Binding Characteristics of ^3H -CGP12177 to β -Adrenoceptors in Rat Myocardial Membranes

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Abstract—The present study was designed to examine the selectivity of ^3H -CGP-12177 (4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7- ^3H]benzimidazole-2-one hydrochloride) for β_1 - and β_2 -adrenergic receptors by the Scatchard and the displacement analysis. The plots of specific binding obtained from the Scatchard analysis using ^3H -CGP12177 for the rat myocardium membrane were uniphasic when the non-specific binding was determined by the use of $10\ \mu\text{M}$ *l*-propranolol, and the K_d and B_{max} values were $408.53 \pm 67.20\ \text{pM}$ and $12.27 \pm 0.83\ \text{fmol/mg protein}$, respectively. On the other hand, two binding sites were observed in the displacement curve when *l*-metoprolol was used as a competitor. The existence of these two binding sites implied the selectivity of ^3H -CGP12177 to β -adrenoceptors because ^3H -CGP12177 was 1.8-fold more selective towards β_1 -adrenoceptors than β_2 -adrenoceptors. In addition, these two binding sites could be regarded as β_1 - and β_2 -adrenergic receptors from the evaluation of the binding characteristics of β -adrenoceptors by the displacement analysis using β -selective antagonists. Thus, ^3H -CGP12177, a hydrophilic radioligand, was useful for the binding assay of β -adrenoceptors in rat myocardial membranes.

There is increasing evidence that data obtained by the radioligand binding assay method are useful for evaluating the pharmacological potencies of drugs in relation to β -adrenoceptors (1, 2). In general, the radioligands ^{125}I -iodocyanopindolol (^{125}I -ICYP), ^{125}I -iodohydroxybenzylpindolol (^{125}I -IHYP) and ^3H -dihydroalprenolol (^3H -DHA) are useful for the assessments of β -adrenoceptors in various tissues (1, 2). All of these radioligands are hydrophobic and often more binding sites are displaced by propranolol than isoproterenol, thus suggesting that ^{125}I -ICYP, ^{125}I -IHYP and ^3H -DHA bound to $5\text{HT}_{1\text{B}}$ -serotonergic receptors (3), to β -adrenoceptors (4) and to hydrophobic non-specific binding sites (5, 6), respectively. Our previous reports (7, 9) found that the treatment of the cardiac membrane fraction with neuraminidase resulted in a higher reproducibility of the binding assay using ^3H -DHA as a radioligand than was obtainable without neuraminidase treatment, thus suggesting

that the binding assay using rat myocardial membrane is more difficult and complicated than that using rat brain membrane. Recent studies indicate that ^3H -CGP12177, (4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7- ^3H]benzimidazole-2-one hydrochloride), is a hydrophilic β -adrenoceptor antagonist (10, 11). Thus, the present study was designed to examine the binding characteristics of ^3H -CGP12177 to β -adrenoceptors in rat myocardial membranes. In addition, Neve et al. (12) suggested that the selectivity of a radioligand for β_1 - and β_2 -adrenoceptors could be determined by displacement experiments but not by Scatchard analysis. Thus, the present study was also designed to examine the selectivity of ^3H -CGP12177 for β_1 - and β_2 -adrenoceptors by Scatchard analysis and displacement experiments.

Materials and Methods

Materials: (–)- ^3H -CGP12177 (53.1 Ci/

mmole) was purchased from Amersham Japan Co.

Preparation of the membrane-enriched fractions: The membrane-enriched fraction from rat heart was prepared by using the following method: Male Wistar rats weighing 250–400 g were killed by a blow on the head. After removal of the rat heart, the myocardium (approx. 1 g) was minced with small scissors in 20 ml of 10 mM Tris-HCl, 250 mM sucrose buffer (pH 7.4) and then homogenized using a Polytron homogenizer, twice for 10 sec at setting 8. The homogenate was filtered through 4 layers of gauze. The filtrate was centrifuged at 40,000 g for 30 min, and the resultant pellets were rinsed at once; then they were homogenized with a Polytron homogenizer, twice for 10 sec at setting 8, using 20 ml of 120 mM Tris-HCl, 40 mM MgCl₂ buffer (pH 7.2). The membrane-enriched fraction was frozen in liquid nitrogen, stored at –80°C and diluted to appropriate concentrations immediately before use. Protein concentrations were determined by the methods of Lowry et al. (13) using bovine serum albumin as the standard.

Binding assay: (a) The saturation binding assay was carried out in duplicate with ³H-CGP12177 in the presence (non-specific) and absence (total) of 10 μM l-propranolol. In brief, 0.25 ml of membrane suspension (0.2 mg of protein) was incubated for 60 min at 23°C with various concentrations (0.01–4.0 nM) of ³H-CGP12177 in a total volume of 0.5 ml containing 60 mM Tris-HCl and 20 mM MgCl₂ (pH 7.2). (b) Displacement experiments were carried out in duplicate with various concentrations (0.015–6.0 nM) of ¹²⁵I-ICYP in the presence of various concentrations of antagonists such as l-metoprolol. At the end of the incubation period, the incubation medium was immediately filtered through a GF/C glass fiber filter using the method described by Tsuchihashi et al. (9). The radioactivity was counted with a scintillation counter (Aloka ALC-500). The difference in mean values between the total and non-specific binding was taken as the specific binding.

Kinetic analyses: All kinetic analyses were carried out on an NEC PC-9801 computer system that performs iterative non-linear

regression (14–16). The models of the displacement curves were as follows:

$$\frac{B_1}{B_0} = \frac{A}{1 + \frac{x}{IC50_a}} + \frac{B}{1 + \frac{x}{IC50_b}} + \frac{C}{1 + \frac{x}{IC50_c}} + \dots + \frac{N}{1 + \frac{x}{IC50_n}}$$

where either B₁ or B₀ is the concentration of the radioligand bound with or without the cold ligand, x is the cold ligand concentration; IC50_a, IC50_b, IC50_c and IC50_n are the apparent inhibition constants of a cold ligand for receptors a, b, c and n, respectively; and A, B, C and N are the proportions of the ligand bound with receptors a, b, c and n, respectively.

The models for the determination of the true inhibition constant (K_i) of the cold ligand, the binding affinity (K_d) and the maximum binding capacity (B_{max}) of the radioligand were as follows:

I. Biphasic Scatchard plot (equation 1)

$$\frac{B}{F} = \frac{1}{2} \left[\frac{R_1 - B}{K_{d1}} + \frac{R_2 - B}{K_{d2}} + \sqrt{\left(\frac{R_1 - B}{K_{d1}} - \frac{R_2 - B}{K_{d2}} \right)^2 + 4 \frac{R_1 \cdot R_2}{K_{d1} \cdot K_{d2}}} \right]$$

II. Uniphasic Scatchard plot (equation 2)

$$\frac{B}{F} = \frac{1}{K_{d1}} (R_1 - B)$$

where K_{d1} and K_{d2} are dissociation constants between a radioligand and receptors 1 and 2, and R₁ and R₂ are the total concentrations of receptors 1 and 2 (B_{max}). For the purpose of this report, R₁ and R₂ are expressed as a percentage of the total B_{max} R₁ + R₂.

III. Displacement experiment used with various concentrations of radioligand: When displacement analyses are carried out using various concentrations of free radioligands (L), the values of K_i, R₁ and R₂ can be determined by the following equations with apparent IC50, A and B.

$$IC50 = K_i + \frac{L}{K_d} \times K_i \dots \dots \dots \text{(equation 3)}$$

$$\frac{B}{A} = \frac{R_1}{R_2} \left(\frac{L + K_{d2}}{L + K_{d1}} \right) \dots \dots \dots \text{(equation 4)}$$

IV. Displacement experiment conducted with the concentrations of radioligand as defined below (two receptor site model): When displacement analyses are carried out using a defined concentration of free radioligand (L), the values of K_i , R_1 and R_2 can be determined by the following equations:

(1) L/K_{d1} and $L/K_{d2} > 10$

$$\frac{B_1}{B_0} = \frac{R_1}{1 + \frac{x}{IC50_1}} + \frac{R_2}{1 + \frac{x}{IC50_2}} \quad \dots\dots \text{(equation 5)}$$

(2) L/K_{d1} and $L/K_{d2} < 1/10$

$$\frac{B_1}{B_0} = \frac{z}{1 + x/K_{i1}} + \frac{1-z}{1 + x/K_{i2}} \quad \dots\dots \text{(equation 6)}$$

where $z = R_1 \cdot K_{d2} / (R_1 \cdot K_{d2} + R_2 \cdot K_{d1})$
 $1-z = R_2 \cdot K_{d1} / (R_1 \cdot K_{d2} + R_2 \cdot K_{d1})$

V. Cheng and Prusoff equation

$$K_i = IC50 / (1 + L/K_d)$$

where A, B and z are the proportions of the ligand bound with receptors 1, 2 and 1, respectively. L is the concentration of free radioligand. R_1 and R_2 are expressed as a percentage of the total B_{max} $R_1 + R_2$. In some portions of this report, the K_i values are expressed as pK_i ($-\log K_i$) values.

VI. Determination of K_i values of various drugs using displacement analysis: In addition, the K_i values of various drugs for β_1 - and β_2 -adrenoceptors can be determined by the use of general models for displacement analysis with an appropriate concentration of the free radioligand (L) as in the following equation (equation 7):

$$\frac{B_1}{B_0} = \left(\frac{L \cdot R_1}{L + K_{d1}(1 + x/K_{i1})} + \frac{L \cdot R_2}{L + K_{d2}(1 + x/K_{i2})} \right) / \left(\frac{L \cdot R_1}{L + K_{d1}} + \frac{L \cdot R_2}{L + K_{d2}} \right)$$

where L is the concentration of free radioligand used. R_1 and R_2 are the proportional ratio of receptors 1 and 2 ($R_1 + R_2 = 1$), and K_{d1} and K_{d2} are the dissociation constants between a radioligand and receptors 1 and 2.

The values of R_1 , R_2 , K_{d1} and K_{d2} were preliminarily determined by the use of equations 3–6 and the determined values are substituted in equation 7. By means of these substitutions, the K_i values for two receptor sites can be directly determined by equation 7.

In these non-linear or linear regression analyses, the parameter fitting method, termination of iteration, and justification of the models were carried out by previously described methods (14, 16).

Pharmacological observations: β -Blocking actions were determined as described previously (8, 16, 17). The right and left atria of the guinea pig were used for the assessment of the antagonistic potencies against the positive chronotropic and inotropic actions of isoproterenol (β_1 -effect), respectively. The rate of the spontaneous contraction of the right atria was recorded on an ink-writing oscillograph to test the positive chronotropic action. The left atrium was stimulated electrically by a square-wave stimulator (Nihon Kohden SEN-3101) at the frequency of 1 Hz with voltages 30% above the threshold (duration=1 msec) to evaluate the inotropic effect. The contractile tension was recorded on an ink-writing oscillograph with a strain-gauge transducer and a carrier amplifier. Isolated tracheal smooth muscle preparations of the guinea pig were used to assess the antagonistic effects towards the β_2 -adrenoceptor. Isoproterenol was used as an agonist. Seven tracheal rings removed from the guinea pig were sutured together and mounted vertically in a 12 ml organ bath. The contractile tension of the preparation was recorded on a potentiometric recorder. Drugs were administered in a cumulative fashion, and were calculated using the previously described equation (8). The bathing solution used was Krebs-Henseleit solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 12 mM glucose, which was aerated with a mixture of 95% O_2 and 5% CO_2 . The temperature of the solution was maintained at $32 \pm 0.10^\circ C$.

Results

As shown in Fig. 1, the selectivity of ³H-CGP12177 for β_1 - and β_2 -adrenoceptors

were examined by Scatchard analysis. When Scatchard analysis was carried out in the absence (total) and presence (non-specific binding) of 10 μ M l-propranolol, the curve of specific binding was uniphasic in character, and the K_d and B_{max} values were 408.53 ± 67.20 pM ($n=5$) and 12.27 ± 0.83 fmoles/mg protein ($n=5$), respectively. On the other hand, when l-propranolol or l-metoprolol were used as the competitors, the displace-

ment curves showed uniphasic or biphasic curves, respectively (Fig. 2). Biphasic curves obtained from the displacement curves using l-metoprolol are composed of two binding sites which are expected to correspond to β_1 - and β_2 -adrenoceptor sites.

Table 1 also shows the values of K_d and B_{max} of the β_1 - and β_2 -adrenoceptor sites of

Fig. 1. Scatchard plots of ^3H -CGP12177 binding to the rat myocardium. Specific binding is defined as the difference between total binding and the binding in the presence of 10 μ M l-propranolol (non-specific binding) at ^3H -CGP12177 concentrations between 0.01 and 4 nM. The values of K_d and B_{max} were 408.53 pM and 12.27 fmoles/mg proteins, respectively. The data shown are those from a single experiment performed in duplicate. The experiment shown is representative of five such experiments. The points show specific (●) binding.

Fig. 2. Inhibition of ^3H -CGP12177 binding by l-propranolol (○) and l-metoprolol (●). Membranes were incubated with ^3H -CGP12177 (0.7 nM) in the presence of various concentrations of the antagonists. The values of apparent IC_{50} and proportional percent (%) were respectively: l-propranolol, 14.70 nM and 100%, l-metoprolol, 23.22 nM and 66.2% (β_1 -adrenoceptor sites) and 1.69 μ M and 33.8% (β_2 -adrenoceptor sites). The data shown are those from a single experiment performed in duplicate. The experiment shown is representative of three such experiments.

Table 1. Determination of the values of K_d and B_{max} in the binding of ^3H -CGP12177 to the myocardial membranes and K_i of l-metoprolol using various equations

Equation used	3	4	5	Mean
β_1 -Adrenoceptor binding sites				
n	(3)	(3)	(3)	
K_d (pM)	249.98 ± 66.04	244.62 ± 49.28	234.50 ± 54.05	243.03
B_{max} (%)	—	68.92 ± 0.68	71.87 ± 2.18	70.40
K_i (nM)	23.22 ± 6.10	—	33.70 ± 0.24	28.46
β_2 -Adrenoceptor binding sites				
n	(3)	(3)	(3)	
K_d (pM)	492.68 ± 96.78	510.27 ± 59.07	325.66 ± 76.99	442.87
B_{max} (%)	—	31.08 ± 0.68	28.13 ± 2.18	29.60
K_i (μ M)	1.69 ± 0.32	—	1.49 ± 0.77	1.59

The values in the parentheses indicate the number of experiments. Data are the mean values $\pm$ S.E. Free radioligand concentrations for equations 3 and 4 are in Fig. 3; and for equation 5, it was 6 nM ^3H -CGP12177. The data obtained by equation 6 could not be determined because the number of counts was very small.

³H-CGP12177 determined by the displacement experiments (equations 3–5) using I-metoprolol as the competitor. Data shown in Fig. 3 were calculated by applying equation

Table 2. pK_i values of β -antagonists

	pK _i values		Significance	Selectivity	$\beta_1 K_i / \beta_2 K_i$ ratio
	β_1 -site	β_2 -site			
1. Arotinolol (3)	9.57±0.21	9.65±0.39	N	$\beta_1 = \beta_2$	0.83
2. Pindolol (3)	9.02±0.31	8.55±0.28	N	$\beta_1 = \beta_2$	3.0
3. l-Propranolol (3)	8.92±0.23	9.20±0.41	N	$\beta_1 = \beta_2$	0.52
4. dl-Propranolol (3)	8.81±0.14	9.11±0.12	N	$\beta_1 = \beta_2$	0.50
5. Oxprenolol (3)	8.56±0.06	8.28±0.35	N	$\beta_1 = \beta_2$	3.0
6. Alprenolol (3)	8.15±0.14	9.00±0.18	A	$\beta_1 < \beta_2$	0.14
7. Labetalol (3)	8.03±0.42	7.67±0.47	N	$\beta_1 = \beta_2$	2.3
8. YM-09538 (3)	7.94±0.16	8.02±0.43	N	$\beta_1 = \beta_2$	0.83
9. Betaxolol (3)	7.78±0.07	6.42±0.09	C	$\beta_1 > \beta_2$	23
10. l-Metoprolol (3)	7.46±0.03	5.82±0.25	B	$\beta_1 > \beta_2$	44
11. dl-Metoprolol (3)	6.88±0.17	4.97±0.27	B	$\beta_1 > \beta_2$	81
12. ICI118,551 (3)	6.58±0.06	8.40±0.57	A	$\beta_1 < \beta_2$	0.012
13. Acebutolol (3)	6.53±0.19	5.01±0.36	A	$\beta_1 > \beta_2$	33
14. d-Propranolol (3)	6.41±0.07	6.32±0.03	N	$\beta_1 = \beta_2$	1.2
15. Atenolol (3)	6.07±0.11	5.10±0.05	B	$\beta_1 > \beta_2$	9.3
16. Sotalol (3)	6.04±0.10	6.07±0.67	N	$\beta_1 = \beta_2$	0.93
17. Practolol (3)	5.80±0.14	5.06±0.14	A	$\beta_1 > \beta_2$	5.5
18. Butoxamine (3)	5.05±0.22	6.03±0.16	A	$\beta_1 < \beta_2$	0.10
19. d-Metoprolol (3)	4.86±0.15	4.25±0.07	A	$\beta_1 > \beta_2$	4.1

Values in the parentheses are the number of experiments. Data are the mean values±S.E. These data were obtained by the displacement analysis using 0.7 nM ³H-CGP12177 and calculated by equation 7. A: P<0.05, B: P<0.01, C: P<0.001 and N: not significant.

Fig. 3. Plots of various free radioligand concentrations vs. its IC₅₀ values of l-metoprolol for two binding sites (equation 3). A. β_1 -adrenoceptor binding site ($y = 92.85x + 2.36 \times 10^{-8}$, $K_i = 23.6$ nM, $K_d = 0.25$ nM). B. β_2 -adrenoceptor binding site ($y = 4721.21x + 2.13 \times 10^{-6}$, $K_i = 2.13$ μ M, $K_d = 0.45$ nM). The data shown are those from a single experiment performed in duplicate. The experiment shown is representative of three such experiments.

Table 3. pA_2 values of β -antagonists

	Right atrial strip	pA_2 values Left atrial strip	Trachea
1. Arotinolol*	9.09±0.09 (5)	9.31±0.09 (5)	9.58±0.16 (5)
2. Pindolol*	9.04±0.11 (6)	8.79±0.12 (6)	8.93±0.06 (6)
3. l-Propranolol*	8.92±0.26 (6)	9.08±0.47 (6)	9.25±0.26 (5)
4. dl-Propranolol*	8.41±0.08 (8)	8.36±0.06 (8)	8.41±0.05 (8)
5. Oxprenolol*	8.67±0.05 (5)	8.49±0.04 (6)	8.21±0.08 (5)
6. Alprenolol*	8.34±0.02 (5)	8.26±0.08 (5)	8.87±0.04 (5)
7. Labetalol*	7.62±0.08 (6)	7.84±0.14 (6)	7.63±0.07 (7)
8. YM-09538	7.91±0.22 (3)	7.82±0.23 (4)	7.70±0.47 (4)
9. Betaxolol	8.28±0.26 (6)	8.74±0.12 (4)	7.03±0.50 (5)
10. l-Metoprolol	7.80±0.06 (5)	7.66±0.08 (5)	6.42±0.06 (5)
11. dl-Metoprolol*	7.72±0.04 (5)	7.63±0.09 (5)	5.67±0.16 (5)
12. IC118,551*	7.40±0.16 (6)	7.26±0.17 (6)	9.08±0.14 (5)
13. Acebutolol*	7.22±0.03 (5)	7.12±0.03 (5)	5.57±0.09 (5)
14. d-Propranolol*	7.38±0.31 (4)	6.94±0.77 (4)	6.98±0.03 (2)
15. Atenolol*	7.44±0.04 (5)	7.14±0.03 (5)	5.61±0.06 (5)
16. Sotalol*	6.22±0.12 (5)	6.19±0.05 (5)	6.83±0.08 (6)
17. Practolol*	7.10±0.18 (6)	6.83±0.12 (6)	4.39±0.09 (6)
18. Butoxamine*	6.44±0.04 (4)	6.00±0.09 (5)	6.72±0.13 (5)
19. d-Metoprolol	5.57±0.16 (5)	5.64±0.07 (5)	4.46±0.06 (5)

Values in the parentheses are the number of experiments. Data are the mean values±S.E. *Some of these data were reported previously (8, 16).

3 to the straight line obtained when IC50 values were plotted against various concentrations of free radioligand. The proportional percentages of B_{max} of these two radioligand. The proportional percentage of B_{max} of these two binding sites (β_1 - and β_2 -adrenoceptors) for 3H -CGP12177 were 70.40:29.60, and the radioligand was 1.8-fold more selective for β_1 -adrenoceptors than β_2 -adrenoceptors. These results suggested that the displacement experiments provided the values of K_d , B_{max} and K_i for l-metoprolol and were able to discriminate between β_1 - and β_2 -adrenoceptors.

Table 2 summarizes the pK_i values, which are directly determined by equation 7, of the β -adrenergic antagonists for β_1 - and β_2 -adrenoceptors in 3H -CGP12177 binding. β_1 -Selective antagonists (acebutolol, atenolol, betaxolol, metoprolol isomers and practolol) were more selective for the β_1 -adrenoceptor binding sites than the β_2 ones, whereas β_2 -selective antagonists (butoxamine and IC118,551) were more selective for the β_2 -adrenoceptor binding sites. These results strongly suggested that these two binding

sites for 3H -CGP12177 corresponded to β_1 - and β_2 -adrenoceptors, respectively. Furthermore, these results suggested that alprenolol, which is usually known as a non-selective β -antagonists, was 7.1-fold more selective to β_2 -adrenoceptors.

Table 3 also summarizes the pA_2 values of these β -adrenergic antagonists, which have already been reported or newly assessed in the present study, with respect to their antagonistic effects for positive chronotropic and inotropic actions and relaxation of tracheal smooth muscle by isoproterenol. Figure 4 depicts the relationship between the K_i values of these drugs for β_1 - and β_2 -adrenoceptors determined by the binding assay in the present study and the pA_2 values determined by the pharmacological observations. For the selective antagonists for β_1 - and β_2 -adrenoceptors, there were especially good correlations between the pK_i values obtained in this study and pA_2 values for the following (Fig. 4): β_1 -sites vs. positive inotropic action (Fig. 4A), β_1 -sites vs. positive chronotropic action (Fig. 4B), and β_2 -sites vs. tracheal relaxation (Fig. 4F).

Fig. 4. Relationship between the potencies of the chemicals for inhibition of ^3H -CGP12177 binding to β_1 - and β_2 -adrenoceptors (pK_i) and the potencies of inhibition of the positive inotropic (A, D), positive chronotropic (B, E) and tracheal relaxation (C, F) effects of l-isoproterenol as assessed by pharmacological methods in guinea pig (pA_2). The pK_i values of non- (●) and selective (○) antagonists that were defined by ^3H -CGP12177 bindings were those of the β_1 -affinity sites (A, B, C) and β_2 -affinity sites (D, E, F) in Table 2. Numbers refer to individual drugs in Tables 2 and 3. A: $y = 0.65x + 3.00$, $r = 0.94$ ($P < 0.001$). B: $y = 0.72x + 2.40$, $r = 0.96$ ($P < 0.001$). C: $y = 0.91x + 0.60$, $r = 0.79$ ($P < 0.001$). D: $y = 0.42x + 4.78$, $r = 0.76$ ($P < 0.001$). E: $y = 0.45x + 4.47$, $r = 0.76$ ($P < 0.001$). F: $y = 0.88x + 1.04$, $r = 0.96$ ($P < 0.001$). Inset, the values of pK_i and pA_2 used were those of the selective antagonists for β_1 - and β_2 -adrenoceptors. A: $y = 0.71x + 2.68$, $r = 0.94$ ($P < 0.001$). B: $y = 0.82x + 1.90$, $r = 0.96$ ($P < 0.001$). C: $y = 0.85x + 0.83$, $r = 0.58$. D: $y = 0.29x + 5.57$, $r = 0.55$. E: $y = 0.30x + 5.40$, $r = 0.50$. F: $y = 1.00x + 0.40$, $r = 0.96$ ($P < 0.001$).

Discussion

Since the plot obtained from the Scatchard analysis was linear, Scatchard analysis can not determine the selectivity of ^3H -CGP-12177 for β_1 - and β_2 -adrenoceptors. On the

other hand, our displacement experiments using l-metoprolol clearly separated the two types of bindings sites for ^3H -CGP12177 on the membrane of rat myocardium. The results suggested that the displacement experiments using equations 3–5 elucidated the detailed

selectivity of radioligands for receptors and the K_i values of the chemicals used in these experiments such as l-metoprolol. In addition, our data indicated that the two binding sites could be regarded as β_1 - and β_2 -adrenoceptors because l-metoprolol inhibited these two adrenoceptors, and good correlations between pK_i by the radioligand binding assay method and pA_2 values obtained from pharmacological experiments using β_1 - and β_2 -antagonists were observed.

The radioligand ^3H -CGP12177 was 1.8-fold more selective for β_1 -adrenoceptors than β_2 -adrenoceptors. The determination of the selectivity of the radioligands is necessary for the following reasons: the determination of the percentage ratio of β_1 - and β_2 -adrenoceptors in the case of a radioligand with different selectivity for β_1 - and β_2 -adrenoceptors is difficult because the ratio changes depending on the concentration of free radioligand. In fact, the apparent observed percentages of l-metoprolol for β_1 - and β_2 -adrenoceptors were 66.2 and 33.8% when the displacement experiments were carried out by 0.7 nM ^3H -CGP12177 (Fig. 2). In this case, the determination of the ratio must be carried out by displacement experiments using free ligand concentrations 10-fold above the K_d values (using equation 5). This binding assay employing ^3H -CGP12177 also provides a very easy and simple procedure for determining the value of K_d for β_1 - and β_2 -adrenoceptors. Thus, K_i values of cold ligands were easily determined by the use of equation 7. Thus, the determination of selectivity of the radioligands is necessary in order to obtain exact assessments of the percentage ratios of receptor subtypes and K_i values of chemical compounds.

The assessments of selectivity of the radioligand, ^3H -CGP12177, were carried out by the displacement experiments in this study. In particular, the evaluations of K_d and $B_{\max}$ for the radioligand to receptors and K_i values of chemical compounds can be performed by the use of equations 3 and 5. These two methods were simple and easy. Thus, one method (equation 3), as depicted in Fig. 3, was carried out by the use of various free radioligand concentrations, and the other method (equation 5) was carried out by using

a large concentration of free radioligand. Because of the increased sensitivity of these methods relative to conventional methods for assessing the selectivity of a radioligand, it is probable that other radioligands that are thought to be nonselective for subtypes of β -adrenoceptors or other receptors will be found to be selective.

^3H -CGP12177 is a hydrophilic β -adrenoceptor antagonist (10, 11). It is now well-known that the non-specific binding of this radioligands to turkey erythrocyte ghosts and C6 glioma cells was less than 5% of the total binding (10). In addition, Lacasa et al. (11) reported that the non-specific binding of this compound to intact rat adipocytes was also less than 30% of the total binding. Thus, these authors indicated that ^3H -CGP12177 did not bind nonspecifically to cells. In the present study, the use of this radioligand resulted in a high reproducibility of the binding assay of rat myocardial membrane. In our previous reports (7, 9), the binding assay of rat myocardial membrane using ^3H -DHA as a radioligand was difficult and complicated, and the myocardial membranes needed to be treated with neuraminidase. Furthermore, the hydrophobic radioligands ^{125}I -ICYP, ^{125}I -IHYP and ^3H -DHA were often bound to β -adrenoceptors and other receptor sites, and the bindings of these ligands were displaced by propranolol rather than isoproterenol (3, 6). In the present study, this treatment was not necessary because ^3H -CGP12177 bound to only β -adrenoceptors, thus suggesting that ^3H -CGP12177, a hydrophilic radioligand, was useful for the binding assay of β -adrenoceptors in rat myocardial membranes.

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