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PHENYLETHANOLAMINOTETRALINES COMPETE WITH [^{3}H]DIHYDROALPRENOLOL BINDING TO RAT COLON MEMBRANES WITHOUT EVIDENCING ATYPICAL β-ADRENERGIC SITES

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Abstract—[^{3}H]Dihydroalprenolol ([^{3}H]DHA) specific binding (determined by the difference in the presence and absence of 20 μM (−)isoprenaline) to rat colon membranes was saturable (B_{max} = 39.6 fmol/mg protein), of high affinity (K_d = 0.87 nM) and stereospecific (IC_{50} 330 and 3510 nM for (−)- and (+)isoprenaline, respectively); the Hill coefficient was close to one, indicating binding homogeneity. [^{3}H]DHA (0.6 nM) specific binding was potently inhibited (K_i range 1.9–3.3 nM) by the non-selective β-adrenoceptor antagonists pindolol, alprenolol and propranolol, but not by the non-adrenergic compounds 5-hydroxytryptamine, 8-hydroxydipropylaminotetraline, methysergide, dopamine and verapamil (K_i > 10,000 nM). The selective β_1- and β_2-adrenoceptor antagonists CGP 20,712A and ICI 118,551 resulted in biphasic competition binding curves, whose low and high affinity components were compatible with two populations of binding sites accounting for about 75 (β_2) and 25% (β_1) of total sites. The relative competing potencies of reference adrenergic agonists also suggested a prevalence of β_2-adrenergic sites. The new agonists phenylethanolaminotetralines (PEATs), highly selective for the atypical β-adrenoceptors whose abundance in rat colon has been confirmed by comprehensive functional studies, had variable affinity for the [^{3}H]DHA-labelled sites depending on chirality, but with no substantial correlation with their pharmacological potency. Only 40% of [^{3}H]DHA binding, at a concentration about 10 times its K_d for high affinity sites (β_1 and β_2), was prevented by saturating concentrations of isoprenaline. Under this condition, the representative PEAT, SR 58611A, highly potent and selective for atypical β-adrenoceptors in functional tests, and its pharmacologically inactive enantiomer, both inhibited the residual binding equipotently. In conclusion, [^{3}H]DHA binding did not detect atypical β-adrenoceptor sites in rat colon membranes, most probably because of its weaker affinity for them than for the coexisting β_1 and β_2 sites. PEAT stereoisomers proved essential for assessing both the stereospecificity and the functional significance of this atypical binding and to compare their affinity for [^{3}H]DHA-labelled sites and pharmacological potency.

The existence of atypical β-adrenoceptors (non β_1, non β_2) mediating a variety of functions in different tissues and organ systems such as fat, intestine, heart and CNS is becoming widely accepted [1–6]. Supporting evidence consists mainly of functional studies in which the responses to specific agonists are unusually resistant or even refractory to currently available β-adrenoceptor antagonists [7–9]. Newly developed adrenoceptor agonists selective for such atypical receptors potently impair the motility of isolated gut segments [10, 11]. The most selective of these new agonists are the phenylethanolaminotetralines (PEATs‡) [12] whose inhibition of the *in vitro* motility of the rat proximal colon involves a new kind of β-adrenoceptor which was differentiated pharmacologically from the well-established β_1 and β_2 subtypes on the basis of agonists' rank order of potency and antagonists' affinities [3]. This adrenoceptor, which bears close similarities to the atypical β-receptor described in rat adipocytes [13, 14], coexists with the β_1 and the β_2 subtypes in the rat colon [3].

In the present study, we set out to detect the atypical β-adrenergic site in the rat colon by competition binding with the radiolabelled ligand, [^{3}H]dihydroalprenolol ([^{3}H]DHA). We had preliminarily reported that several PEATs able to inhibit rat colon *in vitro* motility, also inhibited [^{3}H]-DHA specific binding to rat colon membranes [15]. In the light of the recently claimed difficulties in revealing atypical adrenergic sites in the gut because of unsuitable ligands [2, 16], we further examined the nature of these [^{3}H]DHA-labelled sites. Besides reference β-adrenoceptor agonists and antagonists, we tested three different series of PEATs each consisting of four stereoisomers. The availability of different isomers with nanomolar to virtually no potency in relaxing the rat colon [3], offered an unprecedented opportunity to assess the functional significance and the stereospecificity of the binding to atypical β-adrenergic sites.

MATERIALS AND METHODS

Drugs and chemicals. [^{3}H](−)DHA (98.5 Ci/

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‡ Abbreviations: PEAT, phenylethanolaminotetraline; DHA, dihydroalprenolol.

mmol; radiochemical purity >98%) was obtained from New England Nuclear Co. (Dupont de Nemours Deutschland GmbH, D-6072 Dreieich, Germany). All the PEATs, whose structures and isomers corresponding to appropriate code numbers were reported in a previous study [3], were synthesized in the Chemistry Section of the Sanofi-Midy S.p.A. Research Center. (−)Alprenolol and (+)alprenolol tartrate were also synthesized in our Chemistry Section according to the original method of Brandstrom *et al.* [17].

Some of the reference drugs and chemicals were from the following commercial sources as indicated: Sigma Chemical Co. and Aldrich Corp. (St Louis, MO, U.S.A.): (−)isoprenaline [(−)Iso], (+)isoprenaline [(+)Iso], (−)noradrenaline bitartrate (Nad), (±)propranolol HCl, (±)salbutamol hemisulfate (Salb), verapamil HCl, 5-hydroxytryptamine creatinine sulfate; Lusofarmaco (Milan, Italy): ritodrine (±)erythro HCl (Rit) (Miolene ampoules); Ricerchimica (Milan, Italy): (±)alprenolol HCl; RBI (Natick, MA, U.S.A.): 8-hydroxydipropylaminotetraline HBr (8-OH-DPAT).

The following drugs were kindly provided: Ciba-Geigy (Basel, Switzerland): CGP 20,712A [(±)-[2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-2-propanol]; ICI (Macclesfield, U.K.): ICI 118,551 [erythro(±) - 1- (7 - methylindan - 4 - yloxy)-3-isopropylaminobutan-2-ol hydrochloride]; Janssen (Beerse, Belgium): (−)adrenaline (Adr); Sandoz (Basel, Switzerland): methysergide bimaleate.

All other chemicals were reagent grade of the best quality available.

Animals. Male Crl:CD(SD)BR Charles River rats (Como, Italy) weighing 200–250 g were housed in groups of four in wire-bottomed plastic cages with light on from 6.30 a.m. to 6.30 p.m. They were maintained at room temperature of 22 ± 1° and humidity 55 ± 10% with standard laboratory food and water *ad lib.*

Preparation of membranes. Rats were killed by cervical dislocation and a segment of 6 cm of proximal colon starting from the ileo–cecal junction was carefully dissected and cleaned of adhering adipose tissue. The colon segment was opened longitudinally and the mucosa was removed quickly from the muscularis by scraping with a glass slide on an ice-cold plate. The organ was then weighed and homogenized (Ultra-Turrax TP 18/10) for 20 sec in 5 vol. of 10 mM Tris–HCl buffer at 4° containing 250 mM sucrose in the presence or absence of various concentrations of $MgCl_2$ and $CaCl_2$ ("preparation buffer"). The homogenate was then filtered through a double gauze layer. The filtrate was centrifuged at 1500 *g* for 10 min (Beckman TJ-6) and the supernatant removed and centrifuged at 105,000 *g* for 60 min at 4° (Beckman L5-50B Ultracentrifuge). The resulting pellet was resuspended in 2 vol. of "incubation buffer" made up of 10 mM Tris–HCl (pH 7.4) containing 4 mM $MgCl_2$ and 4 mM $CaCl_2$. This membrane preparation containing approximately 2 mg protein/mL could either be used immediately for binding experiments or frozen at −20° for subsequent use.

Binding assays. In saturation binding studies, the reaction mixture contained 500 μL of membrane preparation, 6–7 different concentrations (range 0.06–4.1 nM) of [^{3}H]DHA and 10 μM phentolamine in a final volume of 1 mL. Incubations, carried out at 25°, were started by the addition of membrane suspension and terminated after 60 min by the addition of 4 mL of ice-cold 10 mM Tris–HCl buffer (pH 7.4) followed by rapid filtration under vacuum on a Brandel Cell Harvester. Ligand bound to membranes was recovered on Whatman GF/B filters that were washed twice with 10 mL of cold buffer and placed in vials containing 10 mL of Ready Protein$^+$ scintillator. Samples were stored overnight at 4° and the amount of [^{3}H]DHA bound was measured by liquid scintillation spectrometry in a Beckman LS 6000 IC with about 57% efficiency. Protein content was determined by the method of Lowry *et al.* [18] using bovine serum albumin as standard.

In kinetic studies, the time course of 0.6 nM [^{3}H]-DHA binding was assessed over a period of 60 min after which (−)isoprenaline (20 μM) was added and the time course of the reverse reaction was followed for a further 60 min.

In competition studies, the reaction mixture contained either 0.6 or 10 nM [^{3}H]DHA and various concentrations of unlabelled competitors.

Non-specific binding was measured in the presence of 20 μM (−)isoprenaline. Specific binding was calculated as the difference between total binding and non-specific binding. All the binding assays were performed at least in triplicate.

Data analysis. Binding kinetic experiments were carried out for calculation of the association (K_{+1}) and dissociation (K_{-1}) constants by pseudo-first-order and first-order rate equations [19].

Saturation curves, from four separate experiments, were analysed either by Scatchard's method, establishing the affinity constant K_d and the maximum number of binding sites B_{max} from the linear regression of the plot [18] or using the computer program for non-linear regression, Accufit® Saturation Two Site (Lundon Software, Beckman, CA, U.S.A.). This program applies the mathematical models of Feldman [20] describing the interaction of a labelled ligand with one or more receptor sites and compares the data fitting to one- and two-site models. The two-site model is accepted if the fit is significantly better ($P < 0.05$) than the one-site analysis.

In competition studies, the IC_{50} values of the competing ligands were calculated from the linear plot of the log of the inhibitor concentration against percentage of binding inhibition [21]. The affinity constants K_i of the competing antagonists were calculated from the IC_{50} using the equation $K_i = IC_{50}/(1 + S/K_d)$ where S is the concentration of [^{3}H]-DHA and K_d is the affinity constant of [^{3}H]DHA obtained from the Scatchard's plot [22].

The competition curves for the selective antagonists ICI 118,551 and CGP 20,712A, which appeared biphasic, were analysed by the computer program Accufit® Competition (Lundon Software, Beckman, CA, U.S.A.) based on the mathematical approach by Feldman *et al.* [23] which evaluates the goodness

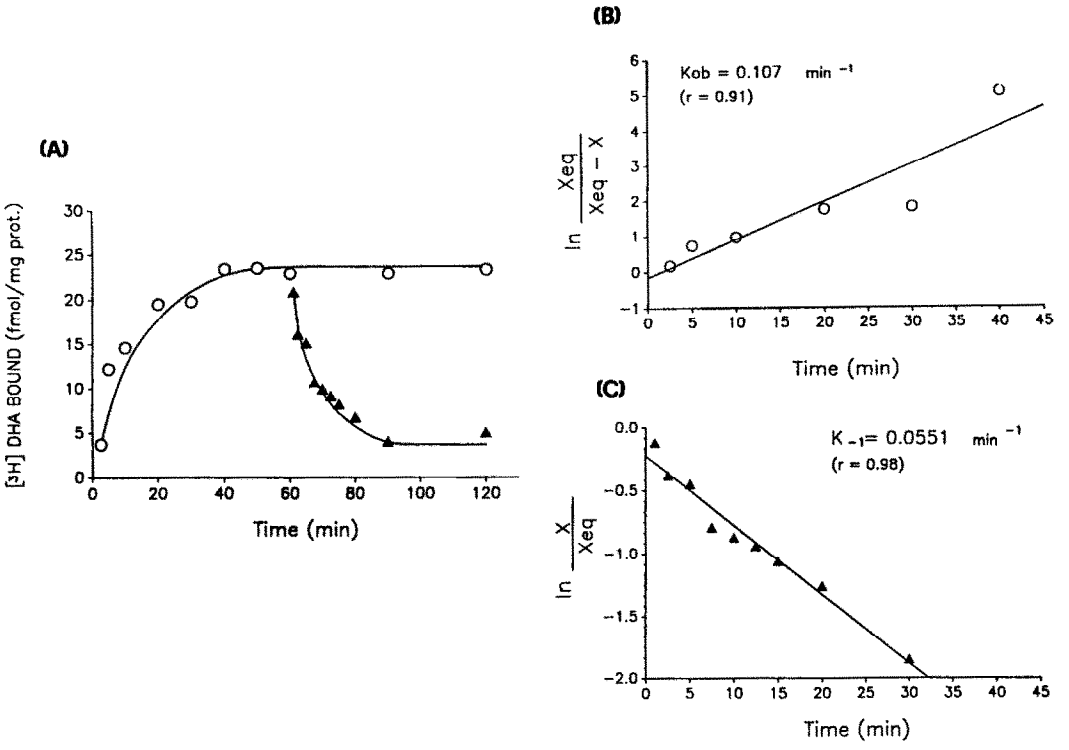

Fig. 1. Kinetic analysis of [^{3}H]DHA specific binding to rat colon membranes. (A) Binding of 0.6 nM [^{3}H]DHA to rat proximal colon membranes plotted against incubation time before (○) and after (▼) addition of 20 μM (−)isoprenaline. (B) Pseudo-first-order plot of rate of specific binding. (C) First-order plot of the dissociation reaction. Data plotted in (B) and (C) are taken from (A). X_{eq} = specific binding at equilibrium and X = specific binding at time t. The rate constant K_{+1} was calculated from the observed association constant K_{ob} and the first-order rate constant for dissociation according to the following equation: $K_{+1} = (K_{ob} - K_{-1})/[^3\text{H-DHA}] = 0.065\ \text{nM}^{-1}\ \text{min}^{-1}$. The $K_d(K_{-1}/K_{+1})$ calculated by kinetic analysis was 0.85 nM.

of fit of the experimental data to the two site-model and calculates the binding parameters (percentage of each site and K_i of the competing ligand for each of the two sites).

RESULTS

Optimization of binding conditions

A variety of conditions were examined during the preparation of membranes in order to obtain the highest percentage of specific [^{3}H]DHA binding. In initial experiments, membranes were homogenized in a "preparation buffer" consisting of Tris–HCl, 10 mM as described by Ek and Nahorski [24] in their study with [^{125}I]pindolol but with 250 mM sucrose added. Specific binding in these experimental conditions was only 36 ± 4% of total. Addition of 4 mM $CaCl_2$ + 4 mM $MgCl_2$ together to the "preparation buffer" virtually doubled the specific binding, to 70 ± 4% of total, whereas separate addition of 4 mM of $CaCl_2$ only gave 49 ± 3% and 4 mM $MgCl_2$ gave 53 ± 5%. This procedure reportedly induces the aggregation of subcellular structures [25] not containing specific binding sites, such as mitochondria, that apparently precipitate during the first centrifugation at 1500 g (in these experimental conditions the mitochondrial fraction normally sedimenting at 10,000 g was not present). Specific binding was further increased (75 ± 8%) by the addition of 10 μM phentolamine during incubation; this reportedly increases the specific binding of β-adrenoceptor ligands [26]. The above conditions were adopted in all subsequent studies.

Since incubation at low temperature has been reported to increase selectively the binding of agonists to β-adrenoceptor sites [27], we carried out a few experiments at 4° and extended the incubation time from 1 to 6 hr to allow the equilibrium attainment (results not reported in detail). However, under these conditions, the potencies of either isoprenaline or SR 58611A in preventing [^{3}H]DHA binding did not increase, suggesting that 25° is a temperature low enough for the agonists to exhibit near maximal affinity for their binding sites [28].

Kinetic studies

Specific binding of [^{3}H]DHA (0.6 nM) to rat colon membranes reached steady state in about 55 min; the addition of a large excess of (−)isoprenaline (20 μM) rapidly displaced the radioligand (Fig. 1A). The pseudo-first-order kinetics may be applied to [^{3}H]DHA which, at equilibrium, is less than 10%

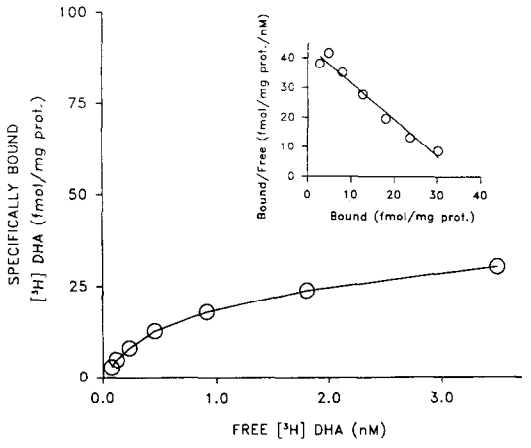

Fig. 2. Specific binding of [³H]DHA to rat colon membranes. Membranes were incubated as described in Materials and Methods with 6–7 increasing concentrations of [³H]DHA in the absence (total binding) and presence (non-specific binding) of 2×10^{-5} M (−)isoprenaline. Specific binding, as the difference between total and non-specific binding, is plotted against the free [³H]DHA concentration. The saturation curve (for descriptive purpose, straight lines connect the mean experimental values) and its Scatchard transformation (inset) are representative of four such experiments performed in triplicate both for total and non-specific binding whose average values were $K_d = 0.87 \pm 0.11$ nM; $B_{max} = 39.6 \pm 2$ fmol/mg protein.

Table 1. Affinity values for competition of β-adrenoceptor antagonists and non-adrenergic compounds with 0.6 nM [³H]DHA specific binding to rat colon membranes

	K_i, nM (95% conf. limits)
(±)Propranolol	3.3 (2.7–3.9)
(±)Alprenolol	2.4 (2.0–2.8)
(−)Alprenolol	1.4 (1.1–1.6)
(+)Alprenolol	28.8 (24.1–34.7)
(±)Pindolol	1.9 (1.6–2.3)
5-Hydroxytryptamine	>10,000
8-OH-Dipropylaminotetraline	>10,000
Methysergide	>10,000
Dopamine	>10,000
Verapamil	>10,000

K_i values were calculated from IC_{50} values using the equation $K_i = IC_{50}/1 + [ligand]/K_d$ [21]. IC_{50} values were calculated by plotting binding data (5–7 concentrations) from three experiments against the log of the inhibitor concentration [20]. K_d was determined by Scatchard analysis (see Fig. 2). The competition curves of the β-adrenergic antagonists were monophasic with apparent pseudo-Hill coefficient (n_H) not significantly different from 1.0 (data not shown).

bound to membranes. The observed rate constant K_{ob}, calculated by linear regression analysis from the association rate plot, was 0.107 min^{-1} (Fig. 1B). The pseudo-first-order rate constant K_{+1} of 0.065 nM^{-1} min^{-1} was determined by the formula $K_{+1} = (K_{ob} - K_{-1})/[^3H\text{-DHA}]$, where K_{-1} was the first-order dissociation constant, 0.0551 min^{-1}, calculated from the dissociation reaction after (−)isoprenaline addition (Fig. 1C). The dissociation constant K_d derived from these values (K_{-1}/K_{+1}) was 0.85 nM.

Saturation and competition studies

The results of the saturation experiments, carried out at equilibrium (60 min incubation), are illustrated in Fig. 2. Scatchard plot analysis of these binding data revealed a single class of binding sites with a dissociation constant K_d of 0.87 ± 0.11 nM, very close to that obtained from kinetic data, and a maximum number of binding sites, B_{max}, of 39.6 ± 2.2 fmol/mg protein. Hill transformation of the saturation curve yielded a coefficient of 1.04 ± 0.01 ($r = 0.99$) indicating the absence of cooperativity. Analysis of the same saturation curve by a non-linear fitting program confirmed a one-site binding model with calculated parameters similar to those obtained by linear plot ($K_d = 0.86 \pm 0.096$ nM; $B_{max} = 35.3 \pm 4.3$ fmol/mg protein).

[³H]DHA specific binding to colon membranes was competitively inhibited by the three non-selective β-adrenoceptor antagonists, (±)propranolol; (±)alprenolol and (±)pindolol, with similar potencies (respective K_i 3.3, 2.4 and 1.9 nM) (Table 1). Binding inhibition was stereo-selective with (−)alprenolol (K_i 1.4 nM) which was approximately 20 times as potent as the corresponding (+)enantiomer (K_i 28.8 nM). Compounds with no affinity for β-adrenergic receptors such as those listed in Table 1 did not compete with [³H]DHA binding even at high concentrations.

Inhibition of [³H]DHA specific binding by the β_1 CGP 20,712A and β_2 ICI 118,551 selective antagonists produced competition curves that, unlike those obtained with the non-selective antagonists, deviated from the mass action law. Analysis of these curves by a computer-assisted non-linear fitting method using a two independent binding sites model, yielded the parameters reported in Table 2. Both antagonists competed for [³H]DHA binding sites with high and low affinity constants (respective K_i for ICI 118,551: 1.5 and 164 nM, for CGP 20,712A: 32.4 and 3703 nM). The relative abundance (percentage of total) of high (72.3) and low (27.7) affinity sites for ICI 118,551 corresponded to that of the low (78.0) and high (22.0) affinity sites for CGP 20,712A.

Table 3 shows the inhibition of [³H]DHA specific binding by reference β-adrenoceptor agonists and by three different series of PEATs, each consisting of four stereoisomers. Agonists were all substantially less potent than antagonists in competing with [³H]-DHA. Competition binding by isoprenaline was stereospecific, the (−)enantiomer being approximately 10 times as effective as the (+)enantiomer; the other agonists, (−)noradrenaline, (−)adrenaline, salbutamol and ritodrine, showed lower affinity than (−)isoprenaline. The PEATs had configuration-dependent affinities, with *RR* and *RS* isomers being definitely more potent in competing with [³H]DHA than their virtually inactive *SS* and *SR* enantiomers.

Table 2. Affinity values for competition of the selective β_1- (CGP 20,712A) and β_2- (ICI 118,551) adrenergic antagonists with 0.6 nM [^{3}H]DHA specific binding to rat colon membranes

	$K_i(\beta_2)$ (nM)	% β_2	$K_i(\beta_1)$ (nM)	% β_1
ICI 118,551	1.5 ± 0.7	72.3 ± 5.3	164 ± 70	27.7 ± 5.3
CGP 20,712A	3703 ± 985	78.0 ± 6.9	32.4 ± 14	22.0 ± 6.9

Affinities (K_i) and proportions of β_1- and β_2-adrenoceptors (mean ± SE) were estimated by three competition experiments, each carried out in triplicate. The values reported were obtained from the best fitting curve of the experimental data estimated by a computer-assisted method using a two independent binding site model (see Materials and Methods).

Table 3. Ability of reference β-adrenergic agonists and PEATs to compete for the binding sites of [^{3}H]DHA (0.6 nM) in rat colon membranes

		[^{3}H]DHA binding IC_{50}, nM (95% conf. limits)	Pseudo-Hill coefficient (n_H ± SE)
(−)Isoprenaline		330 (297–367)	0.74 ± 0.1
(+)Isoprenaline		3510 (2850–4340)	0.79 ± 0.1
(−)Noradrenaline		9600 (7160–12,900)	0.75 ± 0.08
(−)Adrenaline		2860 (2390–3430)	0.83 ± 0.1
Salbutamol		2196 (1920–2514)	0.77 ± 0.01
Ritodrine		4620 (4000–5400)	0.88 ± 0.02
SR 58375A	*RR*	898 (747–1070)	0.85 ± 0.1
SR 58374A	*RS*	5430 (4610–6390)	0.76 ± 0.07
SR 58372	*SR*	28,000 (25,900–30,100)	NE
SR 58373A	*SS*	49,800 (44,600–55,500)	NE
SR 58572A	*RR*	414 (360–480)	0.90 ± 0.1
SR 58589	*RS*	2940 (2146–4039)	0.63 ± 0.1
SR 58590	*SR*	12,930 (11,100–15,000)	NE
SR 58575A	*SS*	30,600 (27,000–34,200)	NE
SR 58612A	*RR*	863 (703–1060)	0.83 ± 0.05
SR 58611A	*RS*	998 (893–1114)	0.71 ± 0.06
SR 58825A	*SR*	14,500 (12,500–17,000)	NE
SR 58613A	*SS*	18,500 (15,000–23,000)	NE

Code numbers indicate HCl salts if followed by the letter "A" but otherwise identify free bases; the first letter in italic characters indicates the configuration of the chiral carbon of the phenylethanolamine and the second that of the tetraline part of the moiety according to the chemical structures reported elsewhere [3].

NE, not evaluated.

Among the *RR* isomers, compound SR 58572A was the most potent followed by SR 58375A and SR 58612A which displayed similar potencies.

The results of the competition studies in the presence of concentrations of [^{3}H]DHA about 10 times higher than its K_d (0.9 nM) are illustrated in Figs 3 and 4. About 40% of total binding of 10 nM [^{3}H]DHA was prevented by (−)isoprenaline concentrations ranging from 3×10^{-6} to 10^{-3} M. SR 58611A, the most selective PEAT for the atypical receptors in functional studies, prevented more than 80% of [^{3}H]DHA total binding, unlike isoprenaline (Fig. 3). In the presence of a saturating concentration of (−)isoprenaline (500 μM), SR 58611A still competed with 10 nM [^{3}H]DHA total binding, but its potency was similar to that of the corresponding enantiomer SR 58825A (Fig. 4), found to be substantially less potent than SR 58611A in inhibiting the binding of a low concentration of [^{3}H]DHA.

DISCUSSION

The present study shows that [^{3}H]DHA binds with high affinity to specific and saturable sites in rat colon membranes. Scatchard transformation of the data indicated that the ligand binds to apparently homogeneous sites as shown by the linearity of the Scatchard plot and the Hill coefficient close to unity. The affinity constant (K_d = 0.9 nM) of [^{3}H]DHA for these sites is similar to those reported for β_1 and β_2 receptors in other tissues [29, 30]. The stereospecificity of the binding is evident from the respectively 10- and 20-fold affinity ratios between the (−)- and (+)isomers of isoprenaline and alprenolol.

In order to characterize the adrenoceptor subtypes labelled by [^{3}H]DHA, we studied the competitive inhibition of the specific binding by selective and non-selective β-adrenoceptor antagonists. The non-

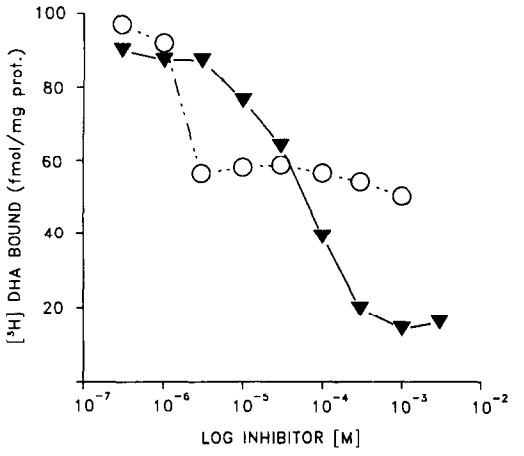

Fig. 3. Competition of (−)isoprenaline (○) or SR 58611A (▼) with 10 nM [^{3}H]DHA binding to rat colon membranes. The data are from one of two separate experiments that gave virtually identical results. For descriptive purpose, straight lines connect the mean experimental values.

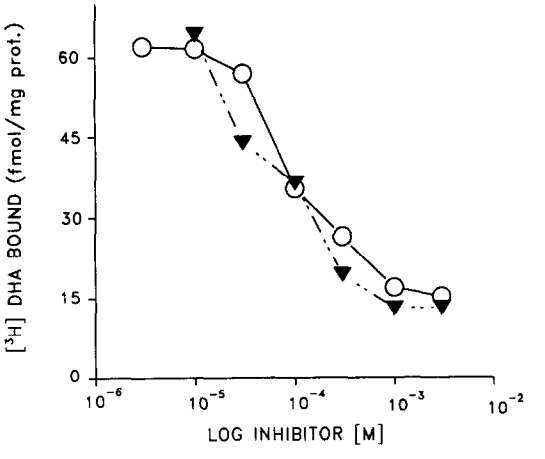

Fig. 4. Competition of SR 58611A (▼) or SR 58825A (○) with 10 nM [^{3}H]DHA binding to rat colon membranes in the presence of a saturating concentration (500 μM) of (−)isoprenaline. The data are from one of two separate experiments that gave virtually identical results. For descriptive purpose, straight lines connect the mean experimental values.

selective antagonists propranolol, alprenolol and pindolol inhibited [^{3}H]DHA binding with similar potencies, resembling their affinities for typical β_1- or β_2-adrenoceptors. However the functionally determined affinities of alprenolol and propranolol in the rat colon were different and both were substantially lower than at established β_1- or β_2-receptor subtypes (pA_2 values around 7.5 and 6.5, respectively) [3].

The inhibition of [^{3}H]DHA specific binding by the highly selective β_1- and β_2-antagonists, CGP 20,712A [31] and ICI 118,551 [32] was better described by two biphasic competition curves. Computer-assisted analysis of these curves showed there were two populations of binding sites. A major population representing about 75% of total sites was recognized with high affinity by ICI 118,551 and with low affinity by CGP 20,712A and presumably corresponded to β_2 binding sites. The smaller population, 25% of the total, made up of β_1 sites, was recognized with high affinity by CGP 20,712A and low affinity by ICI 118,551. Other investigators obtained similar results in rat colon membranes using a different ligand, [^{125}I](−)pindolol, which disclosed a small population of β_1-adrenoceptors, apparently located on enteric neurons, and a larger population of β_2-adrenoceptors on smooth muscle cells [24].

In our study, the prevalence of β_2-adrenergic sites is additionally supported by the characteristic ligand competition profile of the reference adrenergic agonists. Accordingly, the rank order of potencies in competing with [^{3}H]DHA at binding sites of colon membranes [(−)Iso > (−)Adr = Salb > (+)Iso = Rit > (−)Nad] is virtually identical to that previously reported for rat uterus relaxation (a β_2-mediated effect), i.e. (−)Iso > (−)Adr > Salb > (+)Iso = Rit > (−)Nad, but substantially different from the rank orders of potencies for increasing guinea-pig atrial frequency (β_1) or inhibiting rat colon motility (β_1 + β-atypical) [3]. The atypical adrenergic agonists, PEATs, had variable affinities for [^{3}H]-DHA sites which largely depended on the chirality of the molecule. The importance of the chiral carbon of the ethanolamine moiety was apparent from the high potency of the *RS* and *RR* isomers and the virtual lack of affinity of the corresponding *SR* and *SS* enantiomers. Similarly, we had previously found that *RR* and *RS* isomers were several times more potent than their corresponding enantiomers both on rat colon and uterus motility [3]. However, it is unlikely that the competition of PEATs with [^{3}H]-DHA reflects binding at atypical sites since, as with reference agonists, there is no substantial correlation between the present binding data and the functional ones we previously reported on the rat isolated colon [3]. Thus, for instance, considering the three PEATs with *RR* configuration, SR 58612A had similar or slightly lower binding affinity than SR 58375A and SR 58572A, but it was definitely the most potent in inhibiting colonic motility. The inhibition of [^{3}H]-DHA binding by PEATs is more likely to depend on an action at β_2 receptor sites for which the PEATs—according to functional studies that also evidenced their partial agonist nature at these sites—retain variable affinity depending on individual compounds [3].

In view of the acknowledged weak affinity of even the most potent known antagonists for atypical β-adrenoceptors, relative to β_1- and β_2-sites [2, 3, 11], in further binding experiments we employed a concentration of [^{3}H]DHA about 10 times its K_d for high-affinity sites. This higher ligand concentration, was used in an attempt to label atypical β-adrenoceptors, as suggested by De Blasi *et al.* [33]. In this condition, up to 80% of [^{3}H]DHA binding to colon membranes was dose-dependently prevented by SR 58611A, the potent PEATs most selective for

the atypical sites [3], whereas only 40% of this binding no longer occurred in the presence of saturating concentrations of isoprenaline. These findings suggested that atypical binding sites, differently from those corresponding to β_1- or β_2-receptor subtypes, were labelled by [^{3}H]DHA. However, SR 58825, the enantiomer of SR 58611A with virtually no activity on β-adrenergic receptors [3], but by definition having the same physical and chemical properties, inhibited [^{3}H]DHA binding with the same potency as SR 58611A in the presence of an isoprenaline concentration apparently saturating both β_1- and β_2-adrenoceptor sites. This rules out the possibility that even these sites may correspond to atypical β-adrenoceptors. Most likely this competition with [^{3}H]DHA took place at non-stereoselective lipophilic sites already described for this β radioligand in the intestine [2, 16] and in other tissues [34, 35].

Failure by [^{3}H]DHA to reveal atypical sites in rat colon by no means excludes their actual presence which is anyway unequivocally supported by our own functional studies with agonists and antagonists. Several authors have recently addressed the problem of labelling atypical adrenergic sites in a variety of tissues but, as in the present study, no firm evidence for them has yet been produced [2, 16, 36–40]. This apparently depends on the shortcomings of binding techniques, as clearly discussed by Arch [1]. The choice of a radioligand with sufficiently high affinity for atypical receptors is of paramount importance. In binding experiments on rat adipocyte membranes, which reportedly contain atypical β-adrenoceptors presenting significant pharmacological homologies with those in rat colon [3, 13], Bahouth and Malbon [41] used four radioligands with various affinities for different receptors, yet they were unable to detect any atypical β-adrenergic site. However, these authors used only low concentrations of radioligands close to their K_d values for typical β-adrenoceptors, but well below those inhibiting lipolysis and therefore lower than is presumably needed for the detection of atypical sites [33].

A further complication arises if, in a given tissue, the relative abundance of atypical vs typical receptors is low in spite of their functional importance, thus making their identification even more difficult. This problem was circumvented by Emorine *et al.* [42] who recently cloned a gene coding for an atypical human β-adrenoceptor. The receptor protein, called "β_3-adrenergic receptor" was expressed in Chinese hamster ovary cells and the presence of only these atypical sites on these cells made it possible to identify them by [^{125}I]iodocyanopindolol binding that, according to the authors, had a K_d for the atypical sites almost 10 times that of the β_1- or β_2-adrenergic receptors. However, the pharmacological characterization of this receptor which is not labelled with [^{3}H]DHA, did not provide clear evidence that it is the same as any of the other atypical β-adrenoceptors previously described on functional grounds in either adipocytes [5, 43] or the gut [2, 3].

In binding studies, the nature of the material from which membranes are derived is also important. We used membranes from the mucosal-deprived rat proximal colon containing all muscle layers and nerve plexuses. Atypical receptors might be preferentially or exclusively located on longitudinal rather than on circular musculature which appears to contain a high percentage of β_2-receptors [24]. Further studies with selected fractions enriched in membranes possibly containing atypical receptors and the availability of better ligands should facilitate the identification of these sites.

In conclusion, our binding assays with [^{3}H]DHA failed to identify sites corresponding to the atypical β-adrenoceptors clearly evidenced by functional studies in rat colon [3]. Claims of atypical β-adrenoceptors in different tissues based on the results of similar radioligand techniques [36, 39] are still open to question, both because of the aforementioned technical limitations [1] and the lack of assessment of the stereospecific displacement of the allegedly atypical binding. This important stereospecific assessment was possible in our study, since the PEAT stereoisomers offer a unique opportunity for comparison of pharmacological potency with binding affinity.

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