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Selectivity and Potency of Agonists for the Three Subtypes of Cloned Human β-Adrenoceptors Expressed in Chinese Hamster Ovary Cells

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Yanagisawa, T., Sato, T., Yamada, H., Sukegawa, J. and Nunoki, K. *Selectivity and Potency of Agonists for the Three Subtypes of Cloned Human β-Adrenoceptors Expressed in Chinese Hamster Ovary Cells.* Tohoku J. Exp. Med., 2000, **192** (3), 181-193 —— The selectivities, potencies and efficacies of β_3-adrenoceptor (β_3-AR) agonists on human three β-AR subtypes expressed in Chinese hamster ovary (CHO) cells were investigated using radioligand binding assay and cyclic AMP (cAMP) accumulation assay. The three β-AR subtypes showed the nature of G protein-coupled receptors with the constitutive activity. BRL37344, CL-316,243 and a newly synthesized β_3-AR agonist N-5984, 6-[2-(R)-[[2-(R)-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-2, 3-dihydro-1, 4-benzodioxine-2-(R)-carboxylic acid, were compared for the potency and selectivity for the β_3-AR. In the radioligand binding assay, the affinity of N-5984 for β_3-ARs was 14, 70 and 220 times more potent than those of BRL37344, isoproterenol and CL-316, 243, respectively. N-5984 had higher selectivity than BRL37344 for human β_3-ARs compared with either for β_1-ARs or β_2-ARs. N-5984 showed higher potency and intrinsic activity of cAMP production than BRL37344 in CHO cells expressing the β_3-ARs. CL-316, 243 had almost no activity of cAMP production in CHO cells expressing any subtype of β-ARs. These results indicate that N-5984 is the most potent and selective agonist for human β_3-ARs than any other agonists tested. ———— cloned human β_3-adrenoceptor; constitutive activity; isoproterenol; BRL37344; N-5984

Although β-adrenoceptors (β-ARs) have been originally classified into β_1- and β_2-subtypes (Lands et al. 1967), atypical β-AR responses were reported in the gastrointestinal smooth muscles and adipocytes (Arch and Kaumann 1993).

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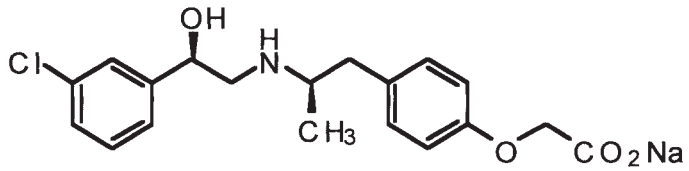

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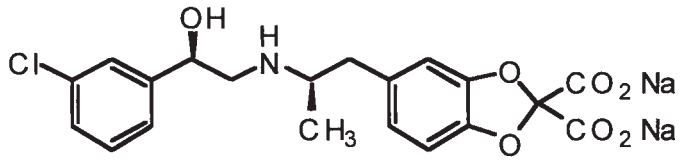

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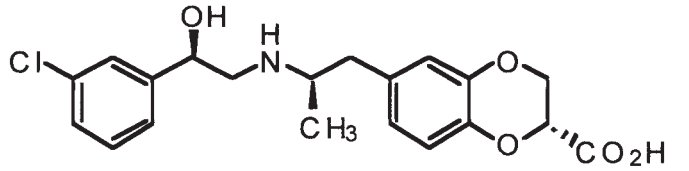

N-5984

Fig. 1. The chemical structures of three β_3-AR agonists.

Human β_3-ARs cDNA was subsequently cloned and sequenced (Emorine et al. 1989). β_3-ARs are selectively expressed in adipose tissues. β_3-AR agonists such as BRL37344 and CL-316, 243 are potent stimulants of lipolysis and thermogenesis, and they increase insulin sensitivity and improve glucose tolerance in animals (Howe 1993; Yoshida et al. 1994; Dow 1997; Weyer et al. 1998, 1999). These lines of evidence suggest that β_3-AR agonists are expected to be promising drugs for the treatment of obesity and diabetes mellitus. However, almost all of the early β_3-AR agonists have been abandoned after clinical trials due to either the lack of sufficient efficacy in human or the adverse effects. One of the reasons of the failure might be the species differences of β_3-ARs between experimental animals and human (Liggett 1992). The development of the newly synthesized β_3-AR agonists has depended on β_3-ARs derived from non-human origins. Another might be the low selectivity for β_3-ARs against β_1- or β_2-ARs, which caused adverse effects such as tachycardia, arrhythmia and tremor. Therefore, it seems important to obtain the information on the affinity, selectivity and agonist efficacy for human β_3-ARs of the compound to be developed for clinical use.

In this report, we describe the construction of the assay system of β_3-AR agonists in which either one of the cloned human β_1-, β_2- or β_3-ARs is expressed in Chinese hamster ovary (CHO) cells, and the evaluation of the affinity, selectivity and efficacy of β_3-AR agonists, BRL37344, CL-316, 243 and N-5984, a newly synthesized β_3-AR agonist, (Fig. 1) using radioligand binding assays and cAMP accumulation assays.

Materials and Methods

DNA constructions and cell culture

The human β_1-AR gene in pTZ18 and the β_2-AR gene in pBC12BI were kind gifts from Dr. Lefkowitz at Duke University Medical Center (Durham, NC, USA). The human β_3-AR gene in pTZ18 was given by Drs. Nagao and Kurose at the University of Tokyo.

Each β-AR gene was inserted into the expression vector pcDNA3.1 (+) (Invitrogen, Groringen, the Netherlands). Each of EcoRI-BamHI fragments of the β_1- and β_3-AR genes was inserted between the NheI and BamHI cloning sites of pcDNA3.1 (+) after the EcoRI sites of the β-AR genes and the NheI restriction site of the vector were filled in with the Klenow fragment of DNA polymerase. The β_2-AR gene was excised from the vector as a NcoI-DraI fragment and the NcoI site was filled in with the Klenow fragment. This fragment was then inserted into the DraI-NheI sites of pcDNA3.1 (+) in which the NheI site was also filled in with the Klenow fragment.

Expression construct of either β_1-, β_2- or β_3-AR gene was introduced into Chinese hamster ovary (CHO) cells by the calcium phosphate precipitation procedure (Graham and van der Eb 1973). The transfected cells were grown to subconfluency in Ham's F-12 nutrient mixture supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B, and 400 μg/ml of geneticin at 37°C. Stable transformants were selected by using [^{125}I]-iodocyanopindolol binding assay and cAMP accumulation assay as described below.

Membrane preparations

The cells of three 75 cm^2 flasks were washed with Dulbecco's phosphate buffer and detached with the buffer containing 0.02% EDTA. The cells were suspended in 5 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA, 5 mg/liter soybean trypsin inhibitor, 5 mg/liter leupeptin and 10 mg/liter benzamidine, and homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland). After removal of nuclei by centrifugation at 500×g for 5 minutes, membrane fraction was centrifuged at 45 000×g for 30 minutes at 4°C. The pellet was suspended in 75 mM Tris-HCl (pH 7.4) containing 12.5 mM $MgCl_2$, 2 mM EDTA, 5 mg/liter soybean trypsin inhibitor, 5 mg/liter leupeptin and 10 mg/liter benzamidine by sonication and stored at −80°C until use. Protein concentration was determined by use of BCA Protein Assay kit (Pierce, Rockford, IL, USA).

Radioligand binding assay

Radioligand binding assays were carried out as described previously (Blin et al. 1993). Aliquotes of membrane preparations (10–20 μg of protein) resuspended in buffer B (50 mM Tris-HCl pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA, 1 mM ascorbic

acid) were incubated for 60 minutes with [^{125}I]-iodocyanopindolol (ICYP) in a final volume of 1.0 ml at 25°C for β_1-AR or 37°C for β_2- and β_3-ARs. For saturation binding assays on β_1-, β_2- and β_3-ARs, concentrations of [^{125}I]-ICYP varied 15–4000, 15–4000 and 31.25–6000 pM, respectively. For competition binding assays, membranes were incubated with [^{125}I]-ICYP at the concentrations of approximate K_d and the various concentrations of competing ligands. Nonspecific binding was determined in the presence of 10 μM (−)-propranolol for the β_1-AR, 5 μM (−)-alprenolol for the β_2-AR and 5 μM unlabeled (−)-cyanopindolol for the β_3-AR. The reactions were terminated by adding 4 ml of cold buffer B and rapid filtration over Whatman GF/C filters. The radioactivity on the filters was determined using a γ scintillation counter (Aloka, Tokyo). Scatchard analysis was performed to calculate K_d and B_{max}. K_i values of drugs were calculated from the Cheng and Prusoff expression as follows: $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is radioligand concentration.

Cyclic AMP (cAMP) accumulation assays

cAMP accumulation in CHO cells expressing human β-ARs was determined by the method of Blin et al. (1993). Cells grown to confluence in 24-well plates were washed with Hank's balanced salt solution (HBSS) containing 20 mM HEPES. Cells were incubated in HBSS containing 1 mM ascorbic acid, 0.1 mM isobutyl-methylxanthine and the various concentrations of agonists for 20 minutes at 37°C. The reaction was stopped by removing the reaction solution and washing with ice-cold Dulbecco's phoshphate buffer. The cAMP generated in the cells was extracted with 5 mM Tris-HCl (pH 7.4) containing 2 mM EDTA. The extract was assayed for cAMP using Amersham cAMP assay kit.

Chemicals and β_3-AR agonists

All chemicals used were reagent grade: EDTA, soybean trypsin inhibitor, leupeptin, benzamidine and ascorbic acid were from Wako Pure Chemical (Osaka), Tris from Nakalai tesque (Kyoto). (−)-Isoproterenol hydrochloride, isobutyl-methylxanthine and BRL37344 sodium, (R*, S*)-(±)-4-[[2-[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl] phenoxyacetic acid were from Sigma (St. Louis, MO, USA). CL-316, 243 disodium, (R, R)-5-[2-[2-(3-chlorophenyl)-2-hydroxyethyl]amino]-propyl-1, 3-benzodioxol-2, 2-dicarboxylate and N-5984, 6-[2-(R)-[(2-(R)-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]-2, 3-dihydro-1, 4-benzodioxine-2-(R)-carboxylic acid were synthesized at Nisshin Flour Milling (Tokyo). BRL37344 and CL-316, 243 were dissolved in distilled water. N-5984 (free, non-salt) was dissolved in 33% DMSO and 0.01 M HCl to make a solution of 6.7 mM, which was then diluted with distilled water to desired concentration. [^{125}I]-iodocyanopindolol and the cAMP assay kit were obtained from Amersham (Little Chalfot, England)

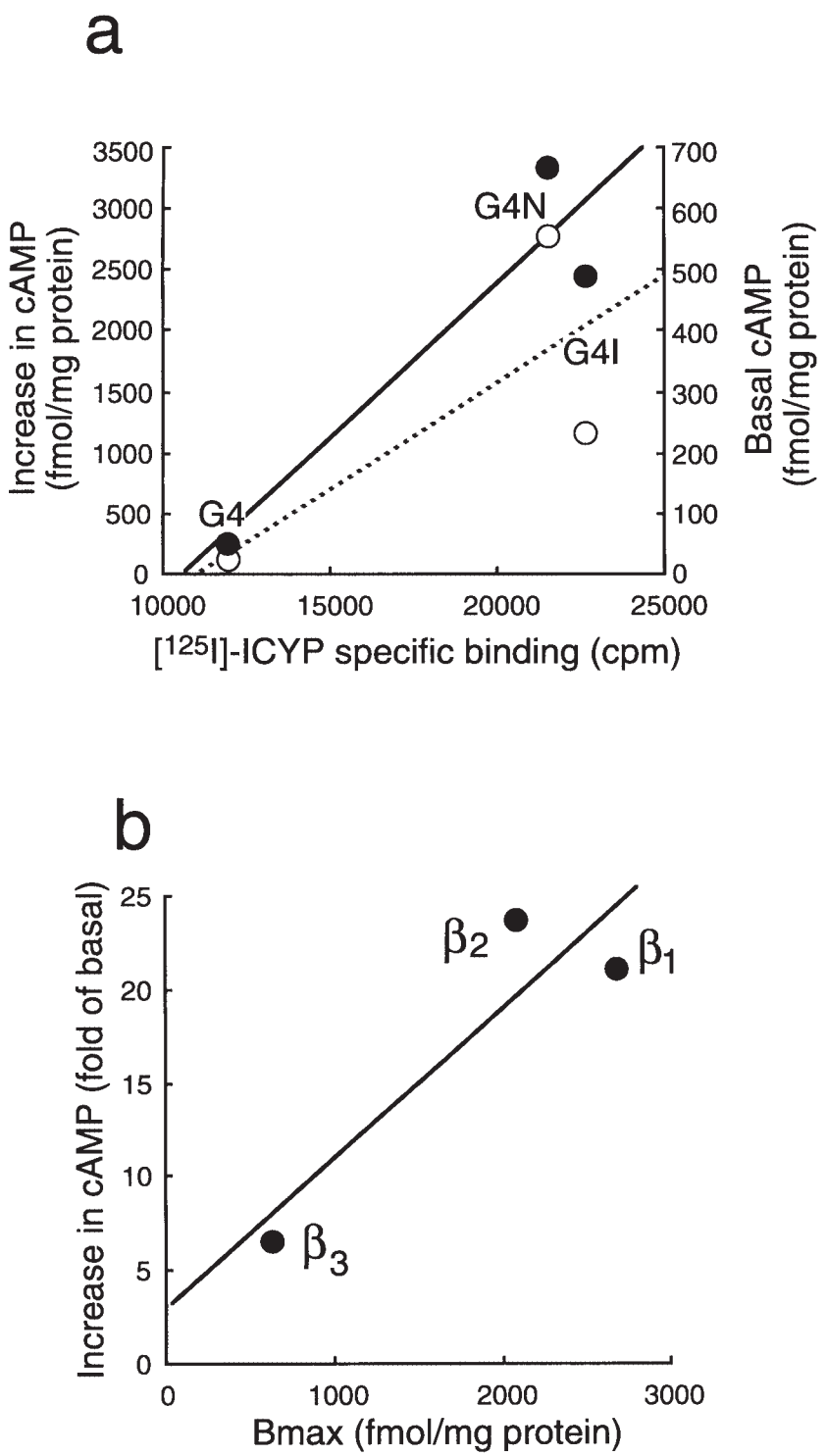

Fig. 2. a: The correlations between specific binding of [^{125}I]-ICYP and basal cAMP level or accumulation of cAMP by 10^{-5} M isoproterenol in the CHO cells expressing human β_3-ARs. Basal cAMP level (○), $Y=0.034X-370$, ($R=0.72$). Increase in cAMP (●), $Y=0.25X-2706$, ($R=0.93$). b: The correation between the B_{max} and the maximum increase in cAMP in CHO cells expressing three subtypes of β-ARs. The maximum increase in cAMP was normalized as a fold of basal cAMP content. $Y=0.0080X+2.73$ ($R=0.91$).

Analysis of concentration-response curves

Each value represents the mean±S.E.M. Concentration-response curves for β-AR agonists on cAMP production were computer-fitted to a logistic equation:

$$E = IA \times A^p / (A^p + EC_{50}{}^p)$$

where E is the normalized response (10^{-5} M isoproterenol: 100%), IA is the intrinsic activity efficacy of each β_3-AR agonist, A is the concentration of β-AR agonists and ^{p} is the slope parameter.

Results

Selection of stable transformants

Stable transformants were selected by using both the [^{125}I]-ICYP (700 pM) binding assay and the cAMP accumulation assay. We selected G4, G4I and G4N cells as stable transformants of β_3-ARs by [^{125}I]-ICYP binding assay. Accumulation of cAMP in each cloned cells was also examined by the response to 10^{-5} M isoproterenol. There were good correlations between the specific binding of [^{125}I]-ICYP and basal cAMP level or the accumulation of cAMP by isoproterenol (Fig. 2a). We finally selected G4I cells as stable transformants of β_3-ARs. Human β_1-CHO 5J10 cells and human β_2-CHO 41101J cells were also selected as stable transformants of β_1- and β_2-ARs, respectively.

Radioligand binding assay

Membrane preparations from CHO cells stably expressing three human β-ARs showed the saturable binding of [^{125}I]-ICYP. The percentages of specific binding of [^{125}I]-ICYP for β_1-, β_2- and β_3-ARs were 93.1, 98.2 and 66.9%, respectively, at the saturating concentrations of the radioligand ($n=1$). The values of K_d and B_{max} obtained from Scatchard analysis for the β_1-, β_2- and β_3-ARs are shown in Table 1.

Isoproterenol displaced [^{125}I]-ICYP binding to β_1-, β_2- and β_3-ARs in a concentration-dependent manner, giving K_i values for each receptors of 0.1, 0.3 and 2.16 μM, respectively (Fig. 3, Table 2). All the β_3-AR agonists examined in

Table 1. *K_d and B_{max} values of [^{125}I]-ICYP binding in CHO cells expressing human β-ARs*

Receptor	K_d (pM)	B_{max} (fmol/mg)
β_1	143.7	2679
β_2	37.2	2077
β_3	634	781

Membrane preparations were incubated with various concentrations of [^{125}I]-ICYP as described in Materials and Methods. K_d and B_{max} were obtained from Scatchard analysis ($n=1$).

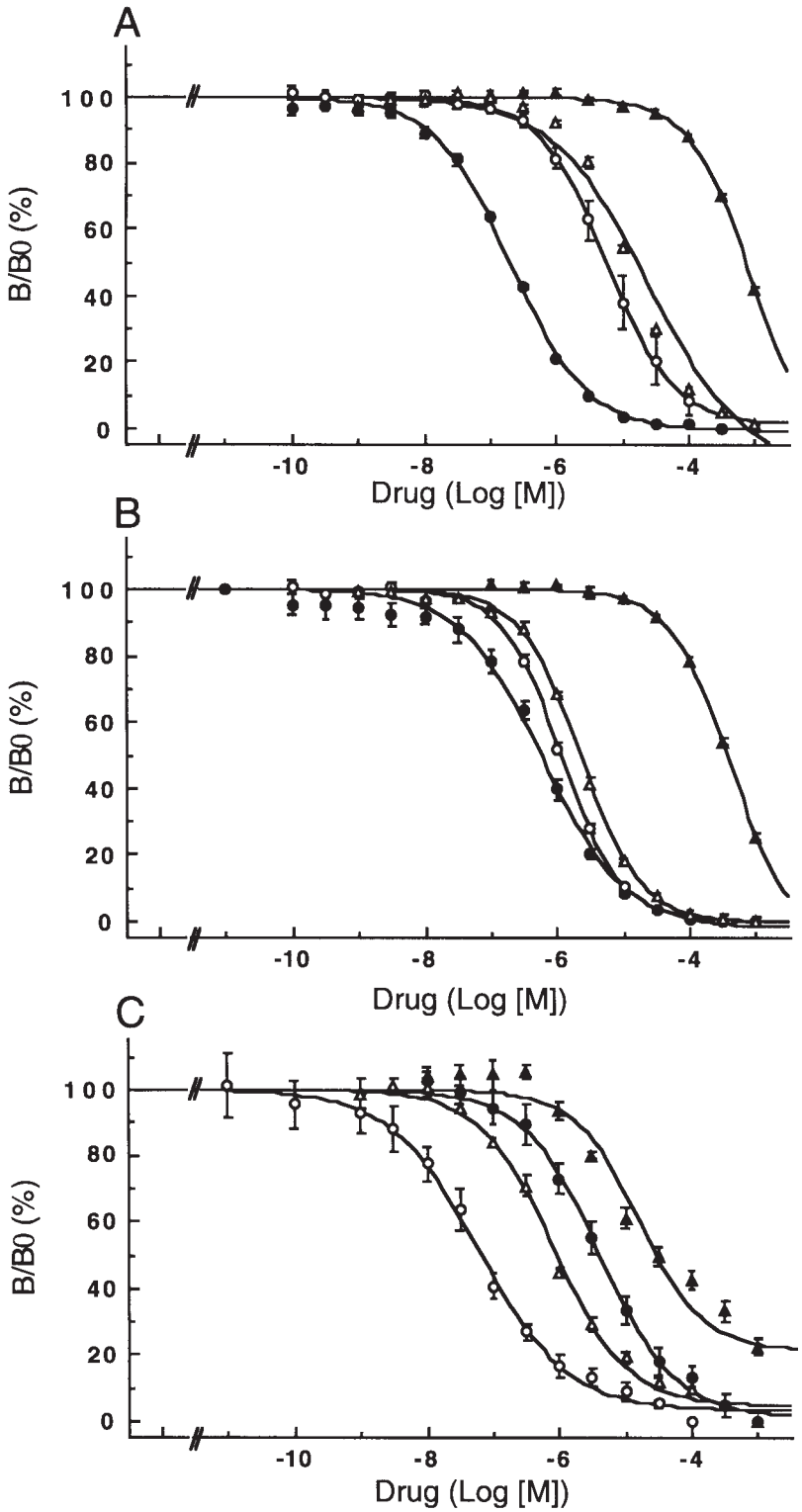

Fig. 3. Displacement curves of isoproterenol and β_3-AR agonists against [^{125}I]-ICYP binding to β_1-, β_2- and β_3-ARs expressed in CHO cells. Membranes were incubated with [^{125}I]-ICYP at approximate K_d concentrations and the indicated concentrations of competing ligands: isoproterenol (●), N-5984 (○), BRL37344 (△) and CL-316, 243 (▲). Nonspecific binding was determined in the presence of 10 μM (−)-propranolol for the β_1-AR, 5 μM (−)-alprenolol for the β_2-AR and 5 μM unradiolabeled (−)-cyanopindolol for the β_3-AR. Values represent means ± S.E.M. of three experiments.

TABLE 2. *Binding activities of β-AR agonists against human β_1-, β_2- and β_3-ARs expressed in CHO cells*

Compounds	K_i (μM)		
	β_1	β_2	β_3
Isoproterenol	0.1±0.01	0.3±0.05	2.16±0.34
N-5984	2.2±0.08	0.6±0.05	0.03±0.01
BRL37344	11.0±3.11	1.1±0.11	0.42±0.04
CL-316,243	720±335	219±17	6.76±1.08

Membrane preparations were incubated with [^{125}I]-ICYP in the absence or presence of various concentrations of test compounds as described in MATERIALS AND METHODS. Values represent means±S.E.M. of three experiments.

the present study also displaced [^{125}I]-ICYP binding to β_1-, β_2- and β_3-ARs in a concentration-dependent manner (Fig. 3). K_i values of N-5984, BRL37344 and CL-316, 243 for β_1-ARs were 2.2, 11.0 and 720 μM, and those for β_2-ARs were 0.6, 1.1 and 219 μM, respectively (Table 2). Affinities of β_3-AR agonists for β_1- and β_2-ARs were weaker than those of isoproterenol. N-5984 and BRL37344 inhibited [^{125}I]-ICYP binding to β_3-ARs at low concentrations, producing K_i values for β_3-ARs of 0.03 and 0.42 μM, respectively. The K_i values of CL-316, 243 were higher than those of isoproterenol and other β_3-AR agonists.

Table 3 showed the selectivity of the compounds for β_3-ARs against β_1-ARs or β_2-ARs. Ratios of β_1- or β_2-ARs against β_3-ARs were calculated from the K_i values in radioligand binding assay. Ratios of β_1-ARs against β_3-ARs for three β_3-AR agonists examined in the study were much larger than that of isoproterenol. The relative order of selectivity was CL-316, 243 > N-5984 > BRL37344 > isoproterenol. The relative order of ratios of β_2-ARs against β_3-ARs was also CL-316, 243 > N-5984 > BRL37344 > isoproterenol. The K_i value of CL-316, 243 for the β_3-ARs, however, was larger than those of other β_3-AR agonists tested (Table 2). Thus, although CL-316, 243 was the most selective for human β_3-ARs among the compounds examined, it was not a potent β_3-AR ligand.

cAMP accumulation assays

Accumulation of cAMP in each cloned cell was examined as functional responses to β-AR agonists. Isoproterenol increased the amount of cAMP in each CHO cell expressing human β_1- β_2- and β_3-ARs in a concentration-dependent manner, giving mean EC_{50} values of 65 ($n=3$), 12 ($n=3$) and 22 nM ($n=3$), respectively. The basal cAMP content and the maximal accumulation of cAMP by 10^{-5} M isoproterenol in each cell were 46 and 967, 55 and 1304, and 419 and 2730 fmol/mg protein, respectively. Although the basal content of cAMP in CHO cells expressing three subtypes of β-ARs and the increases produced by isoproterenol were different among the subtypes, there was a good correlation between the B_{max} and the fold increase in cAMP (Fig. 2b).

TABLE 3. *Comparison of Binding in CHO cells expressing human β_1-, β_2- and β_3-ARs*

Compound	β_1/β_3	β_2/β_3
Isoproterenol	0.05	0.14
N-5984	73.3	20.0
BRL37344	26.2	2.62
CL-316, 243	106.5	32.4

Selectivities of binding were calculated as ratios of K_i (β_1 or β_2) to K_i (β_3).

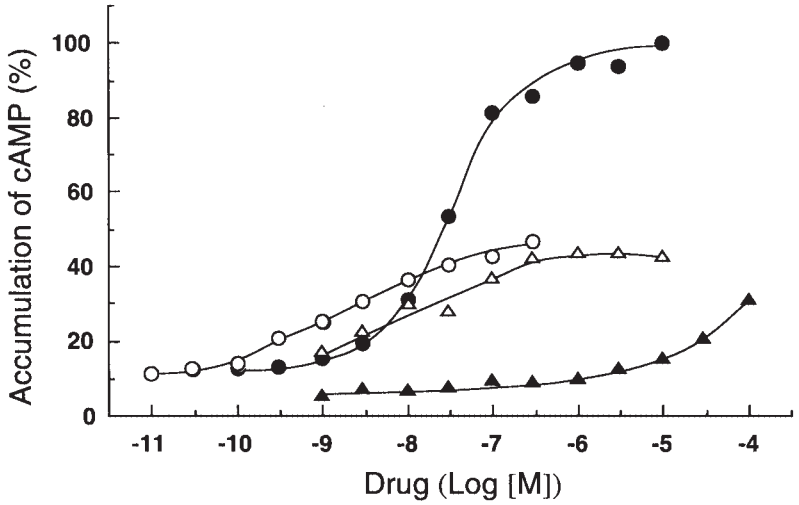

Fig. 4. The accumulation of cAMP in CHO cells expressing human β_3-ARs by isoproterenol and β_3-AR agonists. Cells were exposed to isoproterenol (●), N-5984 (○), BRL37344 (△) and CL-316, 243 (▲). Accumulation of cAMP was determined as described in MATERIALS AND METHODS, and the data are expressed as a percentage of the maximal response to 10^{-5} M isoproterenol. Values represent means of three experiments.

These increases in cAMP by isoproterenol were normalized as 100% when the intrinsic activity (IA) of β_3-AR agonists was calculated. The concentration-response curves for β-AR agonists are shown in Fig. 4. The concentration in 50% of each maximal cAMP accumulation (EC_{50}) and IA for each agonist are presented in Table 4. N-5984 increased cAMP production in the CHO cells expressing human β_3-ARs in a concentration-dependent manner, with EC_{50} values of 1.7 ± 0.3 nM ($n=3$). On the other hand, the EC_{50} value of BRL37344 for β_3-ARs was 21 ± 8 nM ($n=3$), which was 8-fold higher than that of N-5984. N-5984 and BRL37344 were shown to have the IAs of $52\pm 3\%$ and $45\pm 4\%$, which were stronger than that of CL-316, 243 in CHO cells expressing the β_3-AR. EC_{50} value of CL-316, 243 was not determined in the cAMP accumulation assay because this compound had little cAMP production in CHO cells expressing β_3-ARs (Fig. 4, Table 4).

The increases in cAMP production by N-5984, CL-316, 243 and BRL37344 at the highest concentrations tested were less than 10% of that of isoproterenol in the

TABLE 4. *Comparison of agonist activity in CHO cells expressing human β_3-ARs*

Compound	EC_{50} (nM)	IA
Isoproterenol	22	100
N-5984	1.7	52
BRL37344	21	45
CL-316,243	—	—

The concentration-response curves for β_3-AR agonists in Fig. 4 were analysed by the computer curve fitting as described in MATERIALS AND METHODS.

CHO cells expressing β_1-ARs. The increases in cAMP by N-5984 and CL-316, 243 for the β_2-AR were also less than 10% ($n=3$), while that of BRL37344 was $17.1\pm2.7\%$ ($n=3$). EC_{50} values of β_3-AR agonists for β_1- and β_2-ARs in the cAMP accumulation assay were not analysed nor determined because the concentration-response curves for these compounds were not suitable for the analysis in CHO cells expressing β_1- and β_2-ARs.

DISCUSSION

The development of various compounds which have high activities for rodent β_3-ARs and effectiveness in animal models of diabetes mellitus and obesity has been discontinued for clinical trials (Dow 1997). It is well known that the significant species differences exist in β_3-ARs (Liggett 1992). In addition, agonistic activities for β_1- and/or β_2-ARs cause the adverse effects such as tachycardia, arrhythmia and tremor. Therefore, it is important to obtain the information about the affinity, efficacy and selectivity for human β_3-ARs of the compounds to be developed for clinical use.

In the present study, we constructed the cells which stably expressed the cloned human β_1-, β_2- and β_3-ARs and evaluated β_3-AR agonists, BRL37344, CL-316, 243 and N-5984, a newly synthesized β_3-AR agonist, using the radioligand binding assays and cAMP accumulation assays. There was a good correlation between the specific [^{125}I]-ICYP bindings and cAMP production by isoproterenol in the CHO cells expressing β_3-ARs. There was also a good correlation between the B_{max} and the maximum increase in cAMP in CHO cells expressing three subtypes of β-ARs (Fig. 2). These findings indicate that the number of β-ARs determines the basal activity of adenylyl cyclase and the increase in cAMP produced by the agonist. This result supports the concept of "allosteric ternary complex model" about the regulation of G protein-coupled receptors with the constitutive activity (Lefkowitz et al. 1993).

Specific [^{125}I]-ICYP bindings to β_1-, β_2- and β_3-ARs were readily saturable and this proved that the CHO cells expressed specifically each receptor. The K_d value of [^{125}I]-ICYP binding to β_3-ARs was more than 5 times higher than those

of β_1- and β_2-ARs, which was consistent with the results of Tate et al. (1991).

N-5984 and BRL37344 inhibited [^{125}I]-ICYP binding to β_3-ARs at low concentrations and the K_i values of those agonists were lower than that of isoproterenol, while K_i value of CL-316, 243 was much higher than those of isoproterenol and other β_3-AR agonists. It was reported that β_3-adrenergic activity of CL-316, 243 in the stimulation of glycerol release from adipocytes in rat epididymal fat pads was stronger than those of isoproterenol and BRL37344 (Bloom 1992), while the affinity of CL-316, 243 for human β_3-ARs was much lower than that of BRL37344 (Strosberg and Pietri-Rouxel 1996). Although CL-316, 243 was reported to decrease serum glucose level in KKAy mice (Yoshida et al. 1994), it was abandoned to develop as a β_3-AR agonist to treat obesity or diabetes mellitus due to the lack of sufficient efficacy in human (Weyer et al. 1998). On the other hand, although K_i value of BRL37344 for human β_3-ARs was lower than that of isoproterenol in the present study, the development of this compound was also discontinued not due to the efficacy but to the adverse effects in clinical studies, which were resulted from the relatively less selectivity for β_3-ARs (Dow 1997). Therefore, characterization of the compounds using the CHO cells constructed in our study is useful to evaluate the efficacy and selectivity in human.

Relative density of β_3-ARs in CHO cells is known to affect the activity of the agonists. The affinity and the intrinsic activity of β_3-AR agonists were relatively strong in CHO cells highly expressing β_3-ARs (Wilson et al. 1996). This may mislead us to expect that the compounds which have high affinity in CHO cells with higher density level of β_3-ARs are also effective in human. For example, the affinity of ICI-D-7114 for β_3-ARs was higher than that of BRL37344 in CHO cells expressing human β_3-ARs (Strosberg and Pietri-Rouxel 1996), but ICI-D-7114 showed little effects on energy expenditure in healthy, lean men in clinical study (Goldberg et al. 1995). Since the expression levels of the β_3-ARs are not so high in human tissues, the CHO cells expressing low levels of β_3-ARs might be better to predict the efficacy in human. Therefore, we constructed CHO cells expressing relatively lower density level of β_3-ARs than the previously reported ones. Our results that CL-316, 243 showed low affinity for β_3-ARs whereas BRL37344 had relatively high affinity are consistent with the results of clinical efficacy.

N-5984, a newly synthesized β_3-AR agonist, showed the highest affinity in radioligand binding assay among all the β-AR agonists tested including isoproterenol. This result indicates that N-5984 might be a potent agonist of human β_3-ARs and effective in human. From the view point of adverse effects, selectivity for β_3-ARs is another important factor to evaluate the possibility in clinical use. We designed and constructed CHO cells expressing relatively higher density level of β_1- and β_2-ARs than the β_3-ARs. Expression levels of β_1- and β_2-ARs are higher than that of human β_3-ARs, so it is considered favorable for evaluating the effects of the agonists on β_1- and β_2-ARs to use above mentioned CHO cells to predict adverse effects due to those receptor stimulation. As mentioned above,

BRL37344 was abandoned for the clinical use due to adverse effects in human. In the present study, selectivity of BRL37344 was the lowest among all the β_3-AR agonists tested. The lower selectivity of BRL37344 might be related to the incidence of adverse effects in clinical uses. N-5984 was approximately 3 times more selective than BRL37344 for β_3-ARs against β_1-ARs and 7 times more selective than BRL37344 for β_3-ARs against β_2-ARs (Table 3). Furthermore, the affinity of N-5984 for β_3-ARs was higher than that of BRL37344 (Table 2). The agonistic activity (EC_{50} and IA) of N-5984 was more potent than that of BRL37344 in CHO cells expressing β_3-ARs, whereas the IA of N-5984 was smaller than that of BRL37344 in CHO cells expressing β_2-ARs. The agonistic pharmacophore of human β_3-AR (Strosberg 1997) may be stimulated by N-5984 more effectively than CL-316, 243. The side chain of N-5984, 2, 3-dihydro-1, 4-benzodioxine-2-(R)-carboxylic acid, seems to be responsible for a higher potency and efficacy for the human β_3-AR, compared with BRL37344 and CL-316, 243. These findings suggest that N-5984 must be a promising drug for the control or treatment of diabetes mellitus and obesity without undesirable β_1- and β_2-ARs mediated adverse effects.

In conclusion, we constructed CHO cells expressing human β_1-, β_2- and β_3-ARs which may reflect clinical effects. Those cells expressing each cloned human AR could be a powerful tool to evaluate the efficacy and selectivity of β_3-AR agonists for the treatment of human disease such as obesity and diabetes mellitus. N-5984, highly selective and effective for cloned human β_3-ARs, has the promising profiles for being developed as one of the clinically effective drugs for obesity and diabetes mellitus.

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References

1) Arch, J.S.R. & Kaumann, A.J. (1993) β_3- and atypical β-adrenoceptors. *Med. Res. Rev.*, **13**, 663–729.
2) Blin, N., Camoin, L., Maigret, B. & Strosberg, A.D. (1993) Structural and conformational features determining selective signal transduction in the β_3-adrenergic receptor. *Mol. Pharmacol.*, **44**, 1094–1104.
3) Bloom, J.D., Dutia, M.D., Johnson, B.D., Wissner, A., Burns, M.G., Largis, E.E., Dolan, J.A. & Claus, T.H. (1992) Disodium (R, R)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1, 3-benzodioxol-2, 2-dicarboxylate (CL 316, 243). A potent β-adrenergic agonist virtually specific for β_3 receptors. A promising antidiabetic and antiobesity agent. *J. Med. Chem.*, **35**, 3081–3084.
4) Dow, R.L. (1997) β_3 adrenergic agonist: potential therapeutics for obesity. *Expert Opin. Invest. Drugs*, **6**, 1811–1825.

5) Emorine, L.J., Marullo, S., Briend-Sutren, M.-M., Patey, G., Tate, K., Delavier-Klutchko, C. & Strosberg, A.D. (1989) Molecular characterization of the human β_3-adrenergic receptor. *Science*, **245**, 1118–1121.
6) Goldberg, G.R., Prentice, A.M., Murgatroyd, P.R., Haines, W. & Tuersley, M.D. (1995) Effects on metabolic rate and fuel selection of a selective beta-3 agonist (ICI D7114) in healthy lean men. *Int. J. Obes. Related Metab. Dis.*, **19**, 625–631.
7) Graham, F. & van der Eb, A. (1973) A new technical for the assay of infectivity of human adenovirus 5 DNA. *Virology*, **52**, 456–467.
8) Howe, R. (1993) β_3-adrenergic agonists. *Drugs of the Future*, **18**, 529–549.
9) Lands, A.M., Arnold, A., McAuliff, J.P., Luduena, F.P. & Brown, T.G., Jr. (1967) Differentiation of receptor systems activated by sympathomimetic amines. *Nature*, **214**, 597–598.
10) Lefkowitz, R.J., Cotecchia, S., Samama, P. & Costa, T. (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.*, **14**, 303–307.
11) Liggett, S.B. (1992) Functional properties of the rat and human β_3-adrenergic receptors: Differential agonist activation of recombinant receptors in Chinese hamster ovary cells. *Mol. Pharmacol.*, **42**, 634–637.
12) Strosberg, A.D. & Pietri-Rouxel, F. (1996) Function and regulation of the β_3-adrenoceptor. *Trends Pharmacol. Sci.*, **17**, 373–381.
13) Strosberg, A.D. (1997) Structure and function of the β_3-adrenergic receptor. *Annu. Rev. Pharmacol. Toxicol.*, **37**, 421–450.
14) Tate, K.M., Briend-Sutren, M.-M., Emorine, L.J., Delavier-Klutchko, C., Marullo, S. & Strosberg, A.D. (1991) Expression of three human β-adrenergic-receptor subtypes in transfected Chinese hamster ovary cells. *Eur. J. Biochem.*, **196**, 357–361.
15) Weyer, C., Tataranni, P.A., Snitker, S., Danforth, E., Jr. & Ravussin, E. (1998) Increase in insulin action and fat oxidation after treatment with CL-316, 243, a highly selective β_3-adrenoceptor agonist in humans. *Diabetes*, **47**, 1555–1561.
16) Weyer, C., Gautier, J.F. & Danforth, E., Jr. (1999) Development of beta$_3$-adrenoceptor agonists for the treatment of obesity and diabetes-an update. *Diabetes Metab.*, **25**, 11–21.
17) Wilson, S., Chambers, J.K., Park, J.E., Ladurner, A., Cronk, D.W., Chapman, C.G., Kallender, H., Browne, M.J., Murphy, G.J. & Young, P.W. (1996) Agonist potency at the cloned human beta-3 adrenoceptor depends on receptor expression level and nature of assay. *J. Pharmacol. Exp. Ther.*, **279**, 214–221.
18) Yoshida, T., Sakane, N., Wakabayashi, Y., Umekawa, T. & Kondo, M. (1994) Anti-obesity and anti-diabetic effects of CL-316, 243, a highly specific β_3-adrenoceptor agonist, in yellow KK mice. *Life Sci.*, **54**, 491–498.