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Binding and functional affinity of some newly synthesized phenethylamine and phenoxypropanolamine derivatives for their agonistic activity at recombinant human β_3-adrenoceptor

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Abstract

β_3-Adrenoceptor is the predominant β-adrenoceptor in adipocytes and has drawn much attention during the investigation for anti-obesity and antidiabetes therapeutics. Thirteen new compounds have been evaluated for their potencies and efficacies as β_3-adrenoceptor agonists on human β_3-adrenoceptor expressed in COS-7 and Chinese hamster ovary (CHO) cells using radioligand binding assay and cyclic AMP (cAMP) accumulation assay. Phenoxypropanolamine derivatives, SWR-0334NA (([E)-[4-[5-[(3-phenoxy-2-hydroxypropyl)amino]-2-pentene-3-yl] phenoxy]acetic acid sodium salt), SWR-0335SA ((E)-[4-[5-[(3-phenoxy-2-hydroxypropyl)amino]-2-pentene-3-yl] phenoxy] acetic acid ethanedioic acid), SWR-0342SA (*S*-(Z)-[4-[[1-[2-[(2-hydroxy-3-phenoxypropyl)]amino]ethyl]-1-propenyl]phenoxy] acetic acid ethanedioic acid), SWR-0348SA-SITA ((E)-[4-[5-[(3-phenoxy-2-hydroxypropyl)amino]-2-hexene-3-yl] phenoxy]acetic acid ethanedioic acid) and SWR-0361SA ((E)-N-methyl-[4-[5-[(3-phenoxy-2-hydroxypropyl)amino]-2-pentene-3-yl]phenoxy]acetoamide ethanedioic acid) showed higher agonistic activity for the β_3-adrenoceptor. Among the compounds tested, SWR-0334NA exhibited full agonist activity ($\%E_{max}$ = 100.26) despite its lower binding affinity (pK_i = 6.11). Compounds SWR-0338SA ((E)-[4-[5-[(2-phenyl-2-hydroxyethyl)amino]-2-pentene-3-yl]phenoxy]acetic acid ethanedioic acid), SWR-0339SA (*S*-(E)-[4-[5-[(3-phenoxy-2-hydroxypropyl)amino]-2-pentene-3-yl] phenoxy] acetic acid ethanedioic acid), SWR-0345HA ((E)-2-methyl-3-[4-[2-(2-phenyl-2-hydroxyethyl-amino)ethoxy] phenyl]-2-propenoic acid ethyl ester hydrochloride), SWR-0358SA ((E)-(2-methoxy-ethyl)-[4-[5-[(3-phenoxy-2-hydroxypropyl) amino]-2-pentene-3-yl]phenoxy]acetoamide ethanedioic acid) and SWR-0362SA ((E)-1-[[[4-[5-[(3-phenoxy-2-hydroxypropyl)amino]-2-pentene -3-yl]phenoxy]-acetyl]carbonyl]piperidine ethanedioic acid) had moderate agonistic activity and were phenethylamine and phenoxypropanolamine derivatives. Compounds SWR-0065HA ([4-[2-[3-[[(3,4-dihydro-4-oxo-[1,2,4]-triazino(4,5-a)indol)-lyl]oxy]-2-hydroxypropylamino]ethoxy]phenyl]acetic acid methyl ester hydrochloride), SWR-0098NA ((E)-[4-[3-[(2-phenyl-2-hydroxyethyl)amino]-1-butenyl] phenoxy]-acetic acid sodium salt) and SWR-0302HA ([4-[[4-[2-(3-chlorophenoxy-2-hydroxypropyl)amino]-E-2-butenyl]oxy]phenoxy]acetic acid hydrochloride) had very low binding affinity towards β_3-adrenoceptors and they did not induce cAMP accumulation. We concluded that compounds SWR-0334NA, SWR-0335SA, SWR-0342SA, SWR-0348SA-SITA and SWR-0361SA were potential agonists of human β_3-adrenoceptor. Further investigation on their selectivity towards β_3-adrenoceptor could be useful for the exploration of the physiological properties of the β_3-adrenoceptor.

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Introduction

The β-adrenoceptors belong to a large family of G-protein-coupled receptors (GPCRs) that are characterized by seven transmembrane helices. Following the classification of β-adrenoceptor into β_1- and β_2-subtypes (Lands et al 1967), a third member of this family was pharmacologically identified (Arch et al 1984) and was designated β_3-adrenoceptor. The subsequent cloning and characterization of the β_3-adrenoceptor from genomic libraries of several species including man (Emorine et al 1989), mouse

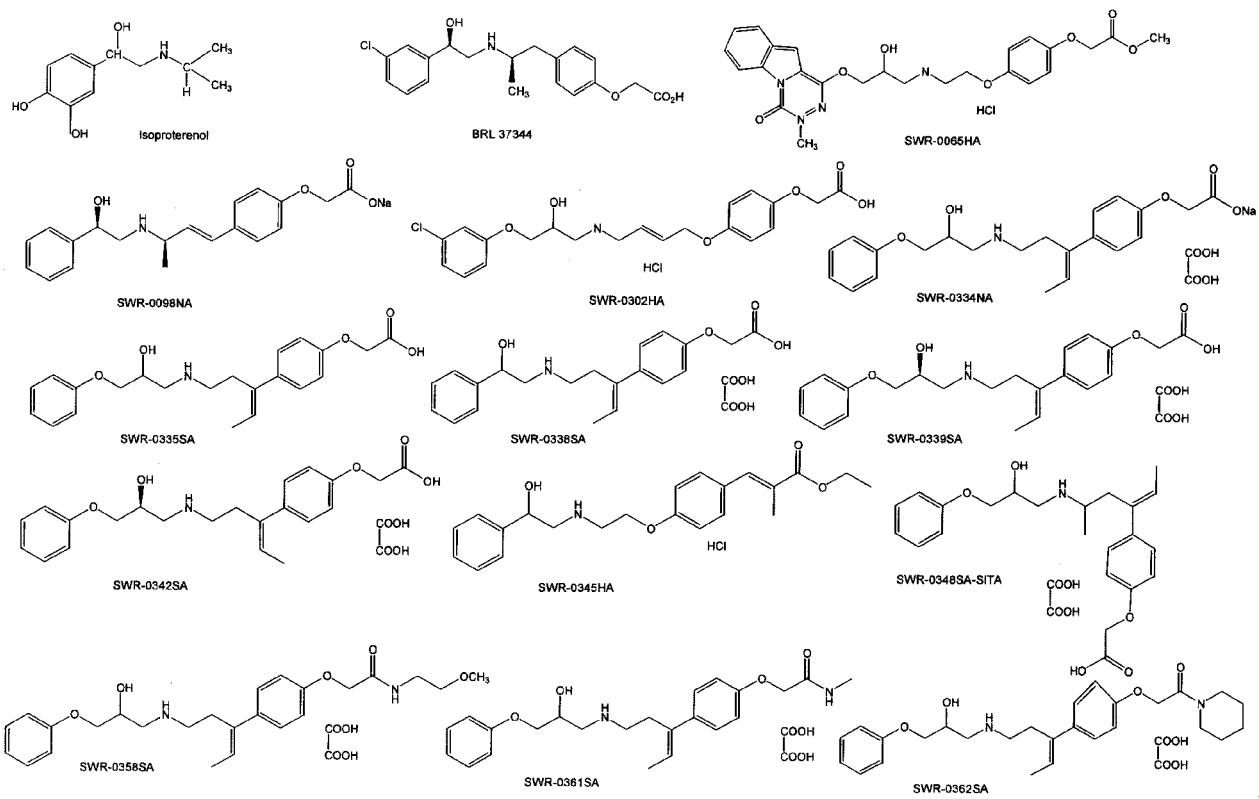

Figure 1 Structures of isoproterenol, BRL-37344 and the newly synthesized compounds.

(Nahamias et al 1991), and rat (Granneman et al 1991) provided conclusive evidence of its existence. Another β-subtype, β_4-adrenoceptor, was reported in the mammalian heart (Kaumann 1997). Granneman (2001) used the β_3-adrenoceptor agonist CGP-12177 to define the novel atypical β-adrenoceptor subtype, the putative β_4-adrenoceptor. β-Adrenergic stimulation activates plasma membrane adenylyl cyclase, resulting in an elevation of intracellular cyclic AMP, which mediates a number of hormone-induced responses. Human β_3-adrenoceptor and its mRNA have been found in the gastrointestinal tract, skeletal muscle and to a greater extent in adipose tissue. Several β_3-adrenoceptor agonists, such as CL-316,243 and BRL-26830A, reduced white adipose and brown adipose tissue mass, and decreased blood glucose and serum insulin levels in obese and diabetic mice (Yoshida et al 1990, 1991, 1994). Their reports indicated that β_3-adrenoceptor agonists could be useful for the treatment of obesity and/or type 2 diabetes. Potent and selective β_3-adrenoceptor agonists could be useful tools in the study of drug–receptor interaction and receptor-mediated physiological responses in tissues.

Several substituted phenethylamines and phenoxypropanolamines have been described that could potently activate the β_3-adrenoceptor. These agents may prove to be very effective in animal models of obesity and diabetes, due to their action on adipocyte β_3-adrenoceptors.

We have performed studies on some substituted phenethylamine and phenoxypropanolamine compounds to obtain potent β_3-adrenoceptor agonists. These experiments have focused upon characteristic pharmacological properties of human β_3-adrenoceptor subtypes, including ligand binding affinity and coupling to cAMP generation. We report on five compounds, SWR-0334NA, SWR-0335SA, SWR-0342SA, SWR-0348SA-SITA and SWR-0361SA (Figure 1), having potent β_3-adrenoceptor agonistic activity.

Materials and Methods

Materials

The SWR compounds were synthesized in the laboratory of Sawai Pharmaceutical Co., Ltd (Osaka, Japan). (−)-Isoproterenol hydrochloride, 3-isobutyl-1-methylxanthine (IBMX) and bovine serum albumin fraction V were purchased from Sigma (St Louis, MO). BRL-37344 [(±)-(*R*,*R*-[4-[2-[[2-3- chlorophenyl]-2-hydroxyethyl]amino]propyl]phenoxy)-acetic acid sodium] was purchased from SmithKline Beecham Pharmaceuticals (Epsom, UK). Ham's F-12 medium was purchased from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). Foetal bovine serum was purchased from Biowhittaker (Maryland). cAMP enzyme immunoassay kit was purchased from Shibayagi Co., Ltd (Gunma, Japan). [^{125}I]Iodocyanopindolol (1.85 TBq mmol^{-1}) was purchased from New England Nuclear Corp. (Tokyo, Japan). All other chemicals used were of the highest purity available.

Expression of β_3-adrenoceptors in COS-7 cells

β_3-Adrenoceptors were transiently expressed in COS-7 cells by the method described by Sato et al (1996). Briefly, human β_3-adrenoceptors in pEF-BOS were transfected into COS-7 cells using the DEAE-dextran method (Cullen 1987). The DNA sequence encoding the human β_3-adrenoceptor was amplified from the genomic DNA prepared from HeLa cells. Any error in the sequence was determined by the polymerase chain reaction (PCR) and was corrected thereafter. The resulting construct was inserted into the mammalian expression vector pEF-BOS (Mizushima & Nagata 1990). The transfected cells were grown as monolayers in 100-mm dishes containing Dulbecco's modified Eagle's medium supplemented with 5% foetal bovine serum and gentamicin (10 μg mL^{-1}) under an atmosphere of 95% air and 5% CO_2 at 37°C.

Membrane preparations from COS-7 cells

Forty-eight hours after the transfection, the COS-7 cells were rinsed with 10 mL ice-cold phosphate-buffered saline (PBS) and mechanically detached by ultrasonication in 5 mL lysis buffer (5 mM Tris-HCl (pH 7.4), 2 mM EDTA, 5 μg mL^{-1} soybean trypsin inhibitor, 5 μg mL^{-1} leupeptin and 10 μg mL^{-1} benzamidine). The COS-7 cells were homogenized using a glass homogenizer and centrifuged at 45000 g for 30 min at 4°C. The resultant membrane fractions were resuspended in lysis buffer and frozen at −80°C until use.

Radioligand binding assay

COS-7 cells were chosen for the binding experiment because of the high receptor expression levels commonly achieved in this system (Samama et al 1993). Radioligand binding studies were carried out in assay buffer containing 75 mM Tris-HCl (pH 7.4), 12.5 mM $MgCl_2$ and 2 mM EDTA at 37°C for 60 min using 5–10 μg membrane protein. The total reaction volume was 250 μL. For saturation isotherms, membranes were incubated with varying concentrations of [^{125}I]iodocyanopindolol (5–250 pM) in the absence (total binding) or presence (nonspecific binding) of 1 μM (±)-propranolol. Competition binding studies were carried out using 100 pM [^{125}I]iodocyanopindolol. The reactions were stopped by dilution with 4 mL cold wash buffer containing 25 mM Tris (pH 7.5) and 1 mM $MgCl_2$ and rapid filtration over Whatman GF/C glass fibre filters. The filters were washed with an additional ice-cold wash buffer (4 mL). The radioactivity remaining on the filters was counted by a γ-scintillation counter.

Expression of β_3-adrenoceptor in CHO cells

Standard cloning techniques were used for the expression of human β_3-adrenoceptor in CHO cells (Maniatis et al 1982). The human β_3-adrenoceptor cDNA were each obtained by reverse transcription and PCR amplification from human placenta mRNA in accordance with the method described by Granneman et al (1991). Each of the PCR products was cloned into expression vector pAP3 neo. The nucleotide sequences of each were verified with that of the human β_3-adrenoceptor cDNA sequences. CHO-K1 cells were transfected with each of the pAP3 neo containing the human β_3-adrenoceptor cDNA using the calcium-phosphate precipitation technique. Stable transfectants with human β_3-adrenoceptor (β_3-CHO cells) were obtained by culture of the cells in Ham's F-12 medium supplemented with penicillium (1000 U mL^{-1}), streptomycin (100 μg mL^{-1}), foetal bovine serum (10% v/v) and G418 (1 mg mL^{-1}).

Assay of cAMP accumulation

G-protein mediated agonist stimulation of adenylyl cyclase can be analysed in the CHO cell system as it does not express measurable levels of β-adrenergic receptor nor does it exhibit catecholamine-stimulated cAMP stimulation (Samama et al 1993). This approach is not feasible in COS-7 cells and therefore for the assay of adenylyl cyclase we used the CHO cell system.

Adenylyl cyclase stimulation was determined by the accumulation of cAMP according to the method of Blin et al (1994) with some modification. Briefly, CHO cells were grown to preconfluence in a 96-well microplate (approximately 5×10^4 cells/well at preconfluence). After washing with Ham's F-12 medium buffered with 20 mM HEPES (pH 7.4) supplemented with 1 mM ascorbic acid and 1 mM IBMX, cell monolayers were incubated for 30min at 37°C in 200 μL of the medium containing 10^{-12}–10^{-5} M SWR-compounds or (−)-isoproterenol. The incubation was terminated by washing once with ice-cold Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS(−)) and by immediate addition of 100 μL 1 M NaOH. After a period of 20 min at 37°C, the dissolved cells were neutralized with 100 μL 1 M acetic acid and centrifuged at 3000 g for 10 min at 4°C. The concentration of cAMP in the supernatant was measured by the cAMP enzyme immunoassay system using the double antibody method. The adenylyl cyclase stimulations to SWR-compounds were expressed as a percentage of the maximum response obtained with (−)-isoproterenol.

Protein assay

The protein content of the membrane concentration was measured by the method of Lowry et al (1951) using bovine serum albumin as the standard.

Data analysis

Results of the experiments are expressed as means ± s.e.m. The inhibition concentration (IC50) in displacement analysis was determined as the concentration of agonists that inhibited [^{125}I]iodocyanopindolol binding by 50%. The inhibition constant (K_i) was calculated by the equation of Cheng & Prusoff (1973) and expressed as pK_i ($-\log K_i$).

EC50 (or K_{act}) parameters (concentration of agonist required to elicit 50% of the maximum response for (−)-isoproterenol in particular clone cell) obtained from adenylyl cyclase activation were determined using computer software (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA). Student's paired *t*-test was performed to assess the significance of any difference. A P value of less than 0.05 was taken as significant.

Results

Newly synthesized compounds SWR-0065HA, SWR-0098NA, SWR-0302HA, SWR-0334NA, SWR-0335SA, SWR-0338SA, SWR-0339SA, SWR-0342SA, SWR-0345HA, SWR-0348SA-SITA, SWR-0358SA, SWR-0361SA, and SWR-0362SA were evaluated for their potency at the β_3-adrenoceptor. The structures of these compounds are given in Figure 1.

Radioligand binding assay

COS-7 cells were transfected with the cDNA of the human β_3-adrenoceptor. The membrane fraction was prepared and saturation binding experiments were performed using increasing concentrations of [^{125}I]iodocyanopindolol. Figure 2 shows representative experiments. Saturation binding was observed in membranes from transfected COS-7 cells. Linear Scatchard transformation is shown also, indicating one receptor population. The K_D and B_{max} values calculated from Scatchard plots (n = 6) were 0.35 ± 0.26 nM and 217.7 ± 114.6 fmol (mg protein)$^{-1}$, respectively, for the β_3-adrenoceptor.

Competition experiments with [^{125}I]iodocyanopindolol (100 pM) and the respective β-adrenoceptor agonists were performed to investigate the binding properties of commonly used β-adrenoceptor agonists and new compounds at homogeneous populations of recombinant human β_3-adrenoceptor. All competition curves fitted into a one-site model with the Hill slopes near unity. Figure 3A illustrates the displacement of [^{125}I]iodocyanopindolol binding from recombinant human β_3-adrenoceptor by BRL-37344, SWR-0065HA, SWR-0098NA, SWR-0302HA, SWR-0334NA, SWR-0335SA and SWR-0338SA. Figure 3B shows the displacement of [^{125}I]iodocyanopindolol binding from recombinant human β_3-adrenoceptor by SWR-0339SA, SWR-0342SA, SWR-0345HA, SWR-0348SA-SITA, SWR-0358SA, SWR-0361SA and SWR-0362SA. The pK_i values of these compounds and two reference compounds are given in Table 1. Compounds SWR-0334NA, SWR-0339SA, SWR-0342SA, SWR-0348SA-SITA, SWR-0358SA, SWR-0361SA and SWR-0362SA showed higher binding affinities for β_3-adrenoceptor, although their binding affinities were weaker than that of (−)-isoproterenol and BRL-37344. SWR-0342SA, being a selective β_3-adrenoceptor agonist (Kiso et al 1999) exhibited the highest affinity among the new compounds.

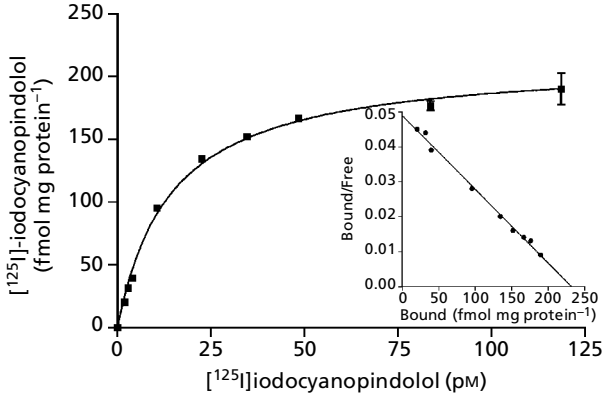

Figure 2 [^{125}I]Iodocyanopindolol saturation binding at recombinant human β_3-adrenoceptor and corresponding Scatchard transformation.

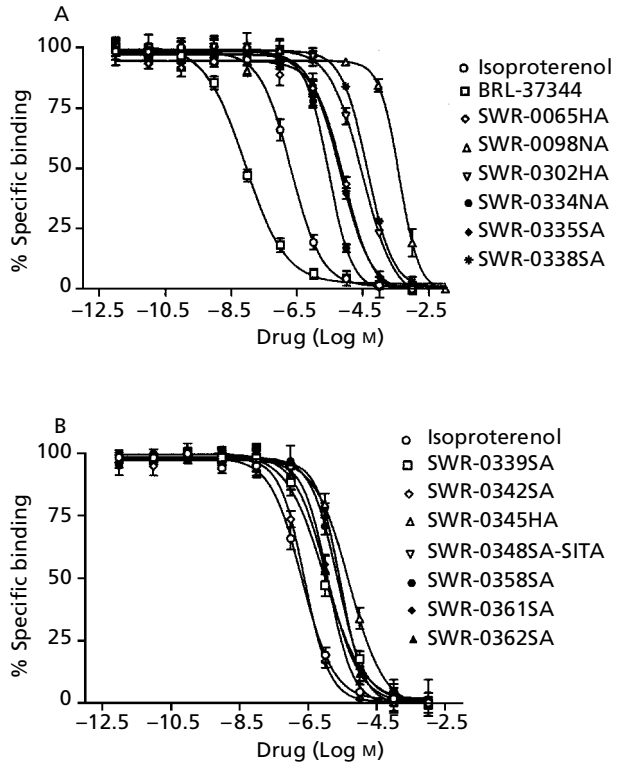

Figure 3 Competition of specific [^{125}I]iodocyanopindolol binding by (A) isoproterenol, BRL-37344 and compounds SWR-0065HA, SWR-0098NA, SWR-0302HA, SWR-0334NA, SWR-0335SA and SWR-0338SA, and (B) SWR-0339SA, SWR-0342SA, SWR-0345HA, SWR-0348SA-SITA, SWR-0358SA, SWR-0361SA and SWR-0362SA, at human recombinant β_3-adrenoceptor COS-7 cells.

cAMP accumulation assays

Accumulation of cAMP in human recombinant β_3-adrenoceptor CHO cells was examined as functional responses to β_3-adrenoceptor agonists. (−)-Isoproterenol increased the

Table 1 Inhibition constants ($-\log K_i$ or pK_i) for [^{125}I]iodocyanopindolol binding in COS-7 cells, maximum cAMP accumulation at 10^{-5} M of the compounds and functional activity constants ($-\log$ EC50 or pK_{act}) for cAMP accumulation in CHO cells expressing human β_3-adrenoceptors.

Compound	pK_i	pK_{act}	cAMP concn (pmol/10^5 cells)	% E_{max}
Isoproterenol	7.26±0.23 (4)	8.30±0.23 (3)	32.70±1.5	100.00±2.1
BRL-37344	8.65±1.28 (4)	7.94±0.19*	ND	ND
SWR-0065HA	5.65±0.15 (5)	ND	0.08±0.6	0.23±4.12
SWR-0098NA	3.97±0.81 (5)	ND	4.95±1.9	15.15±2.18
SWR-0302HA	5.09±0.45 (5)	ND	0.79±0.9	2.41±0.37
SWR-0334NA	6.11±0.37 (4)	7.65±0.81 (3)	32.79±5.4	100.26±8.74
SWR-0335SA	5.76±0.3 (3)	7.60±0.64 (3)	31.77±4.2	97.16±10.41
SWR-0338SA	4.94±0.67 (4)	5.92±0.62 (3)	25.16±4.5	76.95±3.57
SWR-0339SA	6.59±0.14 (4)	7.28±0.46 (3)	23.16±3.4	70.82±1.51
SWR-0342SA	7.18±0.52 (4)	8.20±0.06 (3)	30.80±4.7	94.2±1.4
SWR-0345HA	5.88±0.73 (3)	6.24±0.30 (3)	27.29±2.1	83.47±4.12
SWR-0348SA-SITA	6.14±0.14 (4)	6.20±0.19 (3)	30.53±3.6	93.36±17.28
SWR-0358SA	6.23±0.3 (4)	6.63±0.14 (3)	25.07±2.9	76.66±2.47
SWR-0361SA	6.49±0.28 (5)	6.73±0.15 (3)	29.76±3.0	91.0±2.33
SWR-0362SA	6.52±0.32 (5)	6.15±0.24 (3)	28.03±2.8	85.71±2.54

Values are means±s.e.m. with the number of experiments indicated in parentheses. % E_{max} is the maximum cAMP accumulation stimulated by the compounds relative to that of isoproterenol×100. ND, not determined. *Value obtained from the literature by Mehta et al (2000).

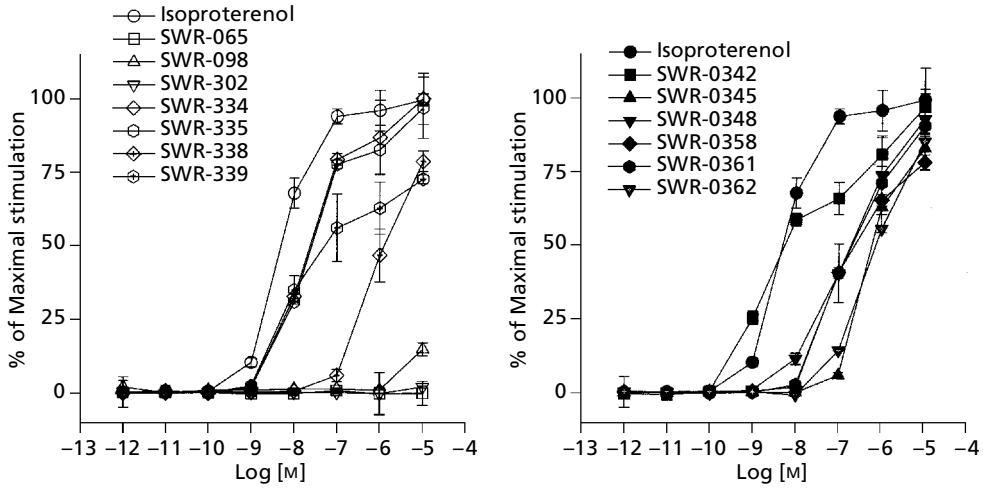

Figure 4 The cAMP accumulation in β_3-adrenoceptor CHO cells exposed to the newly synthesized compounds.

amount of cAMP in the cloned cells in a concentration-dependent manner, giving mean EC50 values of 5.01 nM (n = 3). The increases in cAMP by (−)-isoproterenol were normalized as 100% when the % E_{max} of the β-adrenoceptor agonists was calculated. The concentration–response curves for β-adrenoceptor agonists are shown in Figure 4. The maximal cAMP accumulation responses to the compounds and the negative logarithm of the concentrations in 50% of maximal cAMP accumulation of (−)-isoproterenol ($-\log$ EC50 or pK_{act}) for each agonist are presented in Table 1. The maximal responses to compounds SWR-0334NA (32.79 pmol), SWR-0335SA (31.77 pmol), SWR-0342SA (30.8 pmol), SWR-0348SA-SITA (30.53 pmol) and SWR-0361SA (29.76 pmol) were obtained at 10^{-5} M and they showed more than 90% of the response to isoproterenol and thus can be called full agonists. Others had lower than 90% activity of isoproterenol and were termed partial agonists. Compounds SWR-0065HA, SWR-0098NA, SWR-0302HA were without any functional activity.

Discussion

The development of various compounds that have high activities for human β_3-adrenoceptor is still underway. We constructed cells which transiently express human β_3-adrenoceptor and evaluated some newly synthesized β_3-adrenoceptor agonists.

We examined the displacement potencies of β_3-adrenoceptor agonists for [^{125}I]iodocyanopindolol binding to β_3-adrenoceptor in cloned COS-7 cells. BRL-37344 had the highest affinity for the β_3-adrenoceptor. The newly synthesized compounds such as SWR-0334NA, SWR-0339SA, SWR-0342SA, SWR-0348SA-SITA, SWR-0358SA, SWR-0361SA and SWR-0362SA had high affinities also for this β-adrenoceptor subtype, although their affinities were lower compared with (−)-isoproterenol or BRL-37344.

We examined the functional activity of cAMP accumulation in CHO cells transfected with human β_3-adrenoceptor. The maximum cAMP accumulation by (−)-isoproterenol was taken as 100%. Compounds SWR-0334NA, SWR-0335SA, SWR-0339SA and SWR-0342SA increased the cAMP accumulation in human β_3-adrenoceptor expressed in CHO cells, although the increase was relatively lower than that of (−)-isoproterenol.

In a few instances, the pK_i values and pK_{act} values were very similar but in most cases there were differences that accounted for the maximal activation of adenylyl cyclase by the β-adrenoceptor subtype when relatively few receptors were occupied. Granneman (1995) and Wilson et al (1996) reported that the high efficiency of β_3-adrenoceptor coupling to cAMP generation, indicated by a large discrepancy between K_i and EC50, occurred at various levels of receptor expression and was present in cells that natively expressed the receptor. It implied that binding with few sites on the receptor might promote cAMP accumulation to a large extent. SWR-0334NA had lower binding affinity for β_3-adrenoceptor but it increased cAMP accumulation to virtually that of (−)-isoproterenol and could be considered as a potential agonist for β_3-adrenoceptor.

The results indicated that compounds SWR-0334NA, SWR-0335SA, SWR-0342SA, SWR-0348SA-SITA and SWR-0361SA had comparatively higher potency and efficacy towards β_3-adrenoceptor and that SWR-0334NA could be a potential agonist for the investigation of β_3-adrenoceptor properties.

Conclusions

Using the radioligand binding assay and the cAMP accumulation assay, we have shown that compounds SWR-0334NA, SWR-0335SA, SWR-0342SA, SWR-0348SA-SITA and SWR-0361SA have the potential to be β_3-adrenoceptor agonists. Compound SWR-0334NA was found to be a highly potent β_3-adrenoceptor agonist, with a $\%E_{max}$ value of 100.26 relative to (−)-isoproterenol. Further investigation on their selectivity towards β_3-adrenoceptor could be useful for defining the physiological roles of β_3-adrenoceptor.

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