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RESEARCH PAPER

Lack of effect of the α_{2C}-adrenoceptor Del322-325 polymorphism on inhibition of cyclic AMP production in HEK293 cells

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Background and purpose: The α_{2C}-adrenoceptor has multiple functions, including inhibiting release of noradrenaline from presynaptic nerve terminals. A human α_{2C} polymorphism, Del322-325, a potential risk factor for heart failure, has been reported to exhibit reduced signalling in CHO cells. To further understand the role of the Del322-325 polymorphism on receptor signalling, we attempted to replicate and further study the reduced signalling in HEK293 cells.
Experimental approach: Human α_{2C} wild-type (WT) and Del322-325 adrenoceptors were stably transfected into HEK293 cells. Radioligand binding was performed to determine affinities for both receptors. In intact cells, inhibition of forskolin-stimulated cyclic AMP production by WT and Del322-325 clones with a range of receptor densities (200–2320 fmol·mg^{-1} protein) was measured following agonist treatment.
Key results: Noradrenaline, brimonidine and clonidine exhibited similar binding affinities for WT and Del322-325. Brimonidine and clonidine also had similar efficacies and potencies for both receptors for the inhibition of cyclic AMP production at all receptor densities tested. A linear regression analysis comparing efficacy and potency with receptor expression levels showed no differences in slopes between WT and Del322-325.
Conclusions and implications: The α_{2C} WT and Del322-325 adrenoceptors exhibited similar binding properties. Additionally, inhibition of cyclic AMP production by Del322-325 was similar to that of WT over a range of receptor densities. Therefore, in intact HEK293 cells, the α_{2C}-Del322-325 polymorphism does not exhibit reduced signalling to adenylyl cyclase and may not represent a clinically important phenotype.
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Keywords: adrenoceptor; α_{2C}; Del322-325; polymorphism; signalling; heart failure; adenylyl cyclase

Abbreviations: AMP , adenosine monophosphate; CHO, Chinese hamster ovary; Del322-325, deletion 322-325 ; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HEK293, human embryonic kidney 293; MAP, mitogen-activated protein; WT, wild type

Introduction

The α_2-adrenoceptors are activated by the endogenous hormone adrenaline as well as the neurotransmitter noradrenaline. Upon ligand binding, these G protein-coupled receptors (GPCRs) primarily activate G_i/G_o proteins, initiating downstream signalling pathways such as inhibition of adenylyl cyclase and voltage-gated Ca^{2+} channels, and activation of receptor-operated K^+ channels and the mitogen-activated protein (MAP) kinase cascade (Limbird, 1988; Kobilka, 1992).

The physiological functions of the three α_2-adrenoceptor subtypes, α_{2A}, α_{2B} and α_{2C}, have been studied in receptor knockout mice and include regulatory roles in the cardiovascular, endocrine, ocular and peripheral and central nervous systems (Kable *et al.*, 2000). The α_{2C}-adrenoceptor has been found to be important in locomotion (Sallinen *et al.*, 1998b), stress responses (Sallinen *et al.*, 1999) and the startle reflex (Sallinen *et al.*, 1998a). This receptor also regulates the release of noradrenaline and adrenaline from chromaffin cells via a negative feedback mechanism (Gilsbach *et al.*, 2007). Both α_{2A}- and α_{2C}-adrenoceptors are involved in the inhibition of neurotransmitter release from presynaptic sympathetic nerve terminals (Hein *et al.*, 1999; Trendelenburg *et al.*, 2003). These receptors serve as autoreceptors for both the central and peripheral nervous systems, with α_{2A} mediating inhibition of noradrenaline release at higher stimulation frequencies and

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α_{2C} mediating inhibition at lower stimulation frequencies (Hein *et al.*, 1999). Characterization of α_{2A} and α_{2C} single- and double-knockout mice in the absence and presence of cardiac pressure overload (Hein *et al.*, 1999; Brede *et al.*, 2002) revealed the importance of α_{2A}- and α_{2C}-adrenoceptors for the correct regulation of catecholamine release *in vivo* and suggests that loss of these receptors, or their function, may be associated with heart failure. Similar studies in transgenic mice expressing only one functional copy of the α_{2C}-adrenoceptor gene showed that, in the peripheral sympathetic nervous system, heterozygous expression of the α_{2C}-adrenoceptor was sufficient to inhibit catecholamine release (Gilsbach *et al.*, 2007). These mice did, however, have decreased left ventricular contractility and relaxation after transverse aortic constriction compared with wild-type (WT) animals, suggesting that they are at greater risk for developing heart failure. In the adrenal glands of heterozygous α_{2C} mice, there was an increased release of catecholamines under basal conditions and a roughly 50% reduction in the ability to inhibit catecholamine release, suggesting that in the adrenal glands the α_{2C}-adrenoceptor operates without a receptor reserve (Gilsbach *et al.*, 2007).

These studies with transgenic mice have revealed the critical role of α_2-adrenoceptors in the regulation of hormone and neurotransmitter release. Changes in receptor expression or function, as can occur with genetic polymorphisms, may result in physiological effects leading to various disorders. A variety of characteristics, such as agonist or antagonist binding, protein coupling, and receptor trafficking and regulation can be affected by receptor polymorphisms (Johnson and Lima, 2003; Small *et al.*, 2003). Therefore, polymorphisms may play a role in predisposition for disease, progression of disease, response to pharmacological treatment and the inter-individual variation observed among patients (Buscher *et al.*, 1999, Small *et al.*, 2003; Eichelbaum and Evans, 2006).

The human α_{2C}-adrenoceptor has a twelve nucleic acid deletion polymorphism (Del322-325) within its coding region that results in the loss of four amino acids (Gly-Ala-Gly-Pro) in the third intracellular loop (Small *et al.*, 2000). When expressed in CHO cells, the Del322-325 receptor was reported to have decreased inhibition of membrane adenylyl cyclase activity compared with the WT receptor (Small *et al.*, 2000). The maximum inhibition of adenylyl cyclase activity with brimonidine, also known as UK-14 304, in WT and Del322-325 receptors was approximately 68% and 31%, respectively, in cells expressing 1500–1600 $fmol \cdot mg^{-1}$, resulting in a roughly 50% reduction in signalling with the Del322-325 receptor. The 50% reduction was observed with other full and partial agonists; however, EC_{50} values remained similar. In cells expressing lower receptor levels (500–600 $fmol \cdot mg^{-1}$), the reduction in signalling with the Del322-325 receptor was even greater (10% maximal inhibition versus 73% for WT with adrenaline). The activation of MAP kinase and accumulation of inositol phosphate were also impaired with Del322-325. Competition binding studies with Del322-325 revealed a slight decrease in high-affinity binding of agonist compared with the WT receptor (Small *et al.*, 2000). Because binding of a G protein to the GPCR is required for the receptor to be in its high-affinity state (Maudsley *et al.*, 2005), the decreased high affinity binding of α_2 agonists and the reduced signalling were proposed to be a result of decreased coupling of the receptor to its G protein.

To better understand the mechanism behind the reported decrease in signalling of the α_{2C}-Del322-325 receptor in CHO cells, we attempted to replicate this reduced signalling in HEK293 cells. Because different cell lines are known to have different protein expression levels and different effector molecules present, it was important to show similar effects in a cell line other than CHO cells. Therefore, we compared the inhibition of cyclic AMP production by α_{2C} WT and Del322-325 receptors stably expressed in HEK293 cells at various expression levels.

Methods

Cell culture

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 0.1 $\mu g \cdot mL^{-1}$ gentamicin and 5 $\mu g \cdot mL^{-1}$ amphotericin B, supplemented with 10% fetal bovine serum (FBS). Cells were grown in 5% CO_2 at 37°C.

Creating WT construct and sequencing

The cDNA of the human α_{2C}-adrenoceptor from OriGene contained a 12-nucleotide deletion in the DNA coding for the third intracellular loop and was determined to be that of the previously identified α_{2C}-Del322-325 adrenoceptor polymorphism. A non-conserved C to T mutation at bp 1015, resulting in a serine rather than proline at position 339, was also identified in the cDNA. The α_{2C} cDNA from Missouri S&T cDNA Resource Center was also shown to be the Del322-325 variant; however, the C to T mutation was not present. Site-directed mutatgenesis was performed on the OriGene α_{2C}-Del322-325 adrenoceptor cDNA to convert it to the WT α_{2C}-adrenoceptor.

To create the human WT cDNA, the Quickchange II XL kit (Stratagene, La Jolla, CA, USA) was used to add the 12 nucleotide insert, GGGGGCGGGGCC (nucleotides 963-974; accession number AF280399), into the human α_{2C}-Del322-325 cDNA. Two 44-nucleotide primers were used: 5′-GCGGTGCG GACGGGCAGGGGGCGGGGCCGGGGGCGGCTGAGTCG-3′ (forward) and 5′-CGACTCAGCCGCCCCCGGCCCCGCCCC CTGCCCGTCCGCACCGC-3′ (reverse). For a 50 µL reaction, 20 ng of α_{2C}-Del322-325 template and 125 ng of primers were used. The temperature programme was similar to the Stratagene protocol: 95°C for 1 min (1 cycle), 95°C for 50 s, 60°C for 50 s, 68°C for 15 min (18 cycles), followed by 68°C for 15 min (1 cycle). Dpn I treatment of the reaction product was carried out for 3 h. For conversion of the non-conserved C-T mutation at position 1015 back to the WT C nucleotide, the Quickchange II XL kit was again used. Two 25-nucleotide primers were used: 5′-GACCGCCTCCAGGTCCCCGGGGC CC-3′ (forward) and 5′-GGGCCCCGGGGACCTGGAGGCG GTC-3′ (reverse). For a 50 µL reaction, 10 ng of vector template and 125 ng of primers were used. The temperature programme was similar to the Stratagene protocol: 95°C for 2 min (1 cycle), 95°C for 50 s, 55°C for 50 s, 68°C for 15 min (18 cycles), followed by 68°C for 15 min (1 cycle).

The modified insert was excised from the OriGene pCMV6-XL4 vector using EcoR I and Xba I and ligated into pcDNA3.1(+). DNA sequencing of the WT and Del322-325 constructs was performed using a Perkin-Elmer Applied Biosystems Model 377 DNA Sequencer (Foster City, CA, USA). Sequencing was performed in the forward and reverse directions with the T7 (forward) and BGHR (reverse) primers. The following forward primers were also used: 5′-GCGCC ACAGAACCTCTTC-3′, 5′-TTCCCGCCGCTGGTCTCG-3′, 5′-CAGCGCAGCGGCCGAGAG-3′ and 5′-GAGCAGCAGGC GGCGATG-3′. The sequence of our α_{2C}-Del322-325 adrenoceptor variant was identical to the sequence published in GenBank (accession number AAG28077). Our WT α_{2C}-adrenoceptor sequence was also identical to the WT sequence in GenBank (accession number NP_000674).

Transfection
Human α_{2C} WT and Del322-325 (Missouri S&T cDNA Resource Center, Rolla, MO, USA) adrenoceptor cDNAs were transformed into competent bacterial cells for amplification and then purified by Qiagen Maxi DNA kit (Valencia, CA, USA). Each cDNA was stably transfected into HEK293 cells using the calcium phosphate method. Briefly, 1 μg DNA was added to a 1:10 solution of calcium chloride and nanopure water and mixed. This solution was added dropwise to an equal volume of 2X HEPES buffer with constant mixing. The combined solution was incubated at room temperature for 20 min. The calcium phosphate/DNA solution was added dropwise to HEK293 cells that were approximately 70% confluent with gentle mixing, and the cells were then incubated for 6 h in 5% CO_2 at 37°C. Cells were then washed twice and DMEM containing 0.1 $\mu g \cdot mL^{-1}$ gentamicin, 5 $\mu g \cdot mL^{-1}$ amphotericin B and 10% FBS was added. Stable receptor transfections were selected for and maintained in medium containing 200 $\mu g \cdot mL^{-1}$ G418. Individual clones of α_{2C} WT and Del322-325 were isolated by limiting dilution and maintained in selection media (DMEM containing 0.1 $\mu g \cdot mL^{-1}$ gentamicin, 5 $\mu g \cdot mL^{-1}$ amphotericin B, 10% FBS and 200 $\mu g \cdot mL^{-1}$ G418).

Membrane preparation
Cells were grown to confluence in 150 mm dishes. The medium was aspirated and 20 mL ice-cold phosphate-buffered saline was added to each dish. Cells were scraped from the dish and transferred to a 50 mL conical tube. Cells were centrifuged at 1400 *g* for 10 min. The supernatant was discarded and the cells resuspended in 1.5 mL ice-cold 50 mM sodium phosphate ($NaPO_4$) buffer. Cells were homogenized for 15 s with an Ultra Turrax (A. Daigger & Company, Vernon Hills, IL, USA) at setting 3. The solution was transferred to Sorvall tubes and cells were centrifuged at 44 300 *g* for 10 min. The pellets were resuspended in 1.5 mL ice-cold 50 mM $NaPO_4$ buffer and homogenized for 15 s at setting 3. Membranes were centrifuged at 44 300 *g* for 10 min. The supernatant was discarded and cell membrane pellets were stored at −80°C.

Saturation binding
Saturation binding assays were performed as described previously (Deupree *et al.*, 1996) with some modification. Briefly, 970 μL of cell membranes of clones stably expressing α_{2C} WT or Del322-325 receptors in 25 mM buffer, pH 7.4 (either $NaPO_4$ or Tris-HCl, 10 mM $MgSO_4$ and 0.5 mM EDTA) were incubated without (total binding) or with (non-specific binding) 10 μL noradrenaline (0.2 mM final concentration) and 20 μL of various concentrations of [^{3}H]RX821002 for 1 h with mixing at 30 min. Six concentrations of [^{3}H]RX821002 were used over a 100-fold range. Reaction mixtures were filtered through GF/B glass-fibre filter strips using a 48-well Brandel Cell Harvester (Gaithersburg, MD, USA) to terminate the reactions. The tubes and filters were rinsed three times with 5 mL ice-cold 50 mM Tris HCl pH 8.0. Filters were then placed in scintillation vials with 5 mL Econo-safe liquid scintillation cocktail and quantified in a liquid scintillation counter the next day. Duplicate tubes were used for total and non-specific binding, with 0.2 mM noradrenaline used to determine non-specific binding. Specific binding was calculated as total binding minus non-specific binding. The specific activity of [^{3}H]RX821002 was 49 $Ci \cdot mmol^{-1}$ for experiments using $NaPO_4$ buffer and 60 $Ci \cdot mmol^{-1}$ for experiments using Tris buffer. K_d and B_{max} values were determined using non-linear regression analysis with Prism 4 software (GraphPad, San Diego, CA, USA). K_d values were compared using an unpaired *t*-test. The total amount of protein present in the binding assays was determined by protein assays using the BCA protein assay kit (Pierce, Rockford, IL, USA). The amount of membrane protein per sample used for binding varied between clones, with high-expressing clones requiring less protein than low-expressing clones in order to bind less than 10% of the total added radioligand. For the saturation binding assays, between 0.034 and 0.114 mg protein per assay tube were used. Saturation binding assays were performed on non-transfected HEK293 cells to determine receptor expression level. These cells were found to contain 8 ± 2 $fmol \cdot mg^{-1}$ receptor ($n = 3$).

Competition binding
Competition binding assays were performed as described previously (Deupree *et al.*, 1996) with some modification. Stably transfected clones, one WT clone (B_{max} = 1460 $fmol \cdot mg^{-1}$ protein) and one Del322-325 clone (B_{max} = 2320 $fmol \cdot mg^{-1}$ protein), were used for the standard competition binding experiments (one-site binding). Cell membranes containing α_{2C} WT or Del322-325 receptors in 25 mM $NaPO_4$ buffer, pH 7.4 were incubated with various concentrations of agonist in the presence of [^{3}H]RX821002 (specific activity = 49 $Ci \cdot mmol^{-1}$) for 1 h with mixing at 30 min (final volume of 1.0 mL). Agonists used were noradrenaline, clonidine and brimonidine (UK-14 304). Eleven drug concentrations (one to two per log unit) and one blank (water) were used, giving a total of 12 conditions, and experiments were performed in duplicate. The concentration of [^{3}H]RX821002 used was 0.8 × K_d. Data were analysed with Prism 4 (GraphPad) software using a one-site competition curve to determine IC_{50} values and a sigmoid analysis with a variable slope to determine Hill slopes. The K_i values were calculated from the IC_{50} values by the Cheng–Prusoff equation (Cheng and Prusoff, 1973). Mean pK_i values were calculated from pK_i values obtained from individual experiments.

Table 1 Affinity of [^{3}H]RX821002 for human α_{2C} WT and Del322-325 receptors in $NaPO_4$ and Tris buffers

	Wild type		*Del322-325*		*P*
	K_d ± *SEM (pM)*	*n*	K_d ± *SEM (pM)*	*n*	
$NaPO_4$ buffer	263 ± 8	26	281 ± 8	19	0.51
Tris buffer	1560 ± 140	7	1151 ± 101	4	0.36

Saturation binding assays were performed on HEK293 cell membranes of clones stably expressing various levels of WT or Del322-325 receptors. Experiments were performed in either 25 mM $NaPO_4$ buffer, pH 7.4 or 25 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 0.5 mM EDTA buffer. Data were analysed with GraphPad Prism software using nonlinear regression analysis, from which K_d values were determined. K_d values from all experiments were compared using an unpaired *t*-test.

For experiments designed to detect high- and low-affinity agonist binding, competition binding assays were performed as described previously (Small *et al.*, 2000) with some modifications. Stably transfected clones, one WT clone (B_{max} = 170 fmol·mg^{-1} protein) and one Del322-325 clone (B_{max} = 140 fmol·mg^{-1} protein), were used for these experiments. Cell membranes containing α_{2C} WT or Del322-325 receptors in 25 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 0.5 mM EDTA were incubated with [^{3}H]RX821002 (specific activity = 60 Ci·mmol^{-1}) and 22 concentrations of agonist in the absence or presence of 100 μM Gpp(NH)p for 1 h with mixing at 30 min (final volume of 1.0 mL). Agonists used were noradrenaline, clonidine and brimonidine, with experiments performed in duplicate. Two blank (water) and 22 drug concentrations were used, for a total of 24 points. Drug concentrations ranged from 10 pM to 300 μM. Two drug concentrations per log unit were used at low (<3 nM) and high (>30 μM) ends of the curve, while four drug concentrations per log unit were used for the rest of the curve (10 nM to 10 μM). The concentration of [^{3}H]RX821002 used was approximately $0.8 \times K_d$ (see Table 1). Data were analysed with Prism 5 software (GraphPad) using a global one- or two-site competition curve. For the global fit analysis, Prism 5 uses the K_d and the concentration of the radioligand to calculate shared pK_i and R_H (percentage of receptors in the high-affinity state) values and compares the fit of the data to one-site or two-site models using the extra sum-of-squares *F* test.

Inhibition of cyclic AMP

The inhibition of forskolin-stimulated cyclic AMP production in intact cells was measured as described previously (Shimizu *et al.*, 1969; Johnson and Toews, 1990) with some modification. HEK293 cells stably expressing either α_{2C} WT or Del322-325 receptors were grown in poly-D-lysine-coated 6-well plates. When the cells were approximately 80% confluent, each well was rinsed once with HEPES-buffered DMEM and then 1 mL medium containing 2 μCi·mL^{-1} [^{3}H]adenine was added. The plates were then incubated at 37°C in a non-CO_2 incubator for 1 h, after which each well was rinsed once with HEPES-buffered DMEM. The medium was aspirated and 1 mL of medium containing 30 μM forskolin alone or with various concentrations of clonidine or brimonidine (but without a phosphodiesterase inhibitor) was added to each well. Plates were incubated at 37°C in a non-CO_2 incubator for 5 min, after which the stimulation solution was aspirated and 1 mL 5% trichloracetic acid/1 mM cyclic AMP was added to stop the reaction. The solution from each well was passed over Dowex and alumina columns to isolate the [^{3}H]ATP and [^{3}H]cyclic AMP as described previously (Johnson and Toews, 1990) and the [^{3}H]ATP and [^{3}H]cyclic AMP were quantified. Experiments were performed in triplicate for each condition. Data were expressed as percent conversion of [^{3}H]ATP to [^{3}H]cyclic AMP and analysed with Prism 5 (GraphPad) using sigmoidal dose-response curves to obtain pEC_{50} values. Data were normalized to forskolin-stimulated cyclic AMP production and percent inhibition was calculated as 100-(bottom of curve). Mean pEC_{50} and mean percent inhibition values were derived from sigmoidal dose-response curves of the combined data from all experiments. Binding studies with non-transfected HEK293 cells indicated a very low level of endogenous α_2-adrenoceptors (<10 fmol·mg^{-1} protein). We were also able to detect inconsistent inhibition of forskolin-stimulated cyclic AMP production by brimonidine, confirming the presence of a low density of α_2 receptors in non-transfected cells.

Statistical analysis

For binding, K_d and pK_i values were analysed using an unpaired *t*-test, with significance at $P < 0.05$. For functional studies, linear regression analysis was used to determine if the slope of each line was different from zero and whether the slopes for WT and Del322-325 receptors were different, with significance at $P < 0.05$. Data are reported as means ± SEM.

Materials

HEK293 cells were purchased from ATCC (Manassas, VA, USA) (catalogue # CRL-1573). The human α_{2C}-Del322-325 adrenoceptor cDNA was purchased from OriGene (Rockville, MD; catalog number TC124091) and Missouri S&T cDNA Resource Center (catalog number AR0A2C0000). The OriGene construct was in the pCMV6-XL4 vector and subcloned into EcoR I and Sal I sites. The Missouri S&T cDNA Resource Center construct was in pcDNA3.1(+) and subcloned into BamH I and Xho I sites. DMEM was purchased from Gibco, Invitrogen (Carlsbad, CA, USA). Quickchange II XL kits were from Stratagene. Qiagen Maxi DNA kits were purchased from Qiagen. GF/B glass-fiber filter strips were from Whatman (Clifton, NJ, USA). Noradrenaline and guanosine 5′-[β,γ-imido]triphosphate trisodium salt hydrate [Gpp(NH)p] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brimonidine and clonidine were purchased from Research Biochemicals International (Natick, MA, USA).

Table 2 Potency and efficacy values of brimonidine and clonidine for the inhibition of cyclic AMP production by α_{2C} WT and Del322-325 receptors

Wild type					*Del322-325*				
Expression level		*Inhibition of cyclic AMP*			*Expression level*		*Inhibition of cyclic AMP*		
B_{max} (fmol·mg^{-1})	*n*	*pEC$_{50}$ ± SEM*	*% inhibition ± SEM*	*n*	*B_{max} (fmol·mg^{-1})*	*n*	*pEC$_{50}$ ± SEM*	*% Inhibition ± SEM*	*n*
Brimonidine									
470 [467, 464][a]	2	9.17 ± 0.10	87 ± 3	3	200 [200, 203][a]	2	8.40 ± 0.07	89 ± 3	3
530 ± 50	5	8.45 ± 0.04	93 ± 1	3	670 [706, 637][a]	2	9.32 ± 0.21	78 ± 4	3
1460 ± 104	6	9.37 ± 0.06	96 ± 2	3	1100 [914, 1278][a]	2	9.13 ± 0.05	101 ± 2	4
					1380 ± 292	5	9.44 [9.44, 9.513][a]	100 [97, 100][a]	2
					2320 [2343, 2299][a]	2	9.14 ± 0.10	98 ± 3	4
Clonidine									
470 [467, 464][a]	2	8.49 ± 0.09	84 ± 3	3	200 [200, 203][a]	2	7.86 ± 0.07	82 ± 2	3
530 ± 50	5	8.13 ± 0.10	87 ± 3	3	670 [706, 637][a]	2	9.09 ± 0.21	72 ± 4	3
1460 ± 104	6	8.66 ± 0.06	95 ± 2	3	1100 [914, 1278][a]	2	8.43 ± 0.08	101 ± 3	4
					1380 ± 292	5	8.74 [8.74. 8.99][a]	99 [98, 100][a]	2
					2320 [2343, 2299][a]	2	8.58 ± 0.11	97 ± 3	4

Inhibition of cyclic AMP production was determined at multiple receptor expression levels for each receptor type. HEK293 cells stably transfected with either WT or Del322-325 were treated with forskolin alone or in the presence of various concentrations of agonists, with each condition performed in triplicate. Data were normalized to forskolin-stimulated cyclic AMP production and analysed by non-linear regression. Mean pEC$_{50}$ and mean % inhibition values, which were calculated as 100-(bottom of curve), were derived from sigmoidal dose-response curves of the combined data from all experiments.
[a]At $n = 2$, SEM is not reported. Instead, values from individual experiments are shown in brackets.

Table 3 Receptor binding affinities of adrenoceptor agonists for α_{2C} WT and Del322-325

	Wild type			*Del322-325*		
	K_i (nM)	*pK_i ± SEM*	*Hill slope*	*K_i (nM)*	*pK_i ± SEM*	*Hill slope*
Noradrenaline	843	6.07 ± 0.10	–0.72	654	6.18 ± 0.06	–0.80
Brimonidine	340	6.47 ± 0.03	–0.85	561	6.25* ± 0.06	–0.87
Clonidine	85	7.07 ± 0.05	–0.87	186	6.73* ± 0.06	–0.99

Competition binding assays were performed on WT (B_{max} = 1460 fmol·mg^{-1}) and Del322-325 (B_{max} = 2320 fmol·mg^{-1}) receptors stably transfected into HEK293 cells using the radioligand [^{3}H]RX821002. pK_i values were derived using the Cheng–Prusoff equation with IC$_{50}$ values from one-site competition curves with fixed slopes and were used to calculate mean pK_i. Hill slopes were obtained using a sigmoid analysis with variable slope. Data are from three experiments, each in duplicate.
*$P < 0.05$ compared with WT using an unpaired *t*-test.

The BCA Protein Assay kits were purchased from Pierce. Prism versions 4 and 5 software from GraphPad.

Results

α_{2C} WT and Del322-325 receptor clones
HEK293 cells stably transfected with α_{2C} WT or Del322-325 receptors were characterized by saturation binding using membrane preparations to determine expression levels (B_{max}) and K_d values. The affinity of [^{3}H]RX821002 for each receptor type was determined in $NaPO_4$ and Tris buffers (Table 1). K_d values for WT and Del322-325 did not differ in either buffer; however, K_d values were higher in Tris buffer than in $NaPO_4$. The expression levels of three WT and five Del322-325 clones were determined in $NaPO_4$ buffer (Table 2). WT B_{max} values ranged from 470 to 1460 fmol·mg^{-1} protein and Del322-325 B_{max} ranged from 200 to 2320 fmol·mg^{-1} protein.

Binding characteristics of α_{2C} WT and Del322-325 receptors
The binding characteristics of α_{2C} WT and Del322-325 receptors stably expressed in HEK293 cells were investigated by radioligand competition binding in cell membranes. The affinities of three α_2-adrenoceptor agonists, noradrenaline, brimonidine and clonidine, for both WT and Del322-325 receptors were determined (Table 3). pK_i values for noradrenaline were not significantly different between WT and Del322-325 receptors. K_i values of brimonidine and clonidine were approximately twofold higher for Del322-325 compared with WT, with statistically significant differences in pK_i values.

Additional binding studies were performed with the same ligands under conditions favourable for detecting binding to high- and low-affinity sites of the receptor. A two-site inhibition curve was preferred for all three ligands in the absence of Gpp(NH)p (Table 4). In the presence of Gpp(NH)p, only the brimonidine curve was shifted to a one-site inhibition for both WT (Figure 1A) and Del322-325 (Figure 1B). Gpp(NH)p had a minimal effect on the clonidine inhibition;, however, it increased K_{iH} and K_{iL} values (lowered affinity) for noradrenaline. This trend was observed with both WT and Del322-325 receptors. Under these conditions, noradrenaline, brimonidine and clonidine had similar binding affinities for the high- and low-affinity sites of the WT and Del322-325 receptors.

Table 4 High- and low-affinity receptor binding of adrenoceptor agonists for α_{2C} WT and Del322-325

	Wild type						
	K_{iH} (nM)	*pK_{iH} ± SEM (nM)*	*R_H ± SEM (%)*	*K_{iL} (nM)*	*pK_{iL} ± SEM (nM)*	*P value*	*F*
Noradrenaline	83	7.08 ± 0.13	47 ± 6	912	6.04 ± 0.14	<0.0001	13.93
Noradrenaline + Gpp(NH)p	166	6.78 ± 0.21	49 ± 3	1202	5.92 ± 0.23	0.0011	4.85
Brimonidine	13	7.88 ± 0.21	17 ± 4	513	6.29 ± 0.05	<0.0001	17.01
Brimonidine + Gpp(NH)p	(one-site)	(one-site)	0	447	6.35 ± 0.02	0.1686	1.64
Clonidine	25	7.61 ± 0.33	16 ± 1	407	6.39 ± 0.07	0.0001	6.16
Clonidine + Gpp(NH)p	95	7.02 ± 0.35	21 ± 8	447	6.35 ± 0.13	0.0057	3.82
	Del322-325						
	K_{iH} (nM)	*pK_{iH} ± SEM (nM)*	*R_H ± SEM (%)*	*K_{iL} (nM)*	*pK_{iL} ± SEM (nM)*	*P-value*	*F*
Noradrenaline	49	7.31 ± 0.16	43 ± 4	575	6.24 ± 0.13	<0.0001	11.41
Noradrenaline + Gpp(NH)p	138	6.86 ± 0.22	48 ± 3	871	6.06 ± .21	0.0010	4.92
Brimonidine	26	7.58 ± 0.18	33 ± 4	490	6.31 ± 0.09	<0.0001	15.47
Brimonidine + Gpp(NH)p	(one-site)	(one-site)	0	380	6.42 ± 0.03	0.0945	2.03
Clonidine	13	7.87 ± 0.34	14 ± 3	309	6.51 ± 0.06	<0.0001	6.96
Clonidine + Gpp(NH)p	10	7.98 ± 0.52	5 ± 5	282	6.55 ± 0.05	0.0078	3.62

R_H, percentage of receptors in the high affinity state.
Competition binding assays were performed on HEK293 cell membranes from clones stably expressing WT (B_{max} = 170 fmol·mg^{-1}) or Del322-325 (B_{max} = 140 fmol·mg^{-1}) receptors. In the absence or presence of Gpp(NH)p, α_2-adrenoceptor agonists competed with [^{3}H]RX821002 under conditions suitable for determining binding to high- and low-affinity receptor binding sites. Three experiments were performed for each condition at each receptor type; and each experiment was performed in duplicate. The experiments were first analysed individually comparing one- and two-site fits. For the WT receptor, in the absence of Gpp(NH)p, in 7 out of 9 experiments the data best fit a two-site model, whereas in the presence of Gpp(NH)p 7 of 9 experiments fit best to the one-site model. Similarly, for the Del322-325 receptor in the absence of Gpp(NH)p, in 8 out of 9 experiments, the data fit best to a two-site model, whereas in the presence of Gpp(NH)p, 6 of 9 experiments fit best to the one-site model. Data from all three experiments for each condition were then analysed with GraphPad Prism 5 software using a comparison analysis to determine a global one-site versus global two-site inhibition fit. The mean, shared pK_i value was obtained from the global fit analysis. Shared pK_i values were transformed to mean K_i. The mean R_H values were obtained by averaging R_H from each individual experiments analysed by global two-site inhibition. *P* values and *F*-scores were taken from the Prism global one-site versus two-site inhibition analysis.

Inhibition of cyclic AMP production by α_{2C} WT and Del322-325 receptors

The inhibition of cyclic AMP production in intact HEK293 cells was studied as a measure of functional response of stably transfected α_{2C} WT and Del322-325 receptors. Clones with various levels of receptor expression were used to study the effects of the deletion polymorphism on inhibition by brimonidine (highly efficacious) and clonidine (a less efficacious agonist). In cells expressing high levels of either WT or Del322-325 receptors (1460 and 1100 fmol·mg^{-1}, respectively), both agonists were able to completely inhibit the forskolin-stimulated production of cyclic AMP (Figure 2). The highest concentration used for the brimonidine curves was 1 μM due to a reduced inhibitory effect of brimonidine on cyclic AMP production at concentrations exceeding 1 μM (data not shown), which may reflect a change in coupling of some of the receptors from G_i to G_s proteins (Eason *et al.*, 1992). For brimonidine (Figure 2A) and clonidine (Figure 2B), the functional responses of WT and Del322-325 were essentially identical. These data indicate that WT and Del322-325 receptors have similar efficacies (percentage inhibition) and potencies (pEC_{50} values) for the inhibition of production of cyclic AMP in HEK293 cells.

Additional WT and Del322-325 clones with various expression levels were also studied. Table 2 shows expression levels for all WT and Del322-325 clones, which range from 200 to 2320 fmol·mg^{-1}; % inhibition and pEC_{50} values for brimonidine and clonidine were found to be similar for WT and Del322-325 clones of similar receptor expression levels. The α_{2C} receptor-mediated inhibition of cyclic AMP production was measured in the presence of forskolin-stimulated cyclic AMP production. As expected, the conversion of [^{3}H]ATP to [^{3}H]cyclic AMP in basal conditions was extremely low and variable; however, in the presence of 30 μM forskolin, % conversion values were approximately 10- to 100-fold higher and showed less variation (Table 5).

To test for similarities or differences across the entire range of expression levels, the efficacy and potency values for brimonidine and clonidine were plotted against the log B_{max} for all clones and the data analysed by linear regression (Figure 3 and Table 6). For brimonidine, a comparison of the slopes of the lines for WT and Del322-325 receptors showed no significant differences in either the % inhibition (Figure 3A) or the pEC_{50} values (Figure 3C). Similar results were found for the slopes of the lines for % inhibition (Figure 3B) and pEC_{50} (Figure 3D) with clonidine in WT and Del322-325 receptors. The relatively large error values in slope parameter meant that we could not detect a statistically significant small change in slope. This novel way of expressing the functional data over a range of receptor expression levels shows that there is no difference in the inhibition of cyclic AMP production by the Del322-325 receptor compared with the WT receptor when expressed in HEK293 cells.

Discussion and conclusions

This study used intact HEK293 cells to investigate a functional response mediated by the α_{2C}-Del322-325 adrenoceptor,

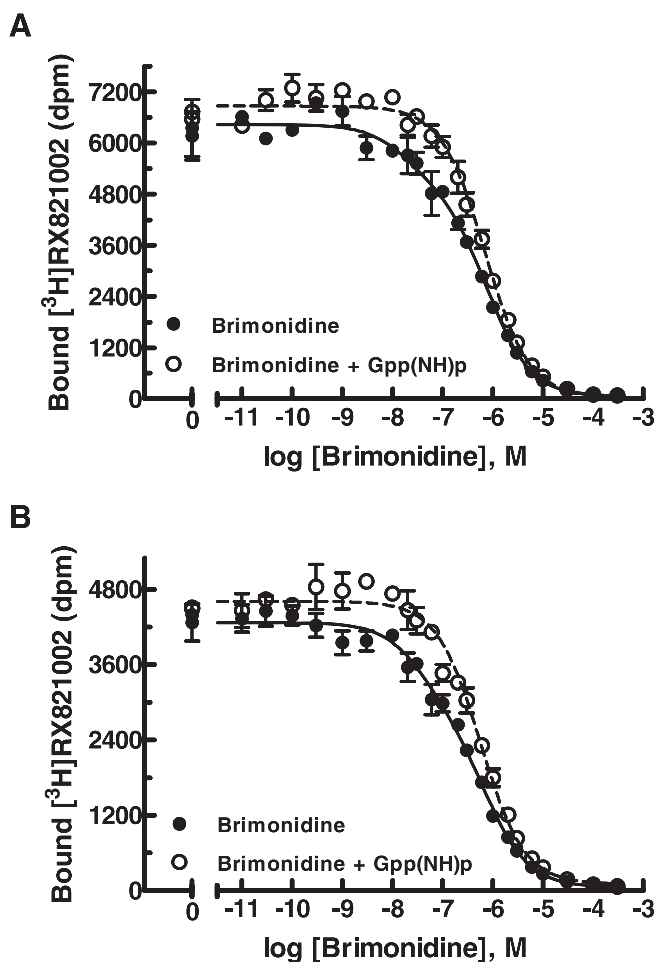

Figure 1 High- and low-affinity competition binding of brimonidine at α_{2C} WT (A) and Del322-325 (B) receptors in the absence or presence of Gpp(NH)p. These curves are from single experiments and are representative of the results from the global fit analysis. Experiments were performed in duplicate and data are plotted as means ± SEMs.

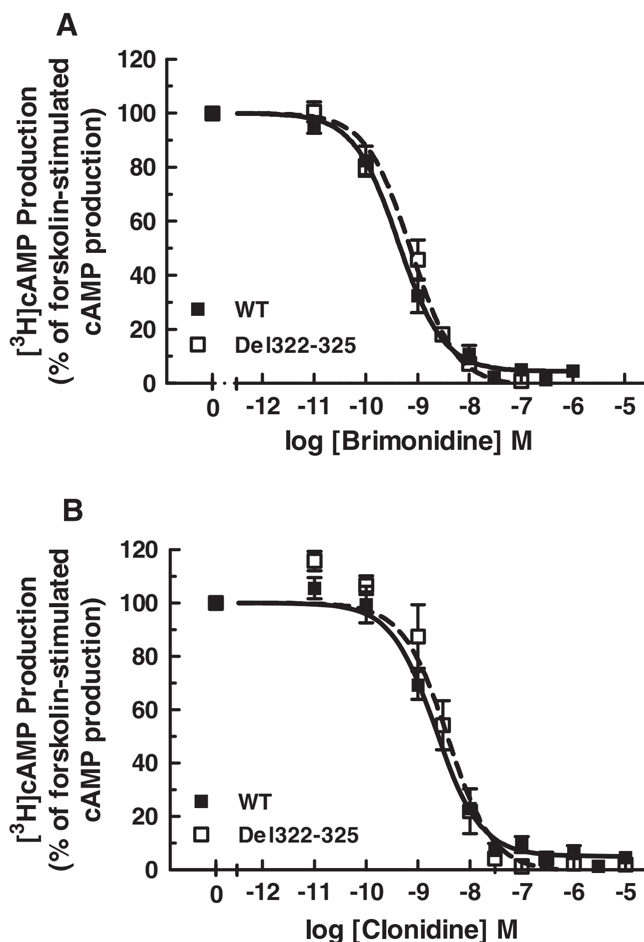

Figure 2 Inhibition of cyclic AMP production by the α_2 agonists brimonidine (A) and clonidine (B) acting at α_{2C} WT and Del322-325 adrenoceptors stably expressed in HEK293 cells. The data are normalized to the maximal forskolin stimulation and the values plotted are the means ± SEM from three to four experiments. WT B_{max} = 1460 fmol·mg^{-1} protein; Del322-325 B_{max} = 1100 fmol·mg^{-1} protein.

which had previously been reported to exhibit reduced signalling compared with the WT receptor when expressed in CHO cells. Ligand binding studies showed that the Del322-325 receptor had similar binding affinities (within about twofold) as compared with the WT receptor for three classic α_2-adrenoceptor agonists: noradrenaline, brimonidine and clonidine. More detailed competition binding experiments in Tris and Mg^{++} buffer revealed high- and low-affinity agonist binding sites. For brimonidine, Gpp(NH)p converted the two-site competition binding to a one-site model; however, with noradrenaline, the percentage of receptors in the high-affinity state remained unchanged in the presence of Gpp(NH)p. It is unclear why such differences were observed, but it may be due to different ligand-specific receptor conformations stabilized by brimonidine and the endogenous ligand noradrenaline (Urban *et al*., 2007). Although Gpp(NH)p was not always able to completely convert the binding to a single site, the WT and Del322-325 receptors behaved identically.

Receptor function was studied with an intact cell assay in which the inhibition of cyclic AMP production was measured. In this system, brimonidine and clonidine had similar efficacies and potencies in clones expressing either WT or Del322-325 receptors. To rule out any differences due to different expression levels, multiple clones of WT and Del322-325 receptors at different densities (B_{max} values) were tested in the functional assay. The relationships between efficacy or potency and receptor density were assessed by linear regression and the slopes of the lines for WT and Del322-325 were compared. If signalling were reduced with the Del322-325 polymorphism, the magnitude of this reduction might be amplified at lower receptor densities, resulting in a steeper (i.e. larger value) slope for the Del322-325 receptor. The slopes of the regression lines for both efficacy and potency, however, were not different between WT and Del322-325 receptors with both brimonidine and clonidine. These data strongly indicate that there is no difference in WT and Del322-325 function across the range of expression levels tested. Therefore, in intact HEK293 cells, the α_{2C}-Del322-325 polymorphism does not affect the signalling of the receptor to adenylyl cyclase.

There are multiple differences between our study and that of Small *et al*. (2000), in which a difference in coupling of

Table 5 Basal and forskolin-stimulated conversion of [^{3}H]ATP to [^{3}H]cyclic AMP

B_{max} (fmol·mg^{-1})	*n*	% conversion (% ± SEM)		*n*
		Basal	*Forskolin stimulation*	
WT				
470 [467, 464][a]	2	0.13 ± 0.03	1.28 ± 0.19	6
530 ± 50	5	0.10 ± 0.02	0.91 ± 0.05	6
1460 ± 104	6	0.02 ± 0.02	0.68 ± 0.16	6
Del322-325				
200 [200, 203][a]	2	0.13 ± 0.03	1.07 ± 0.17	6
670 [706, 637][a]	2	0.11 ± 0.03	0.83 ± 0.10	6
1100 [914, 1278][a]	2	0.12 ± 0.03	2.43 ± 0.44	8
1380 ± 292	5	0.08 ± 0.03	1.89 ± 0.67	4
2320 [2343, 2299][a]	2	0.04 ± 0.01	1.42 ± 0.31	8

To measure α_{2C}-mediated inhibition of cyclic AMP production in clones with various expression levels of WT or Del322-325 receptors, cyclic AMP production was stimulated with forskolin. Basal levels of cyclic AMP production were measured in the absence of forskolin or α_2 agonist. Stimulation with 30 μM forskolin is shown in the absence of α_2 agonist. Percent conversion values represent the % of [^{3}H]ATP converted to [^{3}H]cyclic AMP. Means ± SEMs are reported.
[a]At *n* = 2, SEM is not reported. Instead, values from individual experiments are shown in brackets.

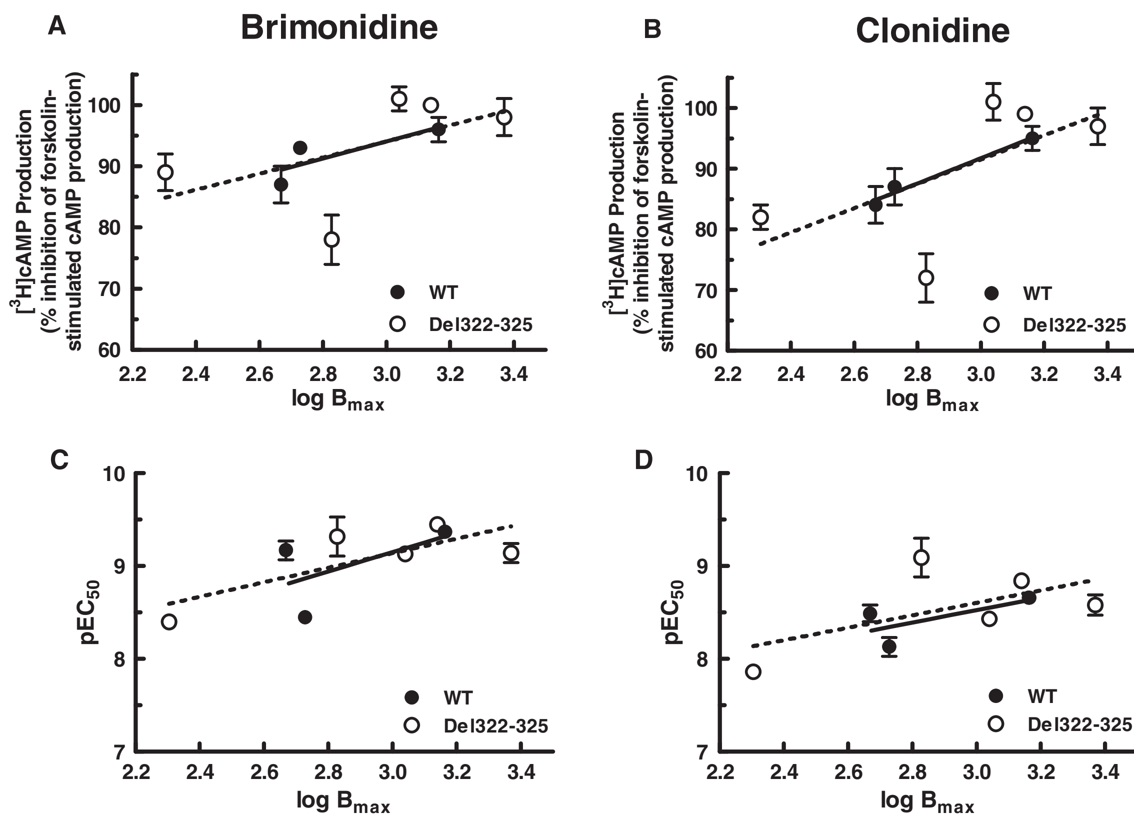

Figure 3 Comparison of the functional responses of α_{2C} WT and Del322-325 adrenoceptors at various receptor expression levels in HEK293 cells. Efficacy and potency values for the inhibition of cyclic AMP production by brimonidine (A and C, respectively) and clonidine (B and D, respectively) are plotted against the log B_{max} values of multiple WT and Del322-325 clones. Plotted values are the means ± SEM. The slopes of the lines for WT and Del322-325 were determined by linear regression analysis and did not differ between the two receptors for either agonist.

Del322-325 was observed. One difference is the cell line used, with the previous study using CHO cells and the current study using HEK293 cells. Due to variations observed from one cell line to another (Chaudhry and Granneman, 1994), validation of the previous findings in a second cell line is necessary before further study of the implications of this polymorphism *in vivo*. Another difference is that the decrease in Del322-325 inhibition of adenylyl cyclase activity in CHO cells was determined in membrane preparations, whereas our results in intact HEK293 cells showed no decrease in the inhibition of cyclic AMP production compared with the WT. Whereas the Small study reported a roughly 50–60% maximal inhibition of adenylyl cyclase activity, the current study achieved >90% inhibition of forskolin-stimulated cyclic AMP production.

Table 6 Slope values for linear regression analysis of efficacy and potency versus log B_{max}

	% inhibition			*pEC₅₀*		
	WT	*Del322-325*	*P*	*WT*	*Del322-325*	*P*
Brimonidine	14.0 ± 9.6	13.2 ± 11.7	0.98	1.04 ± 1.46	0.78 ± 0.37	0.83
Clonidine	20.8 ± 3.3	20.0 ± 13.9	0.98	0.67 ± 0.74	0.67 ± 0.54	1.00

Inhibition of cyclic AMP production by multiple WT and Del322-325 clones of various receptor densities were performed using brimonidine and clonidine. The relationship of average efficacy or potency to log B_{max} was analysed by linear regression and the slope values of WT and Del322-325 clones were compared by linear regression analysis using Prism 5.

These differences in efficacy are due to measuring adenylyl cyclase enzyme activity in membrane preparations versus cyclic AMP production in intact cells. For example, in CHO cells expressing 770 fmol·mg^{-1} of the human α_{2C}-adrenoceptor, it has been shown that the maximum inhibition of adenylyl cyclase activity in membranes is 58, 61 and 47% for noradrenaline, dexmedetomidine and clonidine, respectively, whereas in intact cells the maximum inhibition of cyclic AMP production is 92, 89 and 91% respectively (Pohjanoksa *et al.*, 1997).

Additionally, receptor expression levels differed between the two studies. Small *et al.* (2000) performed functional assays on cells ranging from roughly 500–1600 fmol·mg^{-1} receptor, claiming that the 500 fmol·mg^{-1} level was more similar to that found physiologically. In our study, we tested functional response in cells with a much broader range of receptor expression level, from 200 to 2320 fmol·mg^{-1}. The density of α_2-adrenoceptors in the human brain has been reported as approximately 70–140 fmol·mg^{-1} in the frontal cortex, 105 fmol·mg^{-1} in the cerebellum and 62 fmol·mg^{-1} in the caudate (Petrash and Bylund, 1986; Sastre and Garcia-Sevilla, 1994). By contrast, mouse brain and rat cortex express α_2-adrenoceptors at 200–300 fmol·mg^{-1} (MacMillan *et al.*, 1996; Bucheler *et al.*, 2002) and 200 fmol·mg^{-1} (Wallace *et al.*, 1994) respectively. Our studies include lower, more physiological levels.

These are not the only conflicting studies reported for the α_{2C}-Del322-325 polymorphism. Many studies have investigated the association of this polymorphism with certain clinical conditions, such as heart failure, with inconsistent results. Rosskopf and Michel (2008) present a comprehensive review on the association (or lack thereof) of the α_{2C}-Del322-325 polymorphism with various cardiovascular phenotypes. The rationale for potential cardiovascular effects is that α_{2A}- and α_{2C}-adrenoceptors work as autoreceptors inhibiting the release of noradrenaline in the peripheral nervous system. If this inhibitory effect was reduced, as could occur with loss-of-function receptor polymorphisms, the excess release of noradrenaline would cause increased sympathetic stimulation of the β_1-adrenoceptors of cardiac myocytes. Stimulation of the β_1- and β_2-adrenoceptors results in increased inotropic and chronotropic effects on the heart (Bristow, 1998). Chronic stimulation of β-adrenoceptors leads to the uncoupling of receptors from their G proteins and the down-regulation of β_1-adrenoceptors (Hajjar and MacRae, 2002). Therefore, it is suggested that the progression of heart failure is related to a sustained increase in sympathetic drive.

Although some clinical studies have found an association of the α_{2C}-Del322-325 polymorphism with increased levels of circulating catecholamines, increased risk of heart failure (Rosskopf and Michel, 2008), more progressive heart failure (Brede *et al.*, 2002) and better responses to β-blocker therapy (Regitz-Zagrosek *et al.*, 2006), other studies do not support a role for the Del322-325 variant in cardiovascular diseases. Clonidine and other α_2-adrenoceptors agonists are used in the treatment of hypertension to reduce the sympathetic tone, thereby lowering blood pressure and heart rate. While clonidine effectively lowers blood pressure in Caucasian hypertensive patients, it is not as effective in controlling blood pressure in African Americans (Materson *et al.*, 1993; Materson and Reda, 1994). The allele frequency of the Del322-325 polymorphism is relatively low in the Caucasian population (0.04); however, it is much more prevalent among the African American population (0.38) (Small *et al.*, 2000). Kurnik *et al.* (2008) looked at the association of the Del322-325 polymorphism with variation in pharmacological responses of Caucasian and African American hypertensive patients treated with dexmedetomidine, an α_2-selective agonist. Sympathetic tone decreased similarly in Caucasians and African Americans and there was no association of the Del322-325 variant with dexmedetomidine response. There was also no association of hypertension with the Del322-325 variant, either alone or in combination with a poorly expressed α_{2A} variant (Li *et al.*, 2006). Another study of black South Africans found all control and heart failure patients genotyped to be homozygous for the Del322-325 variant. In patients who were also homozygous for the β_1-Arg389 variant and not undergoing β-blocker treatment, there was no association with heart failure phenotype, severity or progression of the disease (Woodiwiss *et al.*, 2008).

The reason for the conflicting results from these clinical studies may be partially explained by the haplotype expressed in the subsets of patients studied. It has been shown that the Del322-325 variant is found in nine different haplotypes of the α_{2C} gene, with one of these haplotypes found in approximately 48% of Del322-325 carriers (Small *et al.*, 2004). Therefore, other factors may contribute to an association, or lack thereof, of the Del322-325 polymorphism and heart failure. For example, if the Del322-325 variant is found in a haplotype resulting in low receptor expression levels, this could amplify the phenotype resulting in an association with heart failure. However, our studies failed to show different coupling between WT and Del322-325 even at lower expression levels.

Although the Del322-325 polymorphism has no rodent homologue, information obtained from α_{2C} genetically modified mice may give some indications of what occurs physiologically with a loss-of-function variant of this receptor.

Recently, mice expressing just one functional copy of the α_{2C}-adrenoceptor have been created (Gilsbach *et al.*, 2007). These mice show that the loss of one copy of the α_{2C}-adrenoceptor is sufficient to impair the inhibition of catecholamine secretion from the adrenal gland. This differs from catecholamine release in the peripheral nervous system, which was unaffected by loss of one receptor copy. In the rat brain, only 20% of the receptors are required for a full response (Adler *et al.*, 1987; Agneter *et al.*, 1997) whereas in adrenal chromaffin cells of mice, which have approximately 30 fmol·mg^{-1} receptors (Lymperopoulos *et al.*, 2007), no receptor reserve is available. The reduced inhibition of adrenaline release from the adrenal cells of heterozygous mice was shown to result in tachycardia, and these mice developed heart failure after aortic banding (Gilsbach *et al.*, 2007). Therefore, the adrenal cells would be much more sensitive to the effects of an α_{2C} loss-of-function receptor polymorphism and these heterozygous mice could potentially be a good model for such a polymorphism.

Overall, our failure to show reduced function in HEK293 cells compared with the decreased function reported previously in CHO cells adds to the complexities of the phenotypic consequences of the α_{2C}-adrenoceptor Del322-325 genetic polymorphism. It is possible that further studies on the molecular basis for the different results in HEK293 and CHO cells might shed light on the marked differences seen between our studies and those of Small *et al.* (2000). Previous studies on the β_3-adrenoceptor have revealed such cell type-specific differences, where the receptor is desensitized in HEK293 cells but not in CHO cells (Chaudhry and Granneman, 1994; Vrydag *et al.*, 2009). Unexplained variation in receptor subcellular localization, dimerization and downstream signalling components could contribute to the variability observed *in vitro*. Similarly, the inconsistent cardiovascular effects observed with the Del322-325 polymorphism may be due to the presence of additional polymorphisms in other downstream signalling components. Signalling of the α_{2C}-adrenoceptor is linked to multiple downstream effector proteins. Although inhibition of adenylyl cyclase is a classical signalling mechanism of α_2-adrenoceptors, they are also involved in the inhibition of voltage-gated Ca^{++} channels (Limbird, 1988). Activation of receptor-operate K^+ channels and MAP kinase is also an α_2 receptor signalling mechanism (Limbird, 1988; Kobilka, 1992). The $G_{i/o\text{-}\alpha}$ subunit is most likely responsible for these signalling events. However, for the α_{2C} receptor, it has been reported that the $G_{i/o\text{-}\beta\gamma}$ is responsible for a Ca^{2+} mobilization response (Kukkonen *et al.*, 1998). In addition, there is some evidence that α_2-adrenoceptors can couple to the stimulatory G_s protein, leading to stimulation of cyclic AMP production (Eason *et al.*, 1992). Therefore, although the α_{2C}-Del322-325 adrenoceptor polymorphism does not impact the inhibition of cyclic AMP production in HEK293 cells, it is possible that other signalling events are affected by this variant.

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Conflicts of interest

There are no conflicts of interest.

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