Br. J. Pharmacol. (1990), **100**, 831–839

In vitro inhibition of intestinal motility by phenylethanolaminotetralines: evidence of atypical β-adrenoceptors in rat colon

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1 The new compounds phenylethanolaminotetralines (PEAT), unlike the reference β-adrenoceptor agonists isoprenaline (Iso), ritodrine (Ri) and salbutamol (Sal), produced half-maximal inhibition of spontaneous motility of rat isolated proximal colon at substantially lower concentrations (EC_{50} 2.7–30 nM) than those inducing β_2-adrenoceptor-mediated responses (relaxation of guinea-pig isolated trachea and rat uterus) and had virtually no chronotropic action ($EC_{50} > 3 \times 10^{-5}$ M) on the guinea-pig isolated atrium (a β_1-adrenoceptor-mediated response).

2 The nonselective β-adrenoceptor antagonists alprenolol and propranolol prevented the inhibition of rat colon motility by the PEAT with low and different potencies (pA_2 values around 7.5 and 6.5 respectively). Conversely alprenolol and propranolol had a higher and similar potency (pA_2 values around 9.0) in preventing typical β_1- or β_2-responses (increase in atrial frequency by Iso or tracheal relaxation by Ri or Sal).

3 The selective β-adrenoceptor antagonists CGP 20712A (β_1) and ICI 118,551 (β_2) either alone or in combination, did not prevent rat colon motility inhibition by the representative PEAT SR 58611A, which was also fully resistant to α-adrenoceptor, acetylcholine, dopamine, histamine, opioid and 5-hydroxytryptamine antagonists.

4 These results indicate that the PEAT are a new class of β-adrenoceptor agonists and suggest that their preferential intestinal action may be accounted for by selectivity for atypical β-adrenoceptors, abundant in the rat colon and distinct from the currently recognized β_1 and β_2 subtypes.

Introduction

The important physiological role of local intestinal adrenergic mechanisms in reducing gut motility is well established, as discussed in several reviews (Furness & Costa, 1974; Burnstock & Wong, 1981; Daniel, 1982; Bülbring & Tomita, 1987) and substantial clinical evidence (Petri *et al.*, 1971; Neely & Catchpole, 1971; Rees *et al.*, 1980; O'Brien *et al.*, 1985; Lyrenäs *et al.*, 1985; Lyrenäs & Abrahamsson, 1986) supports the potential of this finding for developing pharmacological means for therapeutic applications. However, even the best tolerated β_2-selective adrenoceptor agonists, which otherwise seem promising for treating conditions of abnormally enhanced gastrointestinal motility (Lyrenäs *et al.*, 1985; Lyrenäs & Abrahamsson, 1986), are not recommended for therapy because of their unacceptable concurrent cardiovascular effects (Lyrenäs *et al.*, 1985).

On these grounds, we engaged in a search for gut-specific sympathomimetic agents. We have preliminarily described the relative *in vitro* intestinal specificity of the new putative β-adrenoceptor agonists phenylethanolaminotetralines (PEAT), which had no chronotropic action on the guinea-pig atrium and were considerably more potent in reducing spontaneous motility of the isolated proximal colon of the rat than in relaxing the guinea-pig trachea (Croci *et al.*, 1987; 1988a). *In vivo* studies in the rat have shown that the PEAT, unlike currently available β-adrenoceptor agonists, reduce large intestine motility at doses with no effect on the cardiovascular system (Croci *et al.*, 1989; Giudice *et al.*, 1989).

This paper discusses in detail the nature of the *in vitro* action of PEAT and the possibility that atypical β-adrenoceptors abundant in the proximal colon of the rat and distinct from the currently recognized β_1 and β_2 subtypes may account for the relative intestinal specificity of these new agents.

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Methods

All the isolated preparations, from rats Crl: CD(SD)BR Charles River (250–350 g) of either sex or male DH IFFA-CREDO guinea-pigs (500–700 g), were set up in appropriate solutions (containing 200 μg ml^{-1} ascorbic acid) in a 20 ml organ bath maintained at 37°C and aerated with 95% O_2, 5% CO_2 unless otherwise specified.

The first 3 cm segment of the rat proximal colon, starting from the ileo-caecal junction, was mounted longitudinally in Krebs-Ringer solution with composition identical to that described by Ek *et al.* (1986a). Spontaneous motility (rhythmic phasic contractions) was recorded isotonically under a constant load of 1 g (see Figure 1) in the presence of 10 μM phentolamine, 0.5 μM desmethylimipramine and 30 μM hydrocortisone hemisuccinate to block respectively α-receptors and neuronal and extraneuronal catecholamine uptake, as suggested for other isolated preparations by Kenakin (1984). Phentolamine renders the preparation about ten times more sensitive to the PEAT and to reference β-adrenoceptor agonists and this accounts for a higher EC_{50} than we obtained previously in its absence (Croci *et al.*, 1987; 1988a; Bianchetti *et al.*, 1988).

Quantitative computer analysis was based on a motility index consisting of the area under the pressure waves over a 10 min period. Only one agonist concentration was tested on each preparation either with or without one concentration of antagonist added 30 min before. In some experiments, IBMX and tetrodotoxin, were added to the organ bath 30 min before the agonist.

Guinea-pig spontaneously beating right atria were set up in Krebs solution maintained at 30°C (O'Donnell & Wanstall, 1974) and preincubated (30 min) with 12 μM phenoxybenzamine before addition of the tested compounds by the cumulative method with appropriate log-concentration increments (contact time for each dose 2.5 min); responses were expressed as a percentage of the maximal response. For studying

antagonists (and for calculating pA_2 values), concentration-response curves for the agonist in the absence or presence of one concentration of antagonist, added 30 min before, were obtained in the same preparation.

Guinea-pig tracheal chains, prepared as described by O'Donnell & Wanstall (1974), were set up in Krebs solution and preincubated (30 min) with 12 μM phenoxybenzamine before the addition of the tested compounds by log-concentration increments; only one cumulative concentration-response curve was obtained in each preparation. Drug-induced relaxation was recorded isotonically under a constant load of 0.5 g. The contact time between doses was 5 min or longer until the effect reached a steady state; the shorter contact time we had used in earlier experiments (2.5 min) resulted in lower potencies for several agonists (Croci *et al.*, 1987; 1988a) than those given in this paper. For studying antagonists, tracheal chains from the same animal were mounted in pairs and cumulative concentration-response curves for the agonist with or without one concentration of antagonist (contact time 30 min) were compared directly in the two preparations.

Uterine horns from unprimed oestrus rats were set up in Locke solution aerated with 100% O_2 (Levy & Tozzi, 1963) and preincubated (30 min) with 12 μM phenoxybenzamine before addition of the tested drugs. Spontaneous contractions were recorded isotonically under a constant load of 1 g. Only one agonist concentration was tested on each preparation and allowed to act for at least 5 min. Activity was expressed as the percentage reduction of the amplitude of contractions.

Concentrations of agonists inducing tracheal relaxation or increasing atrial frequency by 50% over the basal value (EC_{50}) were extrapolated from cumulative log-concentration-response curves obtained by the least squares method. In the other preparations (rat colon and uterus) the EC_{50} (concentrations of agonists reducing spontaneous contractions by 50%) were obtained from log-concentration response curves built up by averaging the individual responses in each preparation.

pA_2 values for antagonists, as defined by Arunlakshana & Schild (1959), were obtained from constrained plots (best straight line with a slope of unity) of log (DR − 1) vs negative log[antagonist] according to MacKay (1978). Fitting of the experimental data to a theoretical Schild plot (slope not significantly different from unity), by use of four different concentrations of antagonists was preliminarily assessed. Computer analysis was carried out as described by Tallarida & Murray (1987). Antagonism of responses to Iso by propranolol, which did not fit a simple linear Schild plot with a slope of unity, was analysed by a non-linear fitting programme (Sacchi Landriani *et al.*, 1983) by applying a model for the interaction of competitive antagonists with two receptor sites according to Lemoine & Kaumann (1983).

The phenylethanolaminotetralines (PEAT), whose structures and isomers with appropriate code numbers are presented in Table 1, were synthesized in the Chemistry Section of the SANOFI-MIDY S.p.A. Research Center. Synthesis was completed by R. Boigegrain, S. Boveri, and R. Cecchi (EP 211 721 and pending patent applications).

Some of the reference drugs and other chemicals were from the following commercial sources as indicated: Ciba-Geigy, Varese, Italy – desmethylimipramine HCl, phentolamine methansulphonate (Regitin ampoules); Sigma-Aldrich Corp. St Louis, Missouri, U.S.A. – (−)-isoprenaline HCl (Iso), (+)-Iso, (±)-Iso, (−)-noradrenaline bitartrate (NA), atropine sulphate, propranolol HCl, (±)-salbultamol hemisulphate (Sal), tetrodotoxin, verapamil HCl; Prodotti Gianni, Milan, Italy – ranitidine HCl, indomethacin; RBI Natick, Massachusetts, U.S.A. – spiperone; Rhône-Poulenc, Milan, Italy – mepyramine maleate; Ricerchimica, Milan, Italy – alprenolol HCl; TCI, Tokyo, Japan – phenoxybenzamine HCl; Lusofarmaco, Milan, Italy – ritodrine (±)-erythro HCl (Ri) (Miolene ampoules); Richter-Lepetit, Milan, Italy – hydrocortisone hemisuccinate (Flebocortid ampoules); EGA-Chemie, Albuch, W. Germany – 3-isobutyl-1-methylxanthine (IBMX); S.I.F.A.C., Pavia, Italy – naloxone HCl.

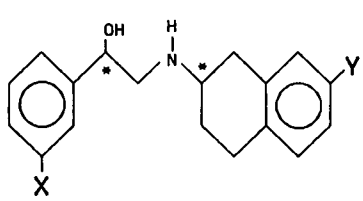
Other chemicals were kindly provided as follows: Ciba-Geigy, Basel, Switzerland – CGP 20712A ((±)-[2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-2-propanol); ICI, Macclesfield, Cheshire, England – ICI 118,551 (erythro (±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol-hydrochloride); Janssen, Beerse, Belgium – (−)-adrenaline (Ad), cisapride and domperidone; Sandoz, Basel, Switzerland – methysergide bamaleate.

Results

Spontaneous motility of the rat isolated proximal colon was reduced concentration-dependently by reference adrenoceptor agonists and by PEAT as illustrated by the representative recordings in Figure 1 and by the log concentration-response curves in Figure 2a. The PEAT and reference compounds were also tested in range of concentrations on the guinea-pig atrium, rat uterus and guinea-pig trachea (Figure 2b, c and d).

The potencies of the compounds tested in relaxing the rat colon *in vitro* and in generating presumably β-adrenoceptor-mediated responses in the other isolated preparations are shown in Table 2. The nonselective β-adrenoceptor agonist Iso and the β_2 selective Sal were the most and the least potent

Table 1 Structural formulae and isomers of phenylethanolaminotetralines (PEAT)



X	Y	Code number and chiral carbons configuration§	
H	−OH	SR 58372	(SR)
		SR 58373A	(SS)
		SR 58374	(RS)
		SR 58375A	(RR)
Cl	−OH	SR 58572A	(RR)
		SR 58575A	(SS)
		SR 58589	(RS)
		SR 58590	(SR)
Cl	$-OCH_2$	SR 58611A	(RS)
	|	SR 58612A	(RR)
	CO	SR 58613A	(SS)
	|	SR 58825A	(SR)
	C_2H_5O		

§ Code numbers, if followed by the letter 'A' indicate the HCl salts, but otherwise identify the free bases; the first letter in parentheses indicates the configuration of the chiral carbon of the phenylethanolamine and the second one that of the tetraline moiety.
* Indicates chiral carbon.

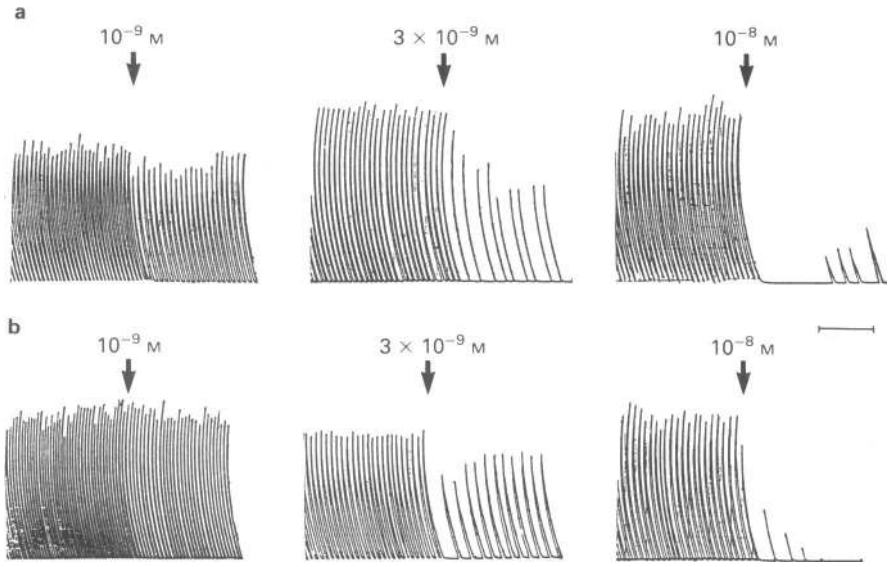


Figure 1 Rat proximal colon spontaneous motility: representative recordings. After stabilization of spontaneous colonic contractions, either isoprenaline (a) or SR 58611A (b) were added (arrows) to the baths (see Methods) at the concentrations indicated. Bar represents five min recording.

(EC_{50} 1.5 nM and 285 nM) on the rat isolated colon. In the same preparation the β_2 selective adrenoceptor agonists Ri, Ad, NA and the PEAT had intermediate potencies, SR 58611A being only about two times less potent than Iso (EC_{50} 3.5 nM).

In the guinea-pig isolated atrium, the PEAT were inactive and Ri had only scant activity at micromolar concentrations; Iso, NA, Ad and Sal were only four to five times less potent in increasing guinea-pig atrial frequency than in relaxing the rat colon. Iso, Ad and Sal were the most potent in relaxing the rat isolated uterus; Ri, NA and the PEAT were substantially less potent in this preparation. SR 58375A and Ri were roughly equally potent in the uterus and in the colon. SR 58572A and SR 58611A, like NA, were less active in relaxing the uterus than the colon, in contrast to Iso, Ad and Sal which were all more potent in the uterus than in the colon. Iso, Ad, Sal, NA and to a slightly less extent Ri (intrinsic activity relative to Iso: 0.85), fully relaxed the guinea-pig isolated trachea. Iso and NA relaxed guinea-pig trachea at concentrations similar to those inhibiting rat colonic motility, whereas a lower concentration of Ad and Sal (about three and 20 times respectively) and five times more Ri than in the rat colon were needed to elicit comparable effects in the guinea-pig trachea. All the PEAT were substantially less effective in the guinea-pig trachea than in the rat colon and even at the highest concentrations tested failed to produce full tracheal relaxation like that observed with the reference adrenoceptor agonists (i.e. in the trachea the PEAT behaved as weak partial agonists).

As shown in Table 3, the action of Iso on the rat isolated colon, rat uterus and guinea-pig atrium was stereospecific, the (−)-isomer being about 56, 116 and 26 times more potent respectively than the (+)-isomer. The PEAT too had configuration-dependent action on the same isolated preparations. This was unequivocal in the case of the phenol compounds SR 58372, SR 58373A, SR 58374 and SR 58375A, the

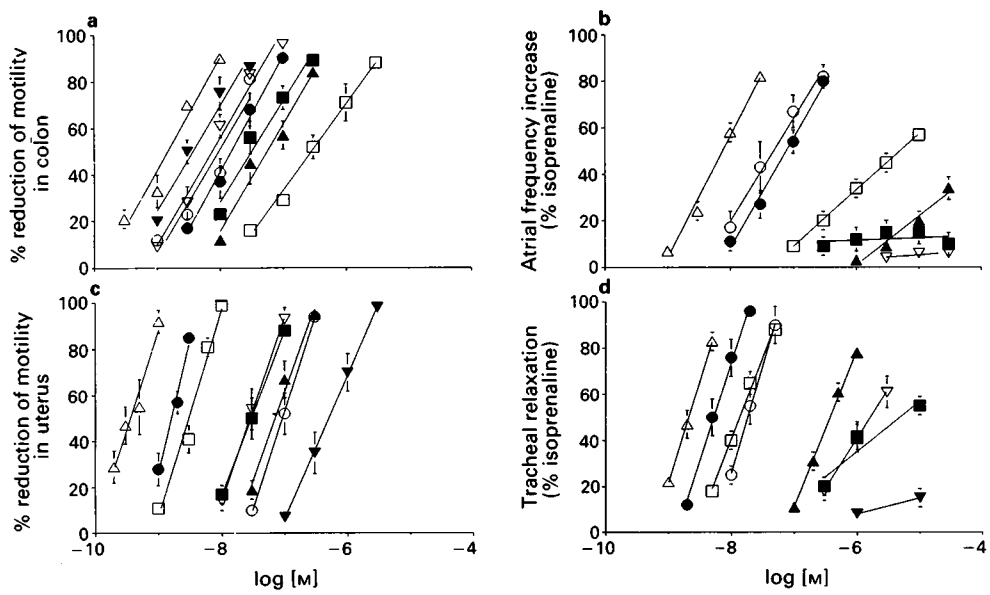


Figure 2 Concentration-response curves of phenylethanolaminotetralines (PEAT) and other adrenoceptor agonists for reduction of rat colon motility (a), stimulation of guinea-pig atrial frequency (b), reduction of rat uterus motility (c) and relaxation of guinea-pig trachea (d). The following symbols are used: (△) (±)-isoprenaline; (●) (−)-adrenaline; (○) (−)-noradrenaline ; (□) (±)-salbutamol; (▲) ritodrine; (■) SR 58375A; (▽) SR 58572A: (▼) SR 58611A. Abscissae: molar concentration of agonists. Ordinates: effects of agonists rated as indicated on each graph. In (a) and (c) 100% reduction corresponds to the maximal effect observed with isoprenaline (not indicated). Vertical bars represent s.e.mean (not shown if smaller than symbol). For further details see Methods.

Table 2 Potencies of phenylethanolaminotetralines (PEAT) and reference adrenoceptor agonists on rat proximal colon and other isolated preparations

	Rat proximal colon EC_{50}	*Rat uterus* EC_{50}	*Guinea-pig trachea* EC_{50}	*IA*	*Guinea-pig atrium* EC_{50}	*IA*
(±)-Isoprenaline	1.5 (1.2–1.9)	0.35 (0.27–0.47)	2.1 (1.8–2.5)	1	7.5 (6.4–8.7)	1
(−)-Noradrenaline	10 (8–14)	90 (75–109)	18 (14–23)	1.07	41 (25–67)	0.98
(−)-Adrenaline	15 (11–21)	1.6 (1.4–1.8)	5.2 (4.3–6.3)	1.01	78 (61–99)	1
Ritodrine	57 (41–78)	68 (53–88)	290 (250–330)	0.85	≅5,000	0.34
(±)-Salbutamol	285 (210–386)	3 (2.6–3.4)	13 (11–15)	0.98	1,030 (750–1,400)	0.67
SR 58375A	30 (22–41)	29 (23–37)	450 (170–1,160)	0.61	>30,000	ND
SR 58572A	7 (6–9)	28 (21–36)	480 (260–880)	0.61	>30,000	ND
SR 58611A	3.5 (2.6–4.7)	499 (372–672)	>10,000	0.15	>30,000	ND

EC_{50} = concentration (nM) producing half-maximal effect. The 95% confidence limits are shown in parentheses.
IA = intrinsic activity (isoprenaline = 1). The maximal dose tested for PEAT was 3×10^{-5} M.
ND = not determined (at 30,000 nM less than 10% of max. effect of isoprenaline).

RR isomer being more potent than its **RS** diastereoisomer and the **SR** and **SS** isomers being virtually inactive. The chlorophenol compounds showed similar configuration-related potency differences, though less pronounced between the **RR** compound SR 58572A and its diastereoisomer SR 58589.

There was a notable exception as far as the chlorophenoxy compounds were concerned. The **RR** isomer SR 58621A and its diastereoisomer SR 58611A were virtually equipotent on the rat colon, although the former was still about eight times more potent than the latter on the uterus.

The data on the quantitative antagonism by alprenolol and propranolol of the action of the representative PEAT SR 58375A and SR 58611A and of reference β-adrenoceptor agents on the rat colon are set out in Table 4. Antagonism with alprenolol was competitive, and roughly equipotent, as shown by the similarity of the pA_2 values (from 7.0 to 7.6), regardless of whether the reference β-adrenoceptor agonists or the PEAT were those selected for inhibiting rat colon motility. However, alprenolol was substantially less potent in antagonizing rat colonic motility inhibition by either the reference agonists or the PEAT than in preventing other reputedly typical β_1- or β_2-adrenoceptor-mediated responses, such as atrial frequency increase by Iso or tracheal relaxation by Ri or Sal. Propranolol antagonized the inhibition of rat colon motility by PEAT and Ri (competitively) and by Iso (noncompetitively), but with lower potency (pA_2 values 6.3 to 6.5) than alprenolol. The pA_2 values of alprenolol and propranolol versus Iso, Ri and Sal in either the guinea-pig atria or trachea were virtually identical and consistently higher (range 8.7–9.1) than in the rat colon (Table 4). Although the antagonism of Iso by propranolol in the rat colon was not compatible with a simple competitive action (Schild plot slope different from one), the experimental data were fitted by a nonlinear function made up of two lines of slope 1.0 joined by an inflection according to a model for two classes of receptor subtypes, which yielded apparent pA_2 values of 8.5 and 6.2 (see legend to Table 4).

We compared the action of nonselective (alprenolol), reputedly β_1-selective (CGP 20712A) (Dooley *et al.*, 1986) and β_2-selective (ICI 118,551) (O'Donnell & Wanstall, 1980) adrenoceptor antagonists on the inhibition of rat colon motility by the representative PEAT SR 58611A and by reference agonists (Figure 3). Motility inhibition by Iso was reduced by all these antagonists (Figure 3a), whereas inhibition by SR 58611A which responded to alprenolol, in agreement with the experiments presented in Table 4, was completely refractory to CGP 20712A and to ICI 118,551, alone or in combination (Figure 3d). Both alprenolol and CGP 20712A antagonized NA whose inhibition of colonic motility was not affected by ICI 118,551 (figure 3b). Sal was antagonized by alprenolol and ICI 118,551, but not by CGP 20712A (Figure 3c).

Data not set out in tables or figures showed that the potency (EC_{50} nM and 95% confidence limits) of the representative PEAT SR 58611A in inhibiting rat colon motility, i.e. 3.5 (2.6–4.7), was not significantly affected by the presence in the organ bath (which always contained the α-adrenoceptor blocking agent phentolamine) of any of various antagonists including methysergide, cisapride, domperidone, spiperone, atropine, mepyramine, ranitidine and naloxone, EC_{50}: 3.6 (2.6–5.1), 3.7 (2.7–5.0), 4.7 (3.4–6.4), 3.5 (2.6–4.6), 3.3 (2.6–4.0), 5.3 (4.0–6.0), 3.3 (2.5–4.4), 5.7 (4.7–6.9) in the given order. Only the nonselective β-adrenoceptor antagonists alprenolol (10^{-6} M) and propranolol (3×10^{-6} M) caused respectively an 8 and 11 fold elevation of the EC_{50} for rat colon motility inhi-

Table 3 Configuration-dependent actions of isoprenaline and phenylethanolaminotetralines (PEAT) on rat colon, uterus and guinea-pig atrium

	*Configuration of chiral carbons**	*Rat colon*	*Rat uterus*	*Guinea-pig atrium*
		EC_{50} (nM) (95% confidence limits)		
(−)-Isoprenaline	**R**	0.5 (0.4–0.6)	0.2 (0.17–0.25)	4.8 (3.6–6.0)
(+)-Isoprenaline	**S**	28 (22–35)	5.2 (4.4–6.1)	79 (72–129)
SR 58375A	**RR**	30 (22–41)	29 (23–37)	>30,000
SR 58374	**RS**	611 (453–824)	947 (715–1,250)	>30,000
SR 58373A	**SS**	>30,000	9,400 (7,600–11,600)	>30,000
SR 58372	**SR**	12,541 (8,328–18,884)	8,300 (6,500–10,600)	>30,000
SR 58572A	**RR**	7 (6–9)	28 (21–36)	>30,000
SR 58589	**RS**	36 (26–48)	340 (260–460)	>30,000
SR 58575A	**SS**	27,069 (21,818–33,583)	9,400 (7,800–11,200)	>30,000
SR 58590	**SR**	>30,000	~15,000	>30,000
SR 58612A	**RR**	2.7 (2.0–3.5)	66 (45–96)	>30,000
SR 58611A	**RS**	3.5 (2.6–4.7)	499 (372–672)	>30,000
SR 58613A	**SS**	11,107 (9,697–12,721)	~25,000	>30,000
SR 58825A	**SR**	2,351 (1,712–3,228)	18,400 (15,000–22,000)	>30,000

* For PEAT the first letter indicates the configuration of the phenylethanolamine chiral carbon and the second that of the tetraline part of the moiety.

Table 4 Quantitative antagonism of responses to phenylethanolaminotetralines (PEAT) and reference β-adrenoceptor agonists: pA_2 values for alprenolol and propranolol

		Antagonist	
Response	*Agonist*	*Alprenolol*	*Propranolol*
Inhibition of rat colonic motility	(±)-Isoprenaline	7.6 (7.4–7.8)	§
	Ritodrine	7.4 (7.2–7.5)	6.5 (6.4–6.7)
	SR 58375	7.5 (7.2–7.8)	6.5 (6.4–6.7)
	SR 58611A	7.0 (6.7–7.3)	6.3 (6.0–6.6)
Increase of guinea-pig atrial frequency	(±)-Isoprenaline	8.7 (7.6–9.7)	8.8 (7.9–9.6)
Relaxation of guinea-pig trachea	Ritodrine	9.0 (7.1–10.8)	9.1 (7.8–10.4)
	(±)-Salbutamol	8.9 (8.3–9.5)	8.7 (8.1–9.2)

Data shown are pA_2 values (and 95% confidence limits) calculated from constrained Schild plots (MacKay, 1978) as described in detail under Methods.

§ Schild plot slope different from one. Data set (six different log-concentrations of antagonists vs log(DR − 1)) was analysed by a non-linear fitting programme by applying a model for the interaction of competitive antagonists with two receptor sites according to Lemoine & Kaumann (1983) as described in Methods. The estimated parameters were as follows. Fractional stimuli originating from the interaction of isoprenaline with the high ($\sigma_Q \pm$ s.d.) and the low ($\sigma_R = 1 - \sigma_Q$) affinity sites inhibiting motility: 0.8 ± 0.28 and 0.2; equilibrium dissociation constants of propranolol for the high and low affinity sites ($K_{BQ} \pm$ s.d. and $K_{BR} \pm$ s.d.): $3.0 \times 10^{-9} \pm 0.47$ M and $5.5 \times 10^{-7} \pm 1.4$ M. The negative logarithms of the respective equilibrium dissociation constants (apparent pA_2) of the antagonist for the two sites yielded 8.5 and 6.2, respectively.

bition by SR 58611A which, conversely, was slightly more potent – EC_{50}: 1.5 (0.9–2.3), $P < 0.01$, Dunnett's t test – in the presence of indomethacin (10^{-6} M). The log concentration-response curve for inhibition of rat colonic motility by either Iso and SR 58611A shifted in parallel about three times leftward when the phosphodiesterase inhibitor IBMX was added to the organ bath (10^{-6} M); the EC_{50} values (nM) with and without IBMX were respectively 1.0 (0.7–1.4) and 3.0 (2.4–3.7) for SR 58611A and 0.7 (0.6–0.9) and 2.2 (1.7–2.9) for Iso. The specificity of this enhancing action was supported by the finding that the EC_{50} of the Ca^{2+} channel blocker verapamil for inhibition of rat colon motility (Manara *et al.*, 1989a) was unchanged in the presence of 10^{-6} M IBMX (data not shown).

Addition of tetrodotoxin (10^{-6} M) to the organ bath had little or no effect on the colonic motility inhibitory potency of either Iso or SR 58611A; EC_{50} values (nM) in the presence of the above agent, 2.5 (2.1–3.1) and 5.3 (4.0–6.0) respectively, tended to be only slightly higher than in its absence, 1.5 (1.2–2.0) and 4.5 (3.5–5.7).

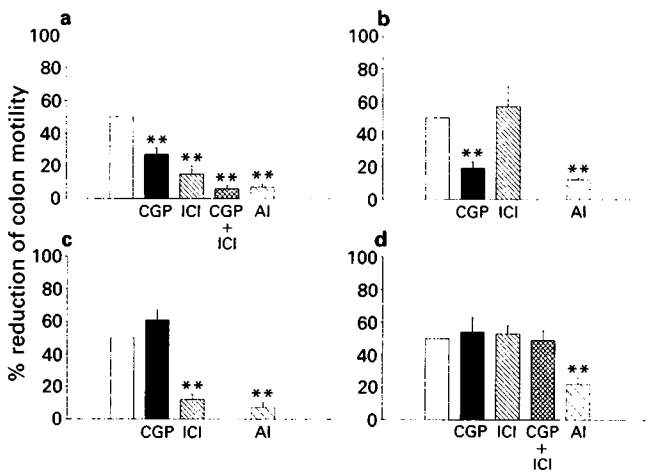


Figure 3 Effects of alprenolol, 10^{-7} M (Al), CGP 20712A, 10^{-8} M (CGP), ICI 118,551, 5×10^{-8} M (ICI) and CGP 20712A + ICI 118,551 (CGP + ICI) on rat proximal colon motility reduced by isoprenaline (a), noradrenaline (b), salbutamol (c) and SR 58611A (d). Open columns indicate 50% motility reduction in the presence of the EC_{50} of each adrenoceptor agonist, calculated from log-concentration-response curves (see Table 2). Statistical analysis of drug effects consisted in comparing the % reduction by the agonist in the presence of each antagonist (average of eight replications) with the theoretical value of 50 (Student's t test). To assess the significance of differences between groups in the isoprenaline experiment (a), Duncan's Multiple Range Test was applied: CGP vs ICI = $P < 0.05$; CGP vs CGP + ICI = $P < 0.05$, CGP vs Al = $P < 0.05$. Vertical bars denote s.e.mean. * $P < 0.05$, ** $P < 0.01$ vs open column (Dunnett's t test).

Discussion

This study shows that the PEAT are potent inhibitors of rat proximal colon motility through a β-adrenoceptor-mediated mechanism that is hard to define in terms of the generally recognized β_1- and β_2-receptor subclassification (Lands *et al.*, 1967).

We preliminarily found that compounds of this class, with no chronotropic action on the guinea-pig atrium, were more potent in reducing spontaneous motility of the rat isolated proximal colon than in relaxing the guinea-pig trachea and suggested that their colonic action might be accounted for by selective stimulation of gut-located atypical β-adrenoceptors distinct from the currently defined β_1 and β_2 subtypes (Bianchetti *et al.*, 1987; Croci *et al.*, 1987; 1988a). This view is further supported by the present results with newer and more selective PEAT and several established adrenoceptor agonists and antagonists.

All the PEAT inhibited rat proximal colon motility by reducing both the frequency and the amplitude of the rhythmic phasic contractions. The importance of the chiral carbon of the ethanolamine moiety was apparent from the substantially higher inhibitory potency of the **RS** and **RR** isomers than the corresponding **SR** and **SS** enantiomers. The configuration-dependent activity of PEAT also indicated that a receptor-mediated event is involved. The potent and most selective compound SR 58611A was chosen as representative of the class to study the mechanism of its intestinal inhibition.

A first line of evidence attests unequivocally to the β-adrenoceptor nature of the mechanism of the effect of PEAT on gut motility. The colonic effects of SR 58611A were competitively antagonized by the nonselective β-adrenoceptor antagonists, propranolol and alprenolol; the latter, as shown previously (Manara *et al.*, 1989a), failed to antagonize relaxation of the rat colon by the calcium channel blocker verapamil, thus attesting to the specificity of the antagonism on SR 58611A. Colonic inhibition by SR 58611A was refractory to addition to the organ baths of various agents affecting specific receptors and/or endogenous mediators. It is therefore unlikely that α-adrenoceptors, H_1 or H_2 histamine, 5-hydroxytryptamine, acetylcholine, dopamine or opioid receptors are responsible for the PEAT intestinal actions. Incidentally, indomethacin potentiated rather than antagonized the action of SR 58611A, indicating that prostanoid synthesis, which accompanies stimulation of β-receptors in some isolated preparations (Omini *et al.*, 1981), somehow interfered with the effects of PEAT on rat colon.

Compounds that increase intracellular cyclic AMP levels, like the phosphodiesterase inhibitor, IBMX, which at suitable concentrations potentiates adenylate cyclase-dependent responses, are often used to probe β-adrenoceptor-mediated effects (Korth, 1978). In the rat isolated colon, IBMX shifted the log-concentration-response curve of SR 58611A to the left and significantly lowered the EC_{50} for motility inhibition. A comparable potentiation by IBMX occurred with Iso but not with the calcium antagonist verapamil, suggesting that SR 58611A, like Iso, affects motility by stimulating an adenylcyclase-coupled β-adrenoceptor.

Additional evidence for the β-adrenoceptor-agonist nature of the PEAT comes from their ability to recognize presumably specific binding sites in rat colon membranes labelled *in vitro* with [^{3}H]-dihydroalprenolol. We have previously shown that several PEAT, including SR 58611A, displace [^{3}H]-dihydroalprenolol with different potencies, to some extent reflecting their respective abilities to induce colon motility inhibition (Manara *et al.*, 1989b) which, however, is substantial at two orders of magnitude lower concentrations than those effective in the binding assay.

Any further interpretation of the PEAT mechanism of action on the rat colon as a result of stimulation of either β_1- or β_2-adrenoceptor subtypes, or both, is open to question.

The rank order of potency of the reference adrenoceptor agonists on rat colon (Iso > NA = Ad > Ri > Sal) was similar to that on guinea-pig atrium (Iso > NA ≥ Ad > Sal > Ri) but quite different from that on rat uterus (Iso > Ad > Sal > Ri ≥ NA) or guinea-pig trachea (Iso > Ad > Sal ≥ NA > Ri). This suggests that stimulation of β_1- rather than β_2-receptor subtypes may determine colonic motility inhibition. The PEAT, however, do not fit into this scheme because, in spite of their substantial potency on the rat colon, they had no effect on guinea-pig atrial frequency (i.e. on β_1-receptors). The PEAT were also generally weak agonists at β_2-receptors as apparent from their relatively low potency on rat uterus, which displays efficient coupling between stimulus and tissue response (Kenakin, 1982), and their low intrinsic activity on guinea-pig trachea, a preparation containing mainly β_2-receptors (O'Donnell & Wanstall, 1979). This profile of mixed agonist-antagonist activity at β_2-receptors presumably accounts for the above mentioned ability of the PEAT to displace [^{3}H]-dihydroalprenolol from rat colon binding sites, whose β_2 nature appears predominant in the light of the high displacing potency of the selective β_2 antagonist ICI 118,551 and the virtual inactivity of the selective β_1 antagonist CGP 20712A (Manara *et al.*, 1989b).

One may argue that bioavailability factors, such as different penetration into the microenvironment surrounding receptor sites, might explain the colon vs uterus selectivity of the PEAT. This explanation appears unlikely in view of the different selectivity ratios (EC_{50} uterus:colon) of SR 58611A and SR 58825A, 143 and 7.8 respectively. These two compounds are in fact enantiomers which, in view of their identical physico-chemical properties, cannot be expected to differ in distribution between tissue compartments. Metabolic factors such as possible hydrolysis of SR 58611A and SR 58612A to their corresponding acids by esterases specifically located in rat colon might account for their preferential actions on this preparation. However, the colon selectivity of chlorophenolic compounds like SR 58572A, as well as the difference in selectivity between the diastereoisomers SR 58611A and SR 58612A, which presumably are equally susceptible to the action of esterases, both argue against this.

Identification of receptor types based on agonist responses in different tissues has inherent shortcomings: agonist action depends on both receptor affinity and efficacy and the lack of specificity of some agonists may derive from multiple actions on different preparations. Therefore compounds which are assumed to be receptor-selective may be merely tissue-selective (Kenakin, 1984). Specific antagonists selective for any given receptor subtype are more dependable than agonists as regards receptor characterization. In the present study both the reputedly highly selective adrenoceptor antagonists CGP 20712A, β_1, and ICI 118,551, β_2, prevented the colonic effects of Iso; CGP 20712A antagonized the action on the colon of NA and ICI 118,551 that of Sal, but neither of these antagonists reduced the inhibition of colonic motility by SR 58611A. The intestinal inhibition by SR 58611A, like that by the other representative PEAT SR 58375A, was antagonized competitively by the nonselective β-adrenoceptor blockers alprenolol and propranolol but, interestingly enough, with different potencies. Alprenolol was more potent on the rat colon although, as expected, both agents had virtually the same (higher) potency in the prevention of typical β_1- or β_2-responses to appropriate reference agonists (Iso, guinea-pig atrium; Ri or Sal, trachea). These results are strongly consistent with the presence in rat colon of atypical β-adrenoceptors (non β_1, non β_2) with lower than conventional affinities for established non-selective β-antagonists and specifically activated by the PEAT.

Ri was also competitively antagonized in the rat colon by alprenolol and propranolol but with substantially lower potency than expected for β_2 receptor stimulation. Unexpectedly, as we have described elsewhere (Manara *et al.*, 1989a), Ri was very poorly susceptible to ICI 118,551 in the same preparation. Thus Ri too, whose structure resembles that of the PEAT, probably inhibits colonic motility by acting preferentially at atypical rather than at β_2-adrenoceptor sites.

Unlike PEAT and Ri, Iso antagonism by propranolol in the rat colon cannot be described by a simple linear Schild plot with a slope of unity. This suggests that mechanisms other than simple competition, such as interaction with more than one receptor subtype, might be involved in the mediation of the effects of Iso (Furchgott, 1972). When a model for two classes of receptor subtypes was applied, apparent pA_2 values of 8.5 and 6.2 were calculated. Thus Iso, at low concentrations, interacts with typical (β_1 and β_2) sites almost exclusively, as also shown by the complete antagonism observed in the presence of both CGP 20712A and ICI 118,551. Conversely, at higher concentrations like those used to build up the Schild plot, the contribution of atypical β-receptors to the effects of Iso becomes predominant. On the other hand antagonism of Iso by alprenolol, unlike that by propranolol, apparently disclosed no involvement of β_1- or β_2-subsites, since it turned out to be competitive with a lower pA_2 (7.6) than at conventional β-adrenoceptors. This apparent inconsistency may depend on the substantially smaller difference in affinities for typical and atypical sites of alprenolol than propranolol (as shown by the pA_2 values obtained in the guinea-pig atrium and trachea and in the rat colon), which probably makes it difficult to demonstrate multiple colonic Iso sites with alprenolol.

Our results with CGP 20712A, ICI 118,551 and propranolol supporting the role of conventional β-adrenoceptors for Iso inhibition of rat colon motility are consistent with those of Ek *et al.* (1986a,b) in isolated strips from rat ascending colon, where either β_1- or β_2-adrenoceptors, presumably located on neuronal elements of the intramural plexuses and smooth muscle cells respectively, apparently accounted for the interactions observed with several established specific agonists and antagonists. As to the cellular location of the putative atypical site mediating PEAT inhibition of colonic motility, our experiments showing comparable potencies of SR 58611A in the presence and absence of the neuronal blocker tetrodotoxin suggest the prevalence of postjunctional non-neuronal structures.

The question of multiple adrenoceptor sites mediating catecholamine inhibition of gut motility and their characterization has been addressed by several authors (Furness & Costa, 1974; Burnstock & Wong, 1981; Daniel, 1982; Ek, 1985). Attempts to interpret mechanical responses in the gut in terms of actions at currently recognized adrenoceptor sites have often been hampered by abnormal conditions, such as the low potencies of established adrenoceptor antagonists in preventing these effects. Thus, relaxant responses to catecholamines

resistant to α- and/or β-adrenoceptor blockers have been described in rabbit stomach (Bristow *et al.*, 1970) and intestine (Wikberg, 1977), in rat oesophageal smooth muscle (Buckner & Cristopherson, 1974), gastric fundus (Dettmar *et al.*, 1986) and proximal colon (Croci *et al.*, 1988a), in guinea-pig gastric fundus (Coleman *et al.*, 1987) and ileum (Bond *et al.*, 1986), in dog distal colon (Grivegnée *et al.*, 1984) and in human colonic circular smooth muscle (McLaughlin *et al.*, 1988). Such observations in general have prompted their authors to suggest that there are atypical adrenoceptors in different gut segments, while others have argued against the creation of further receptor subclasses simply because of observed anomalies (Raper, 1987).

The studies by Bond & Clarke on histamine- or electrically-stimulated longitudinal muscle of guinea-pig ileum (Bond *et al.*, 1986; Bond & Clarke, 1988) are of special interest in this connection. On account of the rank order of potencies of adrenoceptor agonists and of their unresponsiveness or variable resistance to several α- and β-adrenoceptor antagonists in the above preparations, it was suggested that ileal relaxation occurred through a putative adrenoceptor with properties distinct from those of conventional α- and β-subtypes. More recent work by the same group (Blue *et al.*, 1988; 1989) recognized cyanopindolol and alprenolol as the antagonists with the highest affinities for the designated 'β-like' atypical site, which was otherwise resistant to propranolol and to the β_1-selective adrenoceptor blocking agent atenolol. In spite of some similarities, the putative β-adrenoceptor atypical site in the rat colon described in the present study and that described by Blue *et al.* (1988) are unlikely to be the same, because the latter had substantially lower affinity for alprenolol (pA_2 6.5) and was virtually refractory to propranolol rather than just sensitive.

β-Adrenoceptors mediating lipolysis, atypical because of their low sensitivity to specific antagonists, have been found in rat adipocytes (Harms *et al.*, 1977; Arch *et al.*, 1984). Although this finding was recently questioned on the basis of the results of radioligand binding studies (Bahouth & Malbon, 1988) not compatible with those of functional studies (Zaagsma & Nahorski, 1990), novel lipolytic agonists that potently and selectively stimulate receptors in adipocytes have been synthesized (Arch *et al.*, 1984; Wilson *et al.*, 1984). It is worth mentioning that these lipolytic agents also potently relaxed prostaglandin-contracted guinea-pig stomach fundus (Coleman *et al.*, 1987) and histamine-contracted guinea-pig ileum (Bond & Clarke, 1988), apparently by stimulating atypical β-adrenoceptors. The PEAT have been preliminary shown to have considerable *in vitro* lipolytic action on rat white adipocytes (Manara *et al.*, 1989a).

Additional tissues with anomalous responses to adrenoceptor agents that have been tentatively ascribed to atypical, possibly β, sites include the feline myocardium, where a low sensitivity component of the concentration-effect curve for the positive chronotropic action of (−)-pindolol was resistant to combined blockade of β_1- and β_2-adrenoceptors (Kaumann & Lobnig, 1986). Even the proposed antinociceptive action of several β-adrenoceptor agonists in the mouse abdominal constriction test was attributed to stimulation of atypical β-receptors (Bentley & Starr, 1986).

Establishing the exact nature of all these receptors and whether any of them are identical, irrespective of their different tissue and/or precise cellular locations, obviously calls for much more work. The unequivocal demonstration of one or more new β-receptor subtypes as separate individual entities, will depend on the availability of antagonists selective for each of them. Another approach might involve the application of molecular biology techniques like cloning the putative-coding DNA sequence, which is currently used for studying the heterogeneity of adrenoceptors (Frielle *et al.*, 1988; Libert *et al.*, 1989). Quite recently a human gene has in fact been isolated reputedly coding for a 'β_3-adrenoceptor' in eukaryotic cells transfected with it and producing cyclic AMP upon stimulation with several adrenoceptor agonists, whose relative potencies resembled the pattern for eliciting functional responses in preparations currently believed to contain atypical β-adrenoceptors (Emorine *et al.*, 1989). In the cyclic AMP-producing system specific antagonists were tested exclusively against Iso which was inhibited only by CGP 20712A and ICI 118,551 in the micromolar range, but not by alprenolol or propranolol. This pharmacological characterization indicates substantial differences from the site described by us in the rat colon and does not provide firm evidence that any of the other previously described atypical β-adrenoceptor sites is the same as the receptor expressed by the transfected cells.

Further understanding of the mechanism of action of the PEAT is certainly pertinent to the above context and merits special attention for three main reasons: (1) the potency and specificity of the PEAT support their use as proposed selective agonists at the presumed new non β_1- non β_2-adrenoceptor in the rat colon; (2) the action of the PEAT on the colon was antagonized by established nonselective β-adrenoceptor blocking agents at concentrations definitely higher than at conventional β-sites, but still low enough to substantiate the specificity of the finding; (3) whatever the mechanism of the relative specificity for the gut of the PEAT *in vitro*, it is highly likely to be of functional significance because we have already observed in animals the *in vivo* dissociation of their potent inhibition of intestinal motility from cardiovascular and other side effects of potential clinical relevance (Croci *et al.*, 1988b; 1989; Giudice *et al.*, 1989; Manara *et al.*, 1989a).

We gratefully acknowledge the competent technical assistance of G. Aureggi and A. Tarantino who set up all the isolated preparations with the appreciated help of M. Bonalume, M.G. Dozio and E. Mazzoni.

We also thank the Head of our Chemistry Section, Dr Umberto Guzzi whose contribution to the synthesis of PEAT stereoisomers was essential for this study and Dr Maurizio Rocchetti (Istituto di Ricerche Farmacologiche Mario Negri, Milan) for his valuable advice in the computer analysis of data on isoprenaline antagonism by propranolol.

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(Received December 4, 1989
Revised March 6, 1990
Accepted March 27, 1990)