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RESEARCH PAPER

Do gene polymorphisms alone or in combination affect the function of human β_3-adrenoceptors?

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Background and purpose: β_3-Adrenoceptors mediate many important physiological functions, for example, in the urinary bladder. The corresponding gene is polymorphic, and the W64R (Trp64Arg) single nucleotide polymorphism has been associated with disease states such as obesity, type 2 diabetes and bladder dysfunction. While these clinical data suggest that the 64R variant is hypofunctional, previous *in vitro* studies in which this variant was generated by site-directed mutagenesis and subsequent transfection have not consistently confirmed this.
Experimental approach: We transfected the wild-type human β_3-adrenoceptor and the 64R variant and also the more recently discovered 265M and 306F variants as well as 64R/265M and 64R/306F double mutants into human embryonic kidney cells and selected clones expressing the receptors at a density of about 100 fmol mg protein^{-1}. Receptor activation was measured by cAMP accumulation and ligand affinity by radioligand binding. Desensitisation was assessed as alterations of cAMP responses after prolonged agonist treatment.
Key results: Neither mutated receptor exhibited alterations in efficacy or potency for cAMP accumulation for any of five agonists (isoprenaline, noradrenaline, YM 178, FK 4664, CGP 12 177). In competition binding studies, the mutations did not affect the ability of any agonist to bind to the receptor. Wild-type receptors and the 64R variant exhibited similar isoprenaline-induced functional desensitization during a 24 h treatment.
Conclusions and implications: None of the polymorphisms tested here significantly altered the interaction of isoprenaline, noradrenaline, YM 178, FK 4664 or CGP 12 177 with the human β_3-adrenoceptor when expressed at near physiological levels in a human cell line.
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Keywords: Trp64Arg; Thr265Met; Leu306Phe; β_3-adrenoceptor; polymorphism; YM 178; FK 4664; CGP 12 177

Abbreviations: CGP 12,177, (-)-4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazol-2-one; DMEM, Dulbecco's Modified Eagle's Medium; FK 4664, 4′-(2-{[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino}ethyl)-3-methoxybiphenyl-4-carboxylic acid hydrochloride; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; [^{125}I]-CYP, [^{125}I]-iodocyanopindolol; RO 20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone; WT, wild type; YM 178, (R)-2-(2-aminothiazol-4-yl)-4′-{2-[(2-hydroxy-2-phenylethyl)amino]ethyl} acetanilide

Introduction

β_3-Adrenoceptors mediate effects of the endogenous catecholamines adrenaline and noradrenaline in several tissues including adipose tissue and smooth muscle where they can mediate lipolysis and thermogenesis or relaxation respectively (Rozec and Gauthier, 2006; Arch, 2008). While the role of β_3-adrenoceptors in adipocytes is less prominent in humans than in rodents (Arch, 2008), they play a major role in the regulation of smooth muscle tone in the human urinary bladder (Michel and Vrydag, 2006), possibly both at the smooth muscle and the urothelial level (Otsuka *et al.*, 2008).

The human β_3-adrenoceptor gene is located on chromosome 8p11–8p12 (Emorine *et al.*, 1989). More than 10 years ago, this gene was identified as being polymorphic and the W64R (Trp64Arg) single nucleotide polymorphism (SNP) has received considerable attention (Clement *et al.*, 1995; Walton *et al.*, 1995). Although the existing studies are not unequivocal, the overall evidence suggests that the 64R genotype can be associated with disease states such as obesity and type 2 diabetes (Leineweber *et al.*, 2004). A more recent report has also linked the 64R genotype to the overactive bladder syndrome (Honda *et al.*, 2006). All of these

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disease associations would be consistent with the idea that the 64R SNP yields a hypofunctional variant of the β_3-adrenoceptor.

To test this possibility, mechanistic studies have been performed. In some studies the ability of β_3-adrenoceptor agonists to induce lipolysis was compared in samples obtained from subjects stratified according to genotype, and the results were compatible with the proposed hypofunction of the 64R genotype (Hoffstedt *et al*., 1999; Umekawa *et al*., 1999). On the other hand, similar studies have been performed with transfected cells in which the 64R genotype had been generated by site-directed mutagenesis. While some of these studies support the idea of the 64R variant yielding a hypofunctional receptor (Pietri-Rouxel *et al*., 1997; Kimura *et al*., 2000), such differences were not detected in other studies (Candelore *et al*., 1996), and some investigators have even reported an increased function for the 64R variant under certain circumstances, that is, when co-expressed with type III adenylyl cyclase (Isogaya *et al*., 2002).

When considering these somewhat heterogeneous results, three potential explanations come to mind. First, it is possible that a given SNP will selectively affect the response to certain agonists because it has more effect on the binding pocket of a specific compound than on the general receptor function (Michel and Alewijnse, 2007). This possibility is highlighted by findings in human adipocytes (Umekawa *et al*., 1999). Second, the polymorphic allele 64R in humans is the wild type (WT) in almost all animal species including chimpanzees (Vrydag and Michel, 2007), but most expression studies have not been done in human cells but rather in those derived from other species. Therefore, the possibility exists that these cells do not have the correct machinery to fully translate functional effects of this polymorphism. Third, the W64R SNP is not the only polymorphism in the human β_3-adrenoceptor gene. Thus, it has been shown that this SNP forms a haploblock with several polymorphisms in the non-coding region of the receptor which possibly affect transcription of the gene and/or the stability of the corresponding mRNA (Hoffstedt *et al*., 1999). Moreover, an additional SNP in the coding region (T265M) has been reported (Halushka *et al*., 1999), which may be in linkage disequilibrium with the W64R polymorphism but nothing is known about the functional properties of this SNP. Finally, we have recently detected a hitherto unknown SNP in the transmembrane region 6 of the human β_3-adrenoceptor, L306F (data not shown). This SNP is of interest because it resides directly next to an amino acid which is conserved in all nine adrenoceptors and forms part of the binding pocket for catecholamines (Swaminath *et al*., 2005). It is possible that some of these polymorphisms form haplotypes which have a more consistent effect on receptor function than either polymorphism alone.

Against this background, the present study was designed to investigate the following questions: Does the W64R SNP affect β_3-adrenoceptor function when expressed in a human cell? Do any of the more recently discovered T265M and L306F polymorphisms affect receptor function? Is any such alteration specific for a certain chemical class of agonists? Do combinations of these three coding SNPs have effects which extend beyond those of a single SNP in isolation? To address these questions we have used the endogenous agonist noradrenaline, the prototypical agonist isoprenaline, two β_3-selective agonists currently in clinical development, (R)-2-(2-aminothiazol-4-yl)-4′-{2-[(2-hydroxy-2-phenylethyl)amino] ethyl} acetanilide (YM 178) (Takasu *et al*., 2007; Chapple *et al*., 2008) and 4′-(2-{[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino}ethyl)-3-methoxybiphenyl-4-carboxylic acid hydrochloride (FK 4664) (Imanishi *et al*., 2008), and (-)-4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazol-2-one (CGP 12 177), an agonist from a markedly different chemical class than the other compounds.

Methods

Site-directed mutagenesis

pcDNA3.1 (+) containing the entire coding region of the human β_3-adrenoceptor gene was purchased from UMR cDNA Resource Center (Rolla, MO, USA). Site-directed mutagenesis was performed with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to manufacturer guidelines with small modifications, that is, 4% DMSO was added to the PCR solution and reactions were carried out at 66°C on a Biometra T1 thermocycler (Göttingen, Germany). In some cases this procedure was repeated to introduce a second mutation into the receptor. The primers used for the mutagenesis PCR (Table 1) were constructed by use of Primer3 (Rozen and Skaletsky, 2000) and were purchased from Biolegio (Nijmegen, the Netherlands). The sequence identity of the obtained plasmid was checked by Baseclear (Leiden, the Netherlands).

Transfection and cell culture

Human embryonic kidney (HEK) 293 cells were stably transfected with human WT and mutated β_3-adrenoceptors using a pcDNA3.1 (+) vector. Transfection was performed by calcium phosphate co-precipitation according to Graham and van der Eb (1973). Cells were grown and passaged in an atmosphere of 5% CO_2/95% air at 37°C in a 1:1 mixture of Dulbecco's Modified Eagle's Medium with nutrient mixture F12 (DMEM/F12) supplemented with 10% heat inactivated foetal calf serum and penicillin (100 units mL^{-1}) and streptomycin (100 $\mu g\ mL^{-1}$). To maintain selection pressure, the antibiotic geneticin (400 $\mu g\ mL^{-1}$) was added to all growing cells, but was not present during the experiments. The identity of all cell lines was confirmed by sequencing.

Radioligand binding studies

Cells at approximately 80% confluence were harvested by scraping the culture flasks with a cell scraper, washed twice by

Table 1 Primers used for the mutagenesis PCR

W64R – forward	5′-CATCGTGGCCATCGCCCGGACTCCGAGACTCC-3′
W64R – reverse	5′-GGAGTCTCGGAGTCCGGGCGATGGCCACGATG-3′
T265M – forward	5′-CCCCGGTGGGGATGTGCGCTCCGCC-3′
T265M – reverse	5′-GGCGGAGCGCACATCCCCACCGGGG-3′
L306F – forward	5′-CTCTCTGCTGGTTCCCCTTCTTTCTGG-3′
L306F – reverse	5′-CCAGAAAGAAGGGGAACCAGCAGAGAG-3′

centrifugation at 200× *g* and then homogenized in ice-cold buffer (50 mmol·L^{-1} Tris, 0.5 mmol·L^{-1} EDTA, pH 7.5) with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) for 10 s at full speed and then twice for 20 s each at 2/3 speed. The homogenates were centrifuged for 20 min at 50 000× *g* at 4°C. The pellets were resuspended in buffer and stored at –80°C. Protein content was measured according to Bradford (1976), using bovine IgG as the standard.

β_3-Adrenoceptor density in the membrane preparation was determined by [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) saturation binding experiments using approximately 15 µg protein per assay and eight radioligand concentrations as previously described with definition of non-specific binding by 100 µmol·L^{-1} isoprenaline (Niclauß *et al.*, 2006). Clones expressing ≈100 fmol mg protein^{-1} of the receptor were used for all subsequent experiments. In competition binding experiments a radioligand concentration of 500 pmol·L^{-1} and four competitor concentrations per log unit of concentration increment were used. The buffer for the agonist competition binding experiments had been supplemented with 5 mmol·L^{-1} $MgCl_2$.

Cyclic AMP studies

Cells stably expressing the WT or mutated β_3-adrenoceptor were pre-incubated for 1 h in medium without serum. Thereafter, they were detached from the surface using enzyme free cell dissociation buffer and washed once with Hank's balanced salt solution (HBSS). The cells were resuspended in HBSS supplemented with 5 mmol·L^{-1} HEPES and 0.05% bovine serum albumin. The stimulation mixture contained in a twofold concentration the β_3-adrenoceptor agonists, the non-specific cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100 µmol·L^{-1}) and 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone (RO 20-1724, 100 µmol·L^{-1}), a specific type 4 phosphodiesterase inhibitor. Cells were added to the stimulation mixture 1:1 in a 384 well optiplate (625 cells per well in a total volume of 10 µl) and stimulated for 30 min at room temperature. Detection was performed as previously described (Jongsma *et al.*, 2006) using the LANCE® cAMP 384 Kit (Perkin Elmer, Zaventem, Belgium) and measured after 3 h on a Victor plate reader (Wallac, Perkin-Elmer, Zaventem, Belgium).

Data analysis

All data shown are mean values ± SEM. In the cAMP experiments sigmoidal curves were fitted to the experimental data from each experiment to calculate pEC_{50} and E_{max}, the latter being expressed as increase in cAMP accumulation over basal values as determined in that experiment. Saturation and competition radioligand binding experiments were analysed by fitting rectangular hyperbolic and sigmoidal functions, respectively, to the experimental data. The statistical significance of group differences was assessed by two-tailed *t*-tests or one-way analysis of variance as indicated, and $P < 0.05$ was considered significant. All curve fitting and statistical calculations were performed using the Prism programme (Graphpad Software, San Diego, CA, USA). The receptor nomenclature used in this article follows the recommendations of the *British Journal of Pharmacology* (Alexander *et al.*, 2008).

Materials

Untransfected HEK293 cells were kindly provided by Dr M. Schmidt (Groningen, the Netherlands). [^{125}I]-CYP (2220 Ci mmoL^{-1}) was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). DMEM/F12, foetal calf serum, penicillin/streptomycin, and geneticin, enzyme free cell dissociation buffer were from Gibco (via Invitrogen, Breda, the Netherlands). CGP 12 177, HEPES, IBMX, isoprenaline, noradrenaline and RO 20-1724 were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). YM 178 (now known as mirabegron) and FK 4664 were from Astellas Pharma Europe B.V. (Leiderdorp, the Netherlands).

Results

We initially screened multiple clones for each of the six genotypes under investigation by saturation radioligand binding. For each genotype, a clone was picked which expressed approximately 100 fmol receptor mg protein^{-1}. [^{125}I]-CYP had similar affinity (K_d) for all genotypes (Table 2).

The basal cAMP content was about 20–50 fmol per well. None of the agonists increased cAMP accumulation in mock-transfected cells (data not shown), whereas all induced concentration-dependent cAMP accumulation in cells expressing either the WT or a mutated receptor (Fig. 1). The potency and efficacy of each agonist was similar in WT and all mutated receptors (Fig. 1, Table 3). Consistently across all genotypes the order of potency was YM 178 > isoprenaline ≈ noradrenaline > FK 4664 > CGP 12 177, whereas the order of efficacy was isoprenaline ≈ noradrenaline > YM 178 > FK 4664 ≥ CGP 12 177 (Table 3). As CGP 12 177 has been reported to be a partial agonist in several systems (Vrydag and Michel, 2007), a maximally effective concentration of isoprenaline (10 µmol·L^{-1}) was included in each experiment. Relative to this isoprenaline response, the efficacy of CGP 12 177 was 74 ± 9%, 73 ± 8%, 77 ± 10%, 74 ± 9%, 77 ± 9%, 73 ± 3% for WT and the 64R, 265M, 306F, 64R + 265M and 64R + 306F variants respectively ($P < 0.05$ for all groups except 64R and 64R + 265M). Taken the cAMP response to 10 µmol·L^{-1} isoprenaline from these and the concentration-response experiments into account (n = 11–15), again there was no significant different among genotypes (data not shown).

To substantiate these data, we have performed agonist competition binding but have restricted those experiments to

Table 2 Effect of genotype on the affinity of [^{125}I]-CYP in saturation binding experiments

	K_d, pmol·L^{-1}	B_{max}, fmol mg protein^{-1}
Wild type	306 ± 49	121 ± 35
64R	312 ± 14	159 ± 35
265M	295 ± 24	107 ± 19
306F	261 ± 53	133 ± 31
64R + 265M	308 ± 17	119 ± 29
64R + 306F	279 ± 37	162 ± 59

Data are means ± SEM of 4–8 experiments for K_d (including some with clones expressing receptor densities other than 100 fmol mg protein^{-1}) and means ± SEM of three experiments for B_{max}. Values did not differ significantly in a one-way analysis of variance.

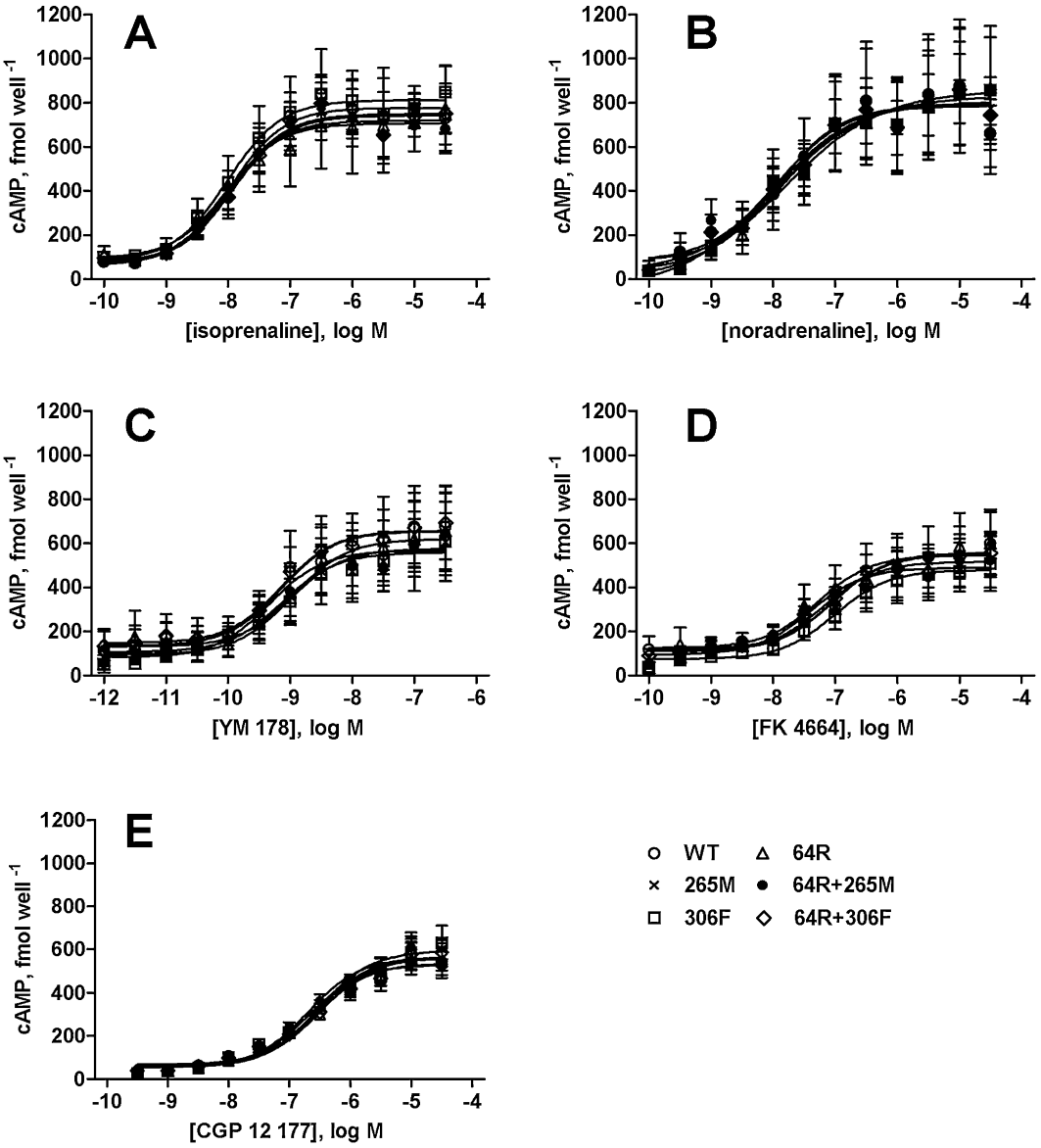

Figure 1 Effect of genotype on cAMP accumulation induced by (A) noradrenaline, (B) isoprenaline, (C) YM 178, (D) FK 4664 and (E) CGP 12 177. Data are means ± SEM of 3–9 experiments, and a quantitative analysis of the data is shown in Table 3.

the WT, the 64R and the 306F genotypes. All five agonists exhibited steep and monophasic competition curves (Fig. 2). A quantitative analysis of these experiments revealed a rank order of potency of YM 178 ≈ CGP 12 177 > FK 4664 > isoprenaline ≥ noradrenaline (Table 4); the differences between binding and cAMP stimulation order of potency would be consistent with a lower efficacy of CGP 12 177 and perhaps FK 4664 as compared with the other agonists.

Finally, we have assessed whether any of the mutations affects the susceptibility of the receptor to agonist-induced functional desensitization. For this purpose cells were exposed to 10 $\mu mol \cdot L^{-1}$ isoprenaline or vehicle in the absence of serum for 24 h prior to the experiment. After washing of the cells with buffer, concentration-response curves for cAMP accumulation induced by freshly added agonist were obtained. Pre-incubation with 10 $\mu mol \cdot L^{-1}$ isoprenaline reduced the maximum cAMP response to freshly added isoprenaline or YM 178 by about 50% but did not significantly affect the potency of either agonist (Table 5, Fig. 3).

Discussion and conclusions

Although the overall evidence from clinical association studies and from those with *ex vivo* samples from patients suggests that the W64R SNP of the β_3-adrenoceptor represents a hypofunctional receptor variant (Leineweber *et al.*, 2004), attempts to link this polymorphism to alterations of receptor function have met with mixed success in assays using site-directed mutagenesis with subsequent heterologous expression. One possible explanation for this discrepancy may be a small overall effect of the 64R receptor variant, as the clinical data are not fully consistent across studies despite, on average, supporting the idea of the 64R variant being hypofunctional.

Table 3 Effect of genotype on agonist efficacy (E_{max}) and potency (pEC_{50}) for stimulating cAMP accumulation

	Noradrenaline		*Isoprenaline*		*YM 178*		*FK 4664*		*CGP 12 177*	
	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}
Wild type	786 ± 236	7.89 ± 0.12	790 ± 212	7.95 ± 0.05	668 ± 168	9.03 ± 0.20	567 ± 146	7.01 ± 0.21	537 ± 41	6.68 ± 0.09
64R	867 ± 296	7.88 ± 0.19	697 ± 194	8.01 ± 0.06	621 ± 133	8.96 ± 0.04	550 ± 122	7.28 ± 0.25	519 ± 66	6.62 ± 0.12
265M	800 ± 37	7.96 ± 0.06	730 ± 91	7.95 ± 0.02	559 ± 121	9.10 ± 0.06	616 ± 46	7.01 ± 0.05	570 ± 92	6.72 ± 0.08
306F	832 ± 238	7.98 ± 0.07	817 ± 91	8.00 ± 0.02	576 ± 152	9.02 ± 0.07	479 ± 105	7.02 ± 0.02	624 ± 92	6.49 ± 0.14
64R + 265M	789 ± 238	7.94 ± 0.04	726 ± 96	8.00 ± 0.03	567 ± 172	9.13 ± 0.11	490 ± 119	7.12 ± 0.04	566 ± 35	6.49 ± 0.06
64R + 306F	803 ± 142	7.89 ± 0.02	745 ± 95	7.95 ± 0.01	659 ± 146	9.15 ± 0.04	611 ± 44	7.15 ± 0.06	569 ± 54	6.56 ± 0.07

Data are mean ± SEM of E_{max} (fmol cAMP formed per well) and pEC_{50} values as measured in 3–9 experiments. A graphical representation of the data is shown in Figure 1. Note that numerical differences in parameter estimates for a given agonist did not significantly differ between genotypes in a one-way analysis of variance.

The present study has addressed several other potential explanations for such discrepancies. Specifically, we have studied not only the W64R but also two more recently discovered SNPs, T265M and L306F, and have also tested potential combinations of either occurring in association with the W64R SNP. Moreover, all of these experiments were performed in a human cell line, HEK293, in order to maximize the chance of detecting functional correlates of receptor polymorphisms. Our data demonstrate that none of the polymorphisms studied here alone or in combination significantly affects the affinity of the radioligand [^{125}I]-CYP in saturation binding studies. All of our experiments were performed in clones expressing a similar receptor density (Table 2) which is close to that expected to exist physiologically based upon studies with other β-adrenoceptor subtypes and that may explain minor differences in observed agonist potency and efficacy. Finally, we have studied not only the endogenous agonist noradrenaline and the prototypical synthetic agonist isoprenaline but also two β_3-selective agonists, YM 178 (Takasu *et al.*, 2007) and FK 4664 (Imanishi *et al.*, 2008), currently in clinical development for the treatment of the overactive bladder syndrome (Chapple *et al.*, 2008) and additional an agonist from a very different chemical family, CGP 12 177. Taken together these factors should allow minimizing the chance of false negative functional differences.

Studies with SNPs in the human β_1-adrenoceptor have demonstrated direct effects on agonist efficacy, for example, the 389G conferring weaker signalling than the 389R WT (Leineweber *et al.*, 2004). Our studies with β_3-adrenoceptor mutants in position 64, 265 and 306 did not reveal an impact of any of these polymorphisms on agonist potency or efficacy for stimulating cAMP accumulation. Studies in human adipocytes have reported that genotype-related differences in β_3-adrenoceptor function may be more prominent for some agonists than for others (Umekawa *et al.*, 1999). Our studies using five different agonists found a similar lack of effect of genotype for all of them. As all previous similar studies had also investigated cAMP accumulation as the functional response to explore effects of genotype (Candelore *et al.*, 1996; Pietri-Rouxel *et al.*, 1997; Kimura *et al.*, 2000; Isogaya *et al.*, 2002), we had focussed on this response as well. However, in light of recent discussions on ligand-directed signalling of β_3-adrenoceptors (Hutchinson *et al.*, 2006; Michel and Alewijnse, 2007; Sato *et al.*, 2007), it should be noted that our findings based upon cAMP accumulation do not exclude distinct findings for other signal transduction pathways which have been linked to β-adrenoceptor signalling such as various types of potassium channels (Bieger *et al.*, 2006; Ferro, 2006; Scherer *et al.*, 2007) or NO synthase (Harmon *et al.*, 2005); however, these signalling pathways are not active in HEK293 cells, and hence we could not evaluate them in our cell lines.

Studies with β_2-adrenoceptor polymorphisms suggest that they may differ functionally when occurring in combination as part of distinct haplotypes (Hahntow *et al.*, 2006). While it is currently unknown whether the polymorphisms in position 64, 265 and 306 of the β_3-adrenoceptor physiologically occur in combination, our data do not support the idea that such combinations yield functional properties distinct from the isolated polymorphisms or the WT.

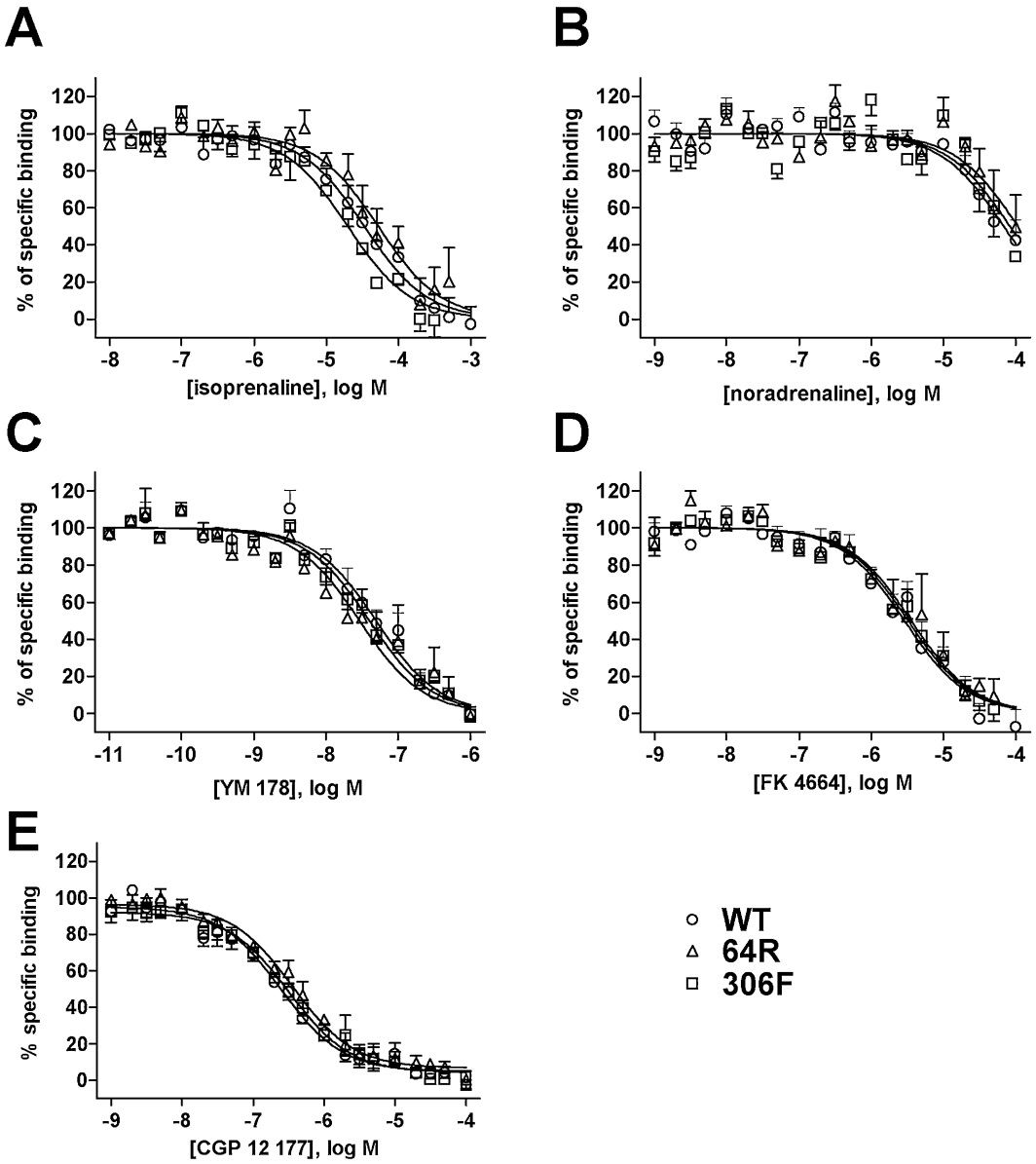

Figure 2 Effect of genotype on competition binding by (A) noradrenaline, (B) isoprenaline, (C) YM 178, (D) FK 4664 and (E) CGP 12 177. Data are means ± SEM of 3–4 experiments, and a quantitative analysis of the data is shown in Table 4.

Table 4 Effect of genotype on the affinity of agonists in [^{125}I]-CYP in competition binding experiments

	Noradrenaline	*Isoprenaline*	*YM 178*	*FK 4664*	*CGP 12 177*
Wild type	4.16 ± 0.06	4.47 ± 0.07	7.26 ± 0.09	5.35 ± 0.08	7.14 ± 0.04
64R	3.97 ± 0.10	4.23 ± 0.10	7.47 ± 0.13	5.37 ± 0.11	6.98 ± 0.08
306F	4.10 ± 0.13	4.74 ± 0.05	7.43 ± 0.06	5.37 ± 0.06	7.03 ± 0.09

Data are mean ± SEM of pK_i values as measured in 3–4 experiments. Note that noradrenaline did not always cause >50% inhibition within the tested concentration range, and hence its pK_i values must be considered estimates. A graphical representation of the data is shown in Figure 2. Note that numerical differences of pK_i values for a given agonist did not significantly differ between genotypes in a one-way analysis of variance.

To further characterize possible genotype effects of agonist interaction with the β_3-adrenoceptor, we have performed agonist competition binding experiments. β_3-Adrenoceptor sequencing studies in 91 Caucasian subjects performed concomitantly with the present *in vitro* experiments did not detect the L265M SNP (data not shown), indicating that it may be relatively rare. Based upon this finding and the apparent lack of a functional effect of this polymorphism, our competition binding experiments were limited to those in position 64 and 306. The latter was of particular interest to us as this amino acid is conserved in all three human β-adrenoceptor subtypes and resides directly next to an amino

Table 5 Effect of genotype on isoprenaline-induced desensitization of agonist-induced cAMP accumulation

	Stimulation of cAMP accumulation with isoprenaline				*Stimulation of cAMP accumulation with YM 178*			
	Vehicle treatment		*Isoprenaline treatment*		*Vehicle treatment*		*Isoprenaline treatment*	
	Wild type	*64R*	*Wild type*	*64R*	*Wild type*	*64R*	*Wild type*	*64R*
E_{max}	671 ± 39	647 ± 35	267 ± 15[a]	261 ± 15[a]	639 ± 57	659 ± 50	247 ± 24[a]	239 ± 23[a]
pEC_{50}	7.86 ± 0.23	7.95 ± 0.20	7.98 ± 0.05	7.92 ± 0.07	9.15 ± 0.27	8.94 ± 0.24	8.78 ± 0.38	8.68 ± 0.35

Data are mean ± SEM of E_{max} (fmol cAMP formed per well) and pEC_{50} values as measured in three experiments. A graphical representation of the data is shown in Figure 3.
[a]$P < 0.05$ in a paired two-tailed *t*-test as compared with vehicle treatment of the same genotype.

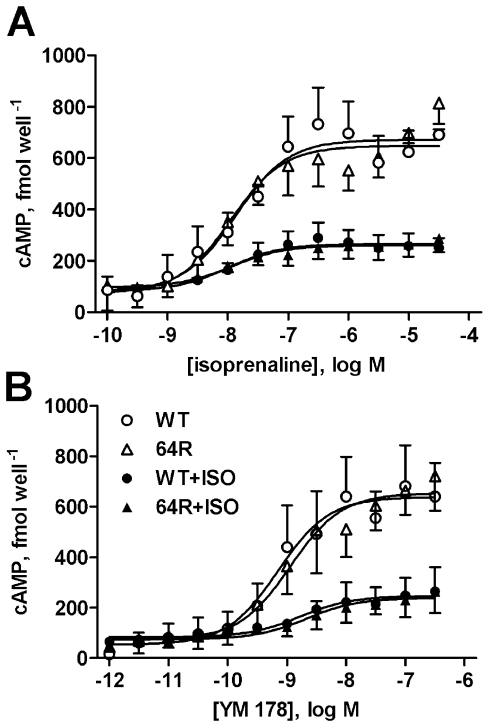

Figure 3 Effect of genotype on isoprenaline-induced desensitization. Data are means ± SEM of three experiments, and a quantitative analysis of the data is shown in Table 5. ISO: cells pre-treated with 10 μmol·L^{-1} isoprenaline for 24 h. The cAMP accumulation is induced by (A) isoprenaline and (B) YM 178.

acid conserved in all nine adrenoceptor subtypes, which is believed to play an important role in catecholamine binding (Swaminath *et al.*, 2005). However, neither the 64R nor the 306F SNP affected the apparent affinity of any of the five agonists or the radioligand, which is an antagonist/weak partial agonist.

Findings from the β_2-adrenoceptor field demonstrate that SNPs may affect the susceptibility to undergo agonist-induced desensitization (Brodde, 2008). Previous literature data indicate that the sensitivity of β_3-adrenoceptors to undergo agonist-induced desensitization is cell type dependent with little desensitization occurring in some cells including transfected Chinese hamster ovary cells (Nantel *et al.*, 1993; Chaudhry and Granneman, 1994; Candelore *et al.*, 1996; Curran and Fishman, 1996). On the other hand, desensitization was observed in HEK293 cells (Chaudhry and Granneman, 1994), a finding which was confirmed in the present study. Our desensitization experiments were limited to the W64R polymorphism because we had meanwhile detected the 306F allele in less than 2% of a large Caucasian population (data not shown). Our findings show that the W64R polymorphism did not affect agonist-induced desensitization in HEK293 cells, which is in line with a previous report that it also did not affect the lack of desensitization in Chinese hamster ovary cells (Candelore *et al.*, 1996).

Taken together our data demonstrate that three SNPs in the coding region of the human β_3-adrenoceptor gene alone or in combination have little effect on ligand recognition, function or regulation of receptor responsiveness. From a pharmacogenetic point of view, our findings with YM 178 and FK 4664, which are currently undergoing clinical development for the treatment of the overactive bladder syndrome (Chapple *et al.*, 2008), suggest that the effectiveness of these agonists is unlikely to be affected by genotype. This finding appears important as the 64R allele was reported to be present in almost 50% of patients with the overactive bladder syndrome (Honda *et al.*, 2006). However, it should be noted that the frequent 64R SNP is in linkage with multiple polymorphisms in the non-coding region of the gene which may have effects on expression levels (Hoffstedt *et al.*, 1999). Such genetic effects on expression levels might explain why *ex vivo* studies have consistently reported the 64R allele to be hypofunctional (Hoffstedt *et al.*, 1999; Umekawa *et al.*, 1999). Our studies with site-directed mutagenesis of the coding region, by definition, cannot address this possibility which merits further investigation.

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Conflicts of interest

M.C.M. is a paid consultant of Astellas. The other authors report no conflict of interest.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to receptors and channels (GRAC), 3rd edn. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Arch JRS (2008). Perspectives from β_3-adrenoceptor agonists on pharmacology, physiology and obesity drug discovery. *Naunyn Schmiedebergs Arch Pharmacol* **378**: 225–240.

Bieger D, Parai K, Ford CA, Tabrizchi R (2006). β-Adrenoceptor mediated responses in rat pulmonary artery: putative role of TASK-1 related K channels. *Naunyn Schmiedebergs Arch Pharmacol* **373**: 186–196.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

Brodde O-E (2008). β-1 and β-2 adrenoceptor polymorphisms: functional importance, impact on cardiovascular diseases and drug responses. *Pharmacol Ther* **117**: 1–29.

Candelore MR, Deng L, Tota LM, Kelly LJ, Cascieri MA, Strader CD (1996). Pharmacological characterization of a recently described human β_3-adrenergic receptor mutant. *Endocrinol* **137**: 2641–5638.

Chapple CR, Yamaguchi O, Ridder A, Liehne J, Carl S, Mattiasson A *et al*. (2008). Clinical proof of concept study (Blossom) shows novel β_3 adrenoceptor agonist YM178 is effective and well tolerated in the treatment of symptoms of overactive bladder. *Eur Urol Suppl* **7**: 239.

Chaudhry A, Granneman JG (1994). Influence of cell type upon the desensitization of the β_3-adrenergic receptor. *J Pharmacol Exp Ther* **271**: 1253–1258.

Clement K, Vaisse C, Manning BStJ, Basdevant A, Guy-Grand B, Riuz J *et al*. (1995). Genetic variation in the β_3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *New Engl J Med* **333**: 352–354.

Curran PK, Fishman PH (1996). Endogenous β_3- but not β1-adrenergic receptors are resistant to agonist-mediated regulation in human SK-N-MC neurotumor cells. *Cell Signal* **8**: 355–364.

Emorine LJ, Marullo S, Briden-sutren M-M, Patey G, Tate K, Delavier-Klutchko C *et al*. (1989). Molecular characterization of the human β_3-adrenergic receptor. *Science* **245**: 1118–1121.

Ferro A (2006). β-Adrenoceptors and potassium channels. *Naunyn Schmiedebergs Arch Pharmacol* **373**: 183–185.

Graham FL, van der Eb AJ (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**: 456–467.

Hahntow IN, Koopmans RP, Michel MC (2006). The β_2-adrenoceptor gene and hypertension: is it the promoter or the coding region or neither? *J Hypertension* **24**: 1003–1007.

Halushka MK, Fan J-B, Bentley K, Hsie L, Shen N, Weder A *et al*. (1999). Patterns of single-nucleotide polymorphisms in candidate genes for blood-pressure homeostasis. *Nat Genet* **22**: 239–247.

Harmon EB, Porter JM, Porter JE (2005). β-Adrenergic receptor activation in immortalized human urothelial cells stimulates inflammatory responses by PKD-independent mechanisms. *Cell Commun Signal* **3**: 10.

Hoffstedt J, Poirier O, Thörne A, Lönnqvist F, Herrmann SM, Cambien F *et al*. (1999). Polymorphism of the human β_3-adrenoceptor gene forms a well-conserved haplotype that is associated with moderate obesity and altered receptor function. *Diabetes* **48**: 203–205.

Honda K, Nomiya M, Shishido K, Yoshimura Y, Yamaguchi O (2006). Mutation of β_3-adrenoceptor gene: a genetic marker for overactive bladder. *Neurourol Urodyn* **25**: 652.

Hutchinson DS, Chernogubova E, Sato M, Summers RJ, Bengtsson T (2006). Agonist effects of zinterol at the mouse and human β_3-adrenoceptor. *Naunyn Schmiedebergs Arch Pharmacol* **373**: 158–168.

Imanishi M, Tomishima Y, Itou S, Hamashima H, Nakajima Y, Washizuka K *et al*. (2008). Discovery of a novel series of biphenyl benzoic acid derivatives as potent and selective human β_3-adrenergic receptor agonists with good oral bioavailability. Part I. *J Med Chem* **51**: 1925–1944.

Isogaya M, Nagao T, Kurose H (2002). Enhanced cAMP response of naturally occurring mutant of human β_3-adrenergic receptor. *Jpn J Pharmacol* **88**: 314–318.

Jongsma M, Hendriks-Balk MC, Michel MC, Peters SLM, Alewijnse AE (2006). BML-241 fails to display selective antagonism at the sphingosine-1-phosphate receptor, $S1P_3$. *Br J Pharmacol* **149**: 277–282.

Kimura K, Sasaki N, Asano A, Mizukami J, Kayahashi S, Kawada T *et al*. (2000). Mutated human β_3-adrenergic receptor (Trp64Arg) lowers the response to β_3-adrenergic agonists in transfected 3T3-L1 preadipocytes. *Horm Metab Res* **32**: 91–96.

Leineweber K, Büscher R, Bruck H, Brodde O-E (2004). β-Adrenoceptor polymorphisms. *Naunyn Schmiedebergs Arch Pharmacol* **369**: 1–22.

Michel MC, Alewijnse AE (2007). Ligand-directed signaling: 50 ways to find a lover. *Mol Pharmacol* **72**: 1097–1099.

Michel MC, Vrydag W (2006). α_1-, α_2- and β-adrenoceptors in the urinary bladder, urethra and prostate. *Br J Pharmacol* **147**: S88–S119.

Nantel F, Bonin H, Emorine LJ, Zelberfarb V, Strosberg AD, Bouvier M *et al*. (1993). The human β_3-adrenergic receptor is resistant to short term agonist-promoted desensitization. *Mol Pharmacol* **43**: 548–555.

Niclauß N, Michel-Reher MB, Alewijnse AE, Michel MC (2006). Comparison of three radioligands for the labelling of human β-adrenoceptor subtypes. *Naunyn Schmiedebergs Arch Pharmacol* **374**: 79–85.

Otsuka A, Shinbo H, Matsumoto R, Kurita Y, Ozono S (2008). Expression and functional role of β-adrenoceptors in the human urinary bladder. *Naunyn Schmiedebergs Arch Pharmacol* **377**: 473–481.

Pietri-Rouxel F, St John Manning B, Gros J, Strosberg AD (1997). The biochemical effect of the naturally occurring Trp64Arg mutation on human beta3-adrenoceptor activity. *Eur J Biochem* **247**: 1174–1179.

Rozec B, Gauthier C (2006). β_3-Adrenoceptors in the cardiovascular system: putative roles in human pathologies. *Pharmacol Ther* **111**: 652–673.

Rozen S, Skaletsky HJ (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**: 365–386.

Sato M, Horinouchi T, Hutchinson DS, Evans BA, Summers RJ (2007). Ligand-directed signaling at the β_3-adrenoceptor produced by SR59230A relative to receptor agonists. *Mol Pharmacol* **74**: 1359–1368.

Scherer D, Kiesecker C, Kulzer M, Günth M, Scholz EP, Kathöfer S *et al*. (2007). Activation of inwardly rectifying Kir2.x potassium channels by β_3-adrenoceptors is mediated via different signaling pathways with a predominant role of PKC for Kir2.1 and of PKA for Kir2.2. *Naunyn Schmiedebergs Arch Pharmacol* **375**: 311–322.

Swaminath G, Deupi X, Lee TW, Zhu W, Thian FS, Kobilka TS *et al*. (2005). Probing the β_2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists. *J Biol Chem* **280**: 22165–22171.

Takasu T, Ukai M, Sato S, Matsui T, Nagase I, Maryama T *et al*. (2007). Effect of YM178, a novel selective β_3-adrenoceptor agonist, on bladder function. *J Pharmacol Exp Ther* **321**: 642–647.

Umekawa T, Yoshida T, Sakane N, Kogure A, Kondo M, Honjyo H (1999). Trp64Arg mutation of β_3-adrenoceptor gene deteriorates lipolysis by β_3-adrenoceptor agonist in human omental adipocytes. *Diabetes* **48**: 117–120.

Vrydag W, Michel MC (2007). Tools to study β_3-adrenoceptors. *Naunyn Schmiedebergs Arch Pharmacol* **374**: 385–398.

Walton J, Silver K, Bogardus C, Knowler WC, Celi FS, Austin S *et al*. (1995). Time of onset of non-insulin-dependent diabetes mellitus and genetic variation in the β_3-adrenergic receptor gene. *New Engl J Med* **333**: 343–347.