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The β-adrenoceptors mediating relaxation of rat oesophageal muscularis mucosae are predominantly of the β_3-, but also of the β_2-subtype

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1 β-Adrenoceptor-mediated relaxation of rat oesophageal smooth muscle was investigated by studying the effects of β_1- and β_2-selective antagonists on the relaxation induced by (−)-isoprenaline, the β_2-selective agonists fenoterol and clenbuterol and the β_3-agonist, BRL 37344.

2 The highly β_1-selective antagonist CGP 20712A did not antagonize (−)-isoprenaline- or BRL 37344-induced relaxations in concentrations up to 10 μM. Only at 100 μM of CGP 20712A were clear rightward shifts of the agonist concentration-response curves (CRCs) observed, with pA_2 values of 4.70 and 4.97 against (−)-isoprenaline and BRL 37344, respectively.

3 ICI 118,551, a potent and selective β_2-antagonist, at 100 nM caused moderate rightward shifts of the CRCs of (−)-isoprenaline, fenoterol and clenbuterol; with fenoterol and clenbuterol, this was accompanied by a clear steepening of the curve. Only at the highest concentration (100 μM ICI 118,551) did the shifts to the right further increase substantially. Resulting Schild-plots were clearly biphasic. BRL 37344-induced relaxations were only antagonized at 100 μM ICI 118,551, yielding a pA_2 value of 5.48.

4 These results clearly demonstrate that the BRL 37344-induced relaxation of rat oesophageal muscularis mucosae is mediated solely through β_3-adrenoceptors, whereas (−)-isoprenaline-, fenoterol- and clenbuterol-induced relaxations were shown to involve both β_2- and, predominantly, β_3-adrenoceptors.

Keywords: β_3-Adrenoceptors; rat oesophagus; muscularis mucosae; BRL 37344; ICI 118,551; CGP 20712A

Introduction

During the past few years, both pharmacological and molecular studies have revealed that β-adrenoceptors are more heterogeneous than believed thus far. In a number of tissues, especially adipose and gastrointestinal tissue, responses appeared to be mediated by a receptor with distinct characteristics, different from classical β_1-and/or β_2-adrenoceptors. Recently, a human gene was cloned that encoded for a third (β_3-) adrenoceptor which, after transfection into Chinese Hamster Ovary (CHO) cells, revealed similar properties (Emorine *et al.*, 1989). Although this receptor was shown to exhibit clear atypical characteristics, it did not completely correspond to functional human and rat adipocyte β-adrenoceptors (Zaagsma & Nahorski, 1990). Furthermore, cloning of the rat β_3-adrenoceptor and subsequent expression in CHO cells revealed a pharmacological profile different from that reported for the human β_3-receptor, but similar to the properties exhibited by the atypical receptors in rat adipocytes (Granneman *et al.*, 1991). This would indicate species differences and/or the existence of multiple atypical receptor subtypes.

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 (Bond & Clarke, 1988); rat proximal colon (Croci *et al.*, 1988); rat distal colon (McLaughlin & MacDonald, 1990); rat jejunum (Van der Vliet *et al.*, 1990); and rat gastric fundus (McLaughlin & MacDonald, 1991). Also, the presence of atypical β-adrenoceptors has been indicated in other non-gastrointestinal tissues, for example in skeletal muscle (Challiss *et al.*, 1988) and in tracheal epithelium (Webber & Stock, 1992).

In rat oesophageal smooth muscle, Buckner & Christopherson (1974) have reported unusually low potencies of β-adrenoceptor antagonists in antagonizing isoprenaline-induced relaxations. Because this is now an established hallmark of an atypical, β_3-type adrenoceptor (Zaagsma & Nahorski, 1990), we decided to study the β-adrenoceptor-mediated relaxation of rat oesophageal muscularis mucosae in detail by comparing the potencies of (−)-isoprenaline, the selective β_3-adrenoceptor agonist BRL 37344 and the β_2-adrenoceptor agonists fenoterol and clenbuterol, using CGP 20712A and ICI 118,551 as selective β_1- and β_2-adrenoceptor antagonists, respectively. The results show that both β_2- and β_3-adrenoceptors are involved in the relaxation of rat oesophageal muscularis mucosae, the β_3-adrenoceptor playing the predominant role.

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Methods

Tissue preparation

Male Wistar rats (250–300 g) were killed by a blow on the head and exsanguinated. Oesophagi were rapidly removed and placed in a water-jacketed preparation dish filled with Krebs-Henseleit solution at 37°C, composed of (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.52, NaH_2PO_4 1.28, $NaHCO_3$ 25.0, glucose 5.5, gassed with 95% O_2 and 5% CO_2, pH 7.4. The preparation was divided into two parts, cervical and thoracic, each with a length of 10–15 mm. Both parts were cut longitudinally and pinned on a silicon mat with the outer, striated muscle coat up. After dissection of the striated muscle, the remaining muscularis mucosae was divided into 4 (5 × 2 mm, thoracic part) and 6 (5 × 1.5 mm, cervical part) strips. Strips from different parts showed no differences in pharmacological behaviour. The preparations were mounted in 20 ml water-jacketed organ baths filled with Krebs-Henseleit buffer solution, gassed with 95% O_2/5% CO_2, pH 7.4, 37°C, for isotonic recording under 0.2 g load. After equilibration for a period of at least 30 min, tissues showed neither resting tone nor spontaneous activity throughout the experiments.

Concentration-response curves

An initial methacholine concentration-response curve (CRC) (0.1, 1, 10 μM) was constructed for each preparation which, after a washing period of 30 min, was followed by a second curve (0.1, 1, 10, 100 μM) to determine the maximal contraction. The preparations were then washed twice and, before the cumulative addition of a β-adrenoceptor agonist, contracted with methacholine in two concentration-steps, starting with 0.1 μM methacholine and supplemented to a final concentration of 1 μM, which induced approximately 50% of the maximal contraction.

With each preparation, only single CRCs to each agonist were constructed. When studying the effects of antagonists, two untreated strips (i.e. without antagonist) always served as controls. Antagonists were added 40 min prior to the beginning of each agonist-CRC.

(−)-Isoprenaline and fenoterol were added in 0.5 log increments. As relaxations by BRL 37344 and clenbuterol developed more slowly, these compounds were added in log intervals. At the end of each CRC, preparations were washed twice to obtain basal tone again.

All experiments were performed in duplicate each day using strips from the same animal, providing one data-set for the mean results.

Data analysis

CRCs of all β-adrenoceptor agonists were expressed as a percentage of the (1 μM) methacholine-induced contraction. Schild plots were constructed according to Arunlakshana & Schild (1959) using the agonist-concentrations producing half-maximal relaxation in the absence and the presence of different concentrations of the antagonist. In cases where the Schild plot was biphasic, the slope was calculated from the steep part of the plot only, discounting the constant log (DR-1) values obtained with low antagonist concentrations (Bond & Clarke, 1988); if the slope was not significantly different from unity (two-tailed Student's t test, $\alpha < 0.05$), pA_2 values were calculated from each antagonist concentration, using the formula $pA_2 = -\log\{[\text{antagonist}]/(DR-1)\}$ (MacKay, 1978). This formula was also used when the Schild-plot consisted of only one data point.

All data are given as mean ± s.e.mean of (n) determinations.

Drugs

(−)-Isoprenaline hydrochloride was purchased from Sigma (St. Louis, U.S.A.). BRL 37344 (4-[2-[(2-hydroxy-2-(3-chlorphenyl)ethyl)amino]-propyl]-phenoxyacetic acid), ICI 118,551 (erythro-1-(7-methylindan-4-yloxy)-3-(isopropylamine)-butan-2-ol), CGP 20712A (1-[2-((3-carbamoyl-4-hydroxy)-phenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-propan-2-ol), fenoterol and clenbuterol were kind gifts from SmithKline Beecham (Epsom, U.K.), ICI (Macclesfield, U.K.), Ciba-Geigy (Basel, Switzerland) and Boehringer Ingelheim (Ingelheim, Germany). Phentolamine was donated by Ciba-Geigy (Arnhem, The Netherlands) and corticosterone was from Organon (Oss, The Netherlands). All buffer salts were from Merck (Amsterdam, The Netherlands).

Results

All β-adrenoceptor agonists produced concentration-dependent relaxations of oesophageal smooth muscle. Addition of phentolamine (1 μM) or corticosterone (10 μM) to block classical α-adrenoceptor effects and extraneuronal uptake, respectively, did not affect agonist-induced relaxations (data not shown).

Relaxations to BRL 37344 were slow compared to (−)-isoprenaline-induced relaxations (Figure 1). In addition, with BRL 37344 a clear tachyphylaxis was observed, a second CRC being shifted to the right 10–30 fold. Therefore, only one CRC to each agonist was constructed per tissue.

With ICI 118,551 at the highest concentration (100 μM) only, some depression of the methacholine-induced contraction was observed, which may indicate some antimuscarinic effect; this depression amounted to 21.6 ± 1.3% (mean ± s.e.mean; $n = 29$) of the maximal (0.1 mM methacholine) con-

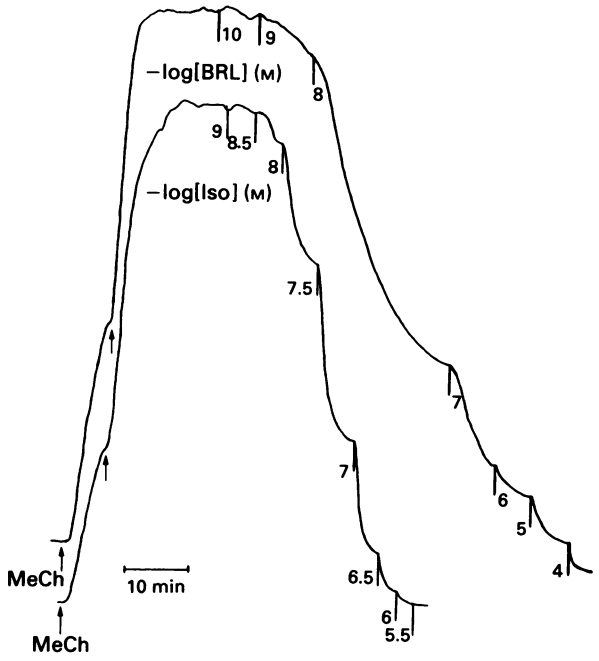

Figure 1 Typical traces showing BRL 37344 (upper curve) and (−)-isoprenaline (Iso) (lower curve)-induced relaxations of rat oesophageal muscularis mucosae. Smooth muscle tone was elevated by step-wise additions of 0.1 and 0.9 μM methacholine (MeCh) (arrows).

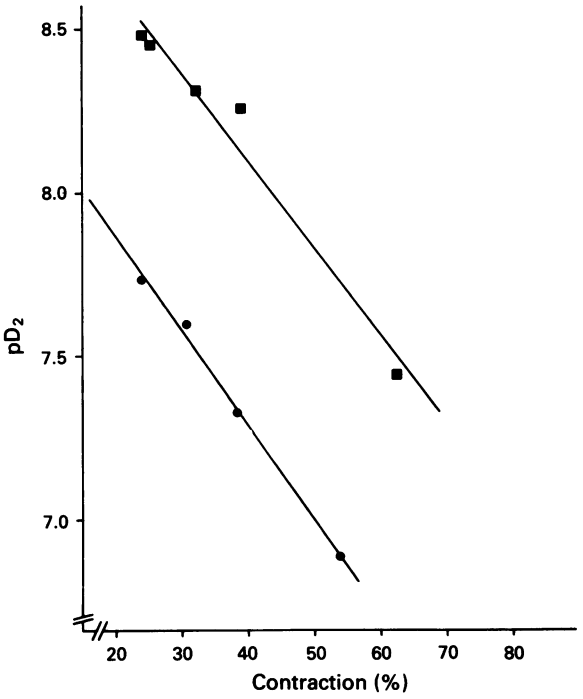

Figure 2 Correlation between methacholine-induced contraction and pD_2 values for BRL 37344 (■) ($r = -0.980$, $P < 0.01$) and fenoterol (●) ($r = -0.996$, $P < 0.01$). Datapoints correspond to methacholine-concentrations (in downward direction) of: 0.2, 0.4, 0.8, 1, and 3 μM (BRL 37344) and 0.4, 0.8, 1, and 3 μM (fenoterol). Data represent the mean from at least three observations.

traction. Because lowering the tone induced by contractile agonists can enhance the responsiveness of a smooth muscle to a β-adrenoceptor agonist, shifting the CRC to the left (Van Amsterdam *et al.*, 1989), the relationship between the size of the methacholine-induced contraction and the pD_2 ($-\log EC_{50}$) value for relaxation induced by the β_3-adrenoceptor agonist BRL 37344, and the β_2-adrenoceptor agonist fenoterol was established (Figure 2). For both compounds linear relationships with identical slopes were found. Hence, the spontaneous leftward-shift, due to the decrease of the methacholine-induced contraction by 100 μM ICI 118,551, could be corrected for. This procedure was applied to all agonists to correct the individual log(DR-1) values, obtained at 100 μM ICI 118,551.

Antagonism of (−)-isoprenaline-induced relaxation by CGP 20712A is shown in Figure 3a. Only at 100 μM CGP 20712A was a clear rightward shift observed, from which a pA_2 value of 4.70 ± 0.16 ($n = 5$) was calculated.

ICI 118,551 at 10 nM caused a moderate shift to the right of the (−)-isoprenaline CRC, which hardly increased at 100 nM to 10 μM. Only at 100 μM did the shift increase substantially (Figure 3b). The resulting Schild-plot (inset) was clearly biphasic. From the steep part of this plot, having a slope of 1.09 ± 0.09, which was not significantly different from unity, a pA_2 value of 5.31 ± 0.10 ($n = 13$) was calculated.

Responses to BRL 37344 were not affected by CGP 20712A or ICI 118,551 in concentrations up to 10 μM. Only at 100 μM of both antagonists was the CRC to BRL 37344 clearly shifted (Figure 4). From the 100 μM data-points pA_2 values were calculated, yielding 4.97 ± 0.12 ($n = 3$) for CGP 20712A and 5.48 ± 0.10 ($n = 12$) for ICI 118,551.

With fenoterol as the (β_2-selective) agonist, a moderate shift and clear steepening of the CRC was seen at 100 nM ICI 118,551 (Figure 5a). A marked further shift was observed only at 100 μM ICI 118,551. From the steep part of the biphasic Schild plot (inset), with a slope not significantly different from unity (1.15 ± 0.05), a pA_2 value of 5.30 ± 0.11 ($n = 10$) was calculated.

Clenbuterol, the least potent of the agonists used, showed a markedly shallow CRC (Figure 5b). As with fenoterol, the CRC to clenbuterol was shifted and clearly steepened at 100 nM ICI 118,551. Again, only at 100 μM of the antagonist was a substantial further shift to the right observed. The inset shows the biphasic Schild plot, of which the steep part has a slope of 1.02 ± 0.23, which was not significantly different from unity, yielding an apparent pA_2 value of 5.48 ± 0.35 ($n = 11$).

Discussion

The existence of atypical or β_3-adrenoceptors has now been generally accepted. Although a selective β_3-adrenoceptor antagonist is still lacking, low potencies and stereoselectivities of classical β-adrenoceptor antagonists (Harms *et al.*, 1977; Bojanic *et al.*, 1985), together with the high potency of a novel class of β-adrenoceptor agonists (Arch *et al.*, 1984) has provided strong support for the occurrence of atypical β-adrenoceptors. These receptors were shown to be abundantly

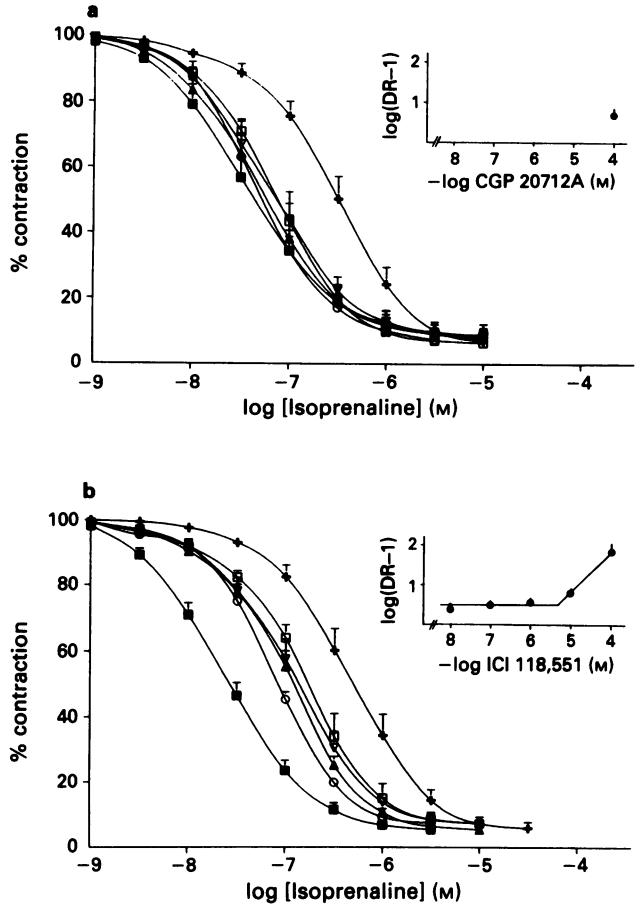

Figure 3 Antagonism of (−)-isoprenaline-induced relaxation by CGP 20712A (a) and ICI 118,551 (b). Control (■), CGP 20712A/ICI 118,551 10 nM (○), 100 nM (▲), 1 μM (▽), 10 μM (□), and 100 μM (+). Shown are the mean of five to eight experiments each performed in duplicate. The inset shows the corresponding Schild plot.

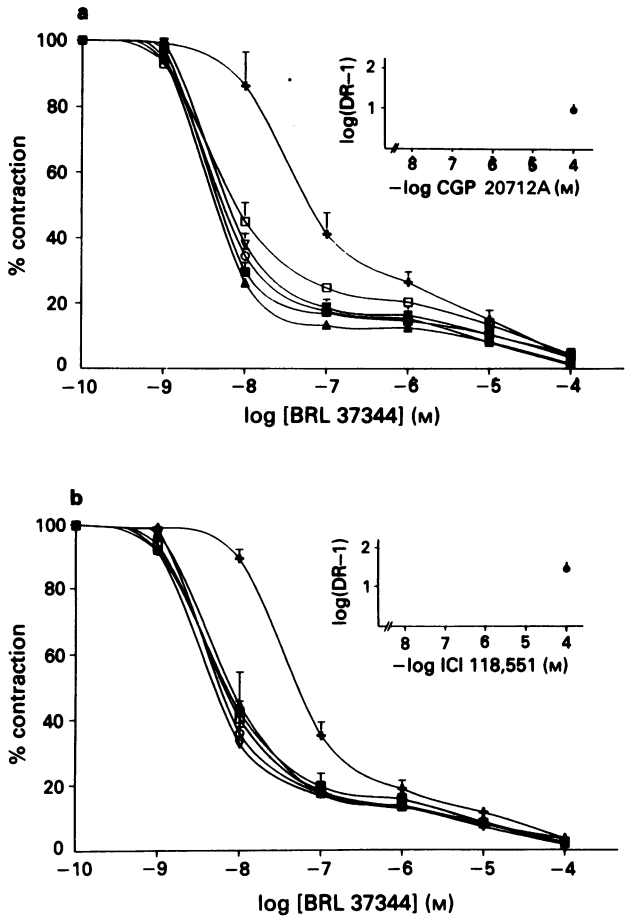

Figure 4 Antagonism of BRL 37344-induced relaxation by CGP 20712A (a) and ICI 118,551 (b). Control (■), CGP 20712A/ICI 118,551 10 nM (○), 100 nM (▲), 1 μM (▽), 10 μM (□), and 100 μM (+). Shown are the mean of three to five (CGP 20712A) and eight to thirteen (ICI 118,551) experiments each performed in duplicate. The inset shows the corresponding Schild plot.

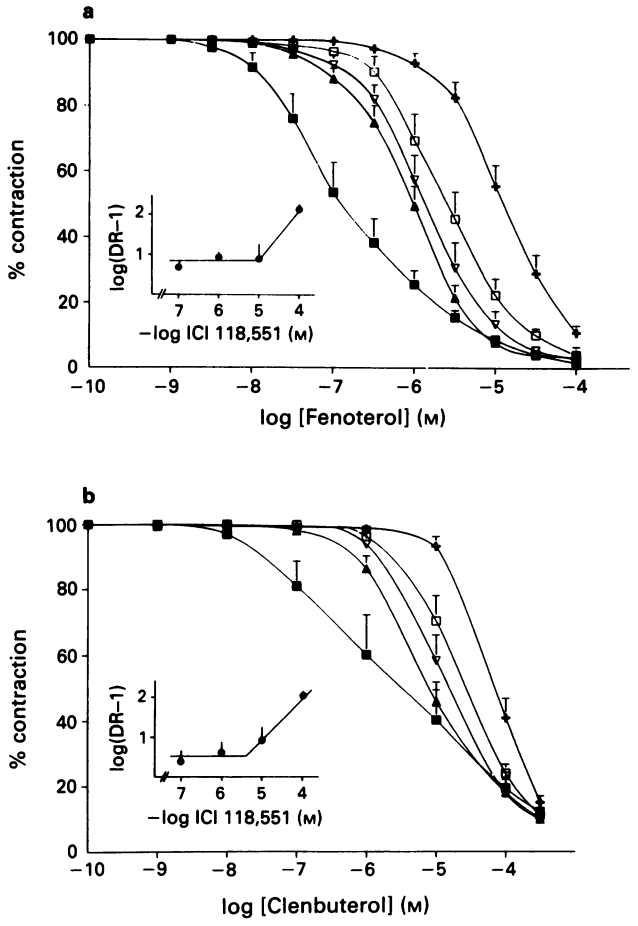

Figure 5 Antagonism of fenoterol- (a) and clenbuterol- (b) induced relaxation by ICI 118,551. Control (■), ICI 118,551 100 nM (▲), 1 μM (▽), 10 μM (□) and 100 μM (+). Shown are the mean of five to seven experiments each performed in duplicate. The inset shows the corresponding Schild plot.

present in adipose and gastrointestinal tissue (for a review, see Zaagsma & Hollenga, 1991).

The present study was undertaken to characterize the β-adrenoceptor(s) mediating relaxation of rat oesophageal muscularis mucosae by performing complete concentration-response curves with (−)-isoprenaline, BRL 37344, fenoterol and clenbuterol. Isoprenaline was almost a full agonist with a potency which was very similar to values reported for lipolysis (Hollenga & Zaagsma, 1989) and colonic relaxation (McLaughlin & MacDonald, 1990). The (−)-isoprenaline-induced relaxation was not antagonized by low CGP 20712A concentrations. Since CGP 20712A is a very potent and highly β_1-selective antagonist (Dooley *et al.*, 1986), it can be concluded that β_1-adrenoceptors are not involved in (−)-isoprenaline-induced relaxation of oesophageal smooth muscle. The pA_2 value of 4.70, derived from the highest CGP 20712A concentration is similar to the value of 4.80 for antagonizing (−)-isoprenaline-induced lipolysis in rat adipocytes (Hollenga & Zaagsma, 1989), indicating the involvement of similar atypical adrenoceptors as in rat adipocytes. With 100 nM ICI 118,551, some steepening and a small rightward shift of the (−)-isoprenaline CRC indicates the involvement of a β_2-adrenoceptor population, particularly at the lower concentrations of the agonist. With the marked rightward shift at 100 μM ICI 118,551, the participation of atypical receptors is clearly indicated as well. The pA_2 value for ICI 118,551 calculated from the steep part of the Schild plot (5.31) is similar to the value of 5.49 for antagonizing (−)-isoprenaline-induced lipolysis in rat adipocytes (Hollenga & Zaagsma, 1989).

BRL 37344-induced relaxation of oesophageal muscularis mucosae was mediated almost exclusively by atypical receptors. As shown in Figure 4, responses to BRL 37344 were not antagonized by either CGP 20712A or ICI 118,551 at concentrations up to 10 μM. Only at 100 μM was a clear rightward shift observed for both compounds. Again, pA_2-values (4.97 and 5.48, respectively) were similar to the pA_2 values for CGP 20712A and ICI 118,551 obtained in rat adipocytes (4.61 and 5.33; Hollenga & Zaagsma, 1989). The relaxing potency of BRL 37344 was 6.5 fold higher than (−)-isoprenaline, which is somewhat lower than the 10 fold potency difference, reported for lipolysis in rat adipocytes (Hollenga & Zaagsma, 1989). This may be explained by the small, but significant contribution of β_2-adrenoceptors in the (−)-isoprenaline-induced relaxation of rat muscularis mucosae, in contrast to the mere subordinate role of the β_1-adrenoceptor population in rat adipocytes.

As compared with the other agonists used, the relaxation by BRL 37344 was less complete. The effects observed at the highest concentrations of BRL 37344 (10 and 100 μM) appeared to be non-specific and not due to a β_2- (or β_3-) adrenoceptor-mediated relaxation, as increasing concentrations of the β_2-antagonist ICI 118,551 were without any effect on this part of the CRC.

BRL 37344-induced relaxations were slow, compared to (−)-isoprenaline-induced relaxations. Complete CRCs to BRL 37344 lasted about 2 times longer than comparable CRCs with (−)-isoprenaline as the agonist. In addition, BRL 37344 caused tachyphylaxis. This phenomenon has also been observed in other gastrointestinal tissues, like guinea-pig gastric fundus with BRL 35135 (Coleman *et al.*, 1987) and with BRL 37344 in rat distal colon (McLaughlin & MacDonald, 1990) and rat gastric fundus (McLaughlin & MacDonald, 1991). The mechanism of this desensitization however, is unclear.

As suggested recently for the cloned human β_3-adrenoceptor (Emorine *et al.*, 1991), agonist-induced regulation of this receptor might be different from that of β_1- and β_2-adrenoceptors, due to the absence of protein kinase A (PKA) phosphorylation sites, together with the absence of several serine and threonine-rich regions in the C-terminal region involved in β-adrenoceptor kinase (βARK)-mediated desensitization (Hausdorff *et al.*, 1990). These structural differences have also been reported for the rat β_3-adrenoceptor (Granneman *et al.*, 1991). According to these findings, the observed desensitization in our study would indicate a mechanism, other than phosphorylation by PKA or βARK, or phosphorylation at different positions within the receptor.

With fenoterol and clenbuterol as β_2-selective agonists, the moderate rightward shifts and clear steepening of the CRCs at 100 nM ICI 118,551 confirmed the presence of a functional β_2-adrenoceptor population, particularly at the lower concentrations of the agonists. This shift increased substantially only at 100 μM, indicating the contribution of a major, atypical β-adrenoceptor population. Interestingly, the pD_2 values for fenoterol and clenbuterol are again very similar to the values reported for rat adipocytes (6.95 and 5.40 for fenoterol and clenbuterol, respectively; Hollenga *et al.*, 1990), suggesting that at concentrations producing half-maximal relaxation, the atypical β-adrenoceptor already predominates. This would be in line with the recent observation in adipocytes that the transduction efficiency (i.e. the relationship between cyclic AMP generation and cellular response) is much higher for the atypical than for the typical β-adrenoceptor (Hollenga *et al.*, 1991). Therefore, it can be predicted that selective blockade of the less efficient β_2-adrenoceptor (by low concentrations of ICI 118,551) in rat oesophagus would steepen the CRCs of β_2-selective agonists like fenoterol and clenbuterol. Thus, although we realize that the construction of Schild plots, in cases where steepening of the CRCs is indicative for the involvement of more than one receptor type, is questionable, it is evident from both the CRCs and the Schild analyses, that β_2-, and predominantly

β_3-adrenoceptors, are involved in the β-adrenoceptor-mediated relaxation of rat muscularis mucosae by fenoterol, clenbuterol as well as (−)-isoprenaline. As expected, the BRL 37344-mediated relaxation was shown to be mediated solely by β_3-adrenoceptors.

Using CGP 20712A as the antagonist, no evidence for any contribution of a β_1-adrenoceptor was found. The pA_2 values of the antagonists as well as the pD_2 values of the agonists clearly indicate that the nature of the β_3-adrenoceptor population in rat oesophageal muscularis mucosae is identical to that of rat adipocytes.

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