubation (37°C, 5% CO_2:95% air), 2 mL of complete media was added to the transfected cells and the cells were incubated overnight. The next morning, the media was changed to fresh complete NG108 media. Cells were harvested 36 h after the start of transfection as described (Salim *et al*., 2003).

Pretreatment
NG108 cells were pretreated with vehicle (medium containing 0.1 mM ascorbate and 1 μM sodium metabisulphite), or vehicle containing 20–1000 μM adrenaline for 8, 12 or 24 h for down-regulation measurements and 5 or 15 min for the phosphorylation and immunofluorescence assays. Ascorbate and sodium metabisulphite are antioxidants included to minimize autoxidation of adrenaline during the treatment. Inclusion of these agents is standard procedure for experiments involving treatment with catecholamines.

Western blot analysis
Cells were washed once with 1X phosphate buffered saline (PBS) solution (pH 7.4), lysed immediately in 100–200 μL of hypotonic lysis buffer (50 mM Tris-HCl pH 7.4, 4 mM ethylenediaminetetraacetic acid (EDTA), 100 $\mu g \cdot mL^{-1}$ PMSF, 1 $\mu g \cdot mL^{-1}$ leupeptin, 1 $\mu g \cdot mL^{-1}$ aprotinin and 1 $\mu g \cdot mL^{-1}$ pepstatin) followed by 5–6 passes through a 23-gauge needle and subsequently centrifuged at 3000× *g* for 10 min to remove cellular debris and nuclei (Salim *et al*., 2003). The lysates thus obtained were examined for protein concentration using Pierce's protein detection kit (Smith *et al*., 1985). The cell lysates were diluted with 4x Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1 $mg \cdot mL^{-1}$ bromophenol blue), resolved on SDS-PAGE (10% gel) and transferred to PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, England). Levels of HA-tagged WT- and $Del_{301–303}$-α_{2B}-adrenoceptor were determined by using anti-HA specific antibody (1:500 dilution). The immunoreactive bands were detected by a horseradish peroxidase-conjugated secondary antibody and the blots were developed using chemiluminescence reagent made by adding *p*-coumaric acid and luminol in 1 M Tris-HCl and hydrogen peroxide solution. Chemiluminescence was detected by an Alpha Innotech imaging system (Alpha Innotech, San Leandro, CA, USA) and densitometrically quantified using Fluorochem FC8800 software. The levels of expression of HA-WT- and $Del_{301–303}$-α_{2B}-adrenoceptor were checked with both anti-rat as well as anti-mouse antibodies. The specificity of the HA antibody was checked by using anti-HA peptide which resulted in disappearance of the signal produced by the HA antibody and by study of non-transfected cells.

Protein estimation
Protein concentrations were determined by the Pierce protein detection kit (Pierce, Rockford, IL Cat# 232009) using BCA protein assay reagent A (Cat# 23223) and reagent B (Cat# 23224) (Smith *et al*., 1985).

Membrane preparation for receptor binding
The membranes for receptor binding were prepared as described (Desai *et al*., 2005). Briefly, the cells were first washed three times with PBS (pH-7.4) and then harvested by gentle scraping. The cells were sedimented by centrifugation at 3000× *g* for 10 min. 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 s). The membranes were incubated for 15 min at 25°C and sedimented by centrifugation (14 300× *g*) for 30 min at 4°C. The membranes were immediately used for binding assay.

Radioligand binding assay to determine receptor number
In order to determine the α_{2B}-adrenoceptor number, binding was performed using the α_2-adrenoceptor antagonist, [^{3}H]rauwolscine. The membranes (0.25–0.30 $mg \cdot protein \cdot mL^{-1}$) were incubated with [^{3}H]rauwolscine (30 nM), in potassium phosphate buffer (25 mM, pH 7.4) at 25°C for 30 min. Assays were performed in triplicate and non-specific binding was defined with 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, Gaithersburg, MD, USA) on a cell harvester. The filter paper was washed three times 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 (Beckerman Coulter Inc., Fullerton, CA, USA).

Receptor down-regulation and phosphorylation
Membrane fractions of cells were analyzed by Western blotting using anti-HA antibody to quantify down-regulation. Receptor phosophorylation was quantified by immunoprecipitation of the receptors with anti-HA antibody, followed by Western blotting and detection with anti-phosphoserine antibody.

Immunoprecipitation
NG108 cells stably expressing HA-WT- or $Del_{301–303}$-α_{2B}-adrenoceptors were grown in 10 cm plates until ~70% confluent, then treated with vehicle/adrenaline (20 μM) for 5 and 15 min. The cells were washed once with cold 1X PBS buffer (pH 7.4) and cell membranes were prepared from the lysates as described above. Immunoprecipitation was carried out as published (Asghar *et al*., 2001) with some modifications. Anti-HA antibody was used to immunoprecipitate receptor protein. Briefly, the cell membranes were added to the IP buffer containing (in mM) 50 mm tris-HCl, pH 8.0, 150 NaCl, 1 EDTA, 1 EGTA, I DTT, 1 PMSF, 1% Triton-X 100, and protease inhibitor cocktail and incubated overnight with anti-HA antibody. The antigen-antibody complex thus formed was incubated with protein A/G agarose beads for 2 h. The ternary complex of antigen-antibody-protein A/G agarose was precipitated by centrifugation, washed once with IP buffer and then with a buffer containing (in mM) 50 Tris-HCl, pH 8.0, 250 NaCl, 1 EDTA, and 0.1% Triton X-100. The complex was

finally washed with another buffer containing (in mM) 50 Tris-$HClO_4$, pH 8.0, 250 NaCl. Bound proteins were eluted from protein A/G agarose beads by addition of 25 μL of SDS sample buffer followed by boiling for 5 min. Samples were analysed by electrophoresis on a 15% SDS-polyacrylamide gel and immunoblotted with anti-HA as well as anti-phosphoserine antibody. The amount of phosphorylated receptor was normalized for the amount of total receptor detected by the anti-HA antibody.

Immunofluorescence

The cells were processed for immunofluorescence as described previously (Salim *et al.*, 2007). Briefly, cells were grown on poly D-lysine coated 20 × 20 mm glass cover slips to 40–70% confluence. The cells were then exposed to vehicle/adrenaline (20 μM) for the indicated time period. Next, the cells were washed with PBS containing 1.2% sucrose (PBSS) and fixed with 4% paraformaldehyde in PBSS at 4°C for 15 min. The following steps were carried out at room temperature. The fixed cells were incubated in 0.034% L-lysine, 0.05% Na meta-periodate for 20 min, washed and permeabilized with 0.2% Triton X-100 for 10 min. After a further wash, the cells were blocked with 10% normal goat serum for 15 min. Primary (anti-HA antibody) and secondary antibody (anti-rat cy3-conjugated secondary antibody) were diluted in PBSS with 0.2% goat serum and 0.05% Triton X-100. The cells were incubated with anti-HA antibody for 1 h at room temperature or overnight at 4°C, followed by cy3-conjugated secondary antibody for 1 h in complete darkness. The cells were washed three times with PBSS before and after incubation with secondary antibody. The cover slips were then mounted on slides with a drop of a 1:1 mixture of Mowiol solution and VECTASHIELD mounting medium with DAPI. Fluorescence examination of at least six fields on the same slide was performed under an oil immersion objective (x 60, 1.4 NA) using a filter selective for cy3 or DAPI using an Olympus IX81 fluorescence deconvolution microscope system. DAPI staining enabled us to determine the area occupied by the nucleus. At each time point, a representative group of cells were assessed for the extent of receptor localization to the plasma membrane and receptor internalization. As a negative control, we stained the cells either with primary (anti-HA) or secondary (cy3 conjugated goat anti-rat IgG) antibody alone in order to determine the specificity of the fluorescence signal. Images were optimized using AutoDeblur and AutoVisualize deconvolution software (AutoQuant Imaging Inc., Watervliet, NY, USA) and transferred to Adobe Photoshop 5.5 (Adobe Systems Incorporated, San Jose, CA, USA) for production of the final figures.

Data analysis

Data are expressed as mean ± SEM. Comparisons between groups 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, USA), and groups were considered significantly different if $P < 0.05$.

Materials

The following were purchased from the indicated sources: (-) Adrenaline bitartrate, phenylmethylsulphonylflouride (PMSF), Dulbecco's modified Eagle's medium (DMEM), HAT® supplement (0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), sodium metabisulphite, theophylline, HEPES, bovine serum albumin, *p*-coumaric acid, luminol and poly-l-lysine hydrobromide, (Sigma Chemical Co., St. Louis, MO, USA); fetal bovine serum and penicillin-streptomycin (Atlanta Biological, Norcross, GA, USA), Pre-stained SDS-PAGE protein marker (Bio-Rad, Hercules, CA, USA, cat# 161-0324), Rat anti-HA antibody (Roche Diagnostics Corporation, IN, USA, cat#1867423), HA-peptide (Roche, cat#1666975), Zeocin (cat#45-0430, Invitrogen, Carlsbad, CA, USA), effectene transfection reagent (#301425, Qiagen Inc. (Valencia, CA, USA), FuGENE 6 transfection reagent (cat#1184-443-001 Roche Biochemicals, Indianapolis, IN, USA), pcDNA3.1/zeo(+) (cat#V86520, Invitrogen), DH5alpha competent cells (cat# 12297016, Invitrogen). Other molecular biology reagents including restriction enzymes, Xho I and Kpn I, DNA ladder, PCR reagents and Wizard SV gel and PCR clean-up system were purchased from Promega (Madison, WI, USA) and mini-prep kit and QIA gel extraction kit were from Qiagen Inc. Anti-phosphoserine antibody was purchased from Calbiochem (cat#525280 San Diego, CA, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-rat secondary antibodies and Protein A/G Plus-Agarose (sc#2003) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). NG108 cells were obtained from Dr. Graeme Milligan, University of Glasgow, Glasgow, Scotland, U.K. Reagents used in immunofluorescence were purchased from the source indicated: Cy3-conjugated goat anti-rat IgG (cat#112-165-1670 Jackson Immunoresearch Laboratory Inc., West Grove, PA, USA), VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (cat#VCH-1500 Vector Lab Inc., Burlingame, CA, USA), paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), Mowiol 4-88 reagent (cat#475904 Calbiochem, San Diego, CA, USA), Poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA), L-Lysine hydrochloride (JT Baker, Phillipsburg, NJ, USA), Sodium periodate (Fisher, Fair Lawn, NJ, USA), Goat serum (cat#G6767, Sigma-Aldrich). Cover slips used to grow cells were from VWR Scientific (West Chester, PA, USA) and the Superfrost microscope slides used to mount the cover slips were purchased from Fischer Scientific (Pittsburgh, PA, USA).

Results

Expression of H-WT- and Del$_{301–303}$-α_{2B}-adrenoceptors in stable clones of NG108 cells

Cell lines were developed from neuroblastoma/glioma hybrid NG108 cells that stably express either N-terminus HA-tagged human wild-type (WT)- or Del$_{301–303}$-α_{2B}-adrenoceptors. Multiple clones were selected using zeocin antibiotic (0.4 mg·mL^{-1}) and the total expression levels of receptor were determined by radioligand binding assay utilizing the α_2-adrenoceptor antagonist, [^{3}H]-rauwolscine (Figure 1A). As endogenous α_{2B}-adrenoceptor expression is approximately 50 fmol·mg^{-1} protein, several stable clones were obtained expressing HA-WT- (50 fmol·mg^{-1} protein) and Del$_{301–303}$-α_{2B}-adrenoceptors (50–600 fmol·mg^{-1} protein). The HA- Del$_{301–303}$-α_{2B}-adrenoceptor clones expressed receptor at levels as high as

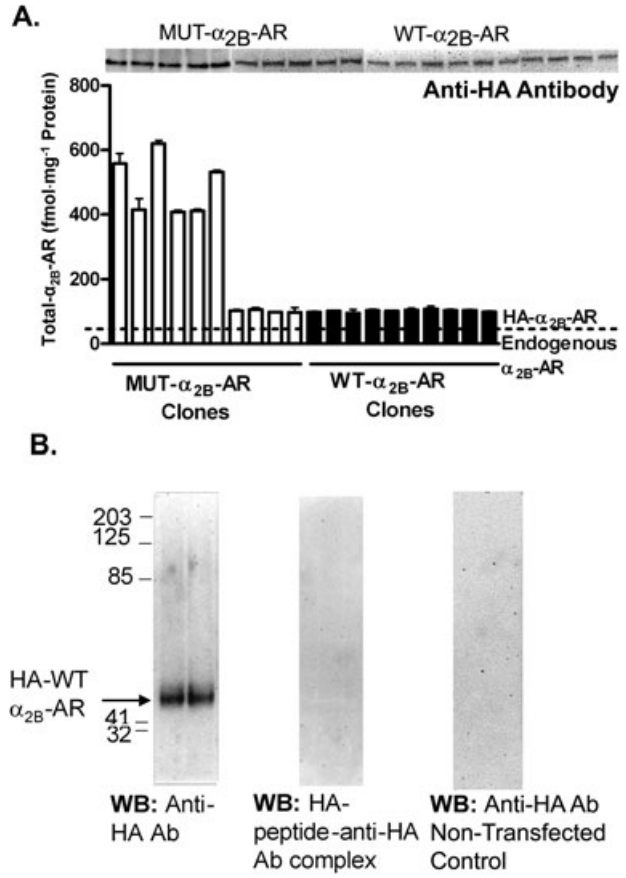

Figure 1 Expression of haemagglutinin-wild type (HA-WT)- and Del$_{301-303}$-α_{2B}-adrenoceptors after stable transfection of NG108 cells evaluated via radioligand binding and western blotting analysis. Total α_{2B}-adrenoceptor (α_{2B}-AR) numbers were determined by radioligand binding in membranes from the stable clones (A). Membrane fractions (0.25–0.30 mg·protein·mL^{-1}) were incubated with the α_2-adrenoceptor antagonist, [^{3}H]rauwolscine (30 nM) at 25°C for 30 min, the reaction was terminated by filtration over Whatman GF/B paper on a cell harvester, and the amount of radioactivity in the filter paper was determined by scintillation spectroscopy in a Beckman LS6000 liquid scintillation counter. The horizontal line in panel A represents endogenous α_{2B}-adrenoceptor expression (50 fmol·mg^{-1} protein) and receptor above this line represents the HA-tagged receptors. The membranes also were analysed for the HA-WT and Del$_{301-303}$-α_{2B} -adrenoceptor (MUT-α_{2B}-AR) protein expression by SDS-PAGE/Western blotting using anti-HA antibody (A) and the specificity of the bands were verified by peptide blocking (B, middle panel) and failure to detect the HA tag in membranes from non-transfected NG108 cells (B, right panel).

12-fold above the levels of endogenous α_{2B}- adrenoceptor expression, but the WT clones express the HA-WT-α_{2B}-adrenoceptors at the same levels as the endogenous α_{2B}-adrenoceptors. While total receptor number including both the endogenous as well as the HA-tagged transfected receptor is detected via radioligand binding, western blotting detects the HA-tagged transfected receptor. Therefore, in addition to determining receptor expression in the clones by radioligand binding, the expression of the transfected HA-WT- or Del$_{301-303}$-α_{2B}-adrenoceptors was detected in membrane preparations using an antibody specific for the HA-epitope on a 15% SDS-PAGE gel (Figure 1B, left panel). Densitometric analysis showed that the Western blots correlated with the level of receptor expression obtained via radioligand binding (data not shown). The specificity of the band corresponding to the expected size of the HA-α_{2B}-adrenoceptor (~50 KDa) was confirmed by peptide blocking (Figure 1B, middle panel). In the presence of the HA antigen (HA peptide taken 10 times in excess of the HA antibody), the immunoreactive bands of the HA-tagged WT- and Del$_{301-303}$-α_{2B} -adrenoceptor forms were undetectable (Figure 1B, middle panel). In addition, in membranes prepared from non-transfected NG108 cells, no labelling was observed with the anti-HA antibody (Figure 1B, right panel).

HA-Del$_{301-303}$-α_{2B}-adrenoceptors did not undergo down-regulation on prolonged adrenaline treatment or increasing adrenaline concentration

Next, we determined the effect of adrenaline treatment on the HA-WT- and Del$_{301-303}$-α_{2B} -adrenoceptor levels in membranes from NG108 cells that stably express the two receptors at comparable levels of expression (50 fmol·mg^{-1}). Cells stably expressing the HA-WT- or Del$_{301-303}$-α_{2B}-ARs were treated with vehicle or adrenaline (20 μM) for 8, 12 and 24 h. We had previously observed that 20 μM is the minimum adrenaline concentration required for significant down-regulation of the-α_{2B}-adrenoceptors in NG108 cells. (Desai *et al*., 2005). In addition, some preliminary experiments not included in the manuscript, suggest an EC_{50} concentration of ~20 μM, which is in good agreement with previously reported EC_{50} for the endogenous α_{2B}-adrenoceptors. The concentration 20 μM was chosen taking these two values into account. The cells were harvested, membranes were prepared as described (Desai *et al*., 2005) and subjected to SDS-PAGE. Receptor levels were determined using an anti-HA antibody. Adrenaline treatment (20 μM) significantly down-regulated HA-WT-α_{2B}-adrenoceptor after 8 h (43%). Continued treatment with adrenaline down-regulated the receptor further after 12 h (60%) and 24 h (54%) (Figure 2A). The HA-Del$_{301-303}$-α_{2B}-adrenoceptors failed to down-regulate after 8, 12 or 24 h of adrenaline treatment (20 μM) (Figure 2B). Similar results were obtained in whole cell lysates prepared from NG108 cells stably expressing the HA-WT- or Del$_{301-303}$-α_{2B}-adrenoceptors (data not shown). We also determined the effect of increasing concentrations of adrenaline (20, 100, 1000 μM) on the down-regulation in NG108 cells stably expressing HA-WT- or Del$_{301-303}$-α_{2B}-adrenoceptors (50 fmol·mg^{-1}). Maximum down-regulation (30%) of the HA-WT-α_{2B}-adrenoceptors was observed at 20 μM (Figure 3A). However, increasing the concentration of adrenaline to 1000 μM did not result in down-regulation of the HA-Del$_{301-303}$-α_{2B}-adrenoceptors (Figure 3B).

HA-Del$_{301-303}$-α_{2B} -adrenoceptors exhibit reduced agonist-induced phosphorylation

The extent of adrenaline-induced receptor phosphorylation also was determined for both the HA-WT- and Del$_{301-303}$-α_{2B}-adrenoceptors using an anti-phosphoserine antibody, normalized for total HA-tagged receptor. Phosphorylation was studied in a transfected system as there are no available antibodies to immunoprecipitate the endogenous receptor. At an

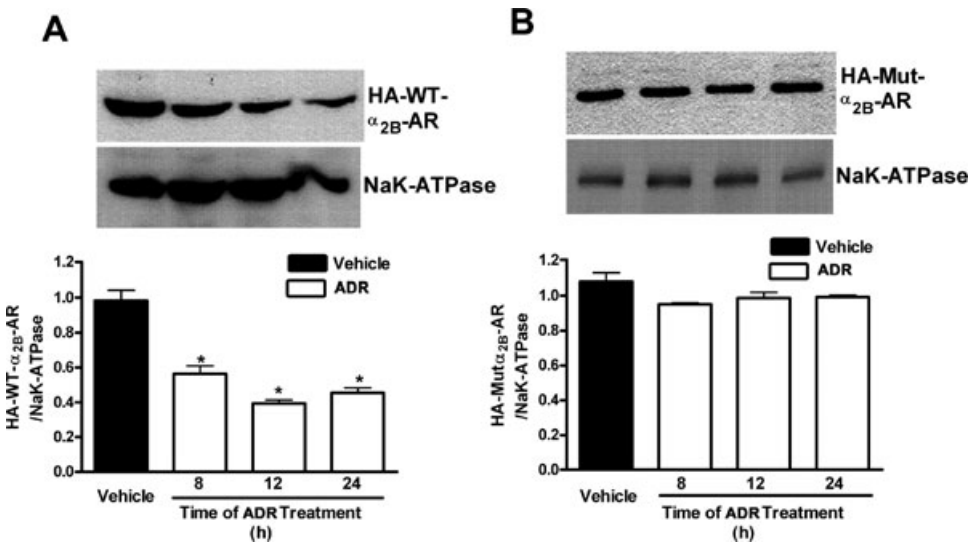

Figure 2 Haemagglutinin-wild type (HA-WT)-α_{2B}-adrenoceptor down-regulated (A) while HA-Del$_{301-303}$-α_{2B}-adrenoceptor did not down-regulate (B) upon adrenaline treatment in stable clones of NG108 cells. Cells expressing HA-WT-α_{2B}-adrenoceptor (HA-WT-α_{2B}-AR) or HA-Del$_{301-303}$-α_{2B}-adrenoceptor (MUT-α_{2B}-AR) were treated with 20 μM adrenaline (ADR) for the times indicated. The cells were harvested, membranes were prepared, subjected to SDS-PAGE/Western blotting and probed with anti-HA antibody. The blots were then stripped and re-probed with NaK-ATPase antibody (loading control). The clones expressing HA-WT-α_{2B}-adrenoceptor or HA-Del$_{301-303}$-α_{2B}-adrenoceptor are representative of at least three clones expressing 50 fmol·mg^{-1} protein. Data are expressed as mean ± SEM. *significantly different at $P < 0.05$, $n = 3$, using Student's *t*-test.

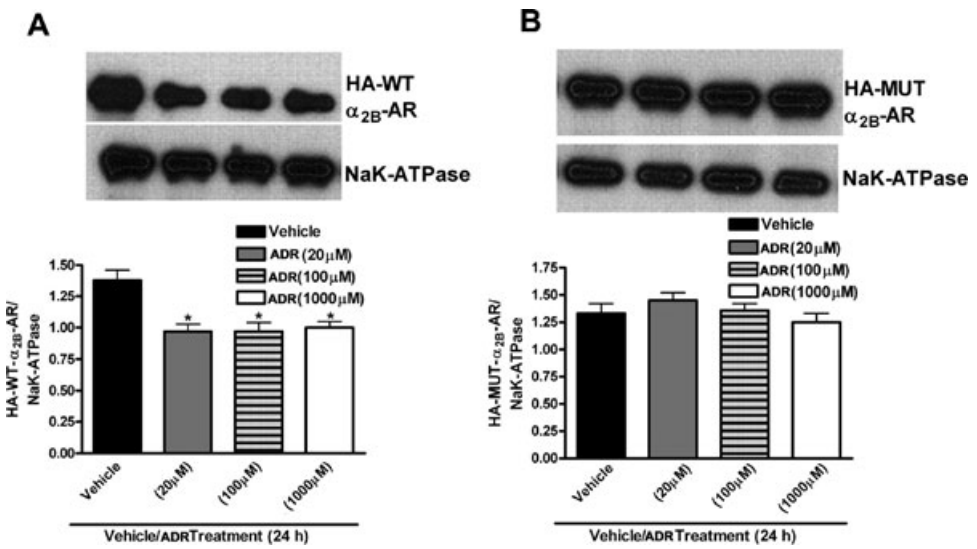

Figure 3 Haemagglutinin-wild type (HA-WT)-α_{2B}-adrenoceptors down-regulate (A) while HA-Del$_{301-303}$-α_{2B}-adrenoceptor does not down-regulate (B) upon chronic adrenaline treatment for 24 h at 20 μM, 100 μM or 1000 μM in stable clones of NG108 cells. Cells expressing HA-WT-α_{2B}-adrenoceptors (HA-WT-α_{2B}-AR) or HA-Del$_{301-303}$-α_{2B}-adrenoceptors (MUT-α_{2B}-AR) were treated with 20, 100 or 1000 μM adrenaline (ADR) for 24 h. The cells were harvested, membranes were prepared, subjected to SDS-PAGE/Western blotting and probed with anti-HA antibody. The blots were then stripped and re-probed with anti-NaK-ATPase antibody (loading control). Data are expressed as mean ± SEM. *significantly different at $P < 0.05$, $n = 3$, using Student's *t*-test.

adrenaline concentration that down-regulated the HA-WT-α_{2B}-adrenoceptors, phosphorylation was readily observed after short-term treatment (5–15 min) with an increase in phosphorylation of 34% at 5 min and 54% at 15 min (Figure 4A). Receptor phosphorylation also was increased after 12 and 24 h of agonist treatment (data not shown). However, adrenaline treatment produced no significant increase in the phosphorylation of the HA-Del$_{301-303}$-α_{2B}-adrenoceptor, which also exhibited greatly reduced phosphorylation (six to ninefold less) compared with the HA-WT-α_{2B}-adrenoceptors in the basal state (Figure 4B). To confirm that the anti-phosphoserine antibody was specifically detecting phosphorylation of the HA-tagged receptor, immunoprecipitation was conducted using membranes from NG108 cells transfected with the HA-WT-α_{2B}-adrenoceptor (Figure 4C, left panel) and from non-transfected NG108 cells (Figure 4C, right panel) and the immunoprecipitates probed with the anti-phosphoserine antibody. While serine phosphorylation

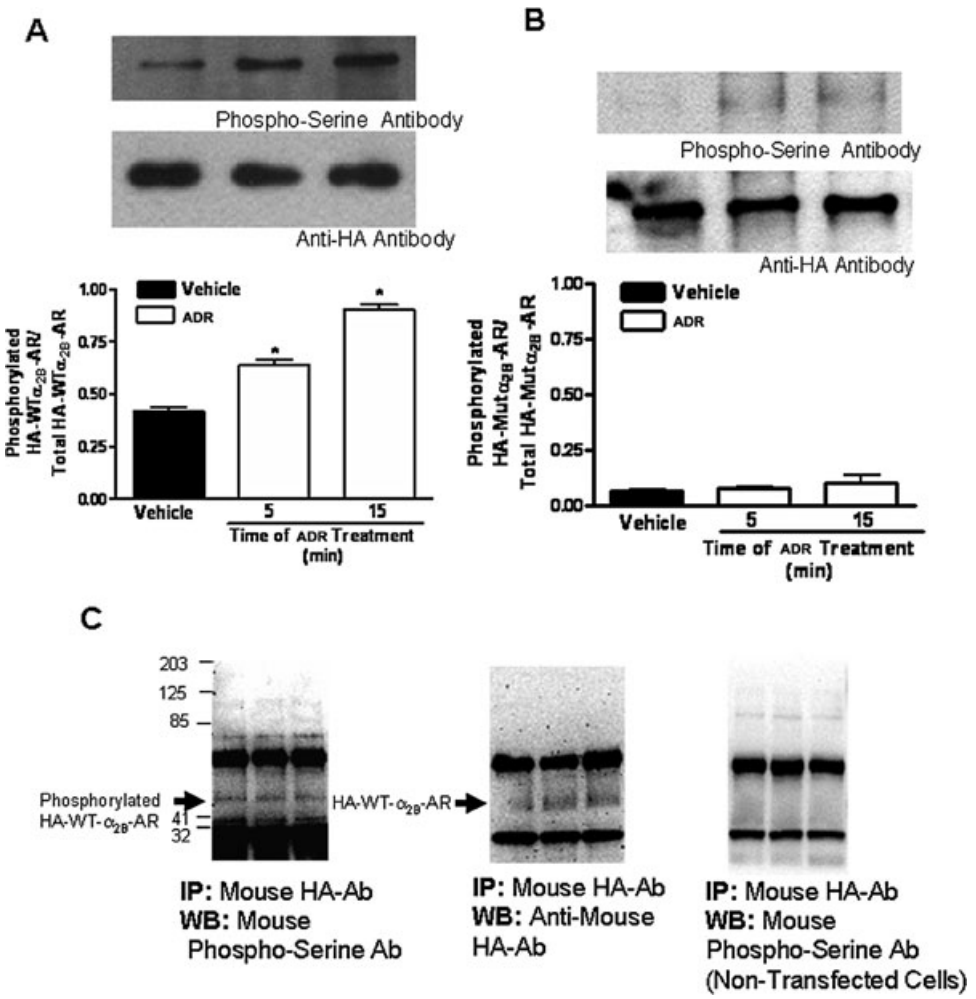

Figure 4 Haemagglutinin-wild type (HA-WT)-α_{2B}-adrenoceptor was phosphorylated (A) while HA-Del$_{301-303}$-α_{2B}-adrenoceptor was weakly phosphorylated (B) under basal conditions as well as after adrenaline treatment in NG108 cells stably expressing HA-WT-α_{2B}-adrenoceptors or HA-Del$_{301-303}$-α_{2B}-adrenoceptors. Cells expressing HA-WT-α_{2B}-adrenoceptors (HA-WT-α_{2B}-AR) or HA-Del$_{301-303}$-α_{2B}-adrenoceptors (MUT-α_{2B}-AR) were treated with 20 μM adrenaline (ADR) for the indicated times. The cells were harvested and membranes were prepared to quantify receptor phosphorylation by immunoprecipitation of the receptors with anti-HA antibody followed by SDS/PAGE/Western blotting and detection with anti-phosphoserine antibody [A, B, C (left panel)]. Data are expressed as mean ± SEM. *significantly different at $P < 0.05$, $n = 3$, using Student's *t*-test. These blots were then stripped and re-probed with anti-HA antibody to detect total HA-tagged receptor [A, B, C (middle panel)]. Immunoprecipitation also was conducted using membranes from non-transfected cells (C, right panel). This blot then was probed with anti-phosphoserine antibody to demonstrate the specificity of the phosphoserine detection for only HA-tagged receptor.

was detected in transfected cells in a band at the expected size of the HA-WT-α_{2B}-adrenoceptors (~50 KDa), no serine phosphorylation was detected in non-transfected cells other than the heavy and light chain immunoglobins. The blot from transfected cells was stripped and then probed with an anti-HA antibody to detect total HA receptor protein in the transfected cells (Figure 4C, middle panel). Combined, these data confirm that the anti-phosphoserine antibody is specifically detecting phosphorylation of the HA tagged receptor.

HA-Del$_{301-303}$-α_{2B}-adrenoceptors did not down-regulate and did not redistribute from the cell circumference, in response to agonist, in contrast to the WT receptor

In addition to studying the effect of adrenaline on receptor number and the degree of phosphorylation, we also attempted to study the trafficking of the HA-WT- and HA-Del$_{301-303}$-α_{2B}-adrenoceptors in stably transfected cells. Unfortunately, at the low levels of expression obtained for the HA-WT-α_{2B}-adrenoceptors (50 fmol·mg^{-1}), we were unable to visualize the WT-α_{2B}-adrenoceptors. However, we could visualize the stably expressed HA-Del$_{301-303}$-α_{2B}-adrenoceptors (600–800 fmol·mg^{-1}) by immunofluorescence. Therefore, in order to qualitatively examine internalization of both α_{2B}-adrenoceptors in intact cells, we utilized transient transfection of NG108 cells with HA-WT-α_{2B}-adrenoceptors or HA-Del$_{301-303}$-α_{2B}-adrenoceptors. To validate this approach, we transiently transfected NG108 cells with HA-WT-α_{2B}-adrenoceptors or HA-Del$_{301-303}$-α_{2B}-adrenoceptors, treated the cells with 20 μM adrenaline for 8, 12 and 24 h and measured down-regulation of the receptors. The transiently transfected receptors behaved similar to the stably transfected receptors. The HA-WT-α_{2B}-adrenoceptors expressed by transient transfection down-regulated upon 8 h (39%) treatment of

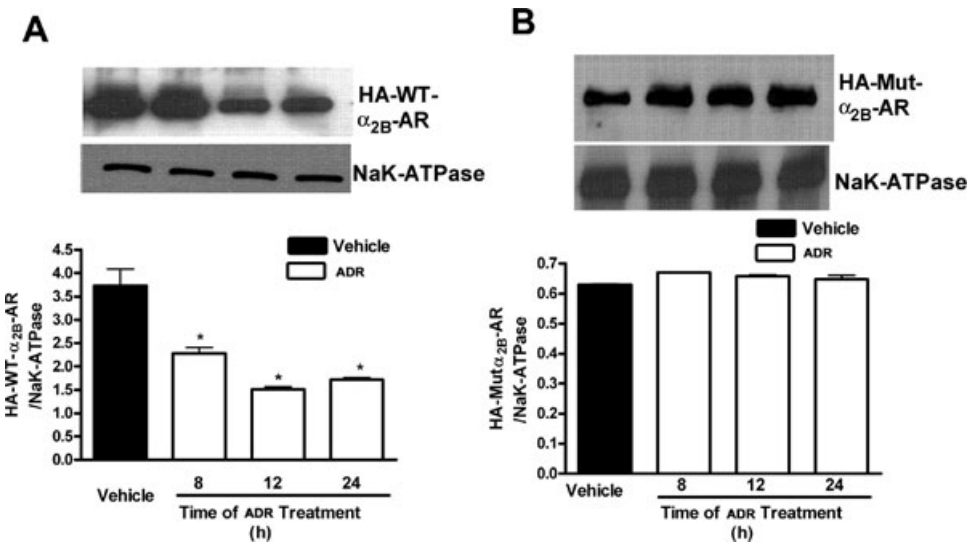

Figure 5 Haemagglutinin-wild type (HA-WT)-α_{2B}-adrenoceptors down-regulate (A) while HA-Del$_{301-303}$-α_{2B}-adrenoceptors did not down-regulate (B) upon adrenaline treatment in transiently transfected NG108 cells. Cells transiently expressing HA-WT-α_{2B}-adrenoceptor (HA-WT-α_{2B}-AR) or HA-Del$_{301-303}$-α_{2B}-adrenoceptor (MUT-α_{2B}-AR) were treated with 20 μM adrenaline (ADR) for the indicated times. The cells were harvested, membranes were prepared, subjected to SDS-PAGE/Western blotting and probed with anti-HA antibody. The blots were then stripped and re-probed with anti-NaK-ATPase antibody (loading control). Data are expressed as mean ± SEM. *significantly different at $P < 0.05$, $n = 3$, using Student's *t*-test.

adrenaline, continued to down-regulate at 12 h (59%) and 24 h (54%) while the HA-Del$_{301-303}$-α_{2B}-adrenoceptors did not (Figure 5A,B). Next, we examined the effect of 5 min adrenaline treatment (20 μM) on the localization of transiently transfected HA-WT-α_{2B}-adrenoceptors and HA-Del$_{301-303}$-α_{2B}-adrenoceptors by immunofluorescence. We observed that the HA-WT-α_{2B}-adrenoceptors were predominantly distributed around the cell circumference in vehicle-treated cells. Upon adrenaline treatment the receptors redistributed inside the cell circumference, with increased punctate staining away from the cell membrane, indicative of receptor internalization (Figure 6A). By contrast, the HA-Del$_{301-303}$-α_{2B}-adrenoceptor remained predominantly localized at the membrane (Figure 6B).

GRK3 over-expression failed to enable agonist-induced down-regulation and redistribution from the cell circumference of the HA-Del$_{301-303}$-α_{2B}-adrenoceptors, in response to agonist

Previous work in our laboratory suggested that an increase in GRK3 expression could greatly increase the sensitivity of the α_{2B}-adrenoceptors to adrenaline-induced down-regulation. Therefore, we examined the effect of adrenaline treatment on HA-WT- and HA-Del$_{301-303}$-α_{2B}-adrenoceptors in cells stably over-expressing GRK3 (3.0-fold) obtained as previously described (Desai *et al.*, 2005). GRK3 over-expressing cells were transiently transfected with HA-WT- or HA-Del$_{301-303}$-α_{2B}-adrenoceptors, treated with adrenaline (20–1000 μM) for 24 h, then harvested and receptor levels were examined in membranes prepared from the cells. The HA-WT-α_{2B}-adrenoceptors down-regulated at all concentrations of adrenaline treatment with 49% at 20 μM, 52% at 100 μM and 48% at 1000 μM adrenaline in GRK3 over-expressing cells (Figure 7A). On the other hand, the HA-Del$_{301-303}$-α_{2B}-adrenoceptors failed to down-regulate at all concentrations of adrenaline (20–1000 μM) in GRK3 over-expressing cells (Figure 7B). We also examined adrenaline-induced receptor internalization in GRK3 over-expressing cells as determined by immunofluorescence. Treatment with adrenaline (1000 μM) for 5 min caused redistribution of the receptor, inside the cell circumference, with increased punctate staining away from the cell membrane, indicative of receptor internalization of transiently transfected HA-WT-α_{2B}-adrenoceptors. Adrenaline had no effect on the distribution of the HA-Del$_{301-303}$-α_{2B}-adrenoceptors along the cell circumference in GRK3 over-expressing cells (Figure 8A,B). These results suggest that the deletion mutation in the α_{2B}-adrenoceptor eliminates agonist-induced down-regulation, an effect that cannot be overcome by increasing agonist concentration or GRK3 over-expression.

GRK3 over-expression failed to enable increased agonist-induced phosphorylation of the HA-Del$_{301-303}$-α_{2B}-adrenoceptor

To confirm that over-expression of GRK3 indeed increased receptor phosphorylation of the HA-WT-α_{2B}-adrenoceptor but not the HA-Del$_{301-303}$-α_{2B} -adrenoceptor, the extent of adrenaline-induced receptor phosphorylation was determined for both receptors in GRK3 over-expressing cells. Agonist-induced phosphorylation was readily observed after short-term adrenaline treatment (5–15 min) with an increase in phosphorylation of 40% at 5 min and 140% at 15 min for the WT receptor (Figure 9A). However, adrenaline treatment produced no significant increase in the phosphorylation of the HA-Del$_{301-303}$-α_{2B}-adrenoceptors (Figure 9B). Therefore, the results suggest that GRK3 over-expression fails to enable agonist-induced down-regulation of the HA-Del$_{301-303}$-α_{2B}-adrenoceptors because even after GRK3 over-expression, adrenaline did not produce a significant increase in receptor phosphorylation.

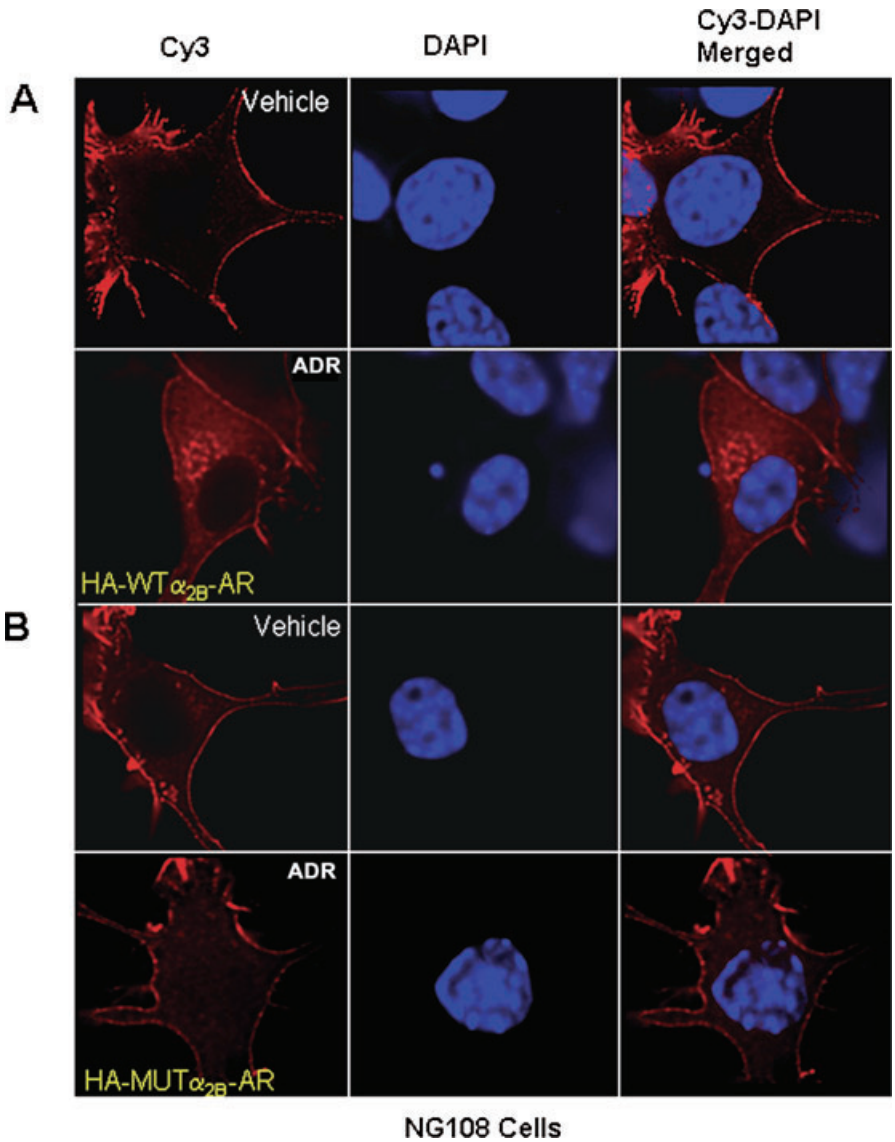

Figure 6 Haemagglutinin-wild type-α_{2B}-adrenoceptors (HA-WT-α_{2B}-AR; upper half) redistributed inside the cell circumference upon adrenaline (ADR) treatment (5 min) while HA-Del$_{301-303}$-α_{2B}-adrenoceptors (MUT-α_{2B}-AR; lower half) remained localized to the cell circumference in transiently transfected NG108 cells. NG108 cells were transiently transfected with WT and Del$_{301-303}$ receptor as described in the Methods section and visualized by probing with rat anti-HA antibody and anti-rat cy3 conjugated secondary antibody on a filter selective for cy3 at 60× magnification under an oil immersion objective, NA1.4. Nuclei are stained with DAPI and visualized under DAPI filter. $n = 3$.

Discussion and conclusions

The present study was undertaken to evaluate the long-term regulation of the WT- and Del$_{301-303}$-α_{2B}-adrenoceptors utilizing HA-epitope tagged receptors under conditions where the receptors are expressed at levels comparable with the endogenous α_{2B}-adrenoceptors. The results suggest that ADR readily down-regulates the HA-WT-α_{2B}-adrenoceptors, similar to the endogenously expressed receptor. However, the HA-Del$_{301-303}$-α_{2B}-adrenoceptors, with a mutation consisting of deletion of three glutamic acids in the third intracellular loop, exhibits reduced agonist-induced phosphorylation, does not down-regulate after treatment with concentrations of adrenaline as high as 1 mM and does not down-regulate when expressed in GRK3 over-expressing cells. This mutant receptor also does not redistribute from the cell circumference upon agonist treatment, suggesting that it does not internalize in response to agonist (adrenaline), in contrast to the WT receptor. Some might consider it a limitation of our phosphorylation experiments, and their relevance to agonist-induced down-regulation, that down-regulation is not observed for 6–8 h while we measured only short-term receptor phosphorylation. However, as short-term agonist-induced receptor phosphorylation, internalization and down-regulation are not observed with the Del$_{301-303}$-α_{2B}-adrenoceptors, we suggest that our conclusion regarding an important role for GRK-mediated receptor phosphorylation in the agonist-induced down-regulation of the α_{2B}-adrenoceptors is well justified.

The observation in the current study that the HA-Del$_{301-303}$-α_{2B}-adrenoceptor does not undergo down-regulation on

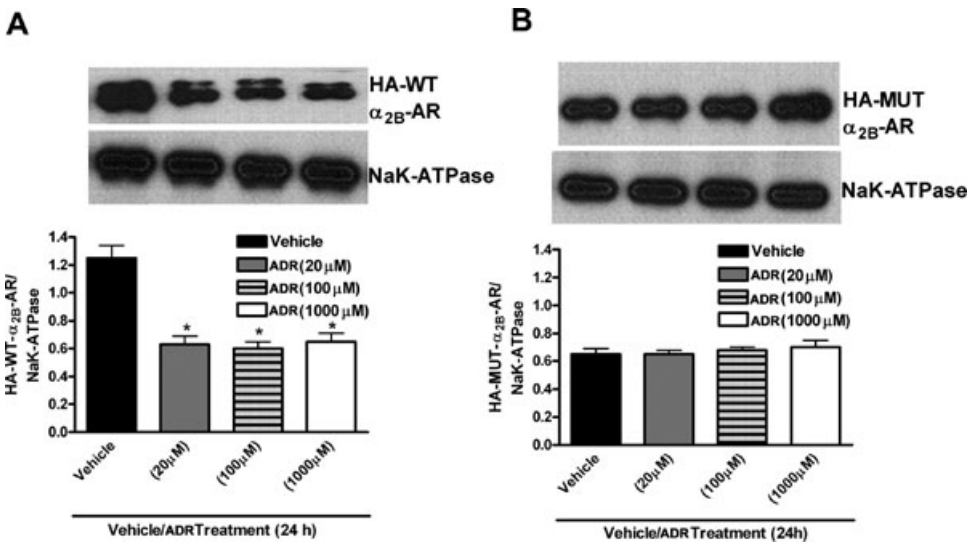

Figure 7 Haemagglutinin-wild type (HA-WT)-α_{2B}-adrenoceptors (A) down-regulate upon adrenaline treatment while HA-$Del_{301–303}$-α_{2B}-adrenoceptors (B) levels remains unchanged in GRK3 over-expressing cells transiently transfected to express the α_{2B}-adrenoceptors. Cells expressing HA-WT-α_{2B}-adrenoceptors (HA-WT-α_{2B}-AR) or HA-$Del_{301–303}$-α_{2B}-adrenoceptors (MUT-α_{2B}-AR) were treated with 20, 100 and 1000 μM adrenaline (ADR) for 24 h. The cells were harvested, membranes were prepared, subjected to SDS-PAGE/Western blotting, and probed with anti-HA antibody. The blots were then stripped and re-probed with anti-NaK-ATPase antibody (loading control). Data are expressed as mean ± SEM. *significantly different at $P < 0.05$, $n = 3$, using Student's *t*-test.

prolonged adrenaline treatment appears to conflict with previous reports. For example, when the down-regulation of two different mutant forms of the α_{2B}-adrenoceptor was examined, it was concluded that receptor phosphorylation was not important for agonist-induced down-regulation of the α_{2B}-adrenoceptor (Jewell-Motz and Liggett, 1995). In this report, amino acids 294–309 (EDEAEEEEEEEEEEEE) were either replaced by glutamine (Q) or deleted from the α_{2B}-adrenoceptor, and both mutants exhibited an approximately 50% reduction in agonist-induced phosphorylation. However, both mutants underwent agonist-induced sequestration and down-regulation comparable with that observed for the WT-α_{2B}-adrenoceptor. It was concluded that sequestration and down-regulation of the α_{2B}-adrenoceptors were regulated by events independent of receptor phosphorylation.

There are several potential reasons for the divergent results in the present study and this previous investigation (Jewell-Motz and Liggett, 1995). First, the amino acid residues deleted from the α_{2B}-adrenoceptor in the previous study (294–309) are different from those in $Del_{301–303}$-α_{2B} -adrenoceptor (301–303) used in the present study. While both deletions include residues 301–303, the consequences of the respective deletions on receptor structure are unknown and may be significantly different. Second, our studies were carried out in neuronal cells that endogenously express the α_{2B}-adrenoceptors while the previous study was performed in Chinese hamster ovary cells that do not endogenously express α_{2B}-ARs. Finally, in the previous study, the WT- and α_{2B}-adrenoceptors with amino acids 294–309 deleted were expressed at 2078–2105 fmol·mg^{-1} protein, as compared with the 50 fmol·mg^{-1} protein for the HA-WT- and HA-$Del_{301–303}$-α_{2B} -adrenoceptors studied herein. Recent studies in our neuronal cells indicate that small (two to threefold) increases in GRK3 levels can increase the sensitivity for agonist-induced α_{2B}-adrenoceptor down-regulation by 70–100 fold (Desai *et al.*, 2005). These results suggest that the basal cellular levels of GRK3 limit the sensitivity to down-regulation, even when receptor is expressed at low levels. Therefore, at high levels of receptor expression, different mechanisms likely may contribute to α_{2B}-adrenoceptor down-regulation. Unfortunately, we were unable to obtain clones that expressed the WT- or $Del_{301–303}$-α_{2B}-adrenoceptors at levels comparable with those in previous studies to evaluate this possibility.

Our evidence that phosphorylation of the α_{2B}-adrenoceptor plays an important role in receptor down-regulation adds to a pool of conflicting evidence regarding the role of phosphorylation in this process for the α_2-adrenoceptor. For example, four consecutive serines in the α_{2A}-adrenoceptor appear critical for short-term agonist-induced desensitization but if two of these serines are substituted by alanines, short-term desensitization is eliminated but receptor sequestration is unaffected (Eason *et al.*, 1995). However, if an 11 amino acid sequence (293–304), including these four serines, is deleted from the α_{2A}-adrenoceptor, short-term desensitization is eliminated with no effect on agonist-induced receptor sequestration or down-regulation (Jewell-Motz *et al.*, 1997). The human α_{2C}-adrenoceptor is structurally similar to the α_{2A}-adrenoceptor, having a region with multiple serines bordered by acidic amino acids in the third intracellular loop (DESSAAAE). However, the human α_{2C}-adrenoceptor does not undergo agonist-induced down-regulation (Eason and Liggett, 1992). The opossum α_{2C}-adrenoceptor, on the other hand, has a slightly different sequence in the same region of the third intracellular loop (EESSTSE) and does undergo agonist-induced down-regulation (Deupree *et al.*, 2002). Thus it appears for both the α_{2A}- and α_{2C}-adrenoceptors that agonist-induced phosphorylation of these four amino acids is required for short-term desensitization. However, phosphorylation of these serines appears to be important for down-regulation only for α_{2C}-adrenoceptors. It is noteworthy that

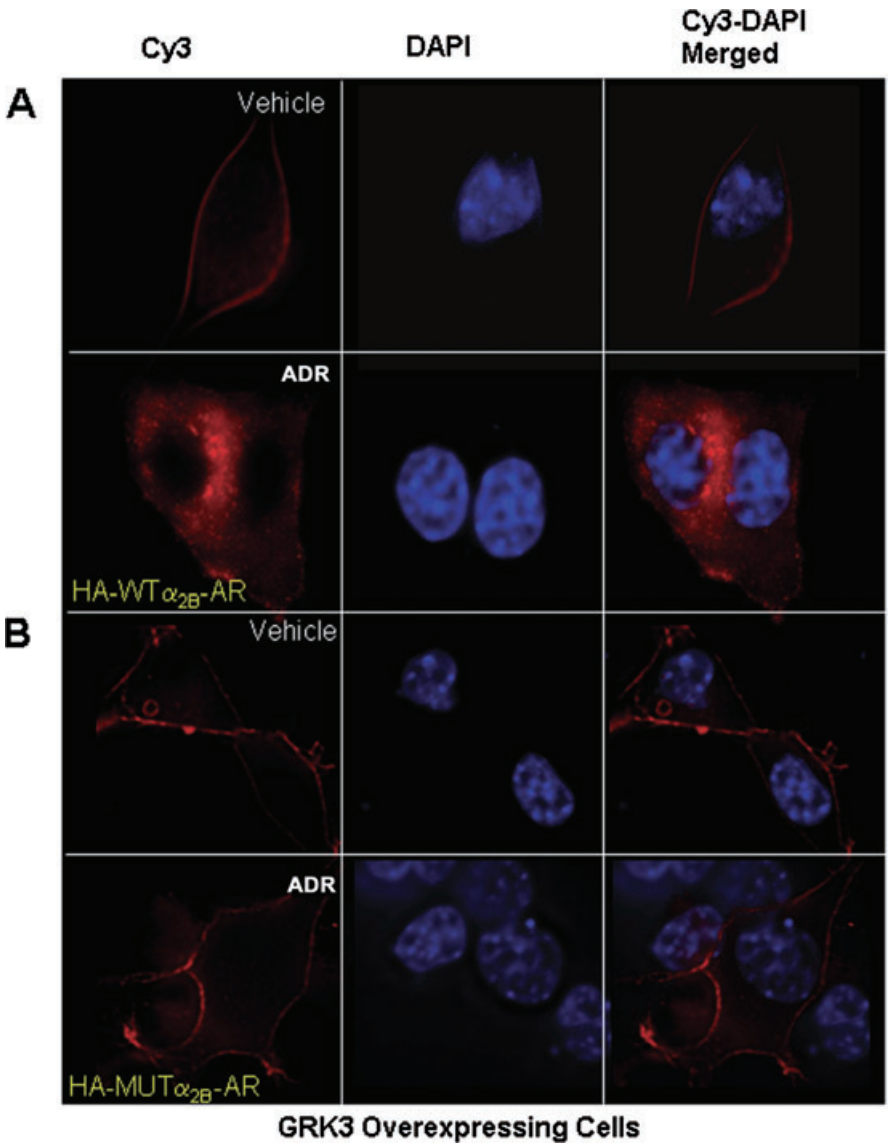

Figure 8 Haemagglutinin-wild type (HA-WT)-α_{2B}-adrenoceptors (upper half) redistributed inside the cell circumference upon adrenaline treatment (5 min) while HA-Del$_{301-303}$-α_{2B}-adrenoceptors (lower half) remained localized to the cell circumference in GRK3 over-expressing cells transiently transfected to express the α_{2B}-adrenoceptors. WT (HA-WT-α_{2B}-AR) and Del$_{301-303}$ (MUT-α_{2B}-AR) receptors were visualized by probing with rat anti-HA antibody and anti-rat cy3 conjugated secondary antibody on a filter selective for cy3 at 60× magnification under an oil immersion objective, NA1.4. Nuclei are stained with DAPI and visualized under DAPI filter. $n = 3$.

all of the studies suggesting a role for receptor phosphorylation in α_2-adrenoceptor down-regulation involved receptors expressed at low levels (<500 fmol·mg^{-1} protein), as in the present study. We hypothesize that the level of receptor expression may significantly contribute to determining whether or not receptor phosphorylation is required for α_2-adrenoceptor down-regulation.

A potential limitation of our study is that we studied the down-regulation of the HA-tagged WT and Del$_{301-303}$-α_{2B}-adrenoceptors in neuronal cells that also endogenously express the WT α_{2B}-adrenoceptor. Therefore, one might argue that using quantitation of the HA-tagged receptor to study the down-regulation of the WT and Del$_{301-303}$-α_{2B}-adrenoceptor does not assess the entire down-regulation process. For this reason, we carefully assessed the relationship between down-regulation of the WT receptor via ligand binding, which measures all receptors, and down-regulation via the HA-tagged receptor. In all cases the down-regulation measured by the two methods correlated, indicating that the down-regulation of the HA-tagged receptor was representative of the behaviour of the entire WT receptor population, and therefore enabled us to use the HA-tagged receptor to assess down-regulation of the WT and Del$_{301-303}$ receptor. Consequently, we suggest that our conclusions regarding the role of receptor phosphorylation and down-regulation for the α_{2B}-adrenoceptor are well supported by the data.

Another objective of the present study was to provide greater insight into the cause of the phosphorylation deficiency of the Del$_{301-303}$ mutant α_{2B}-adrenoceptors. Although there is no consensus site for GRK phosphorylation of GPCRs,

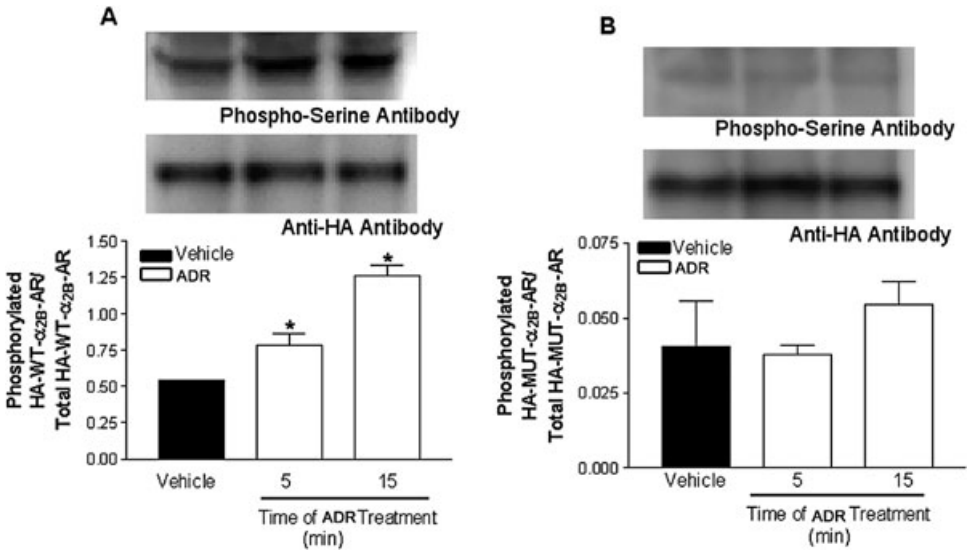

Figure 9 The haemagglutinin-wild type (HA-WT)-α_{2B}-adrenoceptor was phosphorylated (A) while HA-Del$_{301–303}$-α_{2B}-adrenoceptors were weakly phosphorylated (B) under basal conditions as well as upon adrenaline treatment in GRK3-overexpressing cells. GRK3 over expressing cells (~threefold) were transiently transfected with HA-WT-α_{2B}-adrenoceptors (HA-WT-α_{2B}-AR) or HA-Del$_{301–303}$-α_{2B}-adrenoceptors, (MUT-α_{2B}-AR) treated with 20 μM adrenaline (ADR) at the indicated time points. The cells were harvested and membranes were prepared to quantify receptor phosphorylation by immunoprecipitation of the receptors with anti-HA antibody followed by Western blotting and detection with anti-phosphoserine antibody. Data are expressed as mean ± SEM. *significantly different at $P < 0.05$, $n = 3$, using Student's *t*-test.

studies with synthetic peptide substrates suggest that phosphorylation of serines and threonines by GRK3 is favoured by the presence of acidic amino acid residues to the N-terminal side of the phosphorylation sites (Onorato *et al.*, 1991). The Del$_{301–303}$ mutation involves the deletion of three glutamic acid residues in the third intracellular loop of the α_{2B}-adrenoceptor, a region postulated to be in close proximity to the GRK phosphorylation sites in the receptor. Moreover, we had already reported that a two to threefold increase in GRK3 levels made the WT-α_{2B}-adrenoceptor 70–100 times more sensitive to agonist-induced down-regulation by adrenaline. Increasing GRK2 levels two to threefold produced only a sevenfold increase in sensitivity, suggesting GRK3 to be the preferred regulator of α_{2B}-adrenoceptor signaling (Desai *et al.*, 2005). We also have reported that adrenaline-induced down-regulation of the α_{2B}-adrenoceptor requires GRK2/3 recruitment to the membrane as over-expression of GRK3$_{ct}$ prevents GRK3 translocation to the membrane and prevents adrenaline-induced down-regulation of the α_{2B}-adrenoceptor (Desai *et al.*, 2005). Therefore, we hypothesized that higher agonist concentrations and/or increased GRK3 expression might overcome the effects of the deletion of three glutamic acid residues on α_{2B}-adrenoceptor function. However, neither increasing the adrenaline concentration to 1000 μM, nor increasing GRK3 expression by threefold, restored adrenaline-induced down-regulation or phosphorylation of the HA-Del$_{301–303}$-α_{2B}-adrenoceptor. A previous study of the Del$_{301–303}$-α_{2B}-adrenoceptor provided evidence that agonist-binding characteristics, the ratio of high to low affinity receptors, the effect of GTP on agonist binding, and the efficacy and potency of agonists to inhibit cAMP accumulation are minimally affected by the deletion (Small *et al.*, 2001). A slight difference (3.8 vs. 5.1 nM) in K_d for yohimbine binding between the WT and Del$_{301–303}$-α_{2B}-adrenoceptors was reported with no differences in affinity for iodoaminoclonidine. Adrenaline displacement of yohimbine binding revealed no differences in K_H, K_L or in the ratio of high versus low affinity binding sites between the two receptors. There was a small decrease in Ki from 287 to 376 nM in the presence of GTP for the Del$_{301–303}$-α_{2B}-adrenoceptor. Finally, there is a slight decrease in agonist efficacy (28.5% vs. 23.4% inhibition of forskolin-stimulated cAMP) for the Del$_{301–303}$ versus the WT-α_{2B}-adrenoceptor. Therefore, agonist binding characteristics and agonist-mediated receptor function are minimally affected by the deletion. Hence, changes in agonist binding and efficacy to activate the G protein-mediated pathway are unlikely to be responsible for the effect of the deletion on agonist-induced receptor phosphorylation or down-regulation. The simplest explanation is that deletion of these three glutamic acid residues has profound effects on the ability of the receptor to serve as a substrate for GRKs, perhaps by inducing a conformational change in the agonist-activated receptor that reduces the affinity of the receptor for GRK3. Elucidation of the GRK phosphorylation sites in the α_{2B}-adrenoceptor will be required before the role of these three amino acids in the phosphorylation of the α_{2B}-adrenoceptor can be understood.

Finally, we have attempted to delineate a relationship between phosphorylation and redistribution of the α_{2B}-adrenoceptor from the membrane upon agonist treatment. To accomplish this, we relied on transient transfection as higher receptor expression levels (>100 fmol·mg^{-1} protein) were necessary to adequately detect fluorescent labelling. Our results suggest that the HA-WT-α_{2B}-adrenoceptor, stably or transiently expressed, down-regulates similar to the endogenous WT-α_{2B}-adrenoceptor in these cells. Similarly, HA-tagged human and mouse transiently transfected WT-α_{2B}-adrenoceptor are reported to exhibit agonist and antagonist

binding affinities, cellular distribution and internalization similar to endogenously expressed non-epitope tagged WT-α_{2B}-adrenoceptor (Daunt *et al*., 1997; Olli-Lahdesmaki *et al*., 2003). Our immunofluorescent labelling experiments suggest that the HA-WT-α_{2B}-adrenoceptors redistributed from the cell circumference, suggestive of receptor internalization, upon adrenaline treatment while the HA-$Del_{301-303}$-α_{2B}-adrenoceptors did not. It has been suggested that α_{2A}- and α_{2B}-adrenoceptors internalize by similar, although perhaps not identical, mechanisms (Daunt *et al*., 1997; Olli-Lahdesmaki *et al*., 1999; 2003). Given the fact that deletion mutants of the α_{2A}-adrenoceptor which are phosphorylation-deficient reportedly internalize and down-regulate like the WT-α_{2A}-adrenoceptor, one interpretation of our results is that there are significant differences between the mechanisms regulating the internalization and down-regulation of the α_{2A}- and α_{2B}-adrenoceptors. Therefore, significant questions remain regarding the mechanisms which regulate the internalization of both the α_{2A}- and α_{2B}-adrenoceptor subtypes.

In conclusion, the polymorphism resulting in the deletion of three glutamic acid residues from the third intracellular loop of the α_{2B}-adrenoceptor results in loss of agonist-induced down-regulation of the receptor which cannot be overcome by either increasing agonist concentrations or increasing expression of GRK3 in the cells. As the sites of GRK phosphorylation of the α_{2B}-adrenoceptor are not known, future studies will focus on identifying these sites and developing an understanding of how receptor phosphorylation contributes to the down-regulation of the α_{2B}-adrenoceptor.

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Conflicts of interest

None.

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