British Journal of Pharmacology (1996) 119, 1319-1330

Stereoselective recognition of the enantiomers of phenglutarimide and of six related compounds by four muscarinic receptor subtypes

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1 We have compared the binding properties of the enantiomers of phenglutarimide (**1**) and of six related compounds to M_1 receptors in NB-OK-1 cells, M_2 receptors in rat heart, M_3 receptors in rat pancreas and the M_4 receptors of rat striatum, with their functional (antimuscarinic) properties in rabbit vas deferens (M_1/M_4-like), guinea-pig atria (M_2) and guinea-pig ileum (M_3) receptors. The binding properties of the enantiomers of three of the compounds were also measured on cloned human m1-m4 receptors expressed by CHO cells, using [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) as radioligand.

2 The high affinity enantiomers behaved as competitive antagonists in binding and pharmacological studies. (**S**)-phenglutarimide (pK_i-M_1 = 9.0/9.3) and (**R**)-thienglutarimide (pK_i-M_1 = 8.6/9.2) recognized selectively the native $M_1 > M_4 > M_3 > M_2$ receptors in tissues as well as the respective cloned receptors.

3 The pA_2 values at the inhibitory heteroreceptors in the rabbit vas deferens, and at the guinea-pig atria and ileum for the seven more potent enantiomers were compatible with the previous classification of these receptors as M_1/M_4-like, M_2 and M_3, respectively.

4 Replacement of the phenyl by a thienyl ring or of the diethylamino by a piperidino group in the phenglutarimide molecule did not affect markedly the potencies of the high affinity enantiomer. In contrast, replacement of the phenyl by a cyclohexyl ring decreased 20 fold the active enantiomers potency. Methylation of the piperidine-2,6-dione nitrogen also reduced markedly the eutomers' affinities, more on the M_1 than on the other subtypes.

5 The selectivity profiles (recognition of four receptor subtypes) of six of the seven less active enantiomers were different from the corresponding more active enantiomers selectivity profiles, suggesting that the preparations used in this study were pure. However, we cannot not exclude the hypothesis that the batch of (**S**)-thienglutarimide used in this study was contaminated by less than 0.02% of the eutomer.

6 In contrast with the eutomer binding site, replacement of the phenyl ring by a thienyl or cyclohexyl ring did not affect binding of the low affinity enantiomers to the muscarinic receptor or the [^{3}H]-NMS-receptor complex. The replacement of the diethylamino group by a piperidine ring, and N-methylation of the piperidine-2,6 dione moiety increased slightly these enantiomers' potencies.

7 The muscarinic receptors were extremely stereoselective, and had up to 20 000 fold lower affinity for the less active enantiomers. However, the stereochemical requirements of the muscarinic receptor subtypes were different for the enantiomers of compounds **1**-**7**, being most stringent at M_1 receptors.

8 The weaker enantiomers behaved as competitive antagonists in pharmacological studies, at least in the concentration-range investigated.

Keywords: Phenglutarimide derivatives; muscarinic receptor subtypes; structure-affinity relationships; binding/functional correlations; stereoselectivity; muscarinic receptor antagonists

Introduction

Five muscarinic receptor subtypes have been cloned, four of which (the m1-m4 receptors) are widely represented in mammalian tissues (for recent reviews, see Levine & Birdsall, 1993; Brann *et al.*, 1993; Caulfield, 1993).

We were able to identify, in binding studies, four native muscarinic receptors with different selective antagonists binding profiles in rat tissues and in NB-OK-1 cells (Waelbroeck *et al.*, 1990b). The binding properties of these four receptor subtypes correlated with, respectively, the binding profile of the receptors encoded by the m1, m2, m3 and m4 genes (Hulme *et al.*, 1990; Lazareno *et al.*, 1990; Waelbroeck *et al.*, 1990b; Dörje *et al.*, 1991b; Levine & Birdsall, 1993).

Muscarinic receptors are stereoselective, that is, generally able to recognize one enantiomer of chiral ligands with a high affinity (the 'eutomer'), and the other ('distomer') with a lower affinity (Lambrecht *et al.*, 1988; Waelbroeck *et al.*, 1989; 1991; 1992; Feifel *et al.*, 1990; 1991). In functional studies, the enantiomers of the antiparkinsonian drug, phenglutarimide (Lambrecht *et al.*, 1989a) have been shown to display an extremely high stereoselectivity (up to 6000 fold) at muscarinic receptors. In binding studies, muscarinic receptors discriminated very well (eudismic ratio (ratio of the eutomer/distomer affinity constants) up to 10 000) the two enantiomers of a related compound, benzetimide (Waelbroeck *et al.*, 1989). Thus, we were interested in the identification of the structural determinants which allowed such a large stereoselectivity. We decided to characterize the binding and functional properties of the two enantiomers of phenglutarimide and of six related compounds (Figure 1), at muscarinic receptor subtypes.

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In functional studies, the guinea-pig atria and ileum receptors have been clearly identified as, respectively, M_2 and M_3 receptors (for reviews, see Mitchelson, 1988; Levine & Birdsall, 1993; Caulfield, 1993). In contrast, the prejunctional rabbit vas deferens muscarinic receptors mediating inhibition of the neurogenic contractions have not been identified unambiguously. Their pharmacological profile clearly indicates that these receptors do not belong to the M_2, M_3 or m5 subtypes (Eltze, 1988; Eltze *et al.*, 1988; 1993; Lambrecht *et al.*, 1989b; Dörje *et al.*, 1991b; Grimm *et al.*, 1994). Several findings support the hypothesis that these inhibitory heteroreceptors belong to the M_1 subtype. Indeed, they have a high affinity for the $M_1 > M_2$, M_3, M_4-preferring drug, pirenzepine (pA_2 values 8.08–8.52; Lambrecht *et al.*, 1989b; Choo & Mitchelson, 1990; Micheletti *et al.*, 1990; Dörje *et al.*, 1991b; Eltze *et al.*, 1993; Sagrada *et al.*, 1993) and a low affinity for the $M_2 = M_4$-preferring drug, methoctramine (pA_2 value 6.85; Lambrecht *et al.*, 1989b). In contrast with Micheletti *et al.* (1990), we observed a high affinity (pA_2 value 7.0) for the $M_1 > M_4$ selective compound, guanylpirenzepine (Lambrecht *et al.*, unpublished results). It is also worth noting that solubilized muscarinic receptors from the rabbit vas deferens could be immunoprecipitated by antibodies raised against m1 and m2 but not m4 muscarinic receptors (Dörje *et al.*, 1991a) and that the effects of LiCl, phorbol esters and forskolin on the muscarinic receptor responses support the hypothesis that the prejunctional muscarinic receptors are coupled to phosphatidylinositol 4,5-bisphosphate hydrolysis (an effect preferentially induced by the m1, m3 or m5 receptors (Eltze, 1994). On the other hand, the affinity of the M_2, M_4-preferring drug, him-

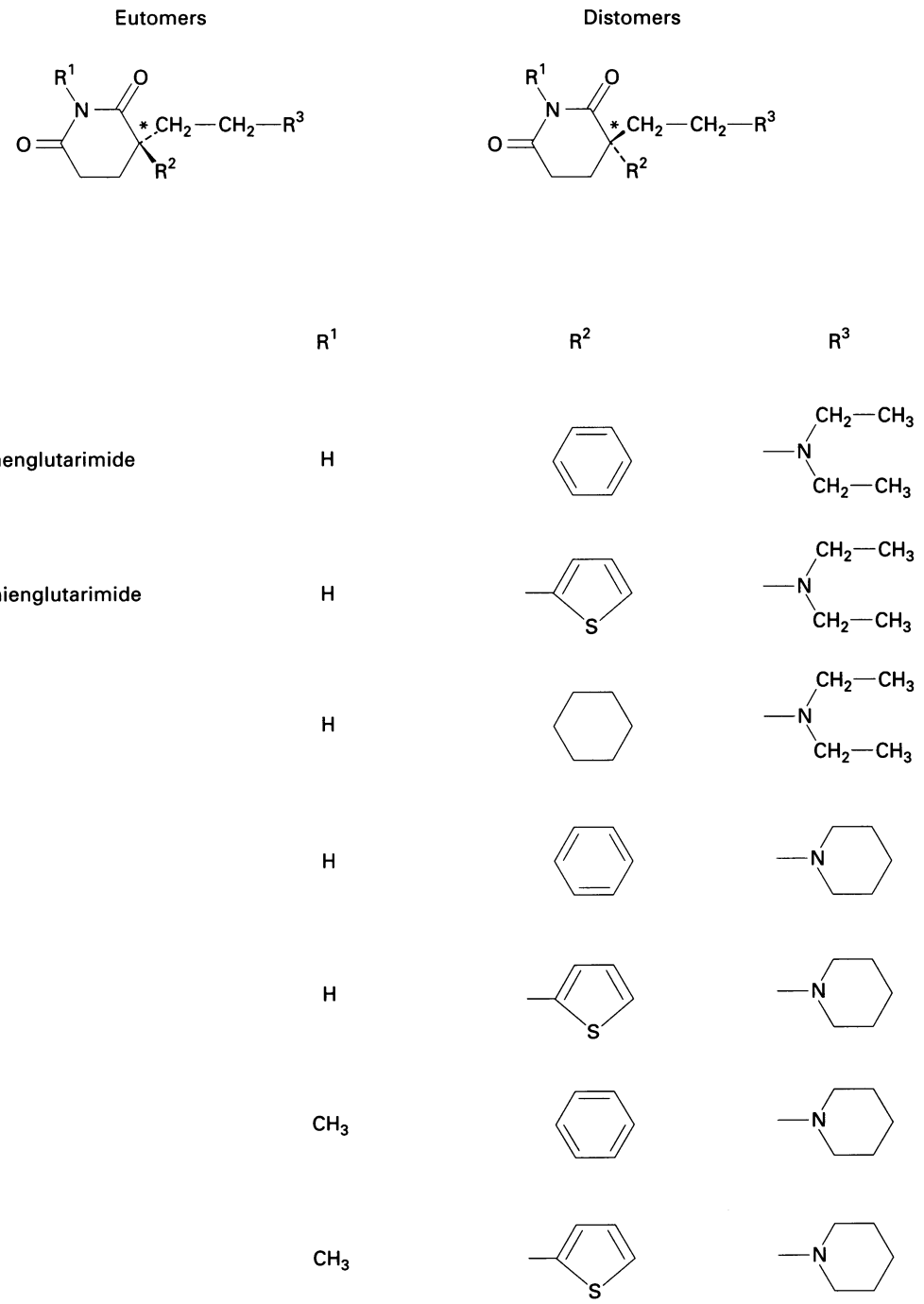


Figure 1 Chemical structures of the enantiomers of phenglutarimide (**1**), thienglutarimide (**2**), and analogues **3** to **7**. The asterisk denotes the centre of chirality.

bacine (Waelbroeck *et al.*, 1990b; Lazareno *et al.*, 1990; Dörje *et al.*, 1991b; Eltze *et al.*, 1993; Sagrada *et al.*, 1993; Caulfield, 1993), was approximately 10 fold higher than expected for M_1 receptors, and more consistent with the affinity expected for M_4 receptors.

We had previously observed that, in functional studies, the eutomer (S)-phenglutarimide is able to discriminate the inhibitory receptors in the rabbit vas deferens from the M_2 and M_3 receptors in heart and ileum, respectively, whereas the distomer (R)-phenglutarimide is non-selective (Lambrecht *et al.*, 1989a). We therefore hoped that a study of the binding properties of these and related compounds might help us to identify the prejunctional, inhibitory rabbit vas deferens muscarinic receptor subtype unambiguously.

Preliminary accounts of this study have been communicated to the German Society for Pharmacology and Toxicology (Pfaff *et al.*, 1993).

Methods

Radioligand binding experiments: general considerations

Protein concentrations were determined according to Lowry *et al.* (1951), with bovine serum albumin used as standard. Male Wistar albino rats were killed by decapitation and the brain, heart or pancreas immediately removed. NB-OK-1 cells were cultured as previously described (Waelbroeck *et al.*, 1988) in RPMI 1640 medium enriched with 10% foetal calf serum, 100 units ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. CHO cells stably expressing cDNA encoding human muscarinic m1–m4 receptors were generously provided by Dr N.G. Buckley (N.I.M.R., London). These were grown in α-MEM medium (GIBCO) containing 10% (v/v) new born calf serum, 50 units ml^{-1} penicillin, 50 $\mu g\ ml^{-1}$ streptomycin and 2 mM glutamine, at 37°C under 5% CO_2.

Preparation of the homogenates or crude membranes used for binding studies

M_1 receptors The NB-OK-1 cells were rinsed, harvested and centrifuged in 20 mM sodium-phosphate buffer (pH 7.4) containing 150 mM NaCl and 1 mM EDTA, resuspended and homogenized in 20 mM Tris/HCl buffer (pH 7.5) enriched with 5 mM $MgCl_2$, and stored in liquid nitrogen.

M_2 receptors The heart was rinsed in isotonic NaCl, then homogenized in 2.5 ml of 20 mM Tris/HCl buffer (pH 7.5), enriched with 250 mM sucrose, with an Ultraturrax homogenizer (maximal speed, for 5 s) followed by addition of 12.5 ml of the same buffer, 7 up and down strokes with a glass-Teflon homogenizer and filtration on 2 layers of medical gauze.

M_3 receptors The pancreas was minced with scissors and homogenized with a glass-Teflon homogenizer (7 up and down strokes in 8 ml of 300 mM sucrose enriched with 0.2 mg ml^{-1} bacitracin and 500 kallikrein inhibitor units (KIU) ml^{-1} of Trasylol). The resulting homogenate was immediately filtered over two layers of medical gauze and diluted 11 fold with the incubation buffer (66 mM sodium phosphate buffer, pH 7.4, enriched with 2.6 mM $MgCl_2$ and with 13 mg ml^{-1} bovine serum albumin, 0.24 mg ml^{-1} bacitracin and 600 KIU ml^{-1} of Trasylol).

M_4 receptors The rat striatum was homogenized in 2 ml of 20 mM Tris/HCl buffer (pH 7.5) enriched with 250 mM sucrose, and stored in liquid nitrogen until use. The homogenate was diluted 20 fold with the homogenization buffer, immediately before the incubation with [^{3}H]-NMS.

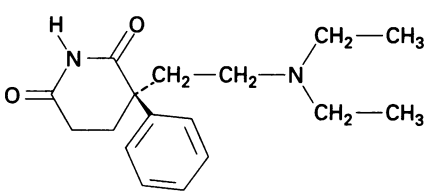
CHO cells expressing muscarinic receptors

Cells were grown to confluence and harvested by scraping in a hypotonic medium (20 mM HEPES + 10 mM EDTA, pH 7.4). Membranes were prepared at 0°C by homogenization with a Polytron followed by centrifugation (40 000 *g*, 15 min), were washed once in 20 mM HEPES + 0.1 mM EDTA, pH 7.4, and were stored at −70°C in the same buffer. The yields of receptor varied from batch to batch but were approximately 5, 1, 7 and 2 pmol mg^{-1} of total membrane protein for the m1, m2, m3 and m4 subtypes, respectively.

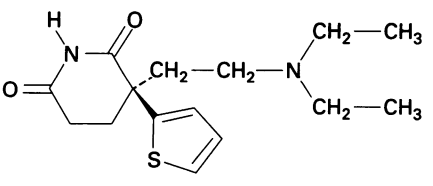
Binding studies

[^{3}H]-NMS binding to NB-OK-1 cells (about 200 μg protein per assay) and rat tissues (30–800 μg protein per assay) was measured in 1.2 ml of a buffer containing 50 mM sodium-phosphate (pH 7.4) enriched with 2 mM $MgCl_2$, and, for experiments in pancreas, 1% bovine serum albumin and 200 kallikrein inhibitory units ml^{-1} of Trasylol. The tracer concentrations used were: 0.25 nM for the experiments on NB-OK-1 cells, rat pancreas and striatum, and 1.0 nM for the experiments on rat heart. Previously estimated [^{3}H]-NMS K_D values were 0.13 nM in NB-OK-1 cells and in rat pancreas, 0.50 nM in rat heart, and 0.04 nM for the M_4 receptors in striatum. An incubation of 2 h at 25°C for rat heart and NB-OK-1 cells, and of 4 h at 25°C with rat pancreas allowed equilibrium binding. In the striatum, [^{3}H]-NMS labelled, after 2 h of incubation at 25°C, M_1, M_4 and very few M_3 and M_2 receptors. To investigate drug binding to the M_4 receptors, we preincubated the receptors with the unlabelled drug, using [^{3}H]-NMS to label the unoccupied receptors. We then added 1 μM atropine to induce tracer dissociation. Since [^{3}H]-NMS dissociates more slowly from the M_4 (and M_3) receptors than from the other subtypes, after 35 min of dissociation, the residual [^{3}H]-NMS labelled mainly (85%) M_4 receptors (Waelbroeck *et al.*, 1990b). [^{3}H]-NMS binding was determined after filtration over Whatman GF/C glass fibre filters presoaked in 0.1% polyethyleneimine. Non-specific binding was measured in the presence of 1 μM atropine.

[^{3}H]-NMS binding to membranes of CHO cells stably transfected with human m1–m4 receptors was measured in 1 ml of a buffer containing 20 mM HEPES (pH 7.4) enriched with 100 mM NaCl and 10 mM $MgCl_2$. The tracer concentrations used were: 0.26 ± 0.02 nM at m1, m3 and m4 receptors and 0.48 ± 0.02 nM at m2 receptors. Previously estimated [^{3}H]-NMS K_D values were 0.19, 0.36, 0.27 and 0.10 nM at m1–m4 receptors, respectively. The incubation was 2 h at 30°C, and terminated by filtration over Whatman GF/B glass fibre filters



(S)-phenglutarimide [(S)-1]



(R)-thienglutarimide [(R)-2]

Figure 2 Comparison of the absolute configuration of the eutomers (S)-phenglutarimide [(S)-1] and (R)-thienglutarimide [(R)-2].

Table 1 pK_i and $p(\alpha K_i)$ values [a] obtained in NB-OK-1 cells and rat tissues homogenates for recognition of the 'empty' receptor $-\log(K_i)$ and of the [^{3}H]-NMS-receptor complex (average of 3 experiments in duplicate)

		NB-OK-1 cells M_1	*Rat heart* M_2	*Rat pancreas* M_3	M_4 *(in rat striatum)*
Phenglutarimide	(R)-1	5.1/3.5[a]	4.6/3.1[a]	4.1	4.7[b]
	(S)-1	9.3	7.5	8.1	8.5
Thienglutarimide	(R)-2	9.2	7.2	7.8	8.2
	(S)-2	6.5	4.9/3.3[a]	5.3	5.5[b]
	(R)-3	5.0/3.6[a]	4.5/3.6[a]	4.0	ND
	(S)-3	8.0	6.3	6.8	7.3
	(R)-4	5.8/3.9[a]	5.2/3.5[a]	5.0	5.5[b]
	(S)-4	8.7	7.1	7.4	8.0
	(R)-5	8.9	7.1	7.3	8.0
	(S)-5	6.1/4.4[a]	5.4	5.3	5.8[b]
	(R)-6	5.4/3.6[a]	5.5/3.4[a]	4.6	5.8[b]
	(S)-6	7.6	6.4	6.5	7.1
	(R)-7	7.1	6.3	6.2	6.8
	(S)-7	5.4/3.9[a]	5.6/3.8[a]	5.0	5.6[b]

[a]All competition curves were fitted to equation (1), assuming either competitive or allosteric inhibition of tracer binding. The two 'best fits' were compared by F test. The error of the pK_i values were always below 0.05 log units. If the allosteric model did not improve significantly the curve fitting, a single value (pK_i) is indicated in the table. In those cases where an allosteric model gave a better fit than the competitive model ($P<0.05$), the error of the $p(\alpha K_i)$ value was always below 0.15 log unit. Two values pK_i ($-\log K_i$) and $p(\alpha K_i)$ [$-\log(\alpha K_i)$] are then indicated successively. [b]Approximate pK_i values, obtained using the experimental data obtained with unlabelled drug concentrations below 200 μM.

Table 2 pK_i and $p(\alpha K_i)$ values[a] obtained in CHO cells expressing the human m1–m4 receptors (mean of two experiments in duplicate)

		m1	*m2*	*m3*	*m4*
Phenglutarimide	(R)-1	4.73/3.60[a,b]	4.87/3.79[a]	4.16/3.54[a,b]	4.40/3.41[a]
	(S)-1	9.04	7.46	7.91	7.95
Thienglutarimide	(R)-2	8.61	6.92	7.39	7.76
	(S)-2	6.01	4.78	4.89	5.27/3.33[a,b]
	(R)-3	4.49/3.06[a]	4.41/3.50[a]	3.73/2.63[a,b]	4.27/3.32[a]
	(S)-3	7.59	6.20	6.58	6.94

[a]See footnote (a) of Table 1. The range/2 of the pK_i values obtained in two experiments in duplicate was always below 0.11 log unit. In those cases where an allosteric model gave a better fit of both experiments, the range/2 of the $p(\alpha K_i)$ values was always below 0.21 log unit. [b]In one experiment out of two the data were better fitted to the allosteric model.

presoaked in 0.1% polyethyleneimine using a 30-place Brandel cell harvester. Non-specific binding was measured in the presence of 1 μM quinuclidinyl benzilate.

Drug effects on the dissociation of [^{3}H]-HMS Rat striatum homogenates were preincubated with 2 nM [^{3}H]-NMS alone for 2 h at 25°C. Following this preincubation, we added simultaneously 1 μM atropine and the indicated concentration of enantiomers, and measured the residual [^{3}H]-NMS binding 1 h after this addition.

Effect of (+)-tubocurarine on the competition curves Rat heart homogenates were incubated for 6 h at 25°C in a 10 mM sodium phosphate buffer (pH 7.4) with 1.0 nM [^{3}H]-NMS, the indicated drug concentrations, in the absence or presence of 100 μM (+)-tubocurarine. [^{3}H]-NMS binding was measured by filtration, as indicated above.

Pharmacological experiments

Rabbit vas deferens Male New Zealand white rabbits (2.5–3.0 kg) were killed by i.v. injection of 120 mg kg^{-1} pentobarbitone sodium. The vasa deferentia were excised, dissected free of connective tissue and divided into four to six segments of approximately 1.5 cm length. The preparations were set up in 7 ml organ baths containing modified Krebs buffer which consisted of (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.0, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and (+)-glucose, 11.1; 1 μM yohimbine was included to block prejunctional α_2-adrenoceptors. The bathing fluid was maintained at pH 7.4, 31°C, and aerated with 95% O_2/5% CO_2. A basal tension of 750 mg was applied. After a 30 min equilibration period, isometric twitch contractions were elicited by electrical field stimulation (0.05 Hz, 0.5 ms, 40 V) using platinum electrodes. These effects were concentration-dependently inhibited by the M_1 receptor agonist, 4-(4-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343) (Eltze, 1988; Eltze *et al.*, 1988; Lambrecht *et al.*, 1993). The neurogenic contractions were measured by a force-displacement transducer connected to a Hellige amplifier and a Rikadenki polygraph.

Guinea-pig atria and ileum Adult guinea-pigs of either sex were killed by cervical dislocation and the organs required were removed. Left atria and strips of ileal longitudinal smooth muscle of 1.5 cm length (Paton & Zar, 1968) were set up in 6 ml organ baths, under 500 mg tension, in oxygenated (95%O_2/5%CO_2) Tyrode solution, composed of (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42 and (+)-glucose 5.6. All experiments were conducted at 32°C (pH = 7.4). The agonist used was arecaidine propargyl ester (Mutschler & Lambrecht, 1984; Barlow & Weston-Smith, 1985; Lambrecht *et al.*, 1993).

Left atria were electrically paced by means of platinum electrodes (2 Hz, 3 ms duration, supramaximal voltage).

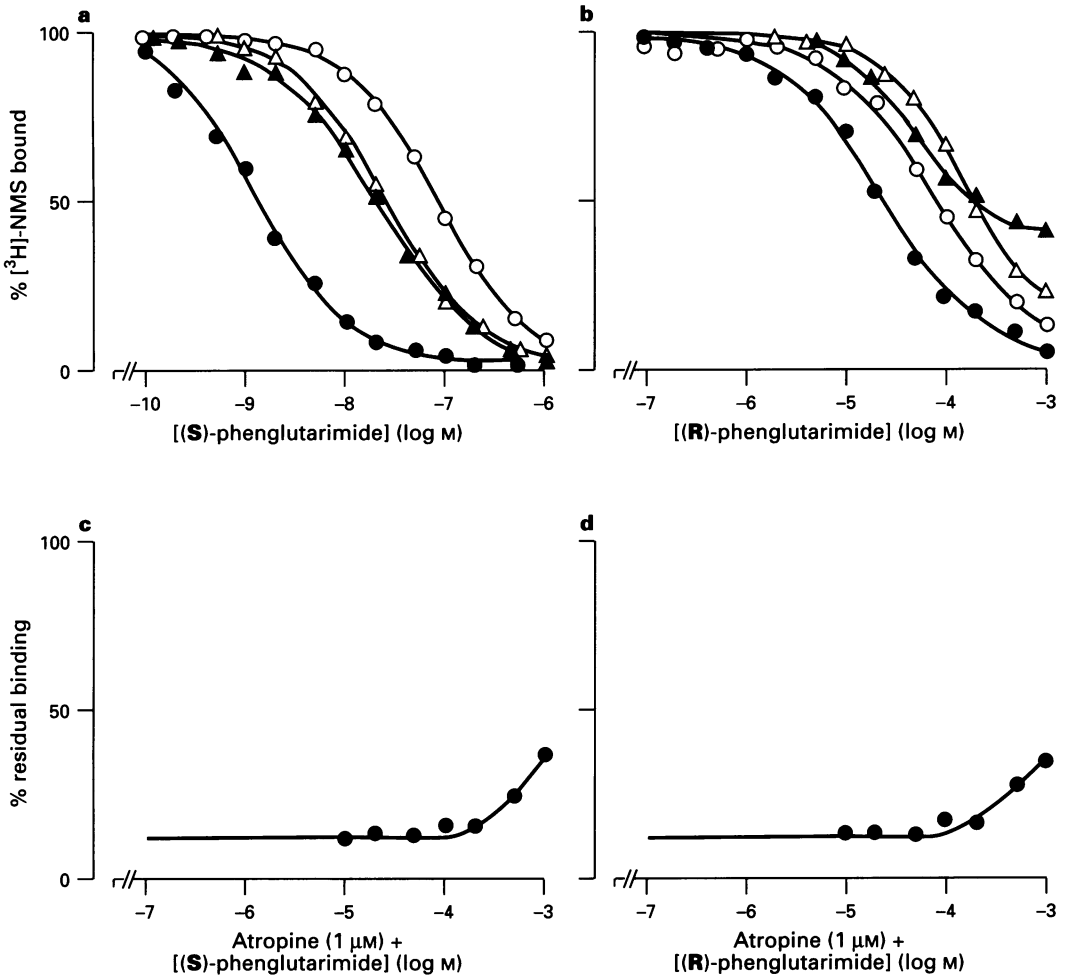


Figure 3 Competition curves obtained at four muscarinic receptor subtypes, and effects on [^{3}H]-NMS dissociation from rat striatum. (a) [^{3}H]-NMS/(**S**)-phenglutarimide [(**S**)-**1**] competition curves were obtained as explained in Methods, using NB-OK-1 cells (●), heart (○), pancreas (△) or striatum M_4 (▲) sites (average of three experiments in duplicate), (b) [^{3}H]-NMS/(**R**)-phenglutarimide [(**R**)-**1**] competition curves were obtained as explained in Methods, using NB-OK-1 cells (●), heart (○), pancreas (△) or striatum M_4 (▲) sites, (average of three experiments in duplicate). (c) The residual [^{3}H]-NMS binding was measured in rat striatum, as explained in Methods, 1 h after the addition of atropine alone (control) or of atropine and the indicated concentrations of (S)-**1** (●) (representative of 2 experiments in duplicate). (d) The residual [^{3}H]-NMS binding was measured in rat striatum, as explained in Methods, 1 h after the addition of atropine alone (control) or of atropine and the indicated concentrations of (**R**)-**1** (●) (representative of 2 experiments in duplicate).

Atrial responses to the agonist were measured as changes in isometric tension, and these effects were expressed as the percentage inhibition of the force of contraction. Responses of ileal longitudinal smooth muscle strips to arecaidine propargyl ester were measured as isotonic contractions. The effects in atria and ileum were recorded as with the rabbit vas deferens.

Antagonist affinities After 1 h equilibration, concentration-response curves were obtained by cumulative addition of the agonists (van Rossum, 1963). When these responses were constant, concentration-response curves were repeated in the presence of at least 3 concentrations (log interval = 0.48) of antagonists, allowing 15–45 min equilibration time. Each concentration of antagonist was tested 3 to 5 times and dose-ratios at EC_{50} values of agonists were calculated.

Data analysis

The slopes of the Arunlakshana and Schild plots (Arunlakshana & Schild, 1959) were determined by linear regression using the method of least squares. pA_2 values were estimated by fitting to the data the best straight line with a slope of unity (Arunlakshana & Schild, 1959; Tallarida *et al.*, 1979).

The competition curves were analysed by non-linear curve fitting to the following equation (adapted from Waelbroeck, 1994):

$$\%B = \frac{100(NMS/K_D)}{NMS/K_D + \frac{(1+I/K_i)}{(1+I/\alpha K_i)}} \quad (1)$$

where: NMS is the tracer concentration, K_D is the tracers' equilibrium dissociation constant, I, the inhibitors' concentration, and K_i and αK_i measure, respectively, the inhibitors' dissociation constants for the receptor and for the tracer-receptor complex. If the inhibitor is a competitive ligand, it does not recognize the tracer-receptor complex, α is infinite, and $I/\alpha K_i$ is equal to zero.

This general equation can therefore be used either to describe competitive inhibition (by taking $1/\alpha K_i = 0$), or allosteric interactions (by taking $1/\alpha K_i$ different from 0). The parameter α is a cooperative factor, measuring the effect of the allosteric drug on tracer binding (see also, Lazareno & Birdsall, 1995).

The pK_i ($-\log K_i$) values were calculated with this equation and the previously estimated [^{3}H]-NMS K_D values. The hypothesis that the allosteric model allowed a statistically significant improvement of the curve fitting ($P > 0.95$) was tested by an F test.

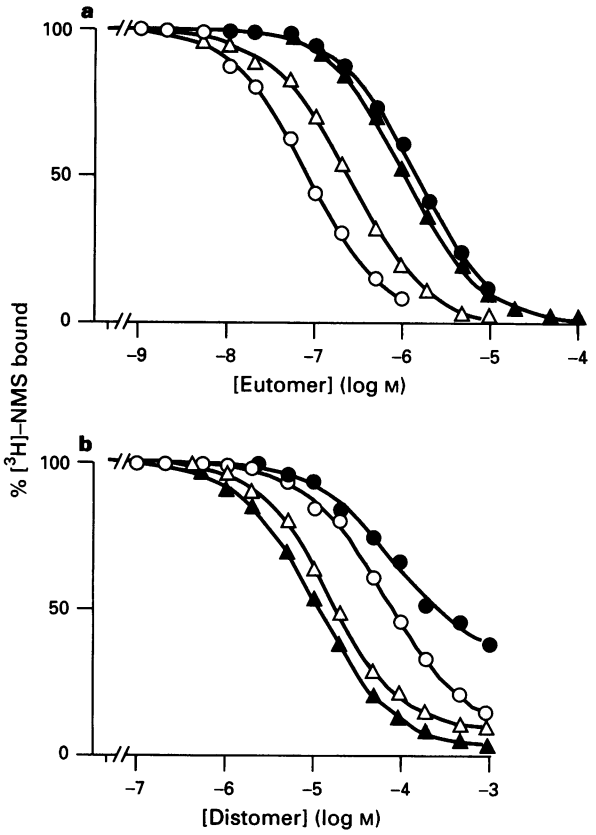


Figure 4 Comparison of the eutomers and distomers structure-activity relationships in rat heart. (a) [^{3}H]-NMS/(S)-1 (○), (S)-3 (●), (S)-4 (△) and (S)-6 (▲) competition curves were obtained in rat heart as explained in Methods. (b) [^{3}H]-NMS/(R)-1 (○), (R)-3 (●), (R)-4 (△) and (R)-6 (▲) competition curves were obtained in rat heart as explained in Methods (average of three experiments in duplicate).

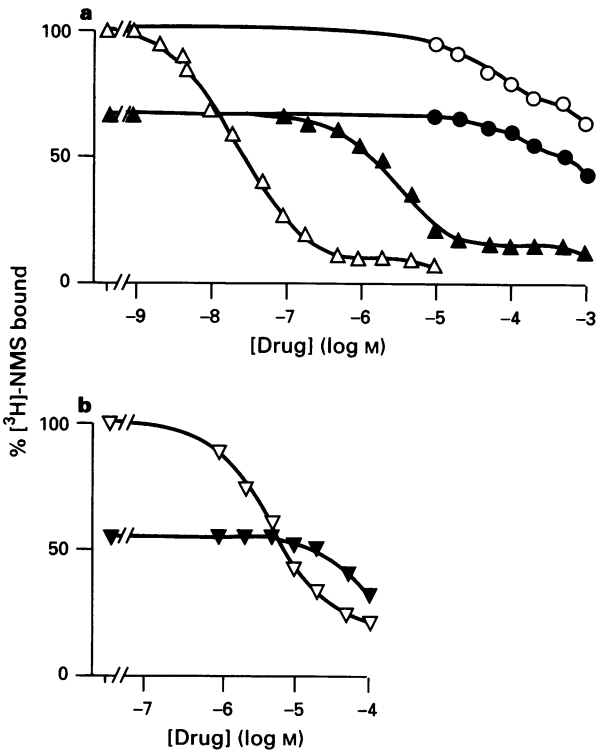


Figure 5 Effect of (+)-tubocurarine on gallamine binding to the allosteric site of M_2 receptors in rat heart and on the recognition of muscarinic receptors by the phenglutarimide analogues distomers. (a) [^{3}H]-NMS/gallamine (△,▲) or (R)-3 (○,●) competition curves were obtained in the absence (open symbols) or presence (solid symbols) of 100 μM (+)-tubocurarine. (b) [^{3}H]-NMS/(S)-7 (▽,▼) competition curves were obtained in the absence (open symbols) or presence (solid symbols) of 100 μM (+)-tubocurarine.

Differences between mean values were tested for statistical significance by Student's t test; $P<0.05$ was accepted as being significant. All data are presented as arithmetic means.

Compounds

1-[^{3}H]-N-methylscopolamine chloride ([^{3}H]-NMS, 80 to 85 Ci mmol^{-1}) was obtained from Amersham International (Bucks, England). Atropine sulphate, gallamine, (+)-tubocurarine chloride, bovine serum albumin (Fraction V) and polyethyleneimine were from Sigma Chemical Co. (St Louis, MO, U.S.A.), and glass fibre filters GF/C and GF/B from Whatman (Maidstone, England). All other reagents were of the highest grade available.

4-(4-Chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343) (Nelson *et al.*, 1976), and arecaidine propargyl ester (Mutschler & Hultzsch, 1973) were synthesized in our laboratories according to the literature.

The enantiomers of phenglutarimide (1) and of the related compounds 2 to 7 were kindly donated by Dr Knabe (University of Saarbrücken, Germany). The structure of these compounds is given in Figure 1, and the absolute configuration of the eutomers (S)-phenglutarimide [(S)-1] and (R)-thienglutarimide [(R)-2], in Figure 2.

Results

Radioligand binding studies

Eutomers binding All the competition curves obtained with the high-affinity enantiomers were compatible with competitive inhibition of tracer binding. Probably due to the different assay buffers and temperatures, the eutomers (S)-1, (R)-2 and (S)-3 had a slightly higher affinity for NB-OK-1 M_1 and rat striatum M_4 receptors as compared to cloned m1 and m4 receptors expressed in CHO cells, respectively. The pK_i values are summarized in Tables 1 and 2.

In binding studies, (S)-phenglutarimide [(S)-1] inhibited [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding to homogenates from rat tissues and human neuroblastoma cells with the following order of potencies: NB-OK-1 (M_1) > rat striatum (M_4) ≥ rat pancreas (M_3) > rat heart (M_2) receptors (Figure 3). The same order of potencies was observed with cloned receptors expressed in CHO cells.

Replacement of the phenyl by a thienyl ring [(S)-1→(R)-2] did not affect markedly the eutomers' potency at the four receptors. In contrast, replacement of the phenyl by a cyclohexyl ring [(S)-1→(S)-3] decreased 20 fold the eutomer potency (Tables 1 and 2). Replacement of the diethylamino by a piperidino group [(S)-1→(S)-4, (R)-2→(R)-5] decreased only slightly the eutomer affinities. Methylation of the piperidine-2,6-dione nitrogen of compounds (S)-4 [→(S)-6] and (R)-5 [→(R)-7] reduced the affinity of the eutomers (up to 100 fold) at the four receptor subtypes. The eutomer affinity decrease upon the structural variations in the parent compound 1 was not identical at all receptor subtypes, and greater at M_1 than at M_2, M_3 or M_4 receptors (Table 1).

Stereoselectivity The four muscarinic receptor subtypes were extremely stereoselective (up to 20 000 fold) (Figures 3 and 4). However, this stereoselectivity was not the same for all subtypes.

The distomers (R)-1, (R)-3, (R)-4, (S)-5, (R)-6 and (S)-7 had very low affinities for the four muscarinic receptor subtypes, with a preference for the M_1, M_2 and M_4 over the M_3 subtype (Tables 1

and 2, Figure 4). Since their binding profile was different from the eutomers' (Tables 1 and 2, Figures 3 and 4), we are confident that we indeed measured the affinity of the distomers, rather than the affinity of a very low contamination by the eutomer. In contrast, the binding profiles of (S)-2 and (R)-2 were qualitatively more similar (Tables 1 and 2). We therefore cannot exclude the hypothesis that the thienglutarimide distomer, (S)-2, was very slightly contaminated, by less than 0.02% of (R)-2.

Distomer binding properties The seven distomers inhibited competively [^{3}H]-NMS binding to the M_3 receptors in pancreas and CHO cells.

The competition curves obtained in NB-OK-1 cells with (R)-1, (R)-3 and (S)-7, and in rat heart, with all the distomers except (S)-5, were better described by an allosteric model (i.e. by introduction of a finite value for 'αK_i' in equation 1). The (R)-1 competition curves obtained in CHO cells expressing the m1 (1 of 2 experiments), m2, m3 (1 of 2 experiments) and m4 receptors were best-fitted to an allosteric model. This was also true for one of the two competition curves obtained with (S)-2 in CHO cells expressing the m4 receptor, and with (R)-3 in CHO cells expressing m1, m2, m3 (one of two experiments) and m4 receptors. This is summarized in Table 2.

Effect of distomers on the [^{3}H]-NMS dissociation rate The previously identified allosteric drugs (gallamine, etc.) slow the dissociation rate of [^{3}H]-NMS from muscarinic receptors, with a half maximal effect at a concentration corresponding to 'αK_i'. We therefore decided to test whether the distomers decreased the tracer's dissociation from striatum receptors (mixed M_1, M_2, M_3 and M_4 receptors). (R)- and (S)-1 to -5 (Figures 3, 4 and results not shown), like dexetimide and levitimide (results not shown), decreased the [^{3}H]-NMS dissociation when added to the dissociation buffer at concentrations above 100 μM. We were, unfortunately, unable to repeat this experiment with (R)- and (S)-6 or -7, due to the lack of material.

Distomer binding properties to rat striatum M_4 receptors Our binding assay for M_4 receptors in the striatum relies on [^{3}H]-NMS dissociation from the other subtypes to improve the tracer selectivity. Our binding assay is therefore not valid if the competing drug is able to alter the [^{3}H]-NMS dissociation.

When we fitted the distomer competition curves in rat heart with a 'competitive inhibition' model, using only the data points obtained below 100 μM unlabelled drugs, the pK_i values were within 0.1 log units of the pK_i values calculated using all the data points and the (correct) allosteric model. This is due to the fact that the drugs had low affinities for the [^{3}H]-NMS-

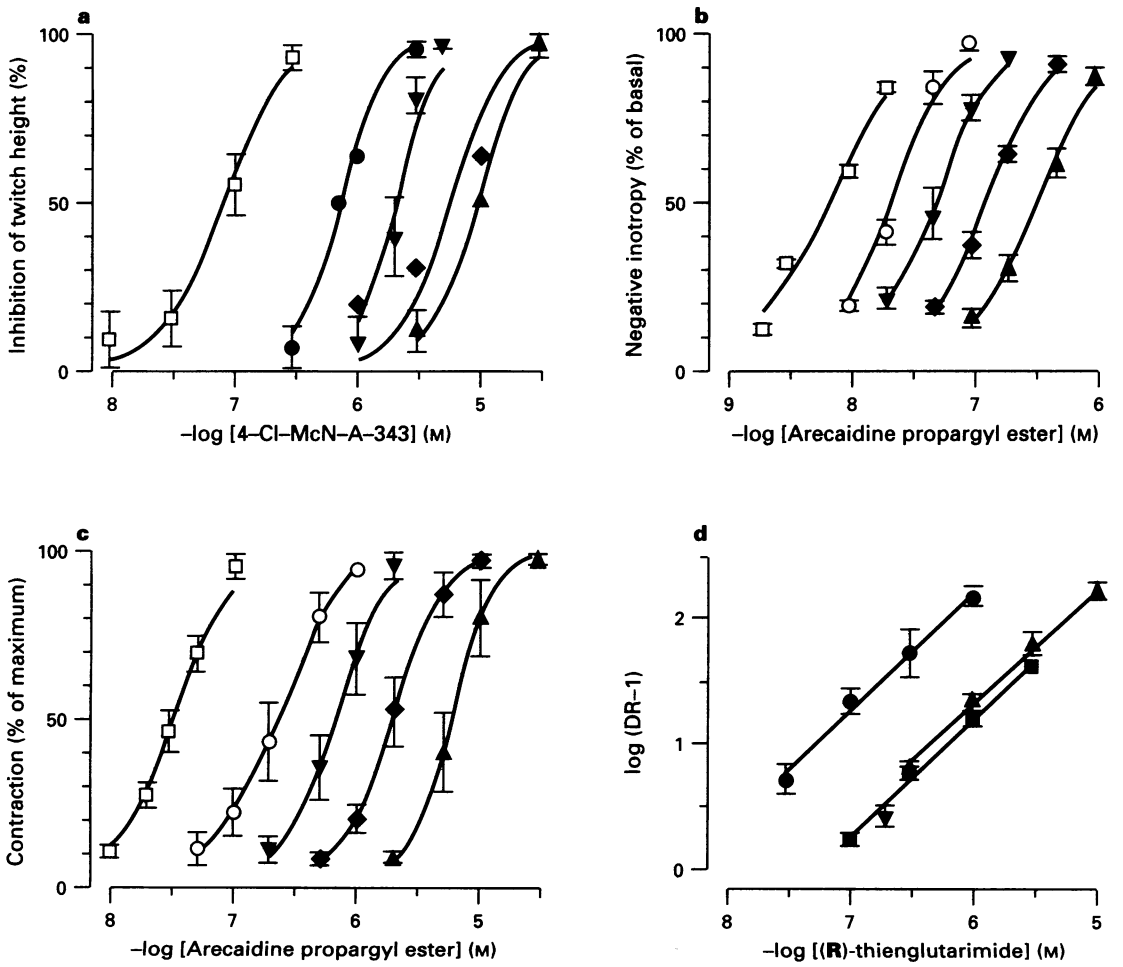


Figure 6 Antagonism of responses to 4-Cl-McN-A-343 in rabbit vas deferens and to arecaidine propargyl ester in guinea-pig paced left atria and ileum by different concentrations of (R)-thienglutarimide ((R)-2). Data are means ± s.e.mean. Error bars falling within the area covered by the symbol are not shown. (a) Concentration-response curves for 4-Cl-McN-A-343-induced inhibition of neurogenic twitch contractions in rabbit vas deferens in the absence (□) and presence of 0.03 (●), 0.1 (▼), 0.3 (◆) or 1 μM (▲) (R)-thienglutarimide ((R)-2). (b) Concentration-response curves for arecaidine propargyl ester-induced negative inotropy in guinea-pig atria in the absence (□) and presence of 0.1 (○), 0.3 (▼), 1 (◆) or 3 μM (▲) (R)-thienglutarimide [(R)-2]. (c) Concentration-response curves for arecaidine propargyl ester-induced contractions of guinea-pig ileum in the absence (□) and presence of 0.3 (○), 1 (▼), 3 (◆) or 10 μM (▲) (R)-thienglutarimide [(R)-2]. (d) Schild regression from experiments in rabbit vas deferens (●), guinea-pig atria (■) and guinea-pig ileum smooth muscle (▲).

Table 3 pA_2 values obtained in rabbit vas deferens, guinea-pig atria and guinea-pig ileum

		Rabbit vas deferens (M_1/M_4-like)	*Guinea-pig atria M_2*	*Guinea-pig ileum M_3*
Phenglutarimide [a]	(R)-1	4.75±0.03	4.73±0.02	4.62±0.03
		(1.13±0.08)	(0.98±0.05)	(0.93±0.09)
	(S)-1	8.53±0.04	7.72±0.03	7.93±0.03
		(0.97±0.08)	(0.96±0.05)	(0.90±0.050)
Thienglutarimide	(R)-2	8.24±0.06	7.21±0.03	7.31±0.02
		(0.96±0.11)	(0.93±0.06)	(0.96±0.04)
	(S)-2	5.69±0.06	4.94±0.06[b]	4.91±0.05
		(0.94±0.12)	(30+100 μM)	(0.81±0.12)
	(R)-3	4.75±0.07	4.55±0.05	4.28±0.02[b]
		(0.77±0.16)	(1.00±0.14)	(100+300 μM)
	(S)-3	7.14±0.03	6.39±0.04	6.56±0.03
		(0.94±0.05)	(0.88±0.06)	(1.00±0.06)
	(R)-4	5.50±0.03[b]	5.37±0.06	5.27±0.04
		(10+30 μM)	(0.86±0.15)	(0.91±0.11)
	(S)-4	8.20±0.05	7.47±0.02	7.59±0.02
		(1.02±0.09)	(0.99±0.05)	(0.91±0.04)
	(R)-5	8.21±0.04	7.17±0.04	7.32±0.03
		(0.96±0.08)	(0.95±0.07)	(1.02±0.06)
	(S)-5	5.88±0.05	5.28±0.01	5.48±0.03
		(0.85±0.08)	(0.86±0.28)	(1.08±0.07)
	(R)-6	5.41±0.04	5.50±0.02	4.84±0.03
		(0.91±0.08)	(1.00±0.05)	(1.14±0.06)
	(S)-6	7.42±0.03	6.65±0.03	6.39±0.04
		(1.00±0.06)	(0.91±0.04)	(1.00±0.08)
	(R)-7	6.98±0.05	6.46±0.03	6.28±0.03
		(1.02±0.09)	(0.91±0.05)	(1.09±0.06)
	(S)-7	5.82±0.05	5.83±0.03	5.34±0.05
		(0.88±0.12)	(1.05±0.06)	(1.07±0.09)

pA_2 values and slopes of Schild plots (in parentheses) are presented as means±s.e.mean ($N=3-6$). [a]Data taken from Lambrecht *et al.* (1989a). [b]Only two antagonist concentrations were investigated. The pA_2 values were therefore calculated from the individual dose-ratios according to Tallarida *et al.* (1979). All other pA_2 values were determined from constrained Schild plots.

receptor complexes (αK_D values above 100 μM). Since we did not observe any alteration of the [^{3}H]-NMS dissociation in striatum at concentrations below 100 μM, we decided to use the same method to estimate approximate pK_i values of the seven distomers for striatum M_4 sites. The pK_i values of **(R)-1**, **(S)-2** and **(R)-3** obtained on cloned m4 receptors (Table 2) were in excellent agreement with the approximate pK_i values estimated in striatum (Table 1), supporting the validity of this approach. This method however did not allow us to estimate the αK_i values at rat M_4 receptors.

Recognition of the gallamine allosteric site? To facilitate the recognition of the allosteric site of M_2 receptors in rat heart, we used a dilute buffer with no divalent ions. Under these conditions, (+)-tubocurarine occupied almost 99% of the muscarinic M_2 receptors and shifted a gallamine competition curve to 70 fold higher concentrations (Figure 5). All the distomer competition curves for M_2 receptors were significantly better fitted with an allosteric model in the 10 mM sodium-phosphate buffer (not shown). We compared distomer binding in the absence and presence of (+)-tubocurarine. The competition curves of **(R)-1**, **(S)-2**, **(R)-4**, **(S)-5**, **(R)-6** and **(S)-7** were either not shifted or shifted to at most 6 fold higher concentrations by (+)-tubocurarine (Figure 5 and results not shown). **(R)-3** did not inhibit [^{3}H]-NMS binding by more than 30% in the absence of (+)-tubocurarine in this buffer. The presence of (+)-tubocurarine however did not prevent **(R)-3** from further inhibiting [^{3}H]-NMS binding (Figure 5). The competition curve obtained with **(S)-7** was parallel to the gallamine competition curve, indicating that **(S)-7** was less potent, but as cooperative as gallamine (similar α-values). In contrast with gallamine, however, the **(S)-7** competition curve was barely shifted in the presence of (+)-tubocurarine. Comparable results were also obtained with the five other distomers (not shown). Taken together, these results suggested that (in contrast with gallamine) **(R)**-phenglutarimide and the distomers of compounds **2** to **7** did not recognize the same allosteric site as (+)-tubocurarine on the muscarinic receptors.

Pharmacological studies

In most experiments, at least three antagonist concentrations could be investigated. All these compounds antagonized the agonist effects of 4-Cl-McN-A-343 and are caidine propargyl ester in rabbit vas deferens as well as in guinea-pig atria and ileum, respectively, without either the basal tension or the maximal agonist responses being affected. There was a concentration-dependent parallel shift to the right of agonist dose-response curves and the Schild plots were linear. The slopes of the Schild plots were not significantly different from unity, indicating that, at least in the concentration-range investigated, the compounds behaved as competitive antagonists (Figure 6 and results not shown). In three cases, however, only two concentrations of antagonists could be used, and the pA_2 values were calculated from individual dose-ratios according to Tallarida *et al.* (1979). The results are summarized in Table 3.

As shown in Figure 7, the pA_2 values obtained in guinea-pig heart and ileum were in excellent agreement with the pK_i values obtained in, respectively, rat heart and CHO-cells expressing m2 receptors, and in rat pancreas and CHO-cells expressing m3 receptors. The receptors responsible for inhibition of neurogenic responses in the rabbit vas deferens (M_1/M_4 receptors) had pA_2 values for the enantiomers of **1**, **2** and **3** closest to the pK_i values obtained at the rat striatum M_4 sites, but also to the pK_i values obtained at m1 receptors expressed in CHO-cells, respectively (Figure 7).

Discussion

Comparison of the native and recombinant receptors

The native muscarinic receptors are currently divided into four subtypes (M_1-M_4). It is possible to identify M_1 to M_4 receptors in binding and functional studies with the cloned m1 to m4 receptors, based on their affinity profiles for a number of selective antagonists (Hulme *et al.*, 1990; Dörje *et al.*, 1991b; Levine & Birdsall, 1993; Caulfield, 1993; Gross *et al.*, 1995; and this study). In contrast, the identity of the prejunctional inhibitory heteroreceptor in the rabbit vas deferens is still controversial (see Introduction). Since we had previously observed that (S)-phenglutarimide [(S)-1] but not (R)-phenglutarimide [(R)-1] could discriminate the M_1/M_4 inhibitory receptors of the rabbit vas deferens from the M_2 and M_3 receptors (Lambrecht *et al.*, 1989a), we decided to compare their functional properties, as well as the properties of other related compounds, with the binding properties at native M_1-M_4 receptors and in CHO cells expressing recombinant m1-m4 receptors. The binding affinities (pK_i values) at M_2 and M_3 receptors (Tables 1 and 2) were very similar to the corresponding antimuscarinic potencies (pA_2 values), summarized in Table 3. The most selective compound on native and cloned muscarinic receptors was (R)-thienglutarimide ((R)-2), with a 7 to 100 fold preference for m1 (M_1) receptors over the other three subtypes. This compound was, in fact, at least as selective as pirenzepine (Waelbroeck *et al.*, 1990b) and 10 fold more potent. The data demonstrate that (R)-thienglutarimide is a useful tool to investigate muscarinic receptor heterogeneity. In addition, it might become the starting point for the development of M_1-selective antagonists useful for quantifying M_1 receptors with positron emission tomography.

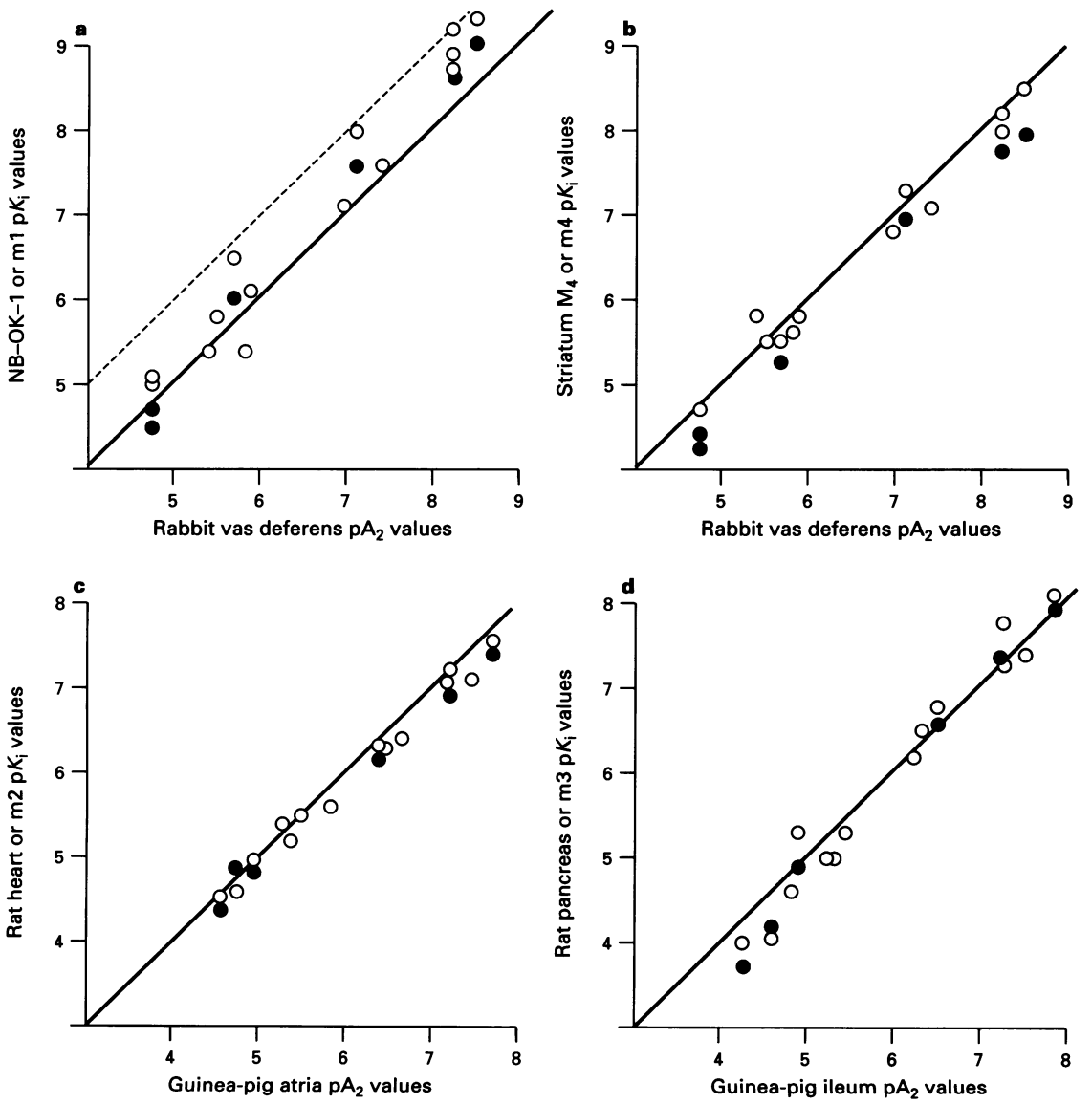


Figure 7 Comparison of the pK_i and pA_2 values of the enantiomers of phenglutarimide and of the related compounds (R)-2-(R)-7 and (S)-2-(S)-7. The full line corresponds to the line of identity ($pK_i = pA_2$); the dashed line in panel (a) corresponds to drugs 10 fold more potent in binding as compared to functional assays ($pK_i = pA_2 + 1$). (a) Comparison of the pK_i values obtained in NB-OK-1 cells (○) and in CHO cells expressing m1 receptors (●) with the pA_2 values obtained in rabbit vas deferens (inhibition of the neurogenic twitch contractions). (b) Comparison of the pK_i values obtained on striatum M_4 sites (○) and in CHO cells expressing m4 receptors (●) with the pA_2 values obtained in rabbit vas deferens (inhibition of the neurogenic twitch contractions). (c) Comparison of the pK_i values obtained on rat heart (M_2 sites) (○) and in CHO cells expressing m2 receptors (●) with the pA_2 values obtained in paced guinea-pig atria. (d) Comparison of the pK_i values obtained on rat pancreas (M_3 sites) (○) and in CHO cells expressing m3 receptors (●) with the pA_2 values obtained in guinea-pig ileum smooth muscle.

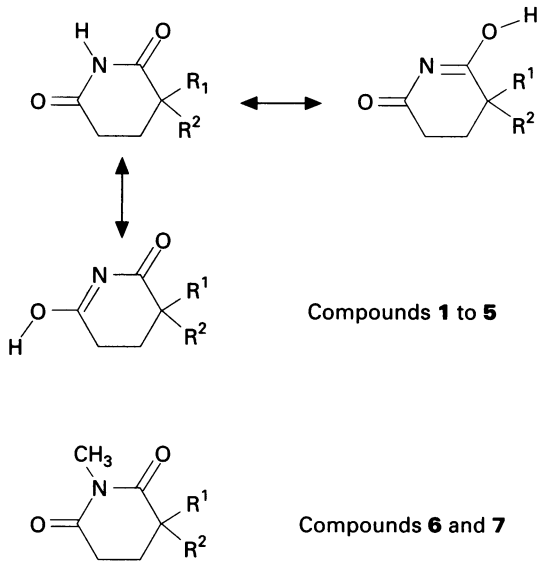


Figure 8 Tautomers of compounds (**R**)- and (**S**)-**1** to -**5**, and absence of tautomers for compounds (**R**)- and (**S**)-**6** and -**7**.

The pA_2 values obtained in the rabbit vas deferens were, on average, similar to pK_i values of M_4 receptors but intermediate between the pK_i values of m1 and m4 receptors. In addition, the binding properties of (**S**)-phenglutarimide, (**R**)-thienglutarimide and (**S**)-**3** on M_1 (m1) and M_4 (m4) receptors apparently depended on the incubation temperature and/or buffer composition or cell type. The results were, therefore, inconclusive.

Structure-activity relationships: eutomers

As shown in Tables 1 and 2 and Figure 3, the four muscarinic receptor subtypes discriminated easily the phenglutarimide enantiomers. In an attempt to evaluate the importance of the groups which surround the asymmetric carbon for eutomer binding, we compared the affinities of several phenglutarimide-related eutomers to four muscarinic receptor subtypes.

The phenyl, 2-thienyl or cyclohexyl rings occupy the same relative position in all the eutomers: (**S**)-phenglutarimide [(**S**)-**1**], (**R**)-thienglutarimide [(**R**)-**2**], (**S**)-**3**, (**S**)-**4**, (**R**)-**5**, (**S**)-**6** and (**R**)-**7** (see, for example, Figure 2). The different (**R**)- and (**S**)-designations of the eutomers are based on the priority rules of the Cahn-Ingold-Prelog nomenclature. Replacement of the phenyl ring of phenglutarimide by a thienyl moiety did not affect the eutomers' binding and functional properties. In contrast, (**S**)-**3**, with a cyclohexyl ring (Figure 1), had a 20 fold lower affinity at the four muscarinic receptor subtypes (Tables 1 and 2). This suggested that the presence of an aromatic ring favoured eutomer binding to the four muscarinic receptors subtypes studied.

Replacement of the diethylaminoethyl group by a piperidinoethyl group [(**S**)-**1**→(**S**)-**4** and (**R**)-**2**→(**R**)-**5**] resulted in a small (2 to 6 fold) eutomer affinity decrease at the four receptor subtypes (Tables 1 and 2).

The piperidine-2,6-dione structure may tautomerize, resulting in a different distribution of the hydrogen atoms, as shown on Figure 8. This reaction can be prevented by modification of the nitrogen atom (Figure 8). It was therefore interesting to investigate the effect of the N-methylation on the binding properties of compounds **4** (→**6**) and **5** (→**7**). As shown in Tables 1 and 3, this modification resulted in a 6 to 60 fold affinity decrease. Further experiments are needed to determine whether the affinity decrease is due to steric hindrance or to the loss of a strong hydrogen bond between one of the two possible enol-like hydroxy groups and the receptor (Waelbroeck *et al.*, 1990a; 1991).

Structure-activity relationships: distomers

As previously stated in the Results section, we are confident that we measured the intrinsic affinity of most distomers, rather than the effect of a (very small) contamination by the eutomer. Indeed, the affinity profile of the eutomers (M_1-selective) and of the distomers (non-selective, with a weaker affinity for M_3 receptors) are significantly different.

The only possible exception was (**S**)-thienglutarimide, [(**S**)-**2**]. Indeed, the affinity constants found with this distomer were higher than the affinity constants of (**R**)-phenglutarimide, [(**R**)-**1**], and its binding profile, qualitatively similar to the eutomer's. These results might be explained by assuming that (**S**)-**2** had the same affinity as (**R**)-**1**, but that the sample of (**S**)-**2** which we used was 'only' 99.98 to 99.99% pure. In view of this uncertainty, we shall discuss the structure-activity relationships of the other six distomers only.

In contrast with eutomer binding, all the distomers had comparable affinities for the four muscarinic receptor subtypes when compared to each other. Replacement of the diethylaminoethyl group by a 1-piperidino-ethyl group [(**R**)-**1**→(**R**)-**4**, (**S**)-**2**→(**S**)-**5**)] increased (3 to 10 fold) the distomers' affinities, but decreased the eutomers' affinities for the four muscarinic receptors. The nature of the hydrophobic (phenyl or cyclohexyl) ring was very important for eutomer, not distomer binding [compare (**R**)/(**S**)-**1** with (**R**)/(**S**)-**3**]. N-methylation of the piperidine-2,6-dione moiety did not change significantly the distomers' affinities in binding or functional studies [compare (**R**)-**4** with (**R**)-**6**, (**S**)-**5** with (**S**)-**7**], but decreased markedly the eutomers' affinities [compare (**S**)-**4**/(**S**)-**6**, (**R**)-**5**/(**R**)-**7**]. These different structure-activity profiles support the hypothesis that the eutomers (at low concentrations) and the distomers do not recognize the same binding site (see below).

Stereoselectivity of muscarinic receptors

It has been suggested (Pfeiffer, 1956) that, with greater potencies of drugs, larger differences in pharmacological effects will be seen between the enantiomers of chiral compounds. The results obtained in this study, like previous results obtained with the enantiomers of trihexyphenidyl, hexahydro-diphenidol, hexbutinol and related antagonists (Lambrecht *et al.*, 1988; Feifel *et al.*, 1990; Waelbroeck *et al.*, 1992), do not substantiate the above suggestion and its implications (Robert *et al.*, 1982; Lehmann, 1986). For example, the stereoselectivity ratios obtained in binding studies at M_1, M_2 and M_3 receptors were higher for compound **3** than for compound **5**, although the eutomer of compound **5** [(**R**)-**5**] was more potent (up to 8 fold) than the eutomer of compound **3** [(**S**)-**3**] (see Table 1). A similar lack of correlation between potency of the eutomer of compounds **3** and **5** and stereoselectivity ratios of the enantiomers was obtained in functional studies (Table 3). This suggests that other structural factors reduced the stereospecificity of some of the enantiomer pairs studied.

Another interesting finding of this study is that the stereoselectivity ratios were not the same for all muscarinic receptor subtypes. Consistently, the eudismic ratios were highest at M_1 and lowest at M_2 receptors. This implies that the stereochemical demands made by the muscarinic receptor subtypes are different for the enantiomers of compounds **1**–**7**, being most stringent at M_1 receptors. These results indicate that stereoselectivity ratios can be successfully used as a parameter to characterize muscarinic receptor subtypes providing information that racemates cannot give (Lambrecht *et al.*, 1988; Feifel *et al.*, 1990; Waelbroeck *et al.*, 1992).

Allosteric effect of distomers on [^{3}H]-NMS binding

In this study, and in contrast with the procyclidine and hexahydro-diphenidol distomers (Waelbroeck *et al.*, 1990a; 1991), we obtained evidence that most distomers did not recognize

the muscarinic site, at least at M_2 (m2) and M_4 (m4) receptors. Their effect on tracer binding was best explained by an allosteric model assuming that the distomers are able to recognize the [^{3}H]-NMS-receptor complex and do not inhibit competitively [^{3}H]-NMS binding (see below).

The distomers often appeared to be unable to inhibit completely [^{3}H]-NMS binding to the muscarinic receptors, especially in rat striatum (M_4 binding assay), in rat heart, and in the low ionic strength buffer. They also decreased the [^{3}H]-NMS dissociation from rat striatum homogenates. These results supported the hypothesis that the distomers recognize an allosteric site on the muscarinic receptors, but did not recognize the [^{3}H]-NMS binding site *per se*. Indeed, compounds which recognize in the same concentration-range an allosteric site and the muscarinic site give steep, complete competition curves reflecting the combination of competitive and allosteric inhibition of [^{3}H]-NMS binding (Waelbroeck, 1994).

(Non)-recognition of the gallamine allosteric site?

We previously demonstrated that (+)-tubocurarine competes with gallamine for recognition of an allosteric site on the muscarinic receptors. Since it does not prevent tracer binding, this compound can be used to differentiate the drugs which recognize the muscarinic site (little affected by (+)-tubocurarine), from the drugs which recognize the gallamine allosteric site (competitively inhibited by (+)-tubocurarine) (Waelbroeck, 1994). The present results indicate that the seven distomers were still able to recognize the muscarinic receptors, even when 98% of the gallamine/(+)-tubocurarine allosteric sites were occupied by (+)-tubocurarine. We conclude that the distomers were either able to recognize a second allosteric site on muscarinic receptors, or altered [^{3}H]-NMS binding by a non-specific mechanism. Detergents are indeed known to affect the tracer binding kinetics to solubilized receptors: interaction of the distomers with the membrane lipids might have the same effect on membrane bound receptors.

In conclusion, muscarinic receptor subtypes, M_1 (m1)–M_4 (m4), discriminate very strongly the enantiomers of phenglutarimide and of related compounds (up to 20000 fold). Our results in fact suggest that the eutomers recognized the muscarinic binding site while the distomers did not recognize the muscarinic site but had allosteric effects on the receptor.

The lipophilic compound (**R**)-thienglutarimide was at least as m1 (M_1)-selective, and 3 to 10 fold more potent than pirenzepine. It is a useful tool to investigate muscarinic receptor heterogeneity in central and peripheral tissues. It might become the starting point for the development of M_1-selective ligands to quantify M_1 receptors with positron emission tomography. However, due in part to small differences in the m1/M_1 and m4/M_4 pK_i values determined in different binding conditions, our data did not allow us to identify the presynaptic, inhibitory muscarinic heteroreceptor in rabbit vas deferens, unequivocally.

This work was supported by the Fund for Medical Scientific Research of Belgium (Grant No. 1.5.011.94F: M.W. and M.T.), the Deutsche Forschungsgemeinschaft (O.P., T.F., G.L.) and the Fonds der Chemischen Industrie, Germany (G.L., E.M.), as well as by Sankyo Co. Ltd., Tokyo (S.L.).

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BARLOW, R.B. & WESTON-SMITH, P. (1985). The relative potencies of some agonists at M2 muscarinic receptors in guinea-pig ileum, atria and bronchi. *Br. J. Pharmacol.*, **85**, 437–440.

BRANN, M.R., ELLIS, J., JORGENSEN, H., HILL-EUBANKS, D. & JONES, S.V.P. (1993). Muscarinic acetylcholine receptor subtypes: localization and structure/function. *Prog. Brain Res.*, **98**, 121–127.

CAULFIELD, M.P. (1993). Muscarinic receptors–characterization, coupling and function. *Pharmacol. Ther.*, **58**, 319–379.

CHOO, L.K. & MITCHELSON, F. (1990). Selective inhibition of responses to carbachol and McN-A-343 in the rabbit vas deferens. *Clin. Exp. Pharmacol. Physiol.*, **17**, 601–611.

DÖRJE, F., LEVEY, A.I. & BRANN, M.R. (1991a). Immunological detection of muscarinic receptor subtype proteins (m1–m5) in rabbit peripheral tissues. *Mol. Pharmacol.*, **40**, 459–462.

DÖRJE, F., WESS, J., LAMBRECHT, G., TACKE, R., MUTSCHLER, E. & BRANN, M.R. (1991b). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.*, **256**, 727–733.

ELTZE, M. (1988). Muscarinic M1- and M2-receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur. J. Pharmacol.*, **158**, 11–19.

ELTZE, M. (1994). Pathways involved in muscarinic M_1 and M_2 receptor stimulation in rabbit vas deferens. *Eur. J. Pharmacol.*, **263**, 31–37.

ELTZE, M., GMELIN, G., WESS, J., STROHMANN, C., TACKE, R., MUTSCHLER, E. & LAMBRECHT, G. (1988). Presynaptic muscarinic receptors mediating inhibition of neurogenic contractions in rabbit vas deferens are of the ganglionic M1-type. *Eur. J. Pharmacol.*, **158**, 233–242.

ELTZE, M., ULLRICH, B., MUTSCHLER, E., MOSER, U., BUNGARDT, E., FRIEBE, T., GUBITZ, C., TACKE, R. & LAMBRECHT, G. (1993). Characterization of muscarinic receptors mediating vasodilation in rat perfused kidney. *Eur. J. Pharmacol.*, **238**, 343–355.

FEIFEL, R., RODRIGUES DE MIRANDA, J.F., STROHMANN, C., TACKE, R., AASEN, A.J., MUTSCHLER, E. & LAMBRECHT, G. (1991). Selective labelling of muscarinic M1 receptors in calf superior cervical ganglia by (3H)(+)-telenzepine. *Eur. J. Pharmacol.*, **195**, 115–123.

FEIFEL, R., WAGNER-RÖDER, M., STROHMANN, C., TACKE, R., WAELBROECK, M., CHRISTOPHE, J., MUTSCHLER, E. & LAMBRECHT, G. (1990). Stereoselective inhibition of muscarinic receptor subtypes by the enantiomers of hexahydro-difenidol and acetylenic analogues. *Br. J. Pharmacol.*, **99**, 455–460.

GRIMM, U., FUDER, H., MOSER, U., BÄUMERT, H.G., MUTSCHLER, E. & LAMBRECHT, G. (1994). Characterization of the prejunctional muscarinic receptors mediating inhibition of evoked release of endogenous noradrenaline in rabbit isolated vas deferens. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **349**, 1–10.

GROSS, J., WAELBROECK, M., MUTSCHLER, E. & LAMBRECHT, G. (1995). Functional characterization of neuronal muscarinic receptors in rabbit anococcygeus muscle. *Life Sci.*, **56**, 1038.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.

LAMBRECHT, G., FEIFEL, R., MOSER, U., AASEN, A.J., WAELBROECK, M., CHRISTOPHE, J. & MUTSCHLER, E. (1988). Stereoselectivity of the enantiomers of trihexyphenidyl and its methiodide at muscarinic receptor subtypes. *Eur. J. Pharmacol.*, **155**, 167–170.

LAMBRECHT, G., FEIFEL, R. & MUTSCHLER, E. (1989a). Stereoselectivity at muscarinic receptor subtypes: Observations with the enantiomers of phenglutarimide. *Chirality*, **1**, 170–173.

LAMBRECHT, G., FEIFEL, R., WAGNER-RÖDER, M., STROHMANN, C., ZILCH, H., TACKE, R., WAELBROECK, M., CHRISTOPHE, J., BODDEKE, H. & MUTSCHLER, E. (1989b). Affinity profiles of hexahydro-sila-difenidol analogues at muscarinic receptor subtypes. *Eur. J. Pharmacol.*, **168**, 71–80.

LAMBRECHT, G., MOSER, U., GRIMM, U., PFAFF, O., HERMANNI, U., HILDEBRANDT, C., WAELBROECK, M., CHRISTOPHE, J. & MUTSCHLER, E. (1993). New functionally selective muscarinic agonists. *Life Sci.*, **52**, 481–488.

LAZARENO, S. & BIRDSALL, N.J.M. (1995). Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: Interactions of strychnine and acetylcholine at muscarinic receptors. *Mol. Pharmacol.*, **48**, 362–378.

LAZARENO, S., BUCKLEY, N.J. & ROBERTS, F.F. (1990). Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG 108-15 cells. *Mol. Pharmacol.*, **38**, 805–815.

LEHMANN, F.P.A. (1986). Stereoisomerism and drug action. *Trends Pharmacol. Sci.*, **7**, 281–285.

LEVINE, R.R. & BIRDSALL, N.J.M. (eds.) (1993). Subtypes of muscarinic receptors V. *Life Sci.*, **52**, 405–597.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MICHELETTI, R., GIUDICI, L., TURCONI, M. & DONETTI, A. (1990). 4-DAMP analogues reveal heterogeneity of M_1 muscarinic receptors. *Br. J. Pharmacol.*, **100**, 395–397.

MITCHELSON, F. (1988). Muscarinic receptor differentiation. *Pharmacol. Ther.*, **37**, 357–423.

MUTSCHLER, E. & HULTZSCH, K. (1973). Über Struktur-Wirkungs-Beziehungen von ungesättigten Estern des Arecaidins und Dihydroarecaidins. *Arzneim.-Forsch.*, **23**, 732–737.

MUTSCHLER, E. & LAMBRECHT, G. (1984). Selective muscarinic agonists and antagonists in functional tests. *Trends Pharmacol. Sci.*, Suppl., **5**, 39–44.

NELSON, W.L. FREEMAN, D.S. & VINCENZI, F.F. (1976). Stereochemical analogs of a muscarinic, ganglionic stimulant. 2. Cis and trans olefinic, epoxide, and cyclopropane analogs related to 4-[N-(3-chlorophenyl)carbamoyloxy]-2-butynyltrimethylammonium chloride (McN-A-343). *J. Med. Chem.* **19**, 153–158.

PATON, W.D.M. & ZAR, M.A. (1968). The origin of acetylcholine release from guinea-pig intestine and longitudinal muscle strips. *J. Physiol.*, **194**, 13–33.

PFAFF, O., FRIEBE, T.P., WAELBROECK, M., CHRISTOPHE, J., CAMUS, J., MUTSCHLER, E. & LAMBRECHT, G. (1993). Stereoselective interaction of phenglutarimide and of related antagonists, with four muscarinic receptor suybtypes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **347**, (Suppl.), R116.

PFEIFFER, C.C. (1956). Optical isomerism and pharmacological action, a generalization. *Science*, **124**, 29–31.

ROBERT, T.A., HAGARDORN, A.N. & DAIGNEAULT, E.A. (1982). Differential stereoselectivity of methotrimepazine enantiomers for selected central nervous system receptor types. *Mol. Pharmacol.*, **21**, 315–319.

SAGRADA, A., DURANTI, P., GIUDICI, L. & SCHIAVONE, A. (1993). Himbacine discriminates between two M_1 receptor-mediated responses. *Life Sci.*, **52**, 574.

TALLARIDA, R.J., COWAN, A. & ADLER, M.W. (1979). pA2 and receptor differentiation: a statistical analysis of competitive antagonism. *Life Sci.*, **25**, 637–654.

VAN ROSSUM, J.M. (1963). Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn.*, **143**, 299–330.

WAELBROECK, M. (1994). Identification of drugs competing with d-tubocurarine for an allosteric site on cardiac muscarinic receptors. *Mol. Pharmacol.*, **46**, 685–692.

WAELBROECK, M., CAMUS, J., TASTENOY, M. & CHRISTOPHE, J. (1988). 80% of muscarinic receptors expressed by the NB-OK 1 human neuroblastoma cell line show high affinity for pirenzepine and are comparable to rat hippocampus M1 receptors. *FEBS Lett.*, **226**, 287–290.

WAELBROECK, M., CAMUS, J., TASTENOY, M., LAMBRECHT, G., MUTSCHLER, E., TACKE, R. & CHRISTOPHE, J. (1990a). Stereoselectivity of procyclidine binding to muscarinic receptor subtypes M_1, M_2 and M_4. *Eur. J. Pharmacol.*, **189**, 135–142.

WAELBROECK, M., CAMUS, J., TASTENOY, M., MUTSCHLER, E., STROHMANN, C., TACKE, R., LAMBRECHT, G. & CHRISTOPHE, J. (1991). Stereoselectivity of (R)- and (S)-hexahydro-difenidol binding to neuroblastoma M_1, cardiac M_2, pancreatic M_3, and striatum M_4 muscarinic receptors. *Chirality*, **3**, 118–123.

WAELBROECK, M., CAMUS, J., TASTENOY, M., MUTSCHLER, E., STROHMANN, C., TACKE, R., SCHJELDERUP, L., AASEN, A., LAMBRECHT, G. & CHRISTOPHE, J. (1992). Stereoselective interaction of procyclidine, hexahydro-difenidol, hexbutinol and oxyphencyclimine, and of related antagonists, with four muscarinic receptors. *Eur. J. Pharmacol.*, **227**, 33–42.

WAELBROECK, M., TASTENOY, M., CAMUS, J. & CHRISTOPHE, J. (1990b). Binding of selective antagonists to four muscarinic receptors (M1 to M4) in rat forebrain. *Mol. Pharmacol.*, **38**, 267–273.

WAELBROECK, M., TASTENOY, M., CAMUS, J., FEIFEL, R., MUTSCHLER, E., STROHMANN, C., TACKE, R., LAMBRECHT, G. & CHRISTOPHE, J. (1989). Stereoselectivity of the interaction of muscarinic antagonists with their receptors. *Trends Pharmacol. Sci.*, **10**, Suppl., 65–69.

(Received June 6, 1996
Revised August 19, 1996
Accepted August 21, 1996)