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Evidence for the existence of 'atypical' β-adrenoceptors (β_3-adrenoceptors) mediating relaxation in the rat distal colon *in vitro*

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1 Experiments were carried out to characterize the adrenoceptors mediating relaxant responses in the rat distal colon. Three agonists were used: noradrenaline, isoprenaline and the β_3-adrenoceptor agonist BRL 37344. Phentolamine, propranolol and (±)-cyanopindolol were tested as antagonists. Tone in the rat distal colon was induced with KCl (30–40 mM) as a spasmogen, and relaxations of this KCl-induced tone produced by the agonists were measured.

2 Relaxant responses to noradrenaline that were obtained in the presence of propranolol (1 μM) were not antagonized by phentolamine (0.01 to 1 μM). Relaxant responses to isoprenaline that were obtained in the presence of phentolamine (1 μM) were antagonized in a concentration-dependent manner by propranolol (0.01 to 3 μM), although this antagonism was weak and non-competitive. Relaxant responses to BRL 37344 that were obtained in the presence of phentolamine (1 μM) were only weakly antagonized by high (1 μM) concentrations of propranolol.

3 Tachyphylaxis to BRL 37344 was observed, a second concentration-response curve being shifted to the right by 15 fold. Exposure of the tissues to BRL 37344 (1 μM) between concentration-response curves also caused rightward shifts in the responses to noradrenaline (18 fold) and isoprenaline (19 fold) but not to papaverine.

4 In the presence of phentolamine (1 μM) and propranolol (1 μM), the rank order of potency of the agonists was: (−)-isoprenaline (1.0) ≥ BRL 37344 (0.93) > (−)-noradrenaline (0.3).

5 Responses to BRL 37344 in the presence of phentolamine (1 μM) and propranolol (1 μM) were antagonized by (±)-cyanopindolol (1 μM), with an apparent pA_2 value of 6.67. Responses to isoprenaline, under the same conditions, were antagonized in a competitive manner by (±)-cyanopindolol (0.1 to 10 μM), with the slope of the Schild plot close to unity and a pA_2 value of 7.12.

6 The resistance of the relaxant responses to antagonism by phentolamine and propranolol, along with the relatively high potency of the β_3-adrenoceptor agonist BRL 37344 and the antagonism of 'resistant' responses by (±)-cyanopindolol would suggest that 'atypical' β-adrenoceptors, similar to the β_3-adrenoceptors of rat adipocytes and other tissues, exist in the rat distal colon.

Introduction

Responses to noradrenaline and isoprenaline which are resistant to blockade of classical α- and β-adrenoceptors have been reported in a number of animal isolated tissues, such as guinea-pig ileum (Wikberg, 1977; Bond *et al.*, 1986; Bond & Clarke, 1987); rabbit stomach (Bristow *et al.*, 1970); rabbit colon (Gillespie & Khoyi, 1977); dog colon (Grivegnee *et al.*, 1984); rat gastric fundus (Dettmar *et al.*, 1986b; Kelly & MacDonald, 1990) and guinea-pig gastric fundus (Coleman *et al.*, 1987). So-called 'atypical' β-adrenoceptors mediating lipolysis in rat adipocytes are also resistant to blockade by classical β-adrenoceptor antagonists (Stanton, 1972; Harms *et al.*, 1977) and in addition are characterized by the high selectivity and potency of a novel group of agonists (Arch *et al.*, 1984; Wilson *et al.*, 1984). It has been proposed that adipocyte β-adrenoceptors should be termed 'β_3-adrenoceptors' (Tan & Curtis-Prior, 1983; Arch, 1989). The finding that selective β_3-adrenoceptor agonists also have high potency at 'atypical' adrenoceptors in the guinea-pig ileum (Bond & Clarke, 1988) and the guinea-pig gastric fundus (Coleman *et al.*, 1987) suggests that β_3-adrenoceptors similar to those present in rat adipocytes may exist in gastrointestinal tissues.

The present study investigated responses to the catecholamines, noradrenaline and isoprenaline and the selective β_3-adrenoceptor agonist, BRL 37344 (Arch *et al.*, 1984) in the rat distal colon. A preliminary account of these findings has been presented to the British Pharmacological Society (McLaughlin & MacDonald, 1989).

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Methods

Tissue preparation

Male Wistar rats (200–300 g), fed *ad libitum*, were killed by a blow to the head and cervical dislocation. The distal 6 cm of the colon was removed and cleared of any faecal material by gentle squeezing of the colon with the fingers. Portions of colon were then immediately placed in cool Krebs physiological saline solution (PSS). Segments (3 cm) of colon were suspended in organ baths containing 30 ml Krebs solution, at 37°C, bubbled continuously with 95% O_2 and 5% CO_2, under an initial tension of 1 g. The composition of the Krebs solution was as follows (mM): NaCl 118, $CaCl_2$ 2.5, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 11.1. The Krebs solution contained cocaine (3 μM) to block neuronal uptake of the catecholamines and ascorbic acid (30 μM) and EDTA (30 μM) to prevent oxidation of the catecholamines. In some experiments, hydrocortisone (30 μM) was also included in the Krebs solution, to block extra-neuronal uptake of isoprenaline. Tissues were allowed to equilibrate for at least 30 min before experimental procedures were begun.

Concentration-response curves to noradrenaline were constructed in the presence of propranolol (1 μM) to allow investigation of the contribution of α-adrenoceptors to responses.

Concentration-response curves to isoprenaline were carried out in the presence of phentolamine (1 μM) to remove any possible contribution from α-adrenoceptors.

Concentration-response curves (CRCs)

The relaxant action of agonists was determined by measuring relaxations of KCl-induced tone evoked by addition of the agonists. Initially a CRC to KCl was constructed for each tissue, to determine a concentration of KCl which gave approximately 75% of maximal tone. Concentrations of 30–40 mM were added hypertonically to the organ baths by adding 0.3–0.5 ml of a 2 M KCl stock solution.

CRCs to adrenoceptor agonists were constructed by cumulative addition (0.5 log unit increments) to KCl-contracted strips at 1–2 min intervals until a stable response was observed. CRCs were constructed at 1 h intervals, allowing 45 min equilibration time for antagonists, which were added at the end of each CRC.

In experiments testing the effect of antagonists on the relaxations evoked by BRL 37344, only one CRC to BRL 37344 was constructed in any one tissue. In these cases paired experiments were carried out. If CRCs to isoprenaline were not significantly different in the paired tissues then one tissue was subsequently exposed to BRL 37344 in the presence of antagonist and a paired tissue exposed to BRL 37344 in the absence of antagonist. The antagonist was present for 45 min before construction of CRCs to BRL 37344.

Schild plots

Agonist concentration-ratios (CRs) were determined from the IC_{50} point on the CRC, with or without antagonist. The plot of log (agonist concentration ratio − 1.0) versus log [antagonist] (Arunlakshana & Schild, 1959) was analysed by linear regression. Antagonism was considered to be competitive if the slope of the regression line was not significantly different from unity. In these cases a mean pA_2 value was obtained from individual estimates using the equation

$$pA_2 = \log(\text{agonist concentration ratio} - 1) - \log[\text{antagonist}]$$

after first verifying that there was no significant regression of pA_2 on antagonist concentration (MacKay, 1978).

In cases where the slope of the Schild plot was significantly different from unity, or in cases where only one concentration of antagonist was tested, 'apparent' pA_2 values were estimated from a single antagonist concentration and the above equation.

Drugs used

The following were dissolved in distilled water, with the exception of hydrocortisone and (±)-cyanopindolol which were dissolved in absolute ethanol and 0.1 M tartaric acid, respectively: BRL 37344 (sodium-4[2[2-hydroxy-2(3-chlorophenyl)ethylamino]propyl]phenoxyacetate) (Beecham Research Laboratories, Great Burgh, Epsom); cirazoline hydrochloride (Synthelabo); cocaine hydrochloride (Thornton and Ross Ltd.); (±)-cyanopindolol (Sandoz); hydrocortisone-21-acetate (Sigma); (−)-isoprenaline (+)-bitartrate (Sigma); (−)-noradrenaline (+)-bitartrate (arterenol) (Sigma); papaverine hydrochloride (Sigma); phentolamine mesylate (Ciba-Geigy); (±)-propranolol hydrochloride (Sigma); UK 14,304 (5-bromo-6[2-imazolin-2-yl-amino]quinoxaline) bitartrate (Pfizer).

Statistical analysis

Results are expressed as mean ± s.e.mean with the number of determinations, n, given in parentheses with the exception of regression line slopes which are expressed as slope ± 95% confidence limits. Statistical significance between two data sets was tested by either Student's t test or a paired t test. A probability level of $P < 0.05$ was considered statistically significant.

Results

Responses to noradrenaline

In the presence of propranolol (1 μM), noradrenaline relaxed KCl-induced tone in the rat distal colon, with a pIC_{50} of 6.3 ± 0.18 ($n = 4$). These relaxant responses to noradrenaline were highly reproducible, CRCs being unchanged when constructed at hourly intervals over a period of 3 h (Table 1).

The CRCs to noradrenaline were unaffected by phentolamine (0.01 to 1 μM, Table 1). Also, in three experiments in the presence of 1 μM propranolol, neither cirazoline nor UK 14,304 relaxed KCl-induced tone in the rat distal colon (in concentrations up to 10 μM).

Responses to isoprenaline

In the presence of phentolamine (1 μM), isoprenaline relaxed KCl-induced tone in the rat distal colon, with a pIC_{50} of 7.66 ± 0.06 ($n = 6$). The relaxant responses to isoprenaline were highly reproducible, CRCs being unchanged when constructed at hourly intervals over a period of 3 h (Figure 1a). The responses to isoprenaline were weakly antagonized in a concentration-dependent manner by propranolol (0.01 to 3 μM, Figure 1b). A Schild plot (Figure 1c) revealed that this antagonism was non-competitive in nature, as demonstrated by the slope of the plot, which was significantly less than unity (0.44 ± 0.18, 95% CL, $n = 36$). An apparent 'pA_2' value for propranolol, calculated on the basis of the shift produced by 1 μM propranolol, gave a value of 6.57 ± 0.18 ($n = 6$).

Hydrocortisone (30 μM) had no effect on the potency of isoprenaline (pIC_{50} of 7.65 ± 0.1, $n = 8$) or on the antagonism by propranolol (apparent pA_2 of 6.48 ± 0.09, $n = 8$).

Responses to BRL 37344

In the presence of phentolamine (1 μM) the β_3-adrenoceptor agonist BRL 37344 relaxed KCl-induced tone in the rat distal colon, with a pIC_{50} of 7.33 ± 0.12 ($n = 4$). Tachyphylaxis to BRL 37344 was observed, a second CRC being shifted to the right by 15 fold, when compared to the control CRC (Figure 2a). The responses to BRL 37344 were not antagonized by low concentrations of propranolol (0.01 to 0.1 μM), but were antagonized to some extent by 1 μM propanolol (mean 8 fold shift of IC_{50} values, $P < 0.05$, Figure 2b). An apparent pA_2 value for propranolol, calculated on the basis of the shift produced by 1 μM propranolol, gave a value of 6.39.

In the presence of phentolamine (1 μM) and propranolol (1 μM), exposure of the tissues to BRL 37344 (1 μM) for 3 min approximately mid-way between CRCs to noradrenaline and isoprenaline produced significant (paired t test, $P < 0.01$) mean rightward 18 and 19 fold shifts of the CRCs to noradrenaline and isoprenaline, respectively (Figures 3a and 3b).

Table 1 Effect of phentolamine on noradrenaline-induced relaxations in rat distal colon in the presence of propranolol (1 μM)

	Phentolamine (μM)		*pIC_{50} of noradrenaline*	
	time		*time*	
CRC No.	*controls*	*treated*	*controls*	*treated*
1	0	0	6.55 ± 0.02	6.30 ± 0.18
2	0	0.01	6.43 ± 0.08	6.35 ± 0.19
3	0	0.1	6.51 ± 0.10	6.37 ± 0.15
4	0	1.0	6.50 ± 0.03	6.33 ± 0.08

Four concentration-response curves (CRCs) to noradrenaline were constructed at hourly intervals, allowing 45 min equilibration with each of the concentrations of phentolamine in the treated tissues. Values shown are means ± s.e.mean for 6 tissues in each case.

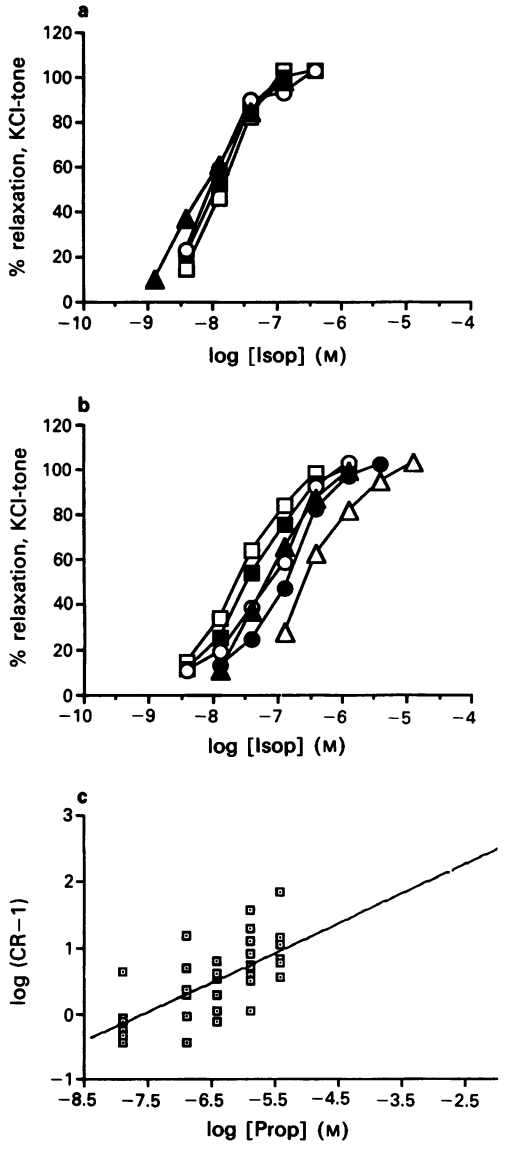

Figure 1 (a) Time control concentration-response curves to isoprenaline (Isop) in the rat distal colon in the presence of 1 μM phentolamine. Tone was raised by the addition of KCl (30–40 mM) to the organ baths. Each of the concentration-response curves was constructed at hourly intervals: the first (□, $n = 6$), second (■, $n = 6$), third (○, $n = 6$) and fourth curves (▲, $n = 6$) are shown. (b) The effect of propranolol, in concentrations 0.01 μM (■, $n = 6$), 0.1 μM (○, $n = 6$), 0.3 μM (▲, $n = 6$), 1 μM (●, $n = 12$) and 3 μM (△, $n = 6$) on control (□, $n = 12$) concentration-response curves to isoprenaline in the rat distal colon is shown. Error bars have been omitted for clarity. Standard errors were less than 15% in each case. (c) Schild plot of the antagonism of isoprenaline action by propranolol (Prop) in the rat distal colon.

The same experimental procedure, however, had no effect on the relaxant effect of papaverine on KCl-induced tone in the rat distal colon (Figure 3c).

Order of potency of agonists under conditions of classical α- and β-adrenoceptor blockade

In the presence of phentolamine (1 μM) and propranolol (1 μM), the pIC_{50}s of isoprenaline, BRL 37344 and noradrenaline were, respectively, 6.85 ± 0.18 (12), 6.82 ± 0.16 (4) and 6.33 ± 0.08 (6), giving a rank order of potency of: isoprenaline (1.0) ⩾ BRL 37344 (0.93) > noradrenaline (0.3).

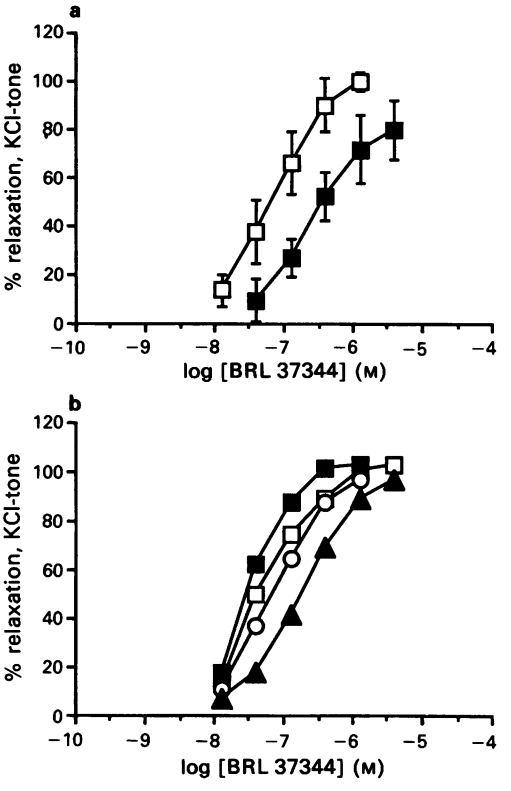

Figure 2 Effect of BRL 37344 on KCl-induced tone in the rat distal colon in the presence of 1 μM phentolamine. (a) Time controls for responses to BRL 37344. Curves represent first (□, $n = 4$) and second (■, $n = 4$) concentration-response curves, constructed 1 h apart. (b) The effect of propranolol, 0.01 μM (■, $n = 4$), 0.1 μM (○, $n = 4$) and 1 μM (▲, $n = 4$) on control (□, $n = 4$) concentration-response curves to BRL 37344. Tone was raised by the addition of KCl (30–40 mM) to the organ baths. Error bars have been omitted from the figure for clarity. In each case, standard errors were less than 15%.

Effect of (±)-cyanopindolol on responses to isoprenaline and BRL 37344 under conditions of α- and β-adrenoceptor blockade

In the presence of phentolamine (1 μM) and propranolol (1 μM), responses to BRL 37344 were antagonized by the β-adrenoceptor antagonist (±)-cyanopindolol (1 μM), with an apparent pA_2 value calculated from the single concentration of cyanopindolol of 6.67 ± 0.25 ($n = 7$). Also, cyanopindolol (0.1 to 10 μM) antagonized responses to isoprenaline in a concentration-dependent manner (Figure 4a). The antagonism of the action of isoprenaline was competitive in nature, since the slope of the Schild plot was not significantly different from unity (1.03 ± 0.17, 95% CL, $n = 12$, Figure 4b). The pA_2 value for cyanopindolol was 7.12 ± 0.06 ($n = 12$). The difference between the pA_2 values for cyanopindolol against BRL 37344 and isoprenaline was statistically significant ($P < 0.05$, Student's t test). Cyanopindolol itself had no effect on the degree of tone induced by KCl (tone in cyanopindolol-treated strips as % of control, $n = 4$; 0.1 μM, 89.7 ± 7; 1 μM, 105 ± 10; 10 μM, 109 ± 7).

Discussion

The action of catecholamines on the rat distal colon was not mediated by α-adrenoceptors, since (a) the responses to noradrenaline were resistant to the classical α-adrenoceptor antagonist phentolamine and (b) the selective α_1- and α_2-adrenoceptor agonists cirazoline (van Meel *et al.*, 1981) and UK 14,304 (Cambridge, 1981) were devoid of any relaxant

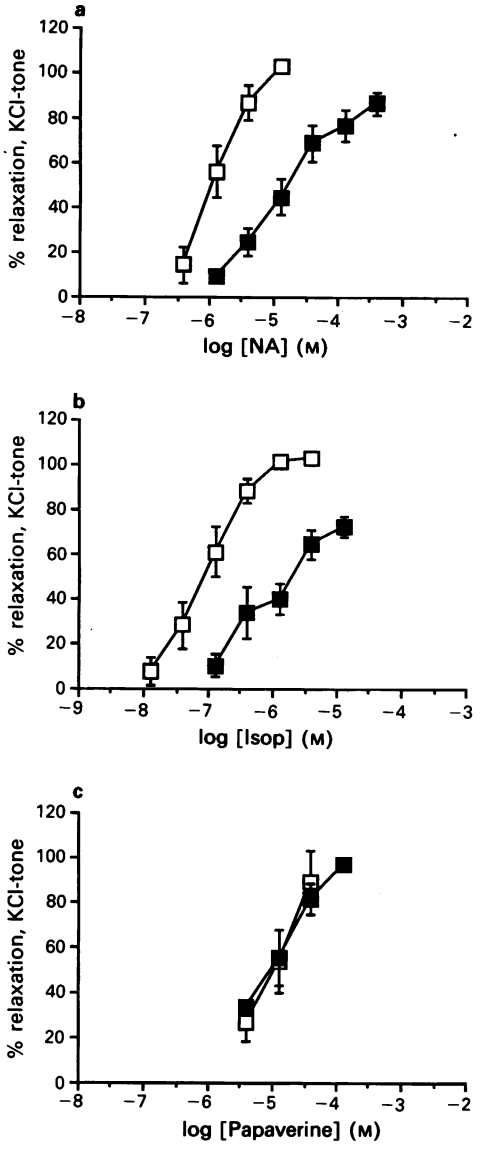

Figure 3 The effect of exposure of the tissues to 1 μM BRL 37344 for a period of 3 min between concentration-response curves to agonists. In each case, first (□, $n = 6$) and second (■, $n = 6$) concentration-response curves are shown. Tone was raised by the addition of KCl (30–40 mM) to the organ baths and phentolamine (1 μM) and propranolol (1 μM) were present throughout. (a) Noradrenaline (NA), (b) Isoprenaline (Isop) and (c) papaverine.

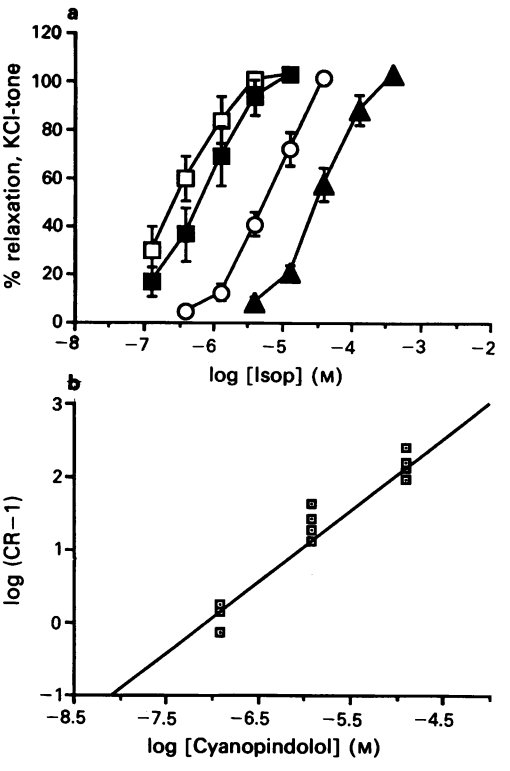

Figure 4 (a) The effect of cyanopindolol, in concentrations 0.1 μM (■, $n = 4$), 1 μM (○, $n = 4$) and 10 μM (▲, $n = 4$) on control concentration-response curves (□, $n = 4$) to isoprenaline in the rat distal colon in the presence of 1 μM phentolamine and 1 μM propranolol. Tone was raised by the addition of KCl (30–40 mM) to the organ baths. (b) Schild plot of the antagonism of isoprenaline action by cyanopindolol.

action. Inhibitory postjunctional α_1-adrenoceptors have previously been shown to exist in the rat distal colon (Dettmar *et al.*, 1986a) and the failure to show their presence in the present study is probably related to the KCl-induced depolarization, since α-adrenoceptor-mediated inhibitory effects in intestinal smooth muscle involve abolition of spontaneous spike discharge and hyperpolarization (Bülbring, 1954; 1957) and are not seen if the tissue is depolarized sufficiently to block spike generation (Magaribuchi & Kuriyama, 1972). Thus the present study does not rule out the presence of inhibitory α-adrenoceptors in this preparation, but their effects are not seen in the presence of KCl-induced tone.

The relaxations to the catecholamines were also elicited in the presence of propranolol (1 μM), conditions under which one would assume that classical β-adrenoceptors had been blocked. Propranolol did produce a shift of the isoprenaline CRC but the antagonism was non-competitive (slope of Schild plot less than unity) and weak with an apparent pA_2 of 6.57 compared with 8.2–8.8 for classical β_1-adrenoceptors mediating atrial stimulation and 8.3–8.6 for β_2-adrenoceptors mediating tracheal relaxation (Wilson *et al.*, 1984). However a similarly low pA_2 value (6.6) for propranolol against the lipolytic effect of isoprenaline was reported (Wilson *et al.*, 1984) and therefore it seems possible that the receptors mediating relaxation of the colon and lipolysis are similar.

Non-competitive antagonism of responses to catecholamines may result from failure to block extraneuronal uptake processes for the catecholamines (Furchgott, 1967). This possibility was ruled out in the present study since both the responses to isoprenaline and the antagonism by propranolol were similar in the presence and absence of the extraneuronal uptake blocker hydrocortisone (Iversen & Salt, 1970).

Relaxation of the rat distal colon mediated by the selective β_3-adrenoceptor agonist BRL 37344 was only weakly antagonized by propranolol, the apparent 'pA_2' value of 6.39 being similar to the reported pA_2 values for propranolol against β_3-adrenoceptor agonists stimulating lipolysis of rat white adipocytes (Wilson *et al.*, 1984). This again suggests that the receptors mediating relaxation in the rat distal colon and the receptors mediating lipolysis in adipocytes may be similar.

The relative potency of BRL 37344 under conditions of α- and β-adrenoceptor blockade, isoprenaline (1.0) $\geq$ BRL 37344 (0.93) > noradrenaline (0.3), compares with rat adipocyte β_3-adrenoceptors where BRL 37344, or its methyl ester BRL 35135, were equipotent with or more potent than isoprenaline (Arch *et al.*, 1984; Wilson *et al.*, 1984). A similar high potency of selective β_3-adrenoceptor agonists was found in guinea-pig gastric fundus (Coleman *et al.*, 1987) and guinea-pig ileum (Bond & Clarke, 1988).

In the present study BRL 37344 caused tachyphylaxis to itself and also to noradrenaline and isoprenaline. This was not simply a non-specific effect on all relaxant responses in the rat distal colon since responses to papaverine, which acts by inhibition of phosphodiesterase (Bar, 1974), were unaffected. Tachyphylaxis to BRL 35135 has also been reported in guinea-pig gastric fundus (Coleman *et al.*, 1987). The mechanism of the tachyphylaxis in the present study and in the

study by Coleman *et al.* (1987) is unclear but the cross-desensitization may be evidence that BRL 37344 and the catecholamines are acting at the same site.

Responses to isoprenaline in the presence of α- and β-adrenoceptor blockade with phentolamine and propranolol were antagonized in a competitive manner by cyanopindolol. Cyanopindolol has previously been shown to be a potent competitive antagonist at propranolol-resistant 'atypical' β-adrenoceptors in guinea-pig ileum (Blue *et al.*, 1989) and the reported pA_2 of 7.63 is similar to that obtained in the present study (7.12). Thus the receptor mediating responses to isoprenaline in rat distal colon appears to be similar to the 'atypical' β-adrenoceptor present in guinea-pig ileum. A Schild regression was not carried out for cyanopindolol against BRL 37344 but the single concentration of cyanopindolol tested (1 μM) gave an apparent pA_2 of 6.67 which was significantly lower than the value obtained with isoprenaline as agonist. Lower pA_2 values for β-adrenoceptor antagonists when BRL 37344 or analogues were the agonists than when isoprenaline was the agonist have previously been found (Stock & Sudera, 1988; Jones *et al.*, 1989; Arch, 1989), suggesting that the interaction of the BRL compounds with the β_3-adrenoceptor may differ from that of isoprenaline. It has been suggested that the bulky N-substituent of the BRL compounds may bind to an accessory site and reduce the affinity of antagonists for the main binding site (Arch, 1989).

There is evidence that some partial adrenoceptor agonists acting on human myocardial tissue, designated unconventional and including (−)-pindolol, cause part of their effects through heart adrenoceptors which are neither β_1- nor β_2-adrenoceptors (Walter *et al.*, 1984; Kaumann, 1989). These agonist effects are seen at concentrations greater than are necessary to produce blockade of β_1- and β_2-adrenoceptors, are resistant to conventional β-blockade and have been attributed to an action at a third heart β-adrenoceptor, termed β_3 (Kaumann, 1989). It may be that the cardiac β_3-adrenoceptors are similar to the β-adrenoceptors of the rat distal colon and other tissues and that there is some relationship between the partial agonist effect of pindolol at β_3-adrenoceptors and the antagonistic effect of cyanopindolol at 'atypical' β-adrenoceptors in the gut. Pindolol analogues may also act as partial agonists at rodent β_3-adrenoceptors (Engel *et al.*, 1981). However, there was no evidence in the present study for a partial agonist effect of cyanopindolol at the concentrations used (up to 10 μM, approximately 100 × pK_a at the 'atypical' β-adrenoceptors in this study).

Recently, a human gene has been isolated that encodes a product referred to as the "β_3-adrenoceptor" (Emorine *et al.*, 1989). Investigation of the properties of this receptor in Chinese Hamster ovary cells transfected with the gene showed low affinities for classical β-adrenoceptor antagonists and a high potency of BRL 37344. It is likely, therefore, that this receptor represents a separate class of β-adrenoceptor which includes the β_3-adrenoceptors of adipocytes, myocardium and gastrointestinal smooth muscle. It has been suggested that these receptors may represent intermediate stages in evolution towards classical β_1- and β_2-adrenoceptors (Bond & Clarke, 1988; Zaagsma & Nahorski, 1990).

In conclusion, the resistance of catecholamine-induced relaxations to propranolol, the relatively high potency of the β_3-adrenoceptor agonist, BRL 37344, and the competitive antagonism of propranolol-resistant responses by cyanopindolol suggest that the rat distal colon contains 'atypical' β-adrenoceptors similar to the β_3-adrenoceptors in rat adipocytes and other tissues. The physiological significance of such receptors in the gastrointestinal tract is at present unknown, although it has been shown that their activation by selective agonists can affect the motility of rat proximal colon *in vivo* (Manara *et al.*, 1989).

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