

Involvement of β_3 -Adrenoceptor in the Relaxation Response in Guinea Pig Taenia Caecum

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Received May 12, 1994 Accepted July 1, 1994

ABSTRACT— β -Adrenoceptors in the guinea pig taenia caecum were investigated by measuring relaxation responses to agonists and by a radioligand binding assay using [3 H]CGP 12177. The rightward shift of the isoprenaline concentration-response curve was observed by butoxamine, a β_2 -selective antagonist, and the pA_2 value for butoxamine was 6.46. In control preparations, catecholamines caused relaxation with the following rank order of potency: isoprenaline > adrenaline > noradrenaline. However, in the presence of 10^{-6} M phentolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine, the rank order of potency of the agonists was: isoprenaline > noradrenaline > adrenaline. CGP 12177 caused graded relaxation of the guinea pig taenia caecum, and this response was not influenced by 10^{-6} M phentolamine, 3×10^{-4} M atenolol, 10^{-4} M butoxamine or 10^{-6} M propranolol. The Scatchard plot of the specific [3 H]CGP 12177 binding to microsomal fractions from the guinea pig taenia caecum showed two affinity sites of the receptor: high affinity ($K_D = 0.64$ nM) and low affinity ($K_D = 142.21$ nM) sites. The pK_D value of the high affinity site of [3 H]-CGP 12177 was in agreement with its pA_2 value, and that of the low affinity site was in agreement with its pD_2 value. These results suggest that isoprenaline-, noradrenaline- and adrenaline-induced relaxations of the guinea pig taenia caecum predominantly involve β_2 - and β_3 -adrenoceptors, whereas CGP 12177-induced relaxation is mediated solely through β_3 -adrenoceptors.

Keywords: Taenia caecum (guinea pig), β -Adrenoceptor, β_2 -Adrenoceptor, β_3 -Adrenoceptor, CGP 12177

β -Adrenoceptors are integral plasma membrane proteins that mediate a wide variety of tissue-specific responses. The multiple effects of catecholamines were thought to be mediated by two receptor subtypes, called β_1 - and β_2 -adrenoceptors (1). However, this early classification is insufficient to account for the thermogenic and lipolytic responses of rat brown and white adipose tissues (2–4).

In addition to adipose tissue, atypical β -adrenoceptors have been shown to exist in a number of gastrointestinal smooth muscle preparations: for example, guinea pig ileum (5), rat proximal colon (6), rat distal colon (7), rat jejunum (8), rat gastric fundus (9), and rat oesophageal muscularis mucosae (10). Also, the presence of atypical β -adrenoceptors has been indicated in other non-gastrointestinal tissues: for example, in skeletal muscle (11, 12) and in tracheal epithelium (13).

Recently, Emorine et al. (14) have successfully cloned a β_3 -adrenoceptor from human tissue. The three

predominant pharmacological characteristics of the β_3 -adrenoceptor are: (i) the high potency of the lipolytic agonist BRL 37344 and related compounds or CGP 12177, (ii) the relatively low affinity of some β -adrenoceptor antagonists and (iii) the higher sensitivity to noradrenaline than to adrenaline (5–8, 14–19).

The aim of this study was to study the β -adrenoceptor-mediated relaxation of the guinea pig taenia caecum in detail by comparing the potencies of isoprenaline, noradrenaline, adrenaline and CGP 12177, the selective β_3 -adrenergic agonist, and by a radioligand binding assay using [3 H]CGP 12177.

MATERIALS AND METHODS

Mechanical responses

Male guinea pigs weighing 300 to 500 g were killed by a blow on the head. A 2- to 3-cm piece of the taenia caecum was isolated and suspended in a 20-ml organ bath filled with a Ringer-Locke solution (154 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 2.1 mM $MgCl_2$, 5.9 mM

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NaHCO₃ and 2.8 mM glucose) kept at 32°C and bubbled with a mixture of 95% O₂ and 5% CO₂. The mechanical responses of the smooth muscle preparations were recorded isotonically under a tension of 0.7 g. The experiments were started after the preparations had been allowed to develop their spontaneous tone for 2 hr. The concentration-response curves of the test drugs were obtained cumulatively, and the relaxation induced by these drugs was expressed as a percentage of the maximal relaxation produced by isoprenaline. To test the antagonism, one of the antagonists was added to the bath 30 min before the addition of the agonist. The concentration-response curves to isoprenaline were then obtained in the presence of an antagonist. The time interval between two consecutive curves was usually set at 60 min. In our previous experiments, after the control concentration-response curves were determined, two or three successive cumulative concentration-response curves of isoprenaline were determined. Curves were nearly superimposable, and the changes in sensitivity (sensitization or desensitization) were small (data not shown). In some experiments, atenolol (3×10^{-4} M), butoxamine (10^{-4} M) and phentolamine (10^{-6} M) were present to inhibit β_1 -, β_2 - and α -adrenoceptors, respectively. The agonistic potency was expressed as the pD₂ value, and the intrinsic activity was expressed as the ratio between the maximum response to a test drug and that to isoprenaline, a reference drug (20). The competitive antagonistic potency was expressed as the pA₂ value. It was calculated according to the method of Tallarida et al. (21), which was originally reported by Arunlakshana and Schild (22).

Data analyses

Numerical results are expressed as means \pm S.E. and statistical analyses were performed by Student's *t*-test and Duncan's new multiple range test as appropriate. A *P* value of less than 0.05 was considered a significant difference.

Preparation of microsomal fractions

Taenia caeci were washed with an ice-cold medium containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4 at 4°C). The isolated tissues were minced with scissors and homogenized with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in 20 vol. of 0.25 M sucrose containing 10 mM Tris-HCl (pH 7.4 at 4°C) with the rheostat set at 9 for 5 sec. The homogenate was centrifuged at $2,500 \times g$ for 10 min. The supernatant was again centrifuged at $15,000 \times g$ for 20 min. Centrifugation of this supernatant at $100,000 \times g$ for 60 min resulted in a pellet that was used as the microsomal fraction (23).

Binding assay

Microsomal fractions were incubated with various concentrations of [³H]CGP 12177 in a total volume of 150 μ l of incubation buffer (50 mM Tris-HCl, pH 7.4 at 35°C) for 60 min. The incubation mixture was rapidly filtered by vacuum through Whatman GF/C glass-fiber filters using the Cell Harvester (Brandel, Geithersburg, MD, USA). The filters were washed 3 times with 3 ml of ice-cold 50 mM Tris-HCl (pH 7.4). The filters were then dried, and the radioactivity was determined in a toluene-based scintillator with a liquid scintillation spectrometer (Aloka LSC-3100, Tokyo).

The nonspecific binding was determined as the radioactivity bound to the microsomal fractions, which was not displaced by isoprenaline (100 μ M). The specific binding was determined as the difference between the total binding and the nonspecific binding.

Protein assay

Protein concentrations were determined by the method of Lowry et al. (24) using bovine serum albumin as a standard.

Data analyses

Saturation binding experiments were analyzed by iterative nonlinear regression analysis using a PC-9801VX (NEC, Tokyo) and the "WBSIKK" programme (25).

Drugs and chemicals

The drugs used were obtained from the following sources: (–)-isoprenaline hydrochloride, (–)-noradrenaline bitartrate, (–)-adrenaline bitartrate, butoxamine hydrochloride, (±)-propranolol hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); (±)-atenolol, (±)-CGP 12177 (4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one) hydrochloride (Research Biochemicals Inc., Natick, MA, USA); phentolamine (Ciba Geigy, Basel, Switzerland); and [³H]CGP 12177 (9.25 MBq; New England Nuclear, Boston, MA, USA). All the drugs were used as solutions in distilled water. The other chemicals used were of analytical grade.

RESULTS

Effects of β_1 - and β_2 -selective antagonists

Isoprenaline caused graded relaxation of the guinea pig taenia caecum where tone had been raised spontaneously. Atenolol (3×10^{-6} – 3×10^{-5} M) competitively antagonized the relaxation responses to isoprenaline (Fig. 1a); The Schild plot of the data gave the pA₂ value of 5.56 ± 0.06 , the slope of the regression line (1.01 ± 0.15) not being significantly different from unity (Fig. 1b). Butox-

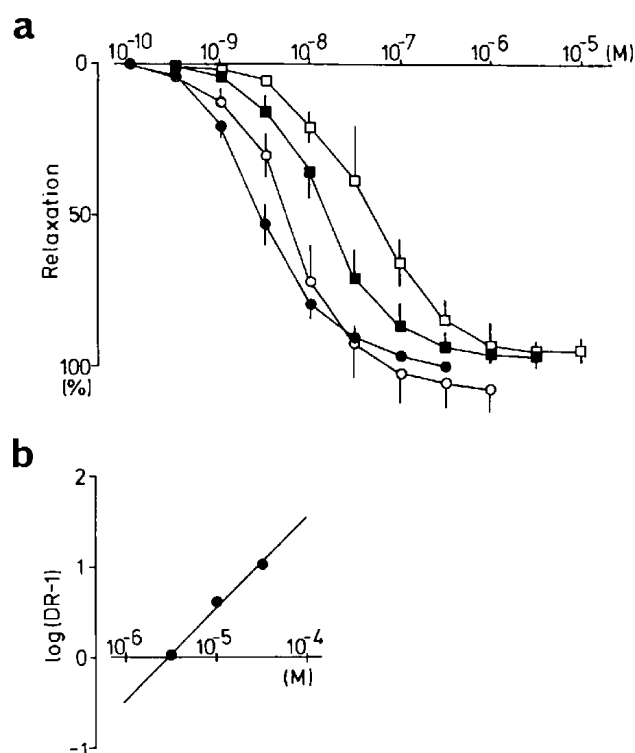


Fig. 1. Determination of the pA_2 value for atenolol. a: Antagonism of isoprenaline-induced relaxation by atenolol. Control (●); atenolol at 3×10^{-6} M (○), 10^{-5} M (■) or 3×10^{-5} M (□). Ordinate: Relaxation (%), expressed as a percentage of the maximal relaxation induced by isoprenaline (3×10^{-7} M). Abscissa: Concentration (M) of isoprenaline. Each point is presented as a mean \pm S.E. of 6 experiments. b: Schild plot for antagonism of isoprenaline by atenolol. The data are taken from experiments shown in a.

amine (10^{-6} – 10^{-5} M) caused competitive antagonism of the relaxant responses (Fig. 2a); The Schild plot of the data gave the pA_2 value of 6.46 ± 0.06 , the slope of the regression line (0.99 ± 0.13) not being significantly different from unity (Fig. 2b).

Effects of isoprenaline, noradrenaline and adrenaline

Isoprenaline, noradrenaline and adrenaline all caused graded relaxation of the guinea pig taenia caecum (Fig. 3). The order of potency was isoprenaline > adrenaline > noradrenaline (Table 1). However, in the presence of 10^{-6} M phentolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine to block α -, β_1 - and β_2 -adrenoceptor effects, respectively, the rank order of potency was isoprenaline > noradrenaline > adrenaline in the presence of blockers (Fig. 3, Table 1). The pD_2 value for noradrenaline was significantly larger than that for adrenaline in the presence of blockers (Table 1). Propranolol ($\sim 10^{-6}$ M) did not significantly affect the relaxant responses to isoprenaline, noradrenaline and

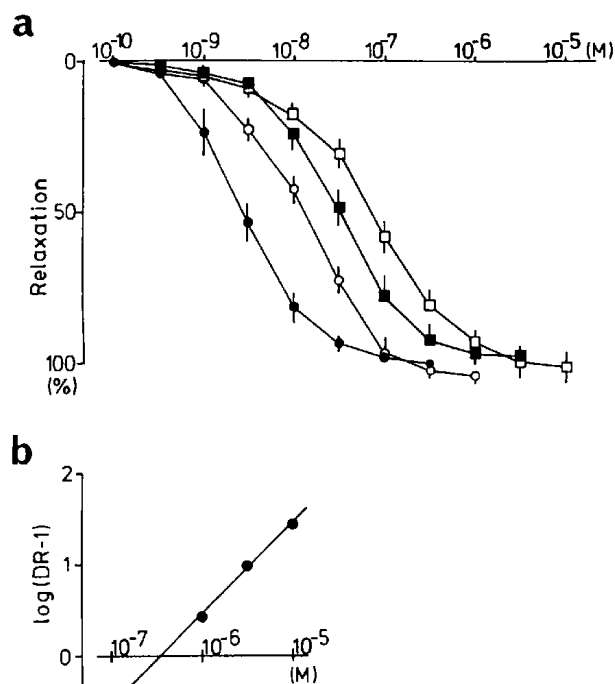


Fig. 2. Determination of the pA_2 value for butoxamine. a: Antagonism of isoprenaline-induced relaxation by butoxamine. Control (●); butoxamine at 10^{-6} M (○), 3×10^{-6} M (■) or 10^{-5} M (□). Ordinate: Relaxation (%), expressed as a percentage of the maximal relaxation induced by isoprenaline (3×10^{-7} M). Abscissa: Concentration (M) of isoprenaline. Each point is presented as a mean \pm S.E. of 6 experiments. b: Schild plot for antagonism of isoprenaline by butoxamine. The data are taken from experiments shown in a.

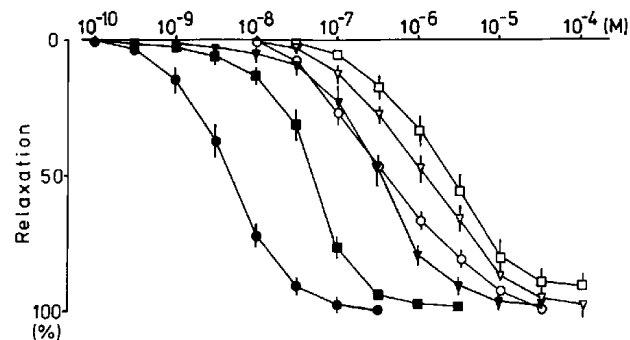


Fig. 3. Concentration-response curves to catecholamines in the presence or absence of 10^{-6} M phentolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine. Isoprenaline (●), adrenaline (■) and noradrenaline (▼) in the absence of blockers. Isoprenaline (○), noradrenaline (▽) and adrenaline (□) in the presence of blockers. Ordinate: Relaxation (%), expressed as a percentage of the maximal relaxation induced by isoprenaline. Abscissa: Concentration (M) of the test drugs. Each point is presented as a mean \pm S.E. of 6 experiments.

Table 1. The pD_2 values for catecholamines in the presence or absence of blockers

Agonists	Absence	Presence
Isoprenaline	8.37 ± 0.06	6.43 ± 0.07
Noradrenaline	6.63 ± 0.11	6.01 ± 0.07
Adrenaline	7.35 ± 0.05	5.70 ± 0.09

Each value is presented as a mean \pm S.E. of 6 experiments. Blockers: 10^{-6} M phentolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine.

adrenaline in the presence of blockers (data not shown).

Effect of CGP 12177

CGP 12177, a selective β_3 -adrenergic partial agonist, caused graded relaxation of the guinea pig taenia caecum (Fig. 4), and the intrinsic activity and the pD_2 value were 0.74 ± 0.06 and 7.24 ± 0.21 , respectively. In the presence of 10^{-6} M phentolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine, the pD_2 value was 7.00 ± 0.17 for CGP 12177 (Fig. 4, Table 2). Propranolol ($\sim 10^{-6}$ M) did not significantly affect the relaxant responses to CGP 12177 in the absence and the presence of blockers (data not shown).

CGP 12177 competitively antagonized the relaxation responses to isoprenaline. The pA_2 value for CGP 12177 was 9.24 ± 0.07 (Table 3).

Radioligand binding experiments

Figure 5a shows the typical specific binding of [3 H]-CGP 12177 to microsomal fractions from the guinea pig

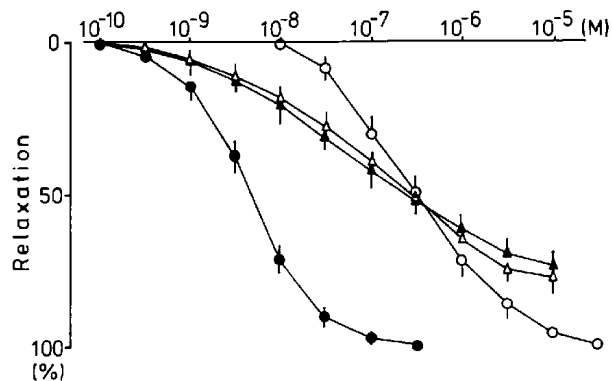


Fig. 4. Concentration-response curve to CGP 12177 in the presence or absence of 10^{-6} M phentolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine. Isoprenaline (●) and CGP 12177 (▲) in the absence of blockers. Isoprenaline (○) and CGP 12177 (△) in the presence of blockers. Ordinate: Relaxation (%), expressed as percentage of the maximal relaxation induced by isoprenaline. Abscissa: Concentration (M) of the test drugs. Each point is presented as a mean \pm S.E. of 6 experiments.

Table 2. The pD_2 values for CGP 12177 in the presence or absence of blockers

Agonists	Absence	Presence
Isoprenaline	8.39 ± 0.06	6.51 ± 0.14
CGP 12177	7.24 ± 0.21	7.00 ± 0.17

Each value is presented as a mean \pm S.E. of 6 experiments. Blockers: 10^{-6} M phentolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine.

Table 3. The intrinsic activity, pD_2 , pA_2 , pK_D and B_{max} values of CGP 12177

From mechanical response ^a	
Intrinsic activity	0.74 ± 0.06
pD_2 value	7.24 ± 0.21
pA_2 value	9.24 ± 0.07
From binding assay ^b	
High affinity site	
pK_D value	9.20 ± 0.04
B_{max} (fmoles/mg protein)	3.11 ± 0.73
Low affinity site	
pK_D value	6.85 ± 0.04
B_{max} (fmoles/mg protein)	104.82 ± 15.34

^a: mean \pm S.E. of 6 experiments, ^b: mean \pm S.E. of 3 separate experiments.

taenia caecum. The binding of [3 H]CGP 12177 to microsomal fractions showed an initial plateau of saturation between 6.25 and 12.5 nM of the radioligand (Fig. 5a), but revealed a second stereospecific and saturable binding component at higher concentrations. The curvilinear Scatchard plot (26) was fitted with a two-binding site model, corresponding to a high ($K_D = 0.64 \pm 0.06$ nM) and a low ($K_D = 142.21 \pm 11.41$ nM) affinity component with respective B_{max} (the maximum binding site) values of 3.11 ± 0.73 and 104.82 ± 15.34 fmoles/mg protein (Fig. 5b).

The intrinsic activity, pD_2 , pA_2 , pK_D and B_{max} values of CGP 12177 are summarized in Table 3. The negative log of the dissociation constant of the high affinity site, pK_D (high) value of CGP 12177, was in agreement with its pA_2 value obtained in the mechanical response and that of the low affinity site, pK_D (low) value of CGP 12177, was in agreement with its pD_2 value obtained in the mechanical response.

DISCUSSION

It is highly probable that the adrenoceptors involved in the responses to the agents used in the current study were

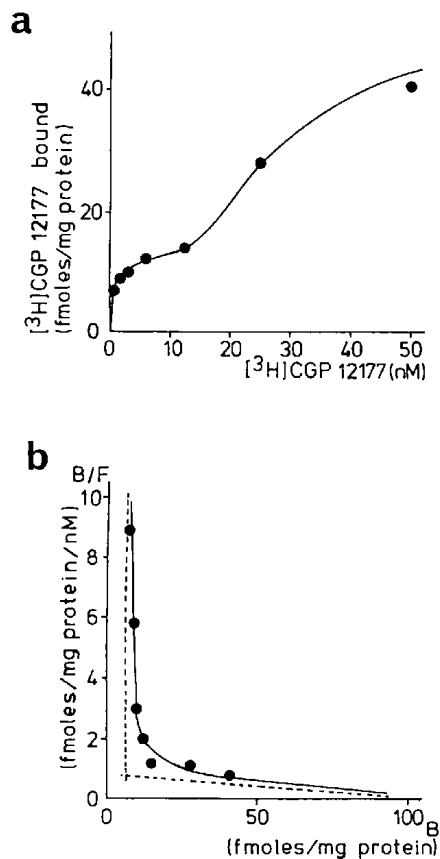


Fig. 5. Characterization of [3 H]CGP 12177 binding sites in microsomal fractions from the guinea pig taenia caecum. a: Typical specific [3 H]CGP 12177 binding with increasing concentrations of [3 H]CGP 12177 to microsomal fractions. Ordinate: [3 H]CGP 12177 (bound) (fmoles/mg protein). Abscissa: Concentration (nM) of [3 H]CGP 12177. b: Typical Scatchard plot of these data. The hyperbola (the solid line) and the two asymptotes (the broken line) were obtained by nonlinear regression analysis. Ordinate: The ratio of bound to free [3 H]CGP 12177 found in the incubation medium. Abscissa: [3 H]CGP 12177 (bound) (fmoles/mg protein).

of the atypical or β_3 -subtype (27–31). For example, the affinity of propranolol (i.e., pA_2 value of 6.5–7.5 versus isoprenaline) was significantly lower than would be expected for effects at classical β -adrenoceptors (i.e., pA_2 typically 8.6–9.0) in guinea pig ileum, rat jejunum and rat colon (7, 8, 19). A possible explanation for these apparent discrepancies could be that a heterogeneous population of β -adrenoceptors (i.e., β_3 -subtype) exists. The present study was undertaken to characterize the β -adrenoceptors mediating relaxation of the guinea pig taenia caecum.

The rightward shift of the isoprenaline concentration-response curve was observed by 10^{-6} – 10^{-5} M butoxamine, a β_2 -selective antagonist. The pA_2 value for butoxamine calculated from the Schild plot (6.46) is in good agreement with the generally accepted value (6.20) (32), indicating a predominance of β_2 -adrenoceptors. As the pA_2

value of butoxamine is eight times greater than that of atenolol, β -adrenoceptors in the guinea pig taenia caecum are predominantly of the β_2 -subtype. In addition, it was shown that catecholamines caused relaxation of the guinea pig taenia caecum with the following order of potency: isoprenaline > adrenaline > noradrenaline. This fact also supports that β_2 -adrenoceptor subtype predominates in the guinea pig taenia caecum.

The existence of atypical β -adrenoceptors or β_3 -adrenoceptors has now been generally accepted (27, 31). Unfortunately, high affinity β_3 -antagonists do not exist at present, but the higher sensitivity to noradrenaline than to adrenaline (14), the high potency of a novel class of β -adrenoceptor agonists (15) and the low potencies and stereoselectivities of classical β -adrenoceptor antagonists (16) have provided strong support for the occurrence of β_3 -adrenoceptors.

In control preparations, isoprenaline, noradrenaline and adrenaline caused relaxation with a rank order of potency suggestive of β_2 -adrenoceptors. However, addition of 10^{-6} M phentolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine to these preparations caused only 4-, 45- and 90-fold decrease in potency for noradrenaline, adrenaline and isoprenaline, respectively, instead of the 500-fold shift expected if the agonists were acting solely at the β_2 -adrenoceptors. Thus, the 500-fold shift in the presence of 10^{-4} M butoxamine should be seen, because the fivefold shift of the concentration-response curve for isoprenaline was caused in the presence of 10^{-6} M butoxamine. Esbenshade et al. (33) suggested that in SK-N-MC human neuroblastoma cells, catecholamines still maximally activated cAMP accumulation with only small decreases in potency when β_1 -adrenoceptors were blocked with CGP 20712A and that two populations of β -adrenergic receptors (β_1 -adrenoceptors and β_3 -adrenoceptors) exist in this cells. Moreover, in our study, the rank order of potency of the agonists in the presence of 10^{-6} M phentolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine was isoprenaline > noradrenaline > adrenaline, suggesting the presence of an atypical β -receptor (or β_3) in the guinea pig taenia caecum. The rank order of potency for catecholamines was the same for various tissues containing β_3 -adrenoceptors (isoprenaline > noradrenaline > adrenaline) (34).

CGP 12177 is generally considered to be a hydrophilic β -adrenergic antagonist (35, 36). [3 H]CGP 12177 has proved to be superior in studies with intact cells, and particularly in studies of the agonist-induced internalization of β -adrenergic receptors (owing to its hydrophilicity; [3 H]CGP 12177 only labels cell-surface receptors) (37). Recently, CGP 12177, a β_1 - and β_2 -adrenoceptor antagonist, behaves as a partial agonist at β_3 -adrenoceptors (27, 31). For example, CGP 12177 distinguishes between brown

adipocyte β_3 -adrenoceptors and classical β -adrenoceptors in that while at low concentrations it is an antagonist at β_1 - or β_2 -adrenoceptors, at higher concentrations, it stimulates oxygen consumption in hamster brown adipocytes and both oxygen consumption and adenylate cyclase in rat brown adipocytes (38–41). The CGP 12177-induced relaxation was not influenced by addition of 10^{-6} M phenolamine, 3×10^{-4} M atenolol and 10^{-4} M butoxamine, which were used at concentrations occupying >95% of α -, β_1 - and β_2 -receptors, respectively, while the isoprenaline concentration-response curves were markedly shifted to the right by addition of blockers. Propranolol, a non-selective antagonist, did not affect the relaxant response to CGP 12177 in the absence or presence of blockers. These results suggest that CGP 12177-induced relaxation in the guinea pig taenia caecum is mediated through β_3 -adrenoceptors.

The discovery of the novel selective agonists has led to further attempts to identify atypical β -adrenoceptors (or β_3 -adrenoceptors) by ligand binding techniques. Apparently by using higher concentrations of [3 H]CGP 12177, Blum-Kaelin et al. (39) were able to detect an atypical (or β_3) binding site. Moreover, the affinity of CGP 12177 for the receptor ($pK_D = 7.4$) was consistent with its EC_{50} value as a respiratory stimulant ($pD_2 = 7.1$). In the present study, the specific [3 H]CGP 12177 binding to microsomal fractions from the guinea pig taenia caecum showed two affinity sites of the receptor by the iterative nonlinear regression analysis: high affinity and low affinity sites. The pK_D value of the high affinity site of [3 H]CGP 12177 was in agreement with its pA_2 value, and that of the low affinity site was in agreement with its pD_2 value. These results suggest that the low affinity site of [3 H]CGP 12177 may be the β_3 -adrenergic binding site and the high affinity site of [3 H]CGP 12177, the β_2 -adrenergic binding site.

We have previously demonstrated (42–46) that β -adrenoceptors contain two different affinity sites: high and low, for which some partial agonists but not isoprenaline (a full agonist) or propranolol (a competitive antagonist) show selectivity. The mechanical response to isoprenaline results from an interaction with the high affinity site. The competitive antagonism shown by propranolol and most partial agonists is due to their ability to compete with isoprenaline for the high affinity site. The mechanical response to the partial agonists results from the interaction with the low affinity site. Moreover, we have showed that the concentration-response curves of the partial agonists (e.g.: carteolol or alprenolol)-induced relaxation of the guinea pig taenia caecum were markedly shifted to the right by propranolol (45). However, CGP 12177-induced relaxation was not affected by propranolol. Emorine et al. (31) have described that the partial agonistic property of several antagonists of β_1 - and β_2 -

adrenoceptors, reflecting intrinsic sympathomimetic activities in certain tissues, is one of pharmacological properties of atypical β -adrenoceptors. Therefore, these observations suggest that atypical β -adrenoceptors or β_3 -adrenoceptors may contain an additional atypical subtype. Further experiments are necessary to clarify the closely related receptor subtypes and the mechanisms of the interactions of the β -adrenergic partial agonists with its receptors.

In conclusion, it is evident from the mechanical responses and the radioligand binding experiments that predominantly β_2 - and partly β_3 -adrenoceptors (atypical β -adrenoceptors) are involved in the β -adrenoceptor-mediated relaxation of the guinea pig taenia caecum. The CGP 12177-induced relaxation of the guinea pig taenia caecum was shown to be mediated solely by β_3 -adrenoceptors.

Acknowledgment

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (No. 04304030).

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