Assessment of \( \beta_2 \) - and \( \beta_3 \) - Adrenoceptors in Rat White Adipose Tissues by Radioligand Binding Assay

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We investigated the characteristics of \( \beta \)-adrenoceptors (\( \beta \)-ARs) in rat white adipose tissues (WAT) with a radioligand receptor binding assay using \((-\rangle\)((\textsuperscript{3}H)CGP12177. Scatchard analysis revealed that there are high- and low-affinity sites for \((-\rangle\)((\textsuperscript{3}H)CGP12177 in WAT. The \((-\rangle\)((\textsuperscript{3}H)CGP12177 bound to a high-affinity site was displaced by \(1 \mu\text{M} \) propranolol. The rank of \( pK_a \) values of catecholamines for the site was norepinephrine \( > \) epinephrine \( > \) isoproterenol. By contrast, BRL37344A, BRL35135A, and SR59230A, \( \beta_2 \)-selective agonists had high affinity for the low-affinity site of \((-\rangle\)((\textsuperscript{3}H)CGP12177, whereas \((-\rangle\)((\textsuperscript{3}H)CGP12177 bound to a low-affinity site was completely displaced by \(100 \mu\text{M} \) butoproanol but not \(1 \mu\text{M} \) propranolol. The \( pK_a \) values of the catecholamines (isoproterenol, norepinephrine, epinephrine) for this site were very low. In addition, the correlation between the \( pK_a \) values of various \( \beta \)-agonists for the low-affinity site of rat WAT and those obtained from rat cloned \( \beta \)-ARs was significant, but those of human cloned \( \beta \)-ARs were not. Consequently, the results suggested that the high- and low-affinity sites were \( \beta_2 \)-ARs and \( \beta_3 \)-ARs in rat WAT, respectively.

Key words: \( \beta_2 \)-adrenoceptor; \((\textsuperscript{3}H)CGP12177 binding; rat white adipose tissue

There is increasing evidence that \( \beta \)-adrenoceptors (\( \beta \)-ARs) can be classified into at least three subtypes. Those are typical \( \beta_1 \)- and \( \beta_2 \)-ARs and \( \beta_3 \)-ARs. The \( \beta_3 \)-ARs were first discovered in rat white adipocytes by Harms et al.,\(^{11}\) as atypical \( \beta \)-ARs, then Tan and Curtis-Prior\(^{k}\) proposed that they should be described as \( \beta_3 \)-ARs. Subsequently, the genes encoding human \( \beta_3 \)-ARs were isolated by Emorine et al.\(^{5,6}\) \( \beta_3 \)-ARs were found in various tissues, for example in adipose tissue,\(^{4,7} \) intestinal tract smooth muscle,\(^{4,7} \) heart,\(^{5,8,10} \) skeletal muscle,\(^{12,13} \) vascular smooth muscle,\(^{14,15} \) liver,\(^{16,17} \) and airway smooth muscle.\(^{19,20} \) \( \beta_3 \)-ARs participate in the modulation of glyceral release, thermogenesis, and glycometabolism; therefore, selective agonists for \( \beta_3 \)-ARs may be useful in treating metabolic disturbances such as obesity and diabetes in which weight control in difficult. Details of the pharmacological properties of \( \beta_3 \)-ARs, however, are unknown. To evaluate the coexistence of \( \beta_2 \)-ARs and the binding characteristics of \((\textsuperscript{3}H)CGP12177 to \( \beta_2 \)-ARs in adipose tissues is important and would be useful in assessing the affinity of new remedies.

The ligands \((\textsuperscript{3}H)\)diiodostryprenol and \((\textsuperscript{125}I)\)iodocyanopindolol have been used to study adiocyte \( \beta \)-ARs. They are highly lipophilic and membrane-permeable, so it is possible that they labeled not only surface, but also internal receptors. Therefore, assessments using these lipophilic radioligands might not necessarily reflect the characteristics of the receptors on the cell surface. On the other hand, \((\textsuperscript{3}H)CGP12177, which is a partial agonist of \( \beta_3 \)-AR, is highly hydrophilic and membrane-impermeable.\(^{20,21} \) In addition, Lönnqvist et al.\(^{22} \) have suggested that CGP12177 is a better tool for studying \( \beta_3 \)-ARs than BRL37344A, which is a selective \( \beta_3 \)-agonist.\(^{23} \) Therefore, it is considered that \((\textsuperscript{3}H)CGP12177 is the most reliable radioligand for assessing \( \beta \)-ARs on the adipocyte surface.\(^{24,25} \)

Thus, we clarified the binding characteristics of \((-\rangle\)((\textsuperscript{3}H)CGP12177 to \( \beta_3 \)-ARs of membranes in rat white adipose tissues (WAT) by Scatchard analysis and competition binding studies.

MATERIALS AND METHODS

Drugs and Chemicals \((-\rangle\)((\textsuperscript{3}H)CGP12177 (Amersham), (\(\pm\))isoproterenol and (\(-\))epinephrine (Sigma Chemical Co., U.S.A.), (\(-\))norepinephrine, serotonin, histamine and atropine (Wako Pure Chemical Industries, Ltd., Japan), yohimbine (Nacalai Tesque Inc., Japan) and butoxamine (Burroughs Wellcome Co., U.S.A.) were purchased. The following drugs were donated: BRL37344A ((\(\pm\))-4-(2-(2-hydroxy-2-(3-chlorophenyl)ethylamino)propyl)phenoxacetic acid sodium salt sesquihydrate) and BRL35135A ((\(\pm\))-methyl-4-(2-(2-hydroxy-2-(3-chlorophenyl)ethylamino)propyl)phenoxacetate hydrobromide) (SmithKline Beecham Pharmaceuticals, England), SR59230A (3-(2-ethylphenoxy)-1-[[15S]-1,2,3,4-tetrahydropraphth-1-ylamino]-2(S)-2-propanol oxalate) (Sanofi Midy S.P.A. Research Center, Italy), butanol metabol (Kaken Pharmaceuticals Co., Ltd., Japan), betaxalom (Mitsubishi Kasei Co., Ltd., Japan), metabolop (Ciba-Geigy Co., Ltd., Japan), naldol (Dainippon Pharmaceuticals, Ltd., Japan), atenolol hydrochloride, ICI-118551 and propranolol (ICI Pharmaceuticals, Japan) bopathidol, pindolol, 18-502 (4-(3-tet-butylamino-2-hydroxypropoxy)-2-methylindole) and 20-785 (4-(3-tet-butylamino-2-hydroxypropoxy)indol-2-carboxylic acid) (Sandoz Pharmaceuticals, Ltd., Switzerland).

Preparation of Membrane-Enriched Fraction from the Rat WAT Membrane-enriched fractions were prepared as described.\(^{16} \) Male Wistar rats weighing about 350 to 450 g were killed by exsanguination and WAT were immediately removed. WAT was then minced with scissors and homogenized in 50 mM Tris-Cl (pH 7.4), 0.25 M sucrose, 1 mM EGTA at 23°C with a Polytron.

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homogenizer.

The homogenates were centrifuged for 10 min at 1000 × g at 23°C. The supernatant was filtered through triple layers of surgical gauze. The filtrates were again centrifuged for 15 min at 10000 × g. The supernatant was transferred to ultracentrifuge tubes and centrifuged for 30 min at 100000 × g. The supernatant was discarded, then the pellets were resuspended in ice-cold buffer (50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 1 mM EGTA). After a second centrifugation for 30 min at 100000 × g, the pellet was resuspended in the incubation buffer (50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 0.15 M NaCl) and stored at −80°C.

The concentration of membrane protein was determined using bovine serum albumin as a standard according to Lowry et al.²⁷¹

**Radioligand Binding Assay**

A) Scatchard Analysis: Saturation experiments were performed in duplicate with (−)[³H]-CGP12177 in the presence (non-specific binding) or absence (total binding) of 1 mM propranolol. In brief, a membrane suspension (20 μg of protein) was incubated for 3 h at 23°C with various concentrations (0.1−40 nM) of (−)[³H]-CGP12177 in a total volume of 500 μl. At the end of the incubation period, the incubation medium was immediately filtered through a Whatman GF/C glass fiber filter as described by Satoh et al.²⁸⁹ The radioactivity level was counted with a liquid scintillation counter (Packard 2200CA). The difference in mean values between the total and non-specific binding was taken as the specific binding.

B) Displacement Experiments: Displacement experiments were performed in duplicate with 10 nM of (−)[³H]-CGP12177 in the presence of various concentrations of β₁- and β₁-agonists. At the end of the incubation period, the incubation medium was immediately filtered through a Whatman GF/C glass fiber filter. The radioactivity level was counted with a liquid scintillation counter. The difference in mean values between the total and non-specific binding was taken as the specific binding.

In the saturation experiments, 1 μM propranolol, 100 μM bupranolol, and 100 μM prazosin and yohimbine (α-blocker) or serotonin was added to the reaction mixture 20 min before the addition of (−)[³H]-CGP12177.

**Data Analysis** All kinetic analyses were performed as described by Satoh et al.²⁸¹ Statistics were analyzed by Student's t-test.

**RESULTS**

**Scatchard Analysis** The Scatchard plot was curvilinear, indicating that there are at least two different affinity sites for (−)[³H]-CGP12177 (Fig. 1A). The $K_d$ values of the high- and low-affinity sites were $0.42 ± 0.06$ and $40.42 ± 10.77$ nM, and the $B_{max}$ values were $321.0 ± 21.7$ and $3655.1 ± 454.3$ fmol/mg protein, respectively. Although 1 μM propranolol completely displaced (−)[³H]-CGP12177 binding for the high-affinity site, it did not affect the binding for the low-affinity site (Fig. 1B). The $K_d$ and $B_{max}$ values of the low-affinity site were not significantly different from those obtained from the

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Fig. 1. Scatchard Plots of (−)[³H]-CGP12177 Binding to Rat WAT

Scatchard Plots of (−)[³H]-CGP12177 binding to rat WAT without displacements (A) or in the presence of 1 mM propranolol (B) and 100 μM bupranolol (C) as displacers.
Table 1. The pKᵢ Values of β-Agonists and Antagonists in Rat White Adipose Tissues

<table>
<thead>
<tr>
<th>Drugs</th>
<th>pKᵢ values</th>
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<tbody>
<tr>
<td></td>
<td>High-affinity site</td>
</tr>
<tr>
<td>Agonists</td>
<td></td>
</tr>
<tr>
<td>Isoproterenol (5)</td>
<td>10.86 ± 0.55</td>
</tr>
<tr>
<td>Epinephrine (7)</td>
<td>9.22 ± 0.32</td>
</tr>
<tr>
<td>Norepinephrine (6)</td>
<td>8.34 ± 0.59</td>
</tr>
<tr>
<td>BRL37344A (5)</td>
<td>4.82 ± 0.20</td>
</tr>
<tr>
<td>BRL35135A (5)</td>
<td>4.79 ± 0.22</td>
</tr>
<tr>
<td>SR59230A (5)</td>
<td>5.14 ± 0.22</td>
</tr>
<tr>
<td>Antagonists</td>
<td></td>
</tr>
<tr>
<td>Propranolol (5)</td>
<td>8.61 ± 0.48</td>
</tr>
<tr>
<td>Pindolol (4)</td>
<td>8.53 ± 0.15</td>
</tr>
<tr>
<td>ICI118551 (5)</td>
<td>9.37 ± 0.34</td>
</tr>
<tr>
<td>Arotinolol (4)</td>
<td>8.83 ± 0.25</td>
</tr>
<tr>
<td>Betaxolol (4)</td>
<td>6.80 ± 0.43</td>
</tr>
<tr>
<td>Bopindolol (4)</td>
<td>9.51 ± 0.83</td>
</tr>
<tr>
<td>Alprenolol (6)</td>
<td>8.84 ± 0.38</td>
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<tr>
<td>Nadolol (7)</td>
<td>8.67 ± 0.50</td>
</tr>
<tr>
<td>Metoprolol (6)</td>
<td>8.28 ± 0.60</td>
</tr>
<tr>
<td>Butoxamine (5)</td>
<td>8.31 ± 0.47</td>
</tr>
<tr>
<td>Bupranolol (6)</td>
<td>9.53 ± 1.05</td>
</tr>
<tr>
<td>CGP12177 (4)</td>
<td>9.11 ± 0.30</td>
</tr>
<tr>
<td>18-502 (4)</td>
<td>10.60 ± 0.17</td>
</tr>
<tr>
<td>20-785 (4)</td>
<td>8.07 ± 0.84</td>
</tr>
</tbody>
</table>

These data represent mean ± S.E. of 4-7 experiments. The concentrations of (−) [³H]-CGP12177 were 10 nM for most drugs and 5 nM for betaxolol, metoprolol and butoxamine. * p < 0.01; high- vs. low-affinity site.

Scatchard plots shown in Fig. 1A. On the other hand, 100 µM bupranolol completely displaced (−)[³H]-CGP12177 binding for the high- and the low-affinity sites (Fig. 1C).

**Displacement Experiments** The competition activities of various agonists against 10 nM (−)[³H]-CGP12177 were examined. The pKᵢ values of isoproterenol, norepinephrine, epinephrine, BRL37344A, BRL35135A and SR59230A are shown in Table 1. For the high-affinity site, the rank order of pKᵢ values of catecholamines was isoproterenol (10.86 ± 0.55) > epinephrine (9.22 ± 0.32) > norepinephrine (8.34 ± 0.59), which corresponded to that of β₁-ARs. On the other hand, higher pKᵢ values of each antagonist for high-affinity sites than those for the low-affinity site were observed (Table 1). In addition, the pKᵢ value of bupranolol for the low-affinity site was 6.17 ± 0.42, and this value of this drug to the low-affinity site was the highest among the β-blockers tested here.

The IC₅₀ values of prazosin, yohimbine and serotonin against high- and low-affinity sites were more than 1 mM (data not shown).

**Relationships between the pKᵢ Values of β-Antagonists Obtained from Rat WAT and from β₁- and β₂-ARs** We have reported on the pharmacological characteristics of β₁- and β₂-ARs in rat heart and bovine trachea. The pKᵢ values of β-antagonists in this study were compared with the values in our previous studies. The correlations between the pKᵢ values of various β-antagonists for the high-affinity sites for (−)[³H]-CGP12177 in rat heart and bovine trachea.

**Fig. 2. Correlation of pKᵢ Values of β-Antagonists between that for the High-Affinity Site of (−)[³H]-CGP12177 in WAT and for β₁- or β₂-ARs in Rat Heart or Bovine Trachea**

The horizontal axis represents the pKᵢ values of β₁- (A, B, C and D)-ARs in rat white adipose tissues and the vertical axis represents β₁- (A and C) or β₂- (B and D)-adrenceptors in rat heart or bovine trachea. Each point was represented as 1, propranolol; 2, pindolol; 3, ICI118551; 4, arotinolol; 5, bopindolol; 6, alprenolol; 7, 1-metoprolol; 8, butoxamine and 9, betaxolol, respectively. A) Y = 0.72X + 1.56, r = 0.52; B) Y = 0.18X + 6.23, r = 0.14; C) Y = 1.03X - 1.31, r = 0.77; D) Y = 0.23X + 5.28, r = 0.20. Data were taken from Sakuma et al.¹⁹⁶ and Tsuchihashi et al.¹⁹⁶ (A and B); and Hosohata et al.¹⁹¹ and Satoh et al.¹⁹¹ (C and D), respectively.
WAT and the values for $\beta_2$-ARs were high (correlation coefficient ($r$) = 0.52 and 0.77 (Fig. 2A and B), but the values for $\beta_2$-ARs ($r$ = 0.14 and 0.20 (Fig. 2C and D)) in rat heart and bovine trachea were not. Furthermore, there was no correlation between the $pK_r$ values of various $\beta$-antagonists for the low-affinity sites for (--)$[^{3}H]$-CGP12177 in rat WAT and the values for rat heart $\beta_2$-ARs ($r$ = 0.31, Fig. 3A) and $\beta_1$-ARs ($r$ = 0.22, Fig. 3B). These results suggest that high-affinity sites for (--)$[^{3}H]$-CGP12177 are dominantly $\beta_2$-ARs.

Relationships between the $pK_r$ Values of $\beta$-Agonists Obtained from Rat WAT and from Cloned $\beta_2$-ARs

The $pK_r$ values of isoproterenol, norepinephrine, epinephrine and BRL37344A in rat WAT were compared with those for $\beta_2$-ARs cloned from rats and humans and with activities ($pK_{act}$) of the $\beta$-agonists on cAMP accumulation in the CHO cells expressing cloned $\beta_2$-ARs. The compared data were taken from Blin et al. $^{32}$ (Fig. 4A), Liggett $^{33}$ (Fig. 4B), and from Muzzin et al. $^{34}$ (Fig. 4C). The $pK_r$ values of the agonists for the low-affinity site of (--)$[^{3}H]$-CGP12177 in rat WAT significantly correlated with those obtained from rat cloned $\beta_2$-ARs, with $r$-values of 0.96 ($p<0.05$), 1.00 ($p<0.01$) and 0.98 ($p<0.05$), respectively. On the other hand, there was no significant relationship between rat WAT and human cloned $\beta_2$-ARs, compared with the data taken from Blin et al. $^{32}$ (Fig. 4D) and from Liggett $^{33}$ (Fig. 4E). In addition, the $pK_r$ values of these drugs for the low-affinity site in rat WAT correlated better with the $pK_{act}$ values obtained from cells expressing rat cloned $\beta_2$-ARs with $r$-values of 0.97 ($p<0.05$) and 0.80 (Fig. 5A and B), than those obtained from cells expressing human $\beta_2$-ARs (Fig. 5C), according to data taken from Nahmias et al. $^{35}$ (Fig. 5A and C) and Muzzin et al. $^{34}$ (Fig. 5B), respectively.

DISCUSSION

It has been reported that $\beta_2$-AR predominantly locates with small amounts of $\beta_2$-AR on rat brown and white adipose membranes, $^{35}$ that additional atypical $\beta$-ARs subtype named $\beta_2$-ARs $^{36}$ locates in colon smooth muscle and the hearts in addition to white and brown rat fat cells, and that $\beta_1$, $\beta_2$, and $\beta_3$-ARs locate in dog fat cells. $^{37}$

In this study, the Scatchard plots indicated that there are at least two binding sites for (--)$[^{3}H]$-CGP12177, and the $K_d$ values of the low-affinity site were 100 times higher than that of the high-affinity site. The results show that there are at least two $\beta$-AR subtypes in rat WAT. It is suggested that (1) the high-affinity sites for (--)$[^{3}H]$-CGP12177 are dominantly $\beta_2$-ARs, because they were inhibited by 1 $\mu$m propranolol, a blocker against $\beta_1$- and $\beta_2$-ARs, and correlations of $pK_r$ values of $\beta$-antagonists between in rat WAT and in other tissues were high, and that (2) the low-affinity sites for (--)$[^{3}H]$-CGP12177 are $\beta_3$-ARs, because they were not inhibited completely by 1 $\mu$m propranolol but almost completely inhibited by
100 μM bupranol, a blocker against β₁-, β₂-, and β₃-ARs at the micro molar order. Arch et al. reported that BRL37344A has more potent anti-obesity action in rats, hamster and dog fat cells than isoproterenol. In addition, Galitzky et al. have shown that in dog adipose tissues, the β₂-AR subtype was stimulated only at high catecholamine concentrations. It is because of the differences in the chemical structure of BRL37344A and BRL35135A from catecholamines that these β₃-agonists have a highly lipophilic Cl-substituent on the catechol ring and bulky 1-(4-methoxybenzyl)ethyl derivatives on the amine substituent. The specificity for β₂-ARs of these compounds might be attributed to their unique structures. BRL37344A, BRL35135A and SR59230A, which are also β₂-agonists, showed high affinity for the low-affinity site of (−)-[³H]-CGP12177 in rat WAT. And isoproterenol (pKᵢ = 4.42), epinephrine (pKᵢ = 3.62) and norepinephrine (pKᵢ = 3.88) had low affinity for the low-affinity site of (−)-[³H]-CGP12177 in rat WAT, in agreement with the studies by Muzzin et al. using Chinese hamster ovary (CHO) cells expressing cloned β₂-ARs. The correlations between the pKᵢ values obtained in rat WAT and the pKᵢ values obtained from rat cloned β₂-ARs or pKᵢ values of β-agonists and BRL37344A obtained from CHO cells expressing rat cloned β₂-ARs were high. Thus, these present results suggest that the low-affinity sites for catecholamines are β₂-ARs in rat WAT. Additionally, the 100 μM α-blockers (prazosin and yohimbine), serotonin, histamine and atropine, had no effect on (−)-[³H]-CGP12177 binding to high- and low-affinity sites for (−)-[³H]-CGP12177 in rat WAT as assessed by Scatchard analysis and displacement studies. These results suggested that (−)-[³H]-CGP12177 binding sites in the high- and low-affinity site of rat WAT were not α-ARs, serotonin, histamine and muscarinic receptors, but β-ARs. The present data show that only β₂-selective agonists have
high selectivity for low affinity sites for \((-\text{H})\)-CGP12177 in rat WAT as well as human and rat cloned $\beta_3$-ARs (Figs. 4 and 5). This supports that this assay system using rat WAT is useful for estimating the $\beta_3$-selectivity of various drugs. Other merits of this assay system are: (1) this system using rat WAT more closely resembles the physiological state than that transfected; (2) it is possible to compare the selectivity of various drugs for a $\beta_3$ subtype with that for other $\beta$ subtypes in this assay.

The correlations between the $pK_a$ values themselves and between the $pK_a$ values and $pK_{act}$ values were evident between the same species, but not between the $pK_a$ values of rat WAT vs. the $pK_a$ and $pK_{act}$ values for human cloned $\beta_3$-ARs (Figs. 4 and 5), although rat WAT $\beta$-ARs have selectivity for the $\beta_3$ agonist. These receptors might have common core sites among species that accept $\beta_3$-selective ligands, and have different modulation sites that are peculiar to species and influence ligand binding profiles. It might be possible to deduce the three-dimensional structures of these receptors by integration and comparison of the information about their structure-activity relationships, the three-dimensional structure of various $\beta$-AR selective agents, and the amino acid sequence of the receptors. As Lafontan et al.\textsuperscript{25,26} suggested, each subtype pharmacologically differs species- and tissue-specifically.

It is considered that $\beta_3$-AR selective agonists could be used to treat not only metabolic disorders such as obesity or diabetes in which weight control is difficult, but also heart failure and bronchial diseases during resistance to typical $\beta$-agonists.\textsuperscript{12,15,26,36} Thus, the assessment of binding to $\beta_2$- and $\beta_3$-ARs by agonists is important and useful in assessing their use in treatment.

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REFERENCES