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Differential contribution of two serine residues of wild type and constitutively active β_2-adrenoceptors to the interaction with β_2-selective agonists

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1 We have studied the difference in receptor binding activity between partial and full β_2-adrenoceptor agonists and the abilities of the agonists to interact with Ser^{204} and Ser^{207} in the fifth transmembrane region of the β_2-adrenoceptor, amino acid residues that are important for activation of the β_2-adrenoceptor.

2 In the binding study with [^{125}I]-iodocyanopindolol, the K_i values of (±)-salbutamol, (±)-salmeterol, TA-2005 and (−)-isoprenaline for the β_2-adrenoceptor expressed in COS-7 cell membranes were 3340, 21.0, 12.0 and 904 nM, respectively. The β_1/β_2 selectivity of these agonists was in the order of (±)-salmeterol (332 fold) > TA-2005 (52.8) > (±)-salbutamol (6.8) > (−)-isoprenaline (1.1), and the β_3/β_2-adrenoceptor selectivity of these agonists was in the order of TA-2005 (150 fold) > (±)-salmeterol (88.6) > (±)-salbutamol (10.4) > (−)-isoprenaline (3.2).

3 The maximal activation of adenylyl cyclase by stimulation of the β_1-, β_2- and β_3-adrenoceptors by TA-2005 was 32, 100 and 100% of that by (−)-isoprenaline, respectively, indicating that TA-2005 is a full agonist at the β_2- and β_3-adrenoceptors and a partial agonist at the β_1-adrenoceptor. (±)-Salbutamol and (±)-salmeterol were partial agonists at both β_1- (8% and 9% of (−)-isoprenaline) and β_2- (83% and 74% of (−)-isoprenaline) adrenoceptors.

4 The affinities of full agonists, TA-2005 and (−)-isoprenaline, were markedly decreased by substitution of Ala for Ser^{204} (S204A) of the β_2-adrenoceptor, whereas this substitution slightly reduced the affinities of partial agonists, (±)-salbutamol and (±)-salmeterol. Although the affinities of full agonists for the S207A-β_2-adrenoceptor were decreased, those of partial agonists for the S207A-β_2-adrenoceptor were essentially the same as for the wild type receptor.

5 The constitutively active mutant (L266S, L272A) of the β_2-adrenoceptor had an increased affinity for all four agonists. The affinities of full agonists were decreased by substitution of Ser^{204} of the constitutively active mutant, whereas the degree of decrease was smaller than that caused by the substitution of the wild type receptor. Although the affinities of (±)-salbutamol and (±)-salmeterol for the S207A-β_2-adrenoceptor were essentially the same as those for the wild type β_2-adrenoceptor, the affinities of (±)-salbutamol and (±)-salmeterol for the constitutively active β_2-adrenoceptor were decreased by substitution of Ser^{207}.

6 These results suggest that Ser^{204} and Ser^{207} of the wild type and constitutively active β_2-adrenoceptors differentially interacted with β_2-selective agonists.

Keywords: TA-2005; (±)-salbutamol; (±)-salmeterol; β_2-adrenoceptor; subtype selectivity; adenlyl cyclase activity; full agonist; partial agonist; constitutively active receptor

Introduction

The β-adrenoceptors are members of a superfamily of the G-protein coupled receptors with seven transmembrane topology. Agonist and antagonist binding domains are assumed to reside within the transmembrane regions (Dixon *et al*., 1987). The protonated amines of agonists and antagonists of the β-adrenoceptors interact with Asp^{113} in the third transmembrane region (Strader *et al*., 1987; 1989a). Molecular pharmacological studies have revealed that Ser^{204} and Ser^{207} in the fifth transmembrane region of the β_2-adrenoceptor serve as hydrogen bond donors for interaction with catecholamine agonists (Strader *et al*., 1989b). It was proposed that the *meta*- and *para*-hydroxyl groups of (−)-isoprenaline or catechol derivatives interact with Ser^{204} and Ser^{207}, respectively. These two serine residues are important for full activation of adenylyl cyclase by agonists. On the other hand, the intracellular loops connecting the transmembrane regions interact with, and activate G-proteins. The intracellular domains close to the transmembrane region are especially important for coupling with G-proteins. The importance of the carboxyl terminus of the third intracellular loop for coupling with G-proteins was further demonstrated by the observation that mutation of a single amino acid in the domain produced a constitutively active β_2-adrenoceptor (Samama *et al*., 1993). The constitutively active receptor is considered to mimic the activated state of the receptor.

Selective β_2-adrenoceptor agonists are clinically the most widely used and effective bronchodilators because they have low cardiac (β_1) side effects. (±)-Salbutamol (Cullum *et al*., 1969) and (±)-salmeterol (Bradshaw *et al*., 1987; Ball *et al*., 1991) are selective β_2-adrenoceptor agonists with a *para*-hydroxymethyl instead of *para*-hydroxy group and are partial agonists that do not fully activate the receptor upon binding (Lemoine *et al*., 1992; Johnson *et al*., 1993; McCrea & Hill, 1993). Recently, we have developed TA-2005 (8-hydroxy-5-[(1**R**)-1-hydroxy-2-[N-[(1**R**)-2-(p-methoxy-phenyl)-1-methylethyl] amino] ethyl] carbostyril hydrochloride, Figure 1) as a β_2-adrenoceptor agonist with a high potency and long duration of action (Kikkawa *et al*., 1994). Although TA-2005 does not

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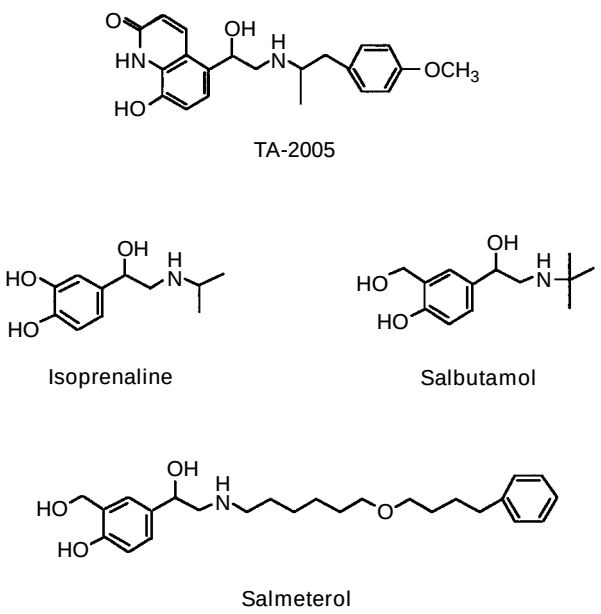

Figure 1 Chemical structures of TA-2005, (−)-isoprenaline, (±)-salbutamol and (±)-salmeterol.

have a catechol moiety, pharmacological studies have revealed that TA-2005 is a full β_2-adrenoceptor agonist in guinea-pig tissues (Kikkawa *et al*., 1991; Voss *et al*., 1992; 1994).

In the present study, we examined the interaction of full and partial β_2-selective agonists with Ser^{204} and Ser^{207} of the wild type and the constitutively active human β_2-adrenoceptors.

Methods

DNA constructions, cell transfection and culture

The plasmid constructs pBC-β_1 and β_2 encoding the entire human β_1- and β_2-adrenoceptors were donated by Dr R. J. Lefkowitz at Duke University. The first exon region of the human β_3-adrenoceptor was amplified from HeLa genomic DNA by polymerase chain reaction (PCR) and sequenced. The second exon region was added to it by PCR and ligated into a mammalian expression vector pEF-BOS (Mizushima & Nagata, 1990) as described by Sato *et al*. (1996). Preliminary experiments showed that the β_1-adrenoceptor with the epitope sequence (YPYDVPDYA) recognized by monoclonal antibody 12CA5 (Wilson *et al*., 1984) at the amino terminus was more stably expressed than the wild type receptor (the reason for this is not clear). Then the epitope sequence was inserted at the amino terminus of the human β_1- and β_2-adrenoceptors by PCR (VonZastrow & Kobilka, 1992; Barak *et al*., 1994; Sato *et al*., 1996). These epitope-tagged β_1- and β_2-adrenoceptors were expressed in COS-7 and CHO cells. The insertion of epitope did not change the binding characteristics of the β_2-adrenoceptors for the ligands examined in the present study (data not shown). The point-mutated receptors of the β_2-adrenoceptor were constructed by PCR with *Taq* or *Pfu* DNA polymerases (Higuchi, 1989).

The constitutively active β_2-adrenoceptor was produced by replacing Leu^{266} with Ser and Leu^{272} with Ala. Two serine residues (Ser^{204} and Ser^{207}) in the fifth transmembrane region of both wild type and constitutively active β_2-adrenoceptors were individually changed to an alanine residue. The identities of the sequences amplified by PCR were confirmed by the dideoxy chain termination method (Sanger *et al*., 1977), and the mutated region was replaced with the corresponding region of the epitope-tagged β_2-adrenoceptor. All constructs used in the present study except the β_3-adrenoceptor and the constitutively active wild type β_2-adrenoceptor had the epitope sequence. These constructs were transfected into either COS-7 cells by the DEAE-dextran method (transient expression) or Chinese hamster ovary (CHO) cells by the calcium phosphate precipitation procedure (stable expression) (Cullen, 1987). Cell lines that stably expressed the epitope-tagged β_1- and β_2-adrenoceptors were selected and maintained by addition of 1 mg ml^{-1} G-418. Expression of β-adrenoceptors was determined by radioligand binding assays with [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) as described below. The CHO cell lines expressing the receptors (220 fmol mg^{-1} protein for β_1-, 880 fmol mg^{-1} protein for β_2- and 140 fmol mg^{-1} protein for β_3-adrenoceptors, respectively) were used for this study. The levels of β_1-, β_2- and β_3-adrenoceptor expression in COS-7 cells were 14500, 4950 and 180 fmol mg^{-1}protein, respectively.

Membrane preparations

The cells were rinsed twice with 10 ml ice-cold PBS and mechanically detached in 1 ml ice-cold buffer containing 5 mM Tris HCl (pH 7.4) and 2 mM EDTA. The lysate was centrifuged at 45,000 × *g* for 10 min at 4°C and the crude membrane fraction was prepared in buffer containing 75 mM Tris HCl (pH 7.4), 12.5 mM $MgCl_2$ and 2 mM EDTA with a Potter type homogenizer and stored at −80°C until use. Protein concentration was determined by the method of Lowry *et al*. (1951).

Radioligand binding assay

Competition binding assays were performed in duplicate with ~10 μg membrane protein, 50 pM (β_1- and β_2-adrenoceptors) or 500 pM (β_3-adrenoceptors) [^{125}I]-CYP and 0 to 100 μM unlabelled ligand in the presence of 100 μM GTP. The mixture was incubated for 60 min at 37°C. The binding reaction was terminated by rapid filtration through Whatman GF/C filters and washed three times with a solution containing 25 mM Tris (pH 7.4) and 1 mM $MgCl_2$. Nonspecific binding was determined in the presence of 5 μM (±)-propranolol (β_1- and β_2-adrenoceptors) or 1 mM (−)-isoprenaline (β_3-adrenoceptors).

Adenylyl cyclase assay

Adenylyl cyclase activity was measured by the method of Johnson & Saloman (1991). Briefly, 20 μl membrane preparation was suspended in buffer containing (mM) HEPES (pH 7.4) 40, $MgCl_2$ 11.6, EDTA 0.8, ATP 0.12, GTP 0.05, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) 0.1, phosphoenolpyruvate 2.8; 1 μCi [α-^{32}P]-ATP, 0.2 unit pyruvate kinase and 1 unit myokinase in a final volume of 50 μl, for 30 min at 37°C. The reactions were terminated by addition of 1 ml ice-cold stop solution containing 0.5 mM ATP, 0.5 mM cyclic AMP and [^{3}H]-cyclic AMP (about 10,000 c.p.m.). Cyclic AMP formed was then isolated by sequential chromatography on Dowex cation exchange resin and aluminum oxide.

Data analysis and statistics

The results are expressed as arithmetic means together with s.e.mean for n determinations except the K_i and K_d values which are expressed as geometric means with 95% confidence limits (CL). Equilibrium dissociation constants were determined from saturation isotherms and competition curves. Radioligand binding data were analysed by nonlinear regression analysis to determine EC_{50} values and K_i values using PRISM software (GraphPAD Software Inc., San Diego, CA). Data of adenylyl cyclase activity were also analysed by a nonlinear curve-fitting technique with this software. Statistical significance was assessed by ANOVA for multiple comparisons; differences with a probability value of $P<0.05$ were considered significant.

Materials

[^{125}I]-iodocyanopindolol was obtained from Amersham International (Lille Chalfont, UK). The plasmid constructs pBC-β_1 and β_2 encoding the human β_1- and β_2-adrenoceptors were donated by Dr R. J. Lefkowitz at Duke University. The mammalian expression vector pEF-BOS was donated by Dr S. Nagata at Osaka University. (−)-Isoprenaline, (±)-propranolol, forskolin, and DEAE-dextran were purchased from Sigma Chemical Co. Ltd. (St. Louis, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), F-12 medium, G418 sulphate and gentamicin were from GIBCO BRL (Gaithersburg, U.S.A.). *Taq* and *Pfu* DNA polymerases were from Takara Shuzo Co. Ltd. (Ohtu, Japan) and Stratagene (La Jolla, U.S.A.), respectively. GTP was from Seikagaku Corporation (Tokyo, Japan). TA-2005 (8-hydroxy-5-[(1**R**)-1-hydroxy-2-[N-[(1**R**)-2-(p-methoxy-phenyl)-1-methylethyl] amino]ethyl] carbostyril, hydrochloride), (±)-salbutamol hemisulphate and (±)-salmeterol hydroxynaphthoate were synthesized at the Lead Optimization Research Laboratory, Tanabe Seiyaku (Saitama, Japan) for the present study.

Results

Affinity and selectivity of β_2-selective agonists for β-adrenoceptor subtypes in binding studies

We determined the affinities of TA-2005 for the β_1-, β_2- and β_3-adrenoceptors by use of the transient COS-7 cell expression system. The K_d values (95% CL) of [^{125}I]-CYP were 89.5 (64.2–125) pM ($n=3$), 55.6 (31.2–99.2) pM ($n=3$) and 201 (151–267) pM ($n=3$) for the β_1-, β_2- and β_3-adrenoceptors, respectively. The K_i value of (−)-isoprenaline for the β_2-adrenoceptor was essentially the same as that for the β_1-adrenoceptor and was slightly lower than that for the β_3-adrenoceptor (Table 1). The affinity of TA-2005 for the β_2-adrenoceptor was higher than that for the β_1-(52.8 fold) and β_3-adrenoceptors (150 fold). Table 1 also shows the high selectivity of (±)-salmeterol for the β_2-adrenoceptor. The ratio of K_i values of the β_1- to β_2-adrenoceptors and the β_3- to β_2-adrenoceptors was 332 and 88.6 for (±)-salmeterol, respectively. (±)-Salbutamol was less selective than TA-2005 and (±)-salmeterol (ratio of K_i values of the β_1- to β_2- and the β_3- to β_2-adrenoceptors was 6.8 and 10.4, respectively).

Stimulation of adenylyl cyclase activity by agonists

In membranes prepared from CHO cells stably expressing the β_1-, β_2- or β_3-adrenoceptors, all four agonists activated adenylyl cyclase in a concentration-dependent manner (Figure 2). The maximum level of (−)-isoprenaline-stimulated adenylyl cyclase activity was 10.3±0.7 ($n=3$), 73.5±2.1 ($n=4$) and 36.5±3.7 ($n=5$) pmol mg^{-1} min^{-1} for the β_1-, β_2- and β_3-adrenoceptors, respectively. Since forskolin activated adenylyl cyclase to a similar degree (126, 171, 192 pmol mg^{-1} min^{-1} for the β_1-, β_2- and β_3-adrenoceptors, respectively), the differences between (−)-isoprenaline-stimulated activities were intrinsic to the receptor subtypes. TA-2005 was a full agonist at the β_2-adrenoceptor and was the most potent of the four agonists (EC_{50} = 1.6 nM). However, the efficacy of TA-2005 was 32% and 100% of that of (−)-isoprenaline at the β_1- and β_3-adrenoceptors, respectively. Thus, TA-2005 was a partial agonist at the β_1-adrenoceptor and a full agonist at the β_3-adrenoceptor. The maximum adenylyl cyclase activities induced by β_2-adrenoceptor stimulation with (±)-salmeterol and (±)-salbutamol were significantly lower than that induced by (−)-isoprenaline (74% ($P<0.01$) and 83% ($P<0.05$) of (−)-isoprenaline, respectively). (±)-Salmeterol was a partial agonist at all three β-adrenoceptors, while (±)-salbutamol was a partial agonist at the β_1- and β_2-adrenoceptors but a full agonist at the β_3-adrenoceptor.

Affinity for Ala^{204}- and Ala^{207}-β-adrenoceptor mutants

(−)-Isoprenaline bound to the S204A- and S207A-β_2-adrenoceptors with 26.7 and 12.5 times higher K_i values than to the wild type β_2-adrenoceptor, respectively (Table 1). The K_i values of TA-2005 for the S204A- and S207A-β_2-adrenoceptors increased 55.9 and 3.7 fold, respectively. The change of affinity of TA-2005 for the S207A-β_2-adrenoceptor mutant was smaller than that for the S204A-β_2-adrenoceptor. There were small but significant increases in the K_i values of (±)-salbutamol (2.4 fold) and (±)-salmeterol (3.2 fold) for the S204A-β_2-adrenoceptor, but not for the S207A-β_2-adrenoceptor (Table 1).

All four agonists had 11.7 to 78.6 times higher affinity for a constitutively active (L266S, L272A) β_2-adrenoceptor than for the wild type β_2-adrenoceptor (Table 2). The affinities of the constitutively active S204A- and S207A-β_2-adrenoceptors for (−)-isoprenaline were decreased 17.7 fold and 13.5 fold, respectively. The affinity of TA-2005 for the constitutively active S204A-β_2-adrenoceptor was decreased less than that for the wild type S204A-β_2-adrenoceptor, but the affinity for the constitutively active S207A-β_2-adrenoceptor was decreased to a greater extent than that for the wild type S207A-β_2-adrenoceptor. The affinities of (±)-salbutamol and (±)-salmeterol for the constitutively active S204A-β_2-adrenoceptor were decreased 2.0 to 6.5 fold. Although substitution of Ser^{207} of the wild type β_2-adrenoceptor did not decrease the affinities of (±)-salbutamol and (±)-salmeterol, substitution of Ser^{207} of the constitutively active β_2-adrenoceptor decreased the affinities of these two agonists 2.8 to 3.7 fold. Thus, the interaction of salbutamol and salmeterol with Ser^{207} was more sensitive in the constitutively active β_2-adrenoceptor than the wild type β_2-adrenoceptor. Consequently, all the agonists including the partial agonists such as (±)-salbutamol and (±)-salmeterol had managed to interact with both Ser^{204} and Ser^{207} of the constitutively active β_2-adrenoceptors to a nearly equal degree (Figure 3).

Table 1 Binding affinity of β-adrenoceptor (AR) agonists in membrane preparations of COS-7 cells expressing human β_1-, β_2- or β_3-adrenoceptors or S204A- or S207A-β_2-adrenoceptors

	K_i (nM)				
β-Agonist	*β_2-AR*	*β_1-AR*	*β_3-AR*	*S204A-β_2-AR*	*S207A-β_2-AR*
TA-2005	12.0 (1.8–81.5)	633** (314–1280)	1800** (15.7–205000)	671** (23.4–19200)	44.6 (2.6–759)
Isoprenaline	904 (205–3980)	951 (629–1440)	2890 (543–15400)	24100* (717–810000)	11300* (667–191000)
Salbutamol	3340 (1190–9380)	22800** (14100–36800)	34700** (4060–297000)	8080* (4370–14900)	2580 (1820–3660)
Salmeterol	21.0 (5.6–78.7)	6970** (4190–11600)	1860** (109–31800)	66.2* (28.1–156)	13.8 (11.5–16.7)

Data are geometric means of 3 experiments with 95% CL in parentheses. *$P<0.05$, **$P<0.01$; significantly different from β_2-AR.

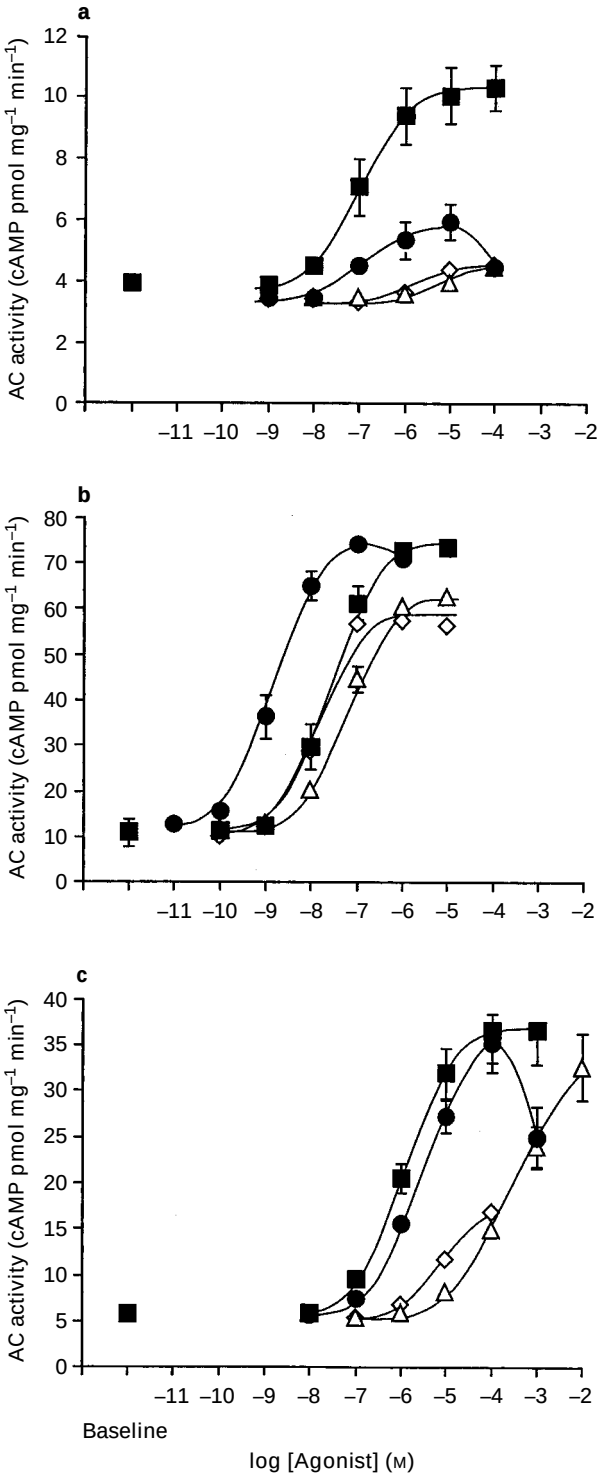

Figure 2 β_2-Adrenoceptor agonist-induced stimulation of adenylyl cyclase activity in CHO cells expressing β_1- (a), β_2- (b) or β_3- (c) adrenoceptors. The enzyme activity was measured in the absence and presence of increasing concentrations of (●) TA-2005, (■) isoprenaline, (△) salbutamol or (◇) salmeterol. Agonist-stimulated adenylyl cyclase (AC) activity is expressed as pmol of cyclic AMP produced min^{-1} mg^{-1} membrane protein. Each point represents mean ± s.e.m. of 3 to 5 duplicate experiments. Some s.e.m. bars are within the symbol.

Table 2 Binding affinity of β-adrenoceptor (AR) agonists in membrane preparations of COS-7 cells expressing constitutively active (CA)-, S204A-CA- or S207A-CA-β_2-adrenoceptors

	K_i (nM)		
β-Agonist	*CA-β$_2$-AR*	*S204A-CA-β$_2$*	*S207A-CA-β$_2$*
TA-2005	0.7 (0.2–1.8)	8.4** (3.5–20.0)	4.1** (2.4–7.0)
Isoprenaline	11.5 (5.5–23.8)	203** (115–358)	155** (95.3–253)
Salbutamol	254 (95.9–675)	1640** (1420–1910)	943** (472–1880)
Salmeterol	1.8 (0.7–4.8)	3.6 (0.4–33.6)	5.1 (1.4–18.5)

Data are geometric means of 3 experiments with 95% CL in parentheses. *$P<0.05$, **$P<0.01$; significantly different from CA-β_2-AR.

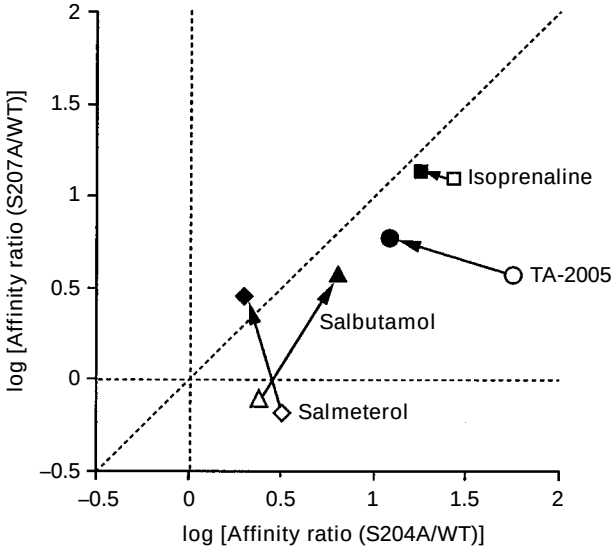

Figure 3 Change in affinity of agonists by S204A- or S207A-mutation in wild type (open symbols) and constitutively active (solid symbols) β_2-adrenoceptors.

Discussion

(−)-Isoprenaline is a non-selective full agonist at β_1, β_2- and β_3-adrenoceptors. TA-2005, (±)-salmeterol and (±)-salbutamol are β_2-adrenoceptor -selective agonists and showed different binding characteristics for the three β-adrenoceptor subtypes. Although the affinity of (±)-salmeterol for the β_2-adrenoceptor was lower than that of TA-2005, (±)-salmeterol bound most selectively to the β_2-adrenoceptor based on the ratio of K_i values of β_2- to β_1- and β_2- to β_3-adrenoceptors. (±)-Salbutamol was less potent and selective for the β_2-adrenoceptor than TA-2005 and salmeterol. (±)-Salmeterol has a long lipophilic N-substituted side chain which is important for its long duration of action. Since the long lipophilic side chain extending from the amino group is different between (±)-salmeterol and (±)-salbutamol, it may contribute not only to the longer duration of action (Bradshaw *et al*., 1987; Coleman *et al*., 1990; Ball *et al*., 1991; Johnson *et al*., 1993; Anderson *et al*., 1994) but also to its higher selectivity for the β_2-adrenoceptor. Recently, it has been shown that (±)-salmeterol interacts with the fourth transmembrane region of the β_2-adrenoceptor (amino acid residues 149–158) which serves as the 'exosite' (Green *et al*., 1996). Green *et al*. also showed that a chimeric receptor, in which a part of the fourth transmembrane region of the β_2-adrenoceptor was replaced with the corresponding region of the β_1-adrenoceptor, lost the long duration of action of (±)-salmeterol. However, a chimeric receptor that did not have the 'exosite' still showed β_2-selectivity for (±)-salmeterol. This suggests that the domain that is important for β_2-selectivity is different from the 'exosite'.

TA-2005 was a full agonist at the β_2-adrenoceptor, whereas (±)-salbutamol and (±)-salmeterol were partial agonists. The selectivity and efficacy of these β_2-adrenoceptor agonists were

in agreement with previous studies in animal tissues (Kikkawa *et al*., 1991; Ball *et al*., 1991; Dougall *et al*., 1991; Lemoine *et al*., 1992) and human bronchial smooth muscle (Nials *et al*., 1993). Although the maximum increase in adenylyl cyclase activity induced by stimulation of the β_2-adrenoceptor by (±)-salmeterol (74% of (−)-isoprenaline) was slightly smaller than that by (±)-salbutamol (83% of (−)-isoprenaline) in the present study, the difference was not statistically significant. Dougall *et al*., (1991) and Ellis *et al*. (1995) have also obtained similar findings. On the other hand, McCrea & Hill (1993) have demonstrated that the maximum response to (±)-salmeterol was only 46% of that to (−)-isoprenaline for cyclic AMP accumulation in a neuronal cell line. Thus, the apparent efficacy varies depending on the cell line. Although TA-2005 fully activated adenylyl cyclase by stimulation of the β_2-adrenoceptor, it partially activated adenylyl cyclase by stimulation of the β_1-adrenoceptor (32% of (−)-isoprenaline). The present results are in good agreement with those obtained by Voss *et al*. (1994). TA-2005 was also a full agonist at the β_3-adrenoceptor. (±)-Salbutamol was a full agonist at the β_3-adrenoceptor but a partial agonist at the β_1- and β_2- adrenoceptors. (±)-Salmeterol was a partial agonist at the three β-adrenoceptor subtypes.

Mutagenesis experiments showed that the binding sites of (−)-isoprenaline on the β_2-adrenoceptor appear to reside in the transmembrane regions (Dixon *et al*., 1987; Dohlman *et al*., 1988). Aspartic acid at position 113 in the third transmembrane region of the β_2-adrenoceptor is assumed to undergo an ionic interaction with the amino group of (−)-isoprenaline (Strader *et al*., 1987; 1989a). It has been proposed that the interaction of *meta*- and *para*-hydroxyl groups of (−)-isoprenaline with the hydroxyl side chain of Ser^{204} and Ser^{207} in the fifth transmembrane region of the β_2-adrenoceptor, respectively, causes conformational changes of the receptor to promote coupling with the G_S protein. (Strader *et al*., 1989b). Strader *et al*. proposed the importance of Ser^{204} and Ser^{207} for activation of adenylyl cyclase based on the following evidence. First, β-adrenoceptor antagonists do not interact with Ser^{204} and Ser^{207}, because the affinities of antagonists for the S204A- and S207A-β_2-adrenoceptors were essentially the same as those for the wild type β_2-adrenoceptor. Second, (−)-isoprenaline-stimulated adenylyl cyclase activity was decreased to ~50% by the substitution of Ser^{204} or Ser^{207}. Third, structure-binding activity analysis demonstrated that maximum intrinsic activity required the presence of the catechol moiety. We found that substitution of Ser^{204} or Ser^{207} reduced the affinities of full agonists such as TA-2005 and (−)-isoprenaline in the present study. In contrast, the affinities of partial agonists such as (±)-salbutamol and (±)-salmeterol for the β_2-adrenoceptor were decreased by substitution of Ser^{204} but not Ser^{207}. These results suggest that Ser^{204} is a requisite for the binding of full ((−)-isoprenaline and TA-2005) and partial agonists ((±)-salbutamol and (±)-salmeterol) with the β_2-adrenoceptor, whereas Ser^{207} is more likely to affect the efficacy as Ser^{204} does. The interaction of (±)-salbutamol and (±)-salmeterol with Ser^{204} seems to be not as strong as that of full agonists, because the binding affinities of these partial agonists for the S204A-β_2-adrenoceptor were decreased less than those of full agonists.

Deletion mutagenesis studies showed that the third intracellular loop, which connects the fifth and sixth transmembrane regions, is important for coupling of the β_2-adrenoceptor with G_S protein (O'Dowd *et al*., 1988). Recent studies, which showed that single amino acid mutation at the carboxyl terminal domain of the third intracellular loop of the α_{1B}-adrenoceptor (Cotecchia *et al*., 1990; Kjelsberg *et al*., 1992), α_{2A}-adrenoceptor (Ren *et al*., 1993) and β_2-adrenoceptor (Samama *et al*., 1993) produced constitutively active receptors, support the importance of the third intracellular loop. Since the conformation of the constitutively active receptors is likely to be locked in the activated state, the constitutively active β_2-adrenoceptor shows increased affinity for full and partial agonists, but not antagonists, with an increased intrinsic activity for partial agonists (Samama *et al*., 1993). We observed an increase of affinity of all agonists by mutation of the carboxyl terminal end of the third intracellular loop, indicating that the mutated β_2-adrenoceptor was constitutively active. Then we examined the interaction of Ser^{204} and Ser^{207} of the constitutively active β_2-adrenoceptor with agonists. Mutation of Ser^{207}, as well as Ser^{204}, of the constitutively active β_2-adrenoceptor decreased the affinity of both full and partial agonists. Thus, (±)-salbutamol and (±)-salmeterol interacted with Ser^{207} of the constitutively active β_2 adrenoceptors, and all the full and partial agonists interacted nearly equally with both Ser^{204} and Ser^{207}. These findings might explain the increased intrinsic activities of partial agonists at the constitutively active β_2-adrenoceptor.

In conclusion, we have demonstrated that full agonists such as TA-2005 and (±)-isoprenaline strongly interacted with Ser^{204} and Ser^{207} of the wild type β_2-adrenoceptors, whereas partial agonists such as (±)-salbutamol and (±)-salmeterol interacted weakly with Ser^{204}. In contrast to the wild type β_2-adrenoceptor, all the agonists interacted nearly equally with Ser^{204} and Ser^{207} of the constitutively active β_2-adrenoceptor. These results suggest that the positions of Ser^{204} and Ser^{207} of the β_2-adrenoceptor in the resting (inactive) state are different from those in the stimulated (active) state.

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