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SPECIAL REPORT

Ser^{203} as well as Ser^{204} and Ser^{207} in fifth transmembrane domain of the human β_2-adrenoceptor contributes to agonist binding and receptor activation

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We examined the contribution of Ser^{203} of the human β_2-adrenoceptor (β_2-AR) to the interaction with isoprenaline. The affinity of (−)-isoprenaline was reduced by substitution of an alanine for Ser^{203}, as well as for Ser^{204} and Ser^{207}. An (−)-isoprenaline derivative with only one hydroxyl group, at the *meta*-position, showed reduced affinity for wild-type β_2-AR and S207A-β_2-AR and even lower affinities for S203A-β_2-AR and S204A-β_2-AR. By contrast, an (−)-isoprenaline derivative with only a *para*-hydroxyl group showed reduced affinity for wild-type β_2-AR but the serine to alanine mutations did not cause further decreases. The EC_{50} value for cyclic AMP generation in response to (−)-isoprenaline was increased, by about 120 fold, for each alanine-substituted β_2-AR mutant. These results suggest that Ser^{203} of the human β_2-AR is important for both ligand binding and receptor activation.

Keywords: β_2-adrenoceptor; catechol hydroxyl group; (−)-isoprenaline; serine residue; Ser^{203}

Abbreviation: AR, adrenoceptor

Introduction The agonist and antagonist binding domains of the human β_2-adrenoceptor (β_2-AR) are thought to reside within the transmembrane domains (Dixon *et al*., 1987). It has been suggested that the protonated amines of β_2-AR agonists and antagonists interact with Asp^{113} in the third transmembrane domain of the receptor (Strader *et al*., 1987). Strader *et al*. (1989) reported that Ser^{204} and Ser^{207} in the fifth transmembrane domain of β_2-AR serve as hydrogen bond donors or acceptors in the interaction with the catechol moiety of each agonist. They proposed that the *meta*- and *para*-hydroxyl groups of (−)-isoprenaline interact with Ser^{204} and Ser^{207}, respectively. However, there is another serine (Ser^{203}) in the fifth transmembrane domain. Strader *et al*. (1989) did not detect the expression in transfected cells of a β_2-AR mutant in which Ser^{203} was replaced by an alanine, so they suggested that Ser^{203} is necessary for proper folding of β_2-AR.

All catecholamine receptors contain two or three serines in the fifth transmembrane domain (Figure 1). It is thought that each of the hydroxyl groups of a catecholamine interacts with one of the receptor's serines in a manner similar to the interaction between Ser^{204} and Ser^{207} of human β_2-AR and isoprenaline. However, rat α_{1A}-AR lacks a serine at the position homologous to Ser^{204} of human β_2-AR. Hwa & Perez (1996) reported that Ser^{188} of rat α_{1A}-AR is homologous to Ser^{203} of human β_2-AR and that it interacts with the *meta*-hydroxyl group of adrenaline. The hamster α_{1B}-AR has three serines (Ser^{207}, Ser^{208} and Ser^{211}) and Cavalli *et al*. (1996) reported that a substitution mutation of Ser^{207}, which is homologous to Ser^{203} of human β_2-AR, decreases the affinity of agonists; they suggested that both Ser^{207} and Ser^{208} of hamster α_{1B}-AR interact with the hydroxyl group in the *meta*-position. The human D_1 dopamine receptor also has three serines, which are homologous to Ser^{203}, Ser^{204} and Ser^{207} of human β_2-AR, and each one is critical for ligand binding and receptor activation (Pollock *et al*., 1992). Similarly, the rat D_2 dopamine receptor has three serines (Ser^{193}, Ser^{194} and Ser^{197}) which are homologous to the serines of human β_2-AR, and all three are necessary for a maximal cyclic AMP response to a partial agonist (Cox *et al*., 1992). Together, these findings indicate that the serines of various catecholamine receptors that are homologous to Ser^{203} of human β_2-AR play important roles in ligand binding and receptor activation.

Here, we report the successful *in vitro* expression of a human β_2-AR mutant in which Ser^{203} was replaced by an alanine. We examined the interactions of this mutant with (−)-isoprenaline and its derivatives.

Methods The plasmid pBC-β_2, which encodes for the human β_2-AR, was kindly provided by Dr R.J. Lefkowitz of Duke University (NC, U.S.A.). The plasmid pEF-BOS was provided by Dr S. Nagata of Osaka University (Osaka, Japan) (Mizushima & Nagata 1990). (−)-Isoprenaline derivatives

rat	α_{1A}	183	GYVLF	**S**	**A**	LG	**S** FY 194
hamster	α_{1B}	202	FYALF	**S**	**S**	LG	**S** FY 213
human	α_{2A}	195	WYILS	**S**	**C**	IG	**S** FF 206
human	β_2	198	AYAIA	**S** (203)	**S** (204)	IV	**S** (207) FY 209
human	D_1	193	TYAIS	**S**	**S**	VI	**S** FY 204
rat	D_2	188	AFVVY	**S**	**S**	IV	**S** FY 199

Figure 1 Alignment of the fifth transmembrane domains of various catecholamine receptors.

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Table 1 Binding characteristics of the wild type, S203A-, S204A- and S207A-β_2-adrenoceptors

	[^{125}I]-CYP		K_i (μM)		
	B_{max} (pmol mg^{-1})	K_D (pM)	(−)-isoprenaline	meta-hydroxyl ISO	para-hydroxyl ISO
wild type	4.58±0.42	24.4±4.1	0.21±0.05	2.2±0.5*	3.4±0.2*
S203A-	1.17±0.07†	46.1±10.8	2.9±0.7	8.3±1.3*†	2.4±0.1
S204A-	2.89±0.34†	17.9±0.8	6.9±0.3	14.8±1.6*†	5.6±1.1
S207A-	2.62±0.11†	20.2±1.2	2.3±0.5	1.4±0.3	2.5±0.6

The wild-type and alanine-substituted mutants were expressed in COS-7 cells, and binding characteristics were determined. The dissociation constant (K_D) and maximal binding activity (B_{max}) of [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) and K_i values of (−)-isoprenaline and its derivatives with one hydroxyl group at *meta*- (*meta*-hydroxyl ISO) or *para*-positions (*para*-hydroxyl ISO) were calculated with the Prism software package. The data are shown as means±s.e.mean from 3–4 separate experiments. Statistical significance was assessed by Dunnett's test: *<0.01, different from (−)-isoprenaline; †<0.01, different from the wild-type β_2-adrenoceptor.

were synthesized at the Drug Discovery Research Laboratory by Tanabe Seiyaku (Saitama, Japan). [^{125}I]iodocyanopindolol ([^{125}I]CYP) (2200 Ci mmol^{-1}), [^{3}H]-adenine (24.0–27.0 Ci mmol^{-1}) and [^{14}C]-cAMP (20.0–50.1 mCi mmol^{-1}) were purchased from DuPont-New England Nuclear Research (Boston, MA, U.S.A.). Lipofectamine and cell culture reagents were from Life Technologies, Inc. (Rockville, MD, U.S.A.).

The construct of the β_2-AR mutants with alanine substitutions for Ser^{204} or Ser^{207}, S204A-β_2-AR and S207A-β_2AR, has been described elsewhere (Kikkawa *et al*., 1997a). Alanine substitution of Ser^{203} was made by Quick change method according to manufacturer's instruction (Stratagene, La Jolla, CA, U.S.A.). S203A-β_2-AR was inserted into pEF-BOS due to low expression when the pCMV5 vector was used.

The transfection procedure, membrane preparation, receptor binding assays, and cyclic AMP accumulation assays were performed as described previously (Kikkawa *et al*., 1997b).

The results are expressed as the mean±s.e.mean for *n* determinations. Statistical significance was assessed by ANOVA for multiple comparisons. ANOVA *post hoc* comparisons were made using Dunnett's test.

Results First, we determined the maximal binding activity (B_{max}) and dissociation constant (K_D) for the wild-type and mutated β_2-ARs (Table 1). The expression levels of the mutants in transfected cells were significantly lower than that of the wild-type receptor, but were much higher than that of endogenous β-AR in COS-7 cells (~10 fmol mg^{-1}) and were sufficient to study the binding characteristics of the mutants. As the K_D values of the mutants for [^{125}I]-CYP were not significantly different from that of the wild-type receptor, we concluded that the substitution of alanine for Ser^{203}, Ser^{204} or Ser^{207} did not cause a global change in the β_2-AR structure.

The affinity of (−)-isoprenaline for β_2-AR was reduced 10–30 fold by the serine to alanine substitutions: by about 30 fold for S204A-β_2-AR, by about 10 fold for S207A-β_2-AR and by about 10 fold for S203A-β_2-AR. To examine the role of each hydroxyl group on the phenyl ring of the ligand in the interactions with the serine residues, (−)-isoprenaline derivatives were synthesized in which the *meta*- or *para*-hydroxyl group was changed to hydrogen. The affinities of these two derivatives for the wild-type receptor were about 10 fold lower than that of (−)-isoprenaline (Table 1). However, they showed at most 6 fold lower affinities for the alanine-substituted mutants than for the wild-type receptor. The affinity of the derivative with one hydroxyl group at the *meta*-position (*meta*-hydroxyl ISO in Table 1) for S203A-β_2-AR and S204A-β_2-AR, but not S207A-β_2-AR was significantly reduced compared to the wild-type receptor. By contrast, the affinity of the derivative with one hydroxyl group at the *para*-position (*para*-hydroxyl ISO in Table 1) was not significantly affected by the substitution of alanine for Ser^{203}, Ser^{204} or Ser^{207}.

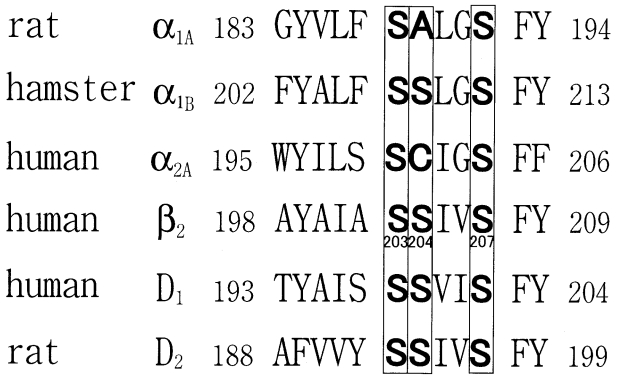

Figure 2 Effect of mutations of Ser^{203}, Ser^{204} or Ser^{207} of human β_2-AR on isoprenaline-stimulated cyclic AMP generation. The CHO cells were transfected with the wild-type, S203A-, S204A- or S207A-β_2-ARs, labelled with [^{3}H]-adenine, and stimulated with the indicated concentration of (−) - isoprenaline for 10 min in the presence of 1 mM isobutyl-1-methylxanthine. The EC_{50} values were calculated with the Prism software package. To compare EC_{50} values between the mutants, the maximal cyclic AMP generation of each experiment was set as 100%, and the other values were normalized to this value.

To examine the functional role of the serine residue at position 203 in the activation of β_2-AR by (−)-isoprenaline, agonist-stimulated cyclic AMP generation was determined in CHO cells. Stimulation of each receptor with (−)-isoprenaline increased the intracellular cyclic AMP content (Figure 2). The EC_{50} values were 7.92±2.71 nM for the wild-type receptor ($n=5$), 361±134 nM for S203A-β_2-AR ($n=5$, $P<0.5$ vs wild-type receptor),1010±120 nM for S204A-β_2-AR ($n=3$, $P<0.01$ vs wild-type receptor) and 430±90 nM for S207A-β_2-AR ($n=4$, $P<0.05$ vs wild-type receptor). These data indicate that Ser^{203} plays a role not only in ligand binding but also in receptor activation.

Discussion All catecholamine receptors contain two or three serine residues in the fifth transmembrane domain. It has been reported that the serine residues of these receptors that are homologous to Ser^{203} of β_2-AR play different roles in ligand binding and receptor activation. Rat α_{1A}-AR has only two serine residues (Ser^{188} and Ser^{192}), which are homologous to Ser^{203} and Ser^{207} of human β_2-AR, and they interact with the *meta*- and *para*-hydroxyl groups of adrenaline (Hwa & Perez, 1996). In addition, Ser^{188} of rat α_{1A}-AR is essential for receptor activation. Human α_{2A}-AR also has only two serine residues (Ser^{200} and Ser^{204}), which are homologous to Ser^{203} and Ser^{207}

of human β_2-AR; a mutation of either Ser^{200} or Ser^{204} decreases both adrenaline binding and the potency with which adrenaline inhibits cyclic AMP generation (Wang *et al*., 1991). However, Wang *et al*. (1991) suggested that Ser^{200} of α_{2A}-AR does not directly participate in adrenaline binding and receptor activation, because mutation of Ser^{200} did not modify the magnitude of inhibition of cyclic AMP generation by adrenaline derivatives with only one hydroxyl group at either the *meta*-position (phenylephrine) or *para*-position (synephrine). These results suggest that the interactions between the hydroxyl groups of adrenaline and the receptor differs between adrenoceptors.

In the present study substitution of an alanine for either Ser^{203} or Ser^{204} significantly decreased the affinity of the (−)-isoprenaline derivative with one hydroxyl group at the *meta*-position for β_2-AR, suggesting that the *meta*-hydroxyl group of (−)-isoprenaline interacts with both Ser^{203} and Ser^{204}. Our functional analysis of the S203A-, S204A- and S207A-β_2-AR mutants indicated that Ser^{203}, as well as Ser^{204} and Ser^{207}, plays an important role not only in ligand binding but also in receptor activation. Cavalli *et al*. (1996) reported that hamster α_{1B}-AR has three serine residues in the fifth transmembrane domain, as does human β_2-AR, and proposed that Ser^{207} of hamster α_{1B}-AR fulfils two roles by forming hydrogen bonds: it is a donor for the *meta*-hydroxyl group and is an acceptor for the *para*-hydroxyl group. Therefore, the simplest explanation of our data is that the *meta*-hydroxyl group of (−)-isoprenaline interacts with both Ser^{203} and Ser^{204} of human β_2-AR in a manner that is similar to the interaction between Ser^{207} of hamster α_{1B}-AR and the *meta*-hydroxyl group.

In conclusion, we have shown that Ser^{203}, as well as Ser^{204} and Ser^{207}, in the fifth transmembrane domain of β_2-AR is involved in ligand binding and receptor activation. However, the precise interactions between each of the serine residues and the hydroxyl groups of (−)-isoprenaline remain to be determined by further analysis of mutant receptors.

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