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Determination of β-adrenoceptor subtype on rat isolated ventricular myocytes by use of highly selective β-antagonists

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1 The relative proportions of β_1- and β_2-adrenoceptors were determined by radioligand binding studies in three different rat myocardial preparations: membranes prepared from rat ventricle (ventricular membranes), membranes prepared from rat isolated ventricular myocytes (myocyte membranes), and myocytes isolated from rat ventricle (myocytes).

2 Competition experiments using CGP 20712A or ICI 118,551 with [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) revealed high- and low-affinity binding sites in ventricular membranes. The concentration at which each β-antagonist occupied 100% of its high-affinity binding sites was 300 nM for CGP 20712A (β_1-adrenoceptor) and 50 nM for ICI 118,551 (β_2-adrenoceptor).

3 The density of high-affinity (β_1-adrenoceptor) and low-affinity (β_2-adrenoceptor) binding sites for CGP 20712A was measured by a saturation experiment using [^{125}I]-ICYP in the presence and absence of 300 nM CGP 20712A. In ventricular membranes, the proportions of high-affinity and low-affinity binding sites for CGP 20712A were 73% and 27%, respectively, whereas in myocyte membranes, the corresponding figures were 90% and 10%, respectively. The density of low-affinity binding sites for CGP 20712A in ventricular membranes, defined as [^{125}I]-ICYP-specific binding in the presence of 300 nM CGP 20712A, was decreased by addition of 50 nM ICI 118,551, whereas that in myocyte membranes was not affected.

4 In myocytes, specific binding of [^{125}I]-ICYP and [^{3}H]-CGP 12177 was not detected by saturation experiments performed in the presence of 300 nM CGP 20712A.

5 In myocytes, the activation of adenylate cyclase caused by β_2-adrenoceptors was not detected in the presence of 10 nM, 100 nM or 1000 nM CGP 20712A, which selectively antagonized β_1-adrenoceptors. Furthermore, the concentration-response curve for isoprenaline-stimulated cyclic AMP accumulation was not shifted by 10 nM or 100 nM ICI 118,551, which selectively antagonized β_2-adrenoceptors, but was shifted to the right by 1000 nM ICI 118,551.

6 These results indicate that β_2-adrenoceptors are not present on rat ventricular myocytes and that β_2-adrenoceptor stimulation does not cause any detectable production of cyclic AMP. We conclude that only β_1-adrenoceptors exist on rat ventricular myocytes.

Keywords: β-Adrenoceptor subtype; myocytes; CGP 20712A; ICI 118,551; adenylate cyclase activity; cyclic AMP accumulation

Introduction

Radioligand binding studies have demonstrated the coexistence of β_1- and β_2-adrenoceptors in the mammalian cardiac ventricle (Brodde, 1991). It has been reported that β_1- and β_2-adrenoceptors are differentially involved in ventricular function. For example, in human ventricle both β_1- and β_2-adrenoceptors mediate a catecholamine-induced positive inotropic effect (Kaumann & Lemoine, 1987; Motomura *et al.*, 1990), whereas, β_2-adrenoceptors are much more efficiently coupled to adenylate cyclase than β_1-adrenoceptors (Bristow *et al.*, 1989). Although the coexistence of β_1- and β_2-adrenoceptors in rat ventricle has been suggested by a binding study (Vago *et al.*, 1984), it is still unknown how each β-adrenoceptor subtype is involved in ventricular function.

Estimation of the relative proportions of β_1- and β_2-adrenoceptors in myocytes is necessary in order to examine the role of β-adrenoceptor subtypes in ventricular function. However, radioligand binding studies with ventricular membranes do not provide an accurate estimate of each subtype in myocytes, since the ventricle also contains non-myocyte cells such as those in coronary arteries and connective tissue. Autoradiographic studies of canine ventricle have revealed that the distribution of β-adrenoceptor subtypes in myocytes is different from that in coronary vessels (Murphree & Saffitz, 1988). Therefore, for experimental purposes, isolated myocytes should be used to avoid contamination with non-myocyte cells.

In the present study we determined the relative proportions of β_1- and β_2-adrenoceptor subtypes in rat isolated ventricular myocytes. To estimate the proportions of the β-adrenoceptor subtypes accurately, we used a highly selective β_1-adrenoceptor antagonist, CGP 20712A, which is one thousand times more selective for β_1- than for β_2-adrenoceptors (Dooley *et al.*, 1986), and the β_2-adrenoceptor-selective antagonist, ICI 118,551 (Bilski *et al.*, 1983). The percentage of β_1- and β_2-adrenoceptors were determined by radioligand binding studies using membranes prepared from rat ventricle (ventricular membranes), membranes prepared from rat isolated myocytes (myocyte membranes), and myocytes isolated from rat ventricle (myocytes). Two different radioligands, the hydrophobic radioligand [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) and the hydrophilic radioligand [^{3}H]-CGP 12177, were used in myocytes.

Furthermore, we measured isoprenaline-stimulated adenylate cyclase activity and adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in isolated ventricular myocytes in the presence and absence of CGP 20712A or ICI 118,551 to examine which β-adrenoceptor subtype is involved in ventricular function.

We found that no β-adrenoceptor was detectable in rat ventricular myocytes by radioligand binding studies and that only β_1-adrenoceptors induced isoprenaline-stimulated cyclic AMP production.

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Methods

Isolation of ventricular myocytes

A male Sprague-Dawley rat (body weight 250–350 g) was anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). The heart was rapidly excised, placed in ice-cold Tyrode solution (composition, mM: NaCl 135, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 2 and glucose 10, pH 7.2) containing 2.6 mM EGTA, and attached via a cannula in the aorta to a Langendorff apparatus. All of the solutions used for perfusion were kept at 37°C and gassed continuously with 100% O_2. The perfusion was performed with Tyrode solution containing 2.6 mM EGTA for 2 min, high-K solution (NaCl 4 mM, K-glutamate 130 mM, KCl 4 mM, $CaCl_2$ 20 μM, $MgCl_2$ 1 mM, HEPES 10 mM, glucose 10 mM, pH 7.2) for 5 min, high-K solution containing trypsin (100 u ml^{-1}) for 15 min, and then with high-K solution containing collagenase (350 u ml^{-1}) for approximately 15–20 min, followed by high-K solution for 2 min to wash out the enzyme. The ventricles were cut into 3–4 sections, and placed in 30 ml high-K solution containing trypsin inhibitor (0.24 mg ml^{-1}) and bovine serum albumin (1 mg ml^{-1}). The ventricle sections were incubated at 37°C for 10 min with gentle shaking (120 cycles min^{-1}). The resulting cell suspension was filtered through a membrane filter and centrifuged at 50 *g* for 90 s. The pellet was resuspended in HEPES-buffered Joklik-modified MEM (HEPES-MEM) containing 2 mM $CaCl_2$ and 10 mM HEPES ($NaHCO_3$ was substituted with equimolar NaCl), and incubated at room temperature for 60 min to select calcium-tolerant cells. The cell suspension was then layerd on a Percoll gradient preformed by centrifugation of 50% (v/v) HEPES-MEM-Percoll solution (pH 7.2) at 30,000 *g* for 60 min. This Percoll gradient was then centrifuged at 1800 *g* for 45 min. Isolated myocytes were collected from the band at a density of 1.063–1.075 (g ml^{-1}), and centrifuged at 50 *g* for 2 min. Finally, we obtained $1-2 \times 10^6$ myocytes, 70–80% of which were rod-shaped and excluded trypan blue.

Membrane preparation

The ventricular membranes were prepared according to the method of U'Prichard *et al.* (1978) with some modifications. A male Sprague-Dawley rat (250–350 g) was anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). The heart was rapidly excised, and placed in ice-cold Tyrode solution containing 2.6 mM EGTA (pH 7.5). All of the subsequent procedures were performed at 0–4°C. The ventricle was minced with scissors in 10 volumes of ice-cold 50 mM Tris-HCl (pH 7.5), and homogenized twice with a Polytron (setting 6, 15 s). The homogenate was filtered through 4 layers of cotton gauze, and centrifuged at 28,000 *g* for 10 min. The pellet was resuspended in ice-cold 50 mM Tris-HCl and recentrifuged as described above. The pellet was again resuspended in ice-cold 50 mM Tris-HCl and then filtered through 4 layers of cotton gauze.

The myocyte membranes were obtained from isolated myocytes, which were collected by centrifugation of the cell suspension at 50 *g* for 30 s and resuspended in 50 mM Tris-HCl (pH 7.5). The membranes were prepared by the method described above without filtration through 4 layers of cotton gauze.

Both membrane samples were used on the day of preparation.

Radioligand binding studies

The membranes (80–100 and 30–40 μg protein/tube for ventricle and myocyte membranes, respectively) or cell suspension (5000 and 30000 myocytes per tube for [^{125}I]-ICYP and for [^{3}H]-CGP 12177, respectively) were incubated with radioligand and β-antagonist in a total volume of 300 μl. In competition experiments, 30 pM [^{125}I]-ICYP (specific activity 2000 Ci mol^{-1}) or 0.6 nM [^{3}H]-CGP 12177 (30–60 Ci mol^{-1}) was used. Incubation was carried out for 90 min ([^{125}I]-ICYP), or 60 min ([^{3}H]-CGP 12177) at 37°C. The binding reaction was terminated by addition of ice-cold 50 mM Tris-HCl (pH 7.5) to the membranes, or ice-cold Tyrode solution (pH 7.2) to the cell suspension, followed by rapid vacuum filtration through Whatman GF/C glass fibre filters using a cell harvester (model M-24, Brandel). The filters were washed with an additional 5 ml of ice-cold 50 mM Tris-HCl or Tyrode solution.

Radioactivity of [^{125}I]-ICYP was counted in a gamma counter (ARC-300, Aloka). [^{3}H]-CGP 12177-bound filters were placed in 5 ml of Aquasol (NEN) for 120 min, then radioactivity was determined with a liquid scintillation counter (LSC-700, Aloka).

All determinations were performed in duplicate. Nonspecific binding was determined with 1 μM propranolol. Specific binding was defined as the difference between total and nonspecific binding. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin used as the standard.

β-Agonist-stimulated adenylate cyclase activity

The isolated myocytes were collected by centrifugation of the cell suspension at 50 *g* for 30 s. The myocytes were then resuspended in Tris-EDTA buffer (Tris 5 mM, EDTA 5 mM, pH 7.4), and centrifuged at 1,800 *g* for 10 min, and resuspended in HEPES-EDTA buffer (HEPES 40 mM, EDTA 0.8 mM, $MgCl_2$ 5 mM, ascorbic acid 1 mM, pH 7.4). The lysate was homogenized with a Teflon homogenizer (600–800 r.p.m.). The membranes were incubated for 30 min at 37°C with a reaction mixture (ATP 0.12 mM, GTP 0.05 mM, phosphoenolpyruvate 2.8 mM, myokinase 1 u/tube, pyruvate kinase 0.2 u/tube, HEPES 40 mM, EDTA 0.8 mM, $MgCl_2$ 5 mM, IBMX 0.5 mM), isoprenaline and CGP 20712A or HEPES-EDTA buffer in a total volume 150 μl. The reaction was terminated by addition of 150 μl 0.1 N HCl, followed by boiling for 2 min. Samples were centrifuged at 1,000 *g* for 20 min at 4°C, and the content of cyclic AMP in the supernatant was assayed by a sensitive radioimmunoassay method (Honma *et al.*, 1977) using a cyclic AMP kit (Yamasa Shoyu, Choshi, Japan).

β-Agonist-stimulated cyclic AMP accumulation

The myocytes were suspended in HEPES-MEM containing 1 mM ascorbic acid, and the cell suspension was incubated with β-antagonist or MEM for 60 min. The reaction was started by addition of β-agonist and carried out at 37°C for 3 min in the presence of 0.5 mM IBMX, which was added 5 min prior to addition of the β-agonist. The reaction was terminated by addition of 30% (w/v) trichloroacetic acid (TCA) containing 50 mM EDTA. The samples were boiled for 2 min, sonicated for 10 s, and centrifuged at 1,000 *g* for 20 min at 4°C. The supernatant was collected in a polypropylene tube. The pellet was resuspended, sonicated and recentrifuged as described above. The supernatant was then added to the supernatant collected in the previous step. Cyclic AMP was extracted three times with water-saturated ether, and the content of cyclic AMP in this sample was assayed.

Data analysis and statistics

The IC_{50} value of the competition curve was calculated by nonlinear regression analysis using a computer programme, LBS (A. Seo, Hiroshima University). The data obtained from the competition experiments were fitted to an equation for a single binding site model (1) or a two binding site model (2):

$$\text{bound} = 1 - I^p/(IC_{50,1}{}^p + I^p) \quad (1)$$

$$\text{bound} = 1 - I/(IC_{50,1} + I) - I/(IC_{50,2} + I) \quad (2)$$

where bound is the occupancy of radioligands, I is the concentration of β-antagonist, p is the Hill coefficient of the

competition curve, and $IC_{50,1}$ and $IC_{50,2}$ are the IC_{50} values of the β-antagonist binding sites. The equilibrium dissociation constant (K_d) and maximal density of binding sites (B_{max}) were determined by fitting the data obtained from the saturation experiments to the Michealis-Menten equation by nonlinear regression analysis using a computer programme, SP123 (H. Ono, Tokyo University). K_i values for β-antagonists were calculated using the equation of Cheng & Prusoff (1973). The EC_{50} value for each concentration-response curve was calculated by fitting the experimental data to the logistic equation by non-linear regression analysis. The K_b value was calculated by equation (3) (Besse & Furchgott, 1976).

$$K_b = I/(r - 1) \quad (3)$$

where I is the concentration of ICI 118, 551, r is the dose-ratio for EC_{50} value. We analysed the shifts of the concentration-response curves induced by CGP 20712A with Schild regression (Arunlakshana & Schild, 1959). Statistical significance was assessed with the Student-Welch t test for simple comparisons or the ANOVA-Bonferroni multiple t test for multiple comparisons; differences at $P<0.05$ were considered significant.

Materials

CGP 20712A (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol methanesulphonate) and ICI 118,551 (erythro-±-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) were kindly provided by Ciba-Geigy (Basal, Switzerland) and Tanabe Seiyaku (Osaka, Japan), respectively. Other drugs used and their sources were as follows: (-)-[^{3}H]-CGP 12177 ((-)-4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one) (NEN, Boston, U.S.A.), (-)-[^{125}I]-iodocyanopindolol (Amersham, Tokyo, Japan), 3-isobutyl-1-methylxanthine (IBMX) (Wako, Osaka, Japan), collagenase S1 (Nitta Zeratin, Osaka, Japan), trypsin type III, trypsin inhibitor type III-O, (-)-isoprenaline hydrochloride, phosphoenolpyruvate, pyruvate kinase, myokinase (Sigma, St. Louis., U.S.A.), Joklik-modified MEM (Gibco, New York, U.S.A.), Percoll (Pharmacia, Uppsala, Sweden).

Results

Radioligand binding studies with ventricular and myocyte membranes

Competition experiments In ventricular membranes, nonspecific binding of [^{125}I]-ICYP was 25% of the total binding. The competition curve for propranolol was monophasic, and had a Hill coefficient of unity, whereas those for CGP 20712A and ICI 118,551 were biphasic (Figure 1a). The proportions of high-affinity and low-affinity binding sites for CGP 20712A, calculated from the competition curve, were 72.6±2.2% and 27.4±2.2%, respectively, whereas those for ICI 118,551 were 10.7±3.2% and 89.3±3.2%, respectively ($n=4$).

As shown in Table 1, the K_i value for CGP 20712A at its high-affinity binding sites was 1200 times higher than that at its low-affinity binding sites, whereas the K_i value for ICI 118,551 at its high-affinity binding sites was 200 times higher than that at its low-affinity binding sites. The selectivity of these β-antagonists corresponded to the results described previously (Brodde *et al.*, 1983; Dooley *et al.*, 1986), and indicated that the high- and low-affinity binding sites for CGP 20712A are β_1- and β_2-adrenoceptors, respectively, whereas those for ICI 118,551 are β_2- and β_1-adrenoceptors, respectively.

In myocyte membranes, nonspecific binding of [^{125}I]-ICYP was 25% of the total binding. The competition curve for propranolol was monophasic and had a Hill coefficient of unity, whereas that for CGP 20712A was biphasic. However,

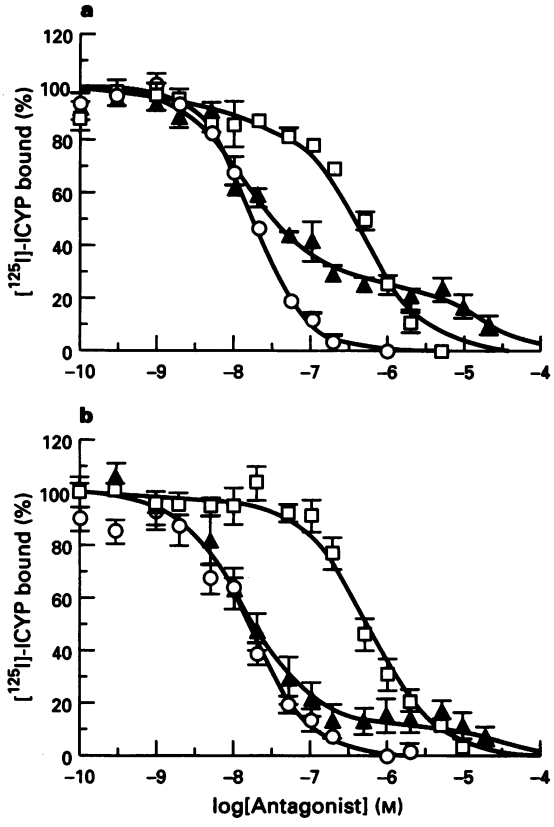

Figure 1 Competition of [^{125}I]-ICYP binding in rat ventricular membranes (a) and myocyte membranes (b) with propranolol (○), CGP 20712A (▲) and ICI 118,551 (□). Each point shows mean±s.e. for four different experiments.

Table 1 Competition of [^{125}I]-ICYP binding with β-antagonists in membranes prepared from rat ventricle and isolated ventricular myocytes

β-*Antagonists*			*Ventricular membrane*		*Myocyte membrane*
Propranolol	K_i (nM)		4.03±0.34		4.63±0.39
	Hill coefficient		1.262±0.12		1.066±0.08
CGP 20712A	K_i (nM)	(High)	3.29±0.20	(High)	3.99±0.85
		(Low)	3995±1407	(Low)	11127±1230*
ICI 118551	K_i (nM)	(High)	0.55±0.30	(High)	ND
		(Low)	107±20	(Low)	182±28

Data are represented as means±s.e. ($n=4$).
* Significantly different from ventricular membrane ($P<0.05$).
ND: not detectable.

in contrast to ventricular membranes, the competition curve for ICI 118,551 was monophasic and no low-affinity binding site for ICI 118,551 binding site was detected (Figure 1b). The proportions of high-affinity and low-affinity CGP 20712A binding sites, calculated from the competition curve, were 87.3±4.7%, and 12.7±4.7%, respectively ($n=4$). The low-affinity CGP 20712A binding sites in the myocyte membranes were less numerous and had lower affinity than those in ventricular myocytes. The K_i value of the low-affinity CGP 20712A binding sites in myocyte membranes was 3 times higher than that in ventricular membranes (Table 1).

The K_i value of CGP 20712A at its high-affinity binding site in myocyte membranes was not significantly different from that in ventricular membranes. Thus the high-affinity CGP 20712A binding site in both membranes is the β_1-adrenoceptor. The K_i value of the low-affinity binding site for ICI 118,551 in myocyte membranes was not significantly different from that in ventricular membranes, indicating that this binding site is the β_1-adrenoceptor.

This difference in proportion and affinity may have been due to enzymatic digestion of the β-adrenoceptors. Thus, in order to check the effects of enzymatic digestion on CGP 20712A binding, we performed competition experiments using membranes prepared from ventricle digested with enzyme under the same conditions as those used for myocyte isolation. In the enzyme-treated ventricular membranes, the proportions of high-affinity and low-affinity sites for CGP 20712A were 64.6% and 37.4%, respectively, and the corresponding IC_{50} values for CGP 20712A from its competition curves were 6.0 nM and 6462 nM, respectively ($n=2$). These values were close to those for untreated ventricular membranes (Table 1), suggesting that the enzymatic treatment did not affect the proportions and affinities of the β-adrenoceptor subtypes.

The concentrations at which each β-antagonist occupied the 100% of its high-affinity binding sites, i.e. β_1-adrenoceptors for CGP 20712A and β_2-adrenoceptors for ICI 118,551, were calculated, using equation (2), to be 300 nM and 50 nM, respectively. On the basis of these results, we defined the low-affinity CGP 20712A binding as the [^{125}I]-ICYP binding detected in the presence of 300 nM CGP 20712A, and the high-affinity binding as [^{125}I]-ICYP total binding minus the low-affinity binding.

Saturation experiments We performed saturation experiments to measure the B_{max} values of high-affinity and low-affinity CGP 20712A binding sites. The saturation curves for [^{125}I]-ICYP in the presence and absence of 300 nM CGP 20712A were monophasic and their Scatchard plots were linear for both ventricular and myocyte membranes (Figure 2). In the absence of CGP 20712A, the K_d and B_{max} value for [^{125}I]-ICYP were 10.3 pM and 17.2 fmol mg^{-1} protein in ventricular membranes, and 13.3 pM and 21.8 fmol mg^{-1} protein in myocytes membranes, respectively. In the presence of 300 nM CGP 20712A, the K_d and B_{max} value for [^{125}I]-ICYP were 16.6 pM and 4.6 fmol mg^{-1} protein in ventricular membranes, and 25.7 pM and 2.28 fmol mg^{-1} protein in myocyte membranes, respectively. The proportions of high-affinity and low-affinity binding sites were 73% and 27% in ventricular membranes, and 90% and 10% in myocytes membranes, respectively (Table 2). These results correspond to those obtained from the competition experiment (Figure 1).

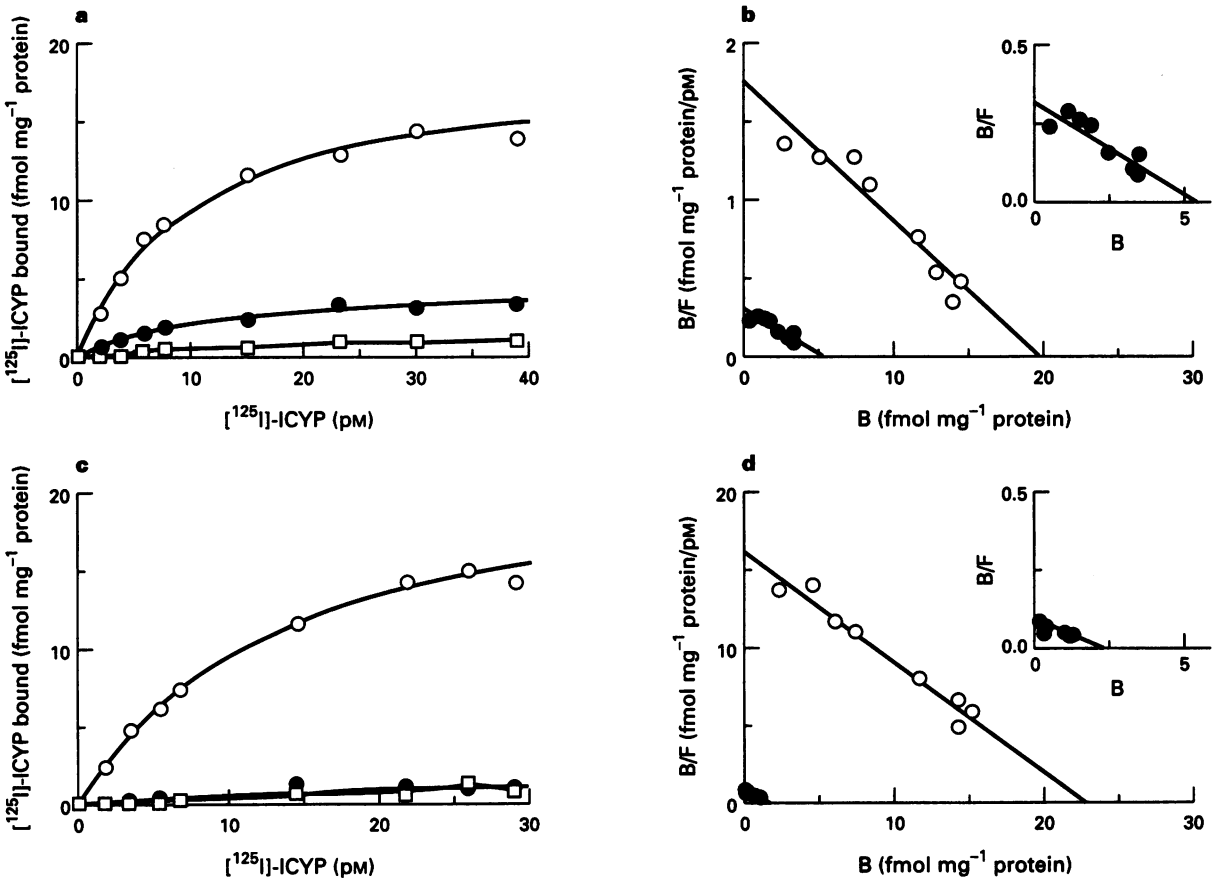

Figure 2 Binding of [^{125}I]-ICYP in rat ventricular membranes (a) and myocyte membranes (c) in the absence (○) and presence of 300 nM CGP 20712A (●) and presence of 300 nM CGP 20712A and 50 nM ICI 118,551 (□). (b,d) Scatchard plots of [^{125}I]-ICYP binding in membranes prepared from rat ventricle (b) and from isolated ventricular myocytes (d). Insets show Scatchard plots of [^{125}I]-ICYP binding in the presence of CGP 20712A. The results shown are representative of four different experiments.

Table 2 Binding of [^{125}I]-ICYP in the absence and presence of CGP 20712A in membranes prepared from rat ventricle and isolated ventricular myocytes

Binding sites		*Ventricular membrane*	*Myocyte membrane*
[^{125}I]-ICYP binding			
Total	K_d (pM)	10.3 ± 0.53	13.3 ± 1.5
	B_{max} (fmol mg^{-1} protein)	17.2 ± 1.1	21.8 ± 0.50
+CGP 20712A	K_d (pM)	16.6 ± 2.6	25.7 ± 6.4*
	B_{max} (fmol mg^{-1} protein)	4.60 ± 0.33	2.28 ± 0.56*
CGP 20712A binding sites			
High affinity	ratio (%)	73.3 ± 0.34	89.6 ± 2.7*
Low affinity	ratio (%)	26.7 ± 0.34	10.4 ± 2.7*

Low-affinity binding sites for CGP 20712A were measured as the [^{125}I]-ICYP binding in the presence of 300 nM CGP 20712A. High affinity binding sites of CGP 20712A were determined by subtraction of low-affinity binding from total binding. Data are represented as mean ± s.e. (n = 4).
* Significantly different from ventricular membrane, $P < 0.05$.

Table 3 Competition of [^{125}I]-ICYP and [^{3}H]-CGP 12177 binding with β-antagonists in rat ventricular myocytes

β-Antagonists		*Radioligands* [^{125}I]-ICYP	[^{3}H]-CGP 12177
Propranolol	K_i (nM)	6.20 ± 0.42	4.03 ± 0.22
	Hill coefficient	0.920 ± 0.07	1.047 ± 0.04
CGP 20712A*	K_i (nM)	2.55 ± 0.47	1.31 ± 0.21
	Hill coefficient	0.613 ± 0.02	0.788 ± 0.04

Data are represented as mean ± s.e. (n = 4). * K_i values were calculated by fitting the data to a 1-site binding model.

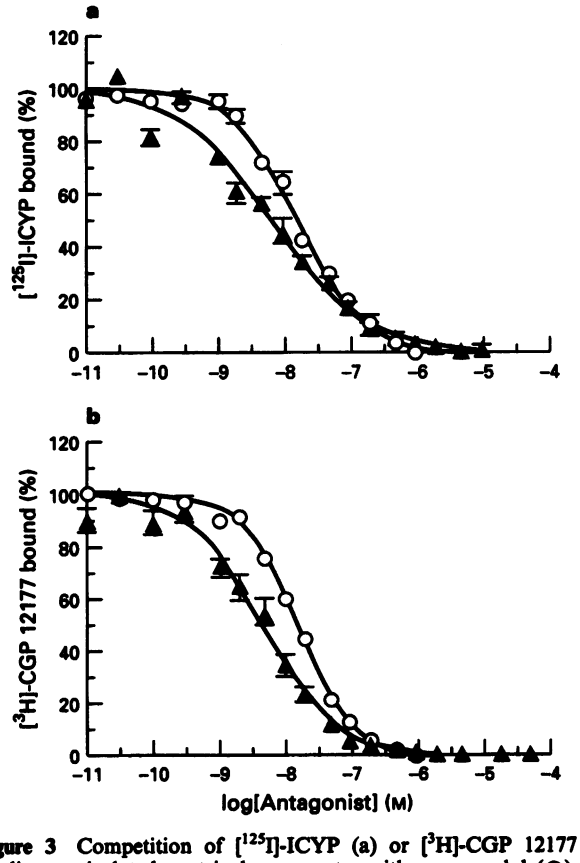

Figure 3 Competition of [^{125}I]-ICYP (a) or [^{3}H]-CGP 12177 (b) binding on isolated ventricular myocytes with propranolol (○) and CGP 20712A (▲). Each point shows mean ± s.e. for four different experiments.

In the absence of CGP 20712A, the K_d value for [^{125}I]-ICYP in ventricular membranes was not significantly different from that in myocyte membranes. On the other hand, in the presence of 300 nM CGP 20712A, the K_d value for [^{125}I]-ICYP in myocyte membranes was 2 times higher than that in ventricular membranes (Table 2).

This difference in the K_d values of [^{125}I]-ICYP at the low-affinity CGP 20712A binding site implies the presence of a receptor other than the β_1- and β_2-adrenoceptors in both membrane preparations. To examine whether the β_2-adrenoceptor exists at the low-affinity binding site for CGP 20712A, we performed saturation experiments in the presence of 300 nM CGP 20712A and 50 nM ICI 118,551.

In ventricular membranes, [^{125}I]-ICYP binding detected in the presence of 300 nM CGP 20712A was decreased by addition of 50 nM ICI 118,551, but unchanged in myocyte membranes (Figure 2). This result showed that the low-affinity CGP 20712A binding site in ventricular membranes contains the β_2-adrenoceptor, whereas that in myocyte membranes does not correspond to the β_2-adrenoceptor. This low-affinity CGP 20712A binding site, which is not a β_1-/β_2-adrenoceptor, was found in both ventricular and myocyte membranes, and the density of the binding was less than 10% of [^{125}I]-ICYP total binding. In fact, this binding was so small that the K_d and B_{max} values could not be calculated.

Radioligand binding study of isolated ventricular myocytes

Competition experiments Nonspecific binding of [^{125}I]-ICYP and [^{3}H]-CGP 12177 to ventricular myocytes was 25% and 10% of the total binding, respectively. The competition curve for propranolol was monophasic and had a Hill coefficient of unity. The K_i value for propranolol in the myocytes obtained from competition experiments using [^{125}I]-ICYP or [^{3}H]-CGP 12177 corresponded to that in ventricular membranes and myocyte membranes (Table 3).

In contrast to ventricular and myocyte membranes, the competition curve for CGP 20712A indicated no low-affinity binding site in myocytes (Figure 3a,b). The Hill coefficient of the competition curve for CGP 20712A was less than unity when [^{125}I]-ICYP or [^{3}H]-CGP 12177 was used as the radioligand. Therefore the competition curve was fitted to a two binding site model. The K_i values of the two CGP 20712A binding sites were 0.48 ± 0.09 nM and 20 ± 2 nM (determined with [^{125}I]-ICYP) and 0.12 ± 0.03 nM and 2.5 ± 0.03 nM (determined with [^{3}H]-CGP 12177), respectively. These K_i values were similar to those for the high-affinity binding site of CGP 20712A, i.e. the β_1-adrenoceptor, obtained from ventricular membranes and myocyte membranes. The proportions of high and low-affinity sites for CGP 20712A obtained with [^{125}I]-ICYP were 53% and 47%, respectively, and those obtained with [^{3}H]-CGP 12177 were 24% and 76%, respectively.

The K_i value for CGP 20712A was calculated by fitting the competition curve to a one binding site model. The K_i value for CGP 20712A on the myocytes was similar to those for the high-affinity CGP 20712A binding sites in ventricular membranes and myocyte membranes (Table 1, Table 3). This result indicated that the binding site for CGP 20712A on myocytes corresponds to the β_1-adrenoceptor.

The concentration of CGP 20712A which occupied 100% of its high-affinity binding sites was 300 nM. This concentration corresponds to that which occupied 100% of the high-affinity CGP 20712A binding sites in ventricular membranes.

Saturation experiments We performed saturation experiments in the presence of 300 nM CGP 20712A to measure the proportion of low-affinity CGP 20712A binding sites.

In the absence of CGP 20712A, the saturation curves for [^{125}I]-ICYP and [^{3}H]-CGP 12177 were monophasic and the Scatchard plots were linear. In myocytes, the K_d and B_{max} values of [^{125}I]-ICYP were 17.5 ± 3.7 pmol, 21.1 ± 0.48 fmol mg^{-1} protein, and those for [^{3}H]-CGP12177 were 0.20 ± 0.02 nM, 17.6 ± 0.88 fmol mg^{-1} protein, respectively. In contrast to myocyte membranes, no specific binding of either radioligand was detected in the presence of 300 nM CGP 20712A (Figure 4). This result indicated that the low-affinity CGP 20712A binding site does not exist on myocytes.

Isoprenaline-stimulated cyclic AMP production in isolated ventricular myocytes

Adenylate cyclase activity The basal and maximal isoprenaline-induced adenylate cyclase activities were 3.59 ± 0.79 and 67.2 ± 12 pmol mg^{-1} protein min^{-1}, respectively.

CGP 20712A did not alter the basal adenylate cyclase activity (data not shown). The concentration-response curve for isoprenaline was monophasic and had an EC_{50} value of 72.9 ± 33 nM. In the presence of 10, 100, and 1000 nM CGP 20712A, the EC_{50} values for isoprenaline were 407 ± 131, 3077 ± 1186, and 9562 ± 532 nM, respectively. The results of the competition experiments showed that CGP 20712A selectively bound to the β_1-adrenoceptor at the concentration employed. Thus, if isoprenaline was able to activate adenylate cyclase through β_2-adrenoceptors, each concentration-response curve would have two components, that is, the response induced by β_1-adrenoceptors at a high concentration of isoprenaline, and that induced by β_2-adrenoceptors at the low concentration.

In the present study, each concentration-response curve was monophasic, and no β_2-adrenoceptor component was detected

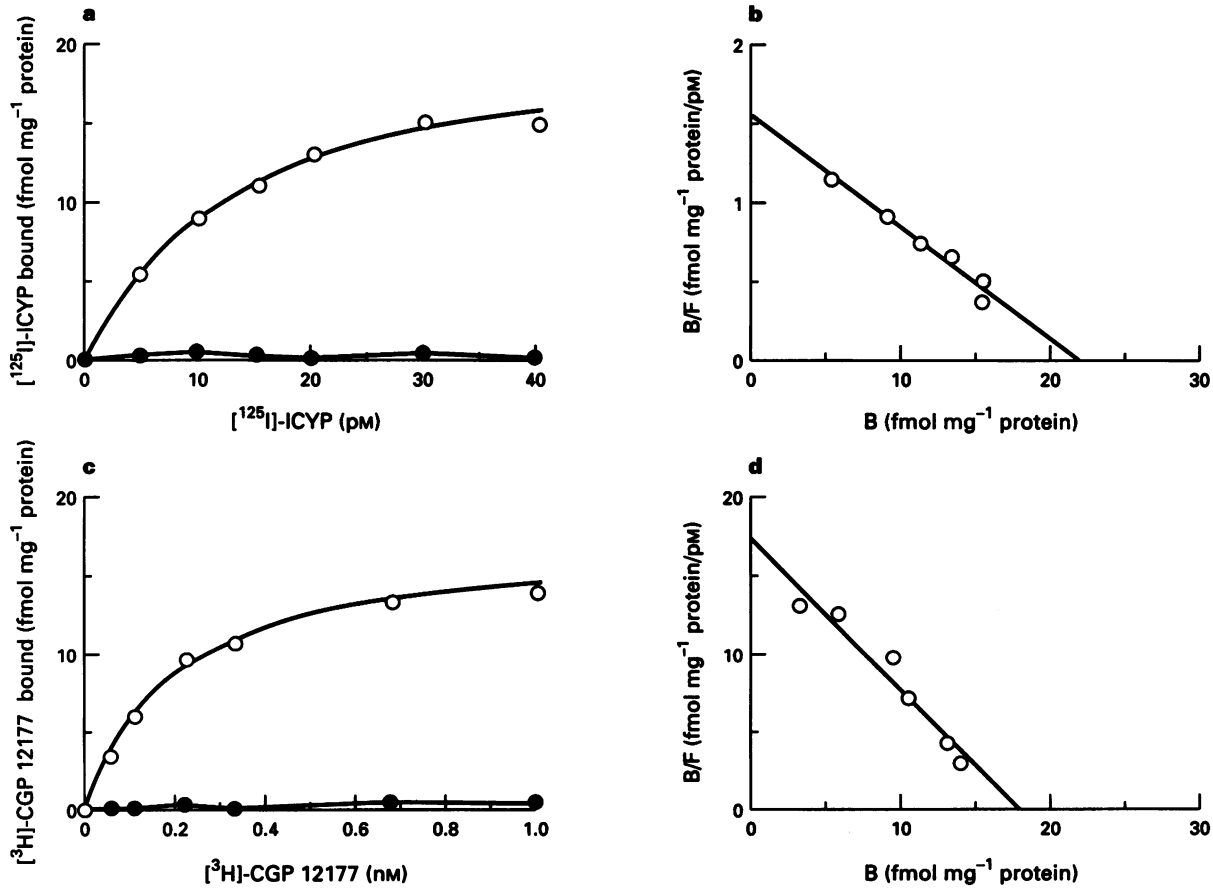

Figure 4 Binding of [^{125}I]-ICYP and [^{3}H]-CGP 12177 in rat isolated ventricular myocytes. (a,c) Saturation curves for [^{125}I]-ICYP (a) and [^{3}H]-CGP 12177 (c) binding in the absence (○) and presence of 300 nM CGP 20712A (●). (b,d) Scatchard plots of [^{125}I]-ICYP (b) and [^{3}H]-CGP 12177 (d) binding in the absence of CGP 20712A. The results shown are representative of four different experiments.

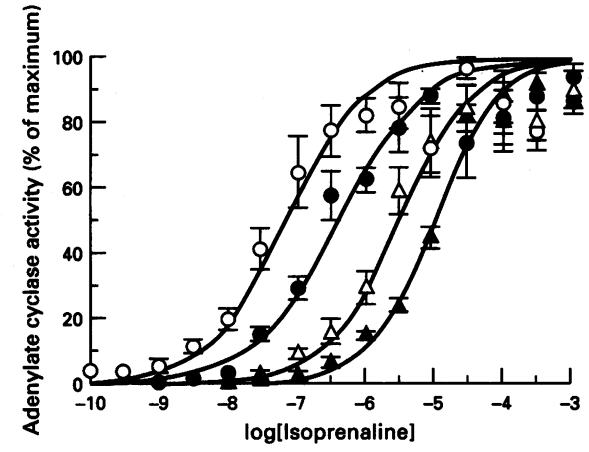

Figure 5 Isoprenaline-stimulated adenylate cyclase activity of rat isolated ventricular myocytes in the absence (○) and presence of 10 nM (●), 100 nM (△), and 1000 nM (▲) CGP 20712A. The results are shown as percentages of the maximal response. In the control preparation the basal and maximal adenylate cyclase activities were 3.59 ± 0.79 and 67.2 ± 12 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, respectively. Each point represents mean ± s.e. for four different experiments.

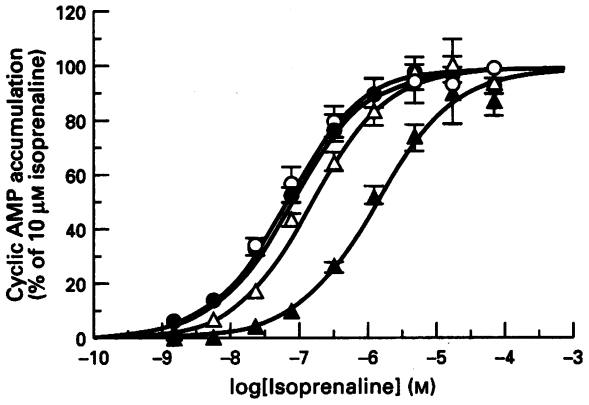

Figure 6 Isoprenaline-stimulated cyclic AMP accumulation in rat isolated ventricular myocytes in the absence (○) and presence of 10 nM (●), 100 nM (△), and 1000 nM (▲) ICI 118,551. The results are shown as percentages of the response induced by 10 μM isoprenaline. In control, the basal and maximal cyclic AMP accumulations were 11.6 ± 1.4 and 289 ± 47 pmol mg^{-1} protein, respectively. Each point represents mean ± s.e. for four different experiments.

(Figure 5). The slope of the Schild plot which was calculated from the shifts of concentration-response curves in Figure 5 was 0.76 ± 0.08, significantly less than unity ($P < 0.05$).

Cyclic AMP accumulation The basal and 10 μM isoprenaline-stimulated cyclic AMP accumulations were 11.6 ± 1.4 and 289 ± 47 pmol mg^{-1} protein, respectively. The concentration-response curve for isoprenaline was monophasic, and had an EC_{50} value of 24.4 ± 4.6 nM (Figure 6).

Procaterol, a β_2-adrenoceptor selective agonist (Yabuuchi, 1977), was used to determine the cyclic AMP accumulation induced by β_2-adrenoceptors in ventricular myocytes. The maximum cyclic AMP accumulation induced by procaterol in the presence of 0.5 mM IBMX was 46.8 pmol mg^{-1} protein, and the EC_{50} value of the concentration-response curve for procaterol was 145 nM ($n = 2$). As the maximum response to procaterol was less than 10% of that to 10 μM isoprenaline, we did not carry out further investigations using procaterol.

We then measured isoprenaline-stimulated cyclic AMP accumulation in the presence of CGP 20712A. CGP 20712A decreased the maximal response to isoprenaline in a concentration-dependent manner (data not shown). Therefore, we used ICI 118,551 to examine the involvement of β_1- and β_2-adrenoceptors in isoprenaline-stimulated cyclic AMP accumulation.

ICI 118,551 did not alter the basal cyclic AMP accumulation in the myocytes. In the presence of 10, 100, and 1000 nM ICI 118,551, the EC_{50} values for isoprenaline were 27.3 ± 4.6, 49.9 ± 10, and 315 ± 47 nM, respectively (Figure 6). The EC_{50} values were increased significantly in the presence of 1000 nM ICI 118,551 ($P < 0.05$), indicating that ICI 118,551 at this concentration causes a 10 fold rightward shift of the concentration-response curve for isoprenaline. The K_b value of ICI 118,551 calculated from the shifts of concentration-response curve in the presence of 1000 nM ICI 118,551 was 89.7 ± 22 nM ($n = 4$). This value was similar to the K_i value of the low-affinity binding sites for ICI 118,551, i.e. β_1-adrenoceptor.

Discussion

It has been shown by radioligand binding studies that only β_1-adrenoceptors exist in rat isolated ventricular myocytes (Buxton & Brunton, 1985; Mauz & Pelzer, 1990). In these studies, competition experiments with β-adrenoceptor subtype-selective antagonists were carried out, and the relative proportions of β_1- and β_2-adrenoceptors were calculated by fitting the data obtained to a theoretical equation (Hancock *et al.*, 1979).

In these previous studies, β-antagonists with relatively low selectivity such as zinterol and bisoprolol, were used. It has been reported that zinterol and bisoprolol have 20 and 80 times greater selectivity for β_1-adrenoceptors, respectively. De Lean *et al.* (1982) showed that a β-antagonist with more than 200 fold greater selectivity for β_2-adrenoceptors is required in order to discriminate β_2-adrenoceptors from a mixture of 90% β_1- and 10% β_2-adrenoceptors. Therefore, zinterol and bisoprolol are inadequate for detecting β_2-adrenoceptors, which have been reported to constitute less than 10% of total β-adrenoceptors on ventricular myocytes. Furthermore, the results of previous studies may have been rather approximate, because they were obtained by fitting of data from competition experiments.

In the present study, we used a highly selective β-antagonist, and also quantified the density of β_1- and β_2-adrenoceptors to determine the proportion of each subtype more accurately by performing saturation experiments in the presence or absence of an appropriate concentration of CGP 20712A, as reported by Beer *et al.* (1988). This method permitted us to quantify the density of β_2-adrenoceptors when they constituted less than 10% of the total. CGP 20712A had adequate selectivity for this method, since it has 1200 fold higher affinity for β_1- than for β_2-adrenoceptors in ventricular membranes (Table 1). In our experiments, 300 nM CGP 20712A occupied 99% of its high-affinity binding sites, β_1-adrenoceptors, and occupied 7% of its low-affinity binding sites, accounting for less than 2% of total binding. This indicates that we can distinguish the β-adrenoceptor subtype using 300 nM CGP 20712A. No binding site corresponding to the β_2-adrenoceptor was detected in myocyte membranes and myocytes (Figures 2b and 4a,c), indicating that only β_1-adrenoceptors exist on isolated ventricular myocytes.

We also measured isoprenaline-stimulated adenylate cyclase activity and cyclic AMP accumulation to examine whether β_2-adrenoceptors play a role in ventricular function. In the presence of 10, 100 or 1000 nM CGP 20712A, which bound only to β_1-adrenoceptors, isoprenaline did not activate adenylate cyclase through β_2-adrenoceptors (Figure 4). Furthermore, ICI 118,551 did not shift the concentration-response curve for cyclic AMP accumulation at concentrations of 10 and 100 nM, at which ICI 118,551 bound selectively to β_2-adrenoceptors (Figure 5). The K_b value of ICI 118,551 was similar to the K_i

value of β_1-adrenoceptor. These results indicated that isoprenaline activates adenylate cyclase only through β_1-adrenoceptors, and supported the results obtained from the binding studies.

Xiao & Lakatta (1993) reported that β_2-adrenoceptors were involved in the function of ventricular myocytes, and showed that the contraction and Ca^{2+} transient evoked by zinterol or isoprenaline in the presence of 300 nM CGP 20712A was antagonized by 100 nM ICI 118,551. The discrepancy between our results and theirs may reflect a difference in the subtype-selectivity of zinterol. They also reported that the β_2-adrenoceptor effect was not mediated through cyclic AMP accumulation. These findings indicate that only β_1-adrenoceptors mediate cyclic AMP-dependent function in rat ventricular myocytes.

In ventricular membranes and myocyte membranes, we detected approximately 10% of [^{125}I]-ICYP binding in the presence of 300 nM CGP 20712A and 50 nM ICI 118,551 (Figure 2a,c). This CGP 20712A and ICI 118,551-resistant [^{125}I]-ICYP binding site was apparently not the β_2-adrenoceptor, since 50 nM ICI 118,551 occupies 99% of β_2-adrenoceptors. This binding site accounted for 10% of total binding in both membrane preparations but was not found in myocytes, suggesting that this type of binding site may result from denaturation of the β_1-adrenoceptor during preparation of ventricular membranes or myocytes. It has been reported that soluble β_1-adrenoceptors are more labile at 22°C than soluble β_2-adrenoceptors (Dickinson & Nahorski, 1981).

There was a possibility that trypsin digested β_2-adrenoceptor, and altered the structure of the binding site for radioligands or β-antagonist, thus making β_2-adrenoceptors undetectable in isolated myocytes and myocyte membranes. However, we ruled out this possibility, because the proportion and affinity of β_2-adrenoceptors were not affected by enzymatic digestion of the ventricle. These results showed that β_2-adrenoceptors found in ventricular membranes were excluded by removal of the cells containing them, such as coronary endothelial cells (Freissmuth *et al.*, 1986) and fibroblasts (Lau *et al.*, 1980).

The CGP 20712A-ICI 118,551-resistant binding site is unlikely to be the β_3-adrenoceptor, as CGP 20712A or ICI 118,551 binds to the β_3-adrenoceptor with low-affinity (Emorine *et al.*, 1989). Although it has been reported that β_3-adrenoceptors exist in adipose tissue, it is still unknown whether they are present in the heart (Kaumann, 1989). The affinity of [^{125}I]-ICYP for β_3-adrenoceptors is 10 times lower than that for β_1- and β_2-adrenoceptors (Emorine *et al.*, 1989; Blin *et al.*, 1993). As the K_d value of [^{125}I]-ICYP was 10–20 pM, the 0–40 pM [^{125}I]-ICYP used in the saturation experiments would not have labelled β_3-adrenoceptors. Furthermore, the β_3-adrenoceptor affinity of propranolol, which was used to determine the nonspecific binding, is 100 times lower than that for β_1-adrenoceptors (Wilson *et al.*, 1984; Blin *et al.*, 1993). Hence, if β_3-adrenoceptors are present on ventricular myocytes, the binding of radioligand to β_3-adrenoceptors would have been recognized as nonspecific binding. Granneman *et al.* (1991) showed that rat β_3-adrenoceptor mRNA was not detectable in rat heart by PCR, suggesting that the density of β_3-adrenoceptors in the heart is very small, if indeed they exist.

In myocytes, the Hill coefficient of the competition curve for CGP 20712A was less than unity (Table 3), suggesting that rat ventricular myocytes may contain two different binding sites for CGP 20712A. However, the difference in the affinity between the two binding sites was much smaller (20–40 fold) than that between β_1- and β_2-adrenoceptors in ventricular membranes (1200 fold). It has been reported that β_1-adrenoceptors on rat ventricular myocytes have two binding sites for bisoprolol, whereas those in myocyte membranes have a single binding site (Mauz & Pelzer, 1990). In the present study, we obtained similar results for CGP 20712A. The slope of Schild plot calculated from the shift of concentration-response curves in Figure 5 was less than unity, suggesting that CGP 20712A may inhibit the effect of isoprenaline at the two different binding sites. However, the results of Figure 4 and Figure 6 suggest that β_2-adrenoceptors are not involved in inhibition by CGP 20712A. One of these binding sites may be denatured or lost during membrane preparation. It has been reported that β_1-adrenoceptors in canine papillary muscle have two binding sites, each having a different affinity for isoprenaline, and having different roles in ventricular function (Yokoyama *et al.*, 1989).

A cell line expressing a single β-adrenoceptor subtype is useful for examining differences in physiological function between the three β-adrenoceptor subtypes. To obtain such cells, cDNA of each human β_1-adrenoceptor subtype has been transfected into Chinese hamster fibroblasts, which have consequently expressed each β-adrenoceptor subtype (Bouvier *et al.*, 1988; Suzuki *et al.*, 1992; Emorine *et al.*, 1989). However, these cell lines might not reflect the intact function of β-adrenoceptors. It is possible that the combination of β-adrenoceptors with various subtypes of adenylate cyclase and G-protein in the transfected cells would be different from that in cells of the original tissue, because the transfected β-adrenoceptor would be expressed at high density. Thus, rat isolated ventricular myocytes are a useful tool for investigating the function of β_1-adrenoceptors, because they express only β_1-adrenoceptors and retain their physiological functions.

In conclusion, in rat myocytes, only β_1-adrenoceptors and not β_2-adrenoceptors were detected by radioligand binding study, and β_2-adrenoceptors were found not to be involved in isoprenaline-stimulated cyclic AMP production. These results suggest that rat ventricular myocytes contain only β_1-adrenoceptors, and that β_1-adrenoceptors mediate cyclic AMP-dependent ventricular function. Rat isolated ventricular myocytes would be a useful tool for further investigation of β_1-adrenoceptor function under physiological conditions.

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