ELSEVIER

European Journal of Pharmacology 303 (1996) 221–226

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Stereoselective interaction of uncharged esters at four muscarinic receptor subtypes

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Received 16 September 1995; revised 4 January 1996; accepted 9 January 1996

Abstract

We investigated the binding and pharmacological properties of the esters of 3,3-dimethylbutan-1-ol (the carbon analogue of choline) with either diphenylglycolic acid, (*R*)-phenylcyclohexylglycolic acid, or (*S*)-phenylcyclohexylglycolic acid [BS-6181, (R)-BS-7826 and (S)-BS-7826, respectively] at muscarinic M_1, M_2, M_3 (Hm3) and M_4 receptors. The three uncharged compounds were muscarinic receptor antagonists, with pA_2 or pK_i values between 7.9 and 5.6. The achiral ester BS-6181 displayed highest affinity for M_1, M_3 (Hm3) and M_4 receptors (pA_2 or $pK_i = 7.2–7.6$) and lower affinity for M_2 receptors (pA_2 or $pK_i = 6.7$ and 6.8). The four muscarinic receptor subtypes were able to distinguish between the two enantiomers of the cyclohexyl derivative of BS-6181 [(R)- and (S)-BS-7826], with a preference for the (*R*)-isomer (up to 79-fold). Interestingly, the (*S*)-enantiomer of BS-7826, being the distomer, was found to be M_4 selective ($pK_i/M_4 = 6.9$; pA_2 or pK_i/M_1-M_3 (Hm3) = 5.6–6.2). These results indicate that uncharged compounds may (stereo)selectively bind to muscarinic receptors via hydrophobic interactions. Thus, an ionic bond between muscarinic ligands and an anionic site of the receptor is not absolutely necessary for recognition of muscarinic receptors.

Keywords: Muscarinic receptor subtype; Muscarinic receptor antagonist; Carbon analog, uncharged; Stereoselectivity, at muscarinic receptor; BS-6181; BS-7826, (*R*)- and (*S*)-enantiomer

1. Introduction

Five muscarinic receptor genes, m1-m5, have been identified in mammalian tissues (Bonner, 1989; Hulme et al., 1990; Caulfield, 1993). Functional and binding experiments allowed the detection of four subtypes, termed M_1-M_4. Comparison of the radioligand binding properties of the native M_1-M_4 receptors with those of the expressed m1-m4 proteins showed a good correlation (Hulme et al., 1990; Caulfield, 1993).

The natural ligand for muscarinic receptors, acetylcholine, is a quaternary ammonium derivative. It was therefore expected that an ionic bond between acetylcholine and an anionic site of the receptor would be very important, if not essential, for binding. An aspartate residue, which is important for drug binding, has indeed been identified in the muscarinic receptor protein (Bonner, 1989; Hulme et al., 1990, 1995; Caulfield, 1993; Spencer et al., 1995) by site-directed mutagenesis (Fraser et al., 1989) as well as by biochemical methods (Spalding et al., 1994). However, 3,3-dimethylbutyl acetate (an uncharged compound isosteric with acetylcholine) is a partial agonist on ileum smooth muscle (Banister and Whittaker, 1951; Barlow and Tubby, 1974) and several isosteric uncharged analogues of 'classical' muscarinic receptor antagonists behave as competitive antagonists in guinea-pig ileum, with only 10- to 1000-fold lower affinities than the charged compounds (Barlow and Tubby, 1974; Barlow et al., 1992). To test the hypothesis that the uncharged compounds can indeed recognize the muscarinic binding site (as opposed to non-specific, 'detergent' effects of these very hydrophobic molecules), we investigated the binding and pharmaco-

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PII S0014-2999(96)00038-6

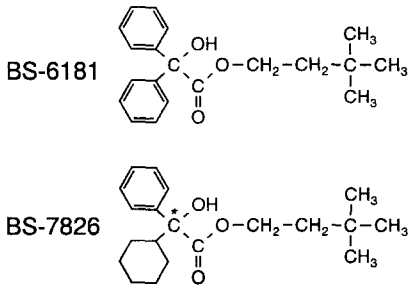

Fig. 1. Chemical structures of BS-6181 and BS-7826. The asterisk denotes the centre of chirality.

logical properties of the achiral ester of 3,3-dimethylbutan-1-ol (the carbon analogue of choline) with diphenylglycolic acid [BS-6181 (Fig. 1)], an uncharged muscarinic receptor antagonist with a pA_2 value in guinea-pig ileum of 7.531 (Barlow and Tubby, 1974), and the chiral esters with (*R*)- and (*S*)-cyclohexylphenylglycolic acid [(R)- and (S)-BS-7826].

The investigation of the enantiomers of BS-7826 was of special importance, since data have accumulated that the antagonist (and agonist) binding sites of muscarinic receptors are asymmetrical, and hence generally capable of distinguishing between optical isomers of chiral ligands (Waelbroeck et al., 1989). Thus, analyses of the binding and pharmacological properties of stereoisomers of chiral muscarinic receptor antagonists can provide information about the binding site geometry that achiral compounds cannot give.

The structurally related charged esters of choline with diphenylglycolic acid (pA_2 value = 8.51; Barlow and Tubby, 1974) and with (*R*)- and (*S*)-cyclohexylphenylglycolic acid [pA_2 values (*R*)/(*S*) = 9.66/7.38; Brimblecombe et al., 1971] were found to be potent muscarinic receptor antagonists at M_3 receptors in guinea-pig ileum.

In this work, we analysed the binding properties of the uncharged compounds BS-6181, (R)-BS-7826 and (S)-BS-7826 at [^{3}H]*N*-methylscopolamine-labelled M_1 receptors in NB-OK-1 cells, M_2 receptors in rat heart, recombinant human m3 receptors expressed in CHO-K1 cells and M_4 receptors in rat striatum (Waelbroeck et al., 1994). The affinity of the compounds for rat pancreas M_3 receptors (the 'classical' M_3 assay used in our laboratory) could not be determined for technical reasons. The very strong binding of the three carbon analogues to bovine serum albumin, which is necessary for binding studies in this tissue, prevents recognition of muscarinic receptors. The binding affinity profiles of BS-6181, (R)-BS-7826 and (S)-BS-7826 were compared with the pharmacological properties of the compounds at prejunctional M_1-like receptors in rabbit vas deferens (Eltze, 1994; Waelbroeck et al., 1994), M_2 receptors in guinea-pig atria and M_3 receptors in guinea-pig ileum (Caulfield, 1993).

2. Materials and methods

2.1. Receptor binding studies

Binding at native M_1, M_2 and M_4 receptors was measured as previously described (Waelbroeck et al., 1994). Briefly, [^{3}H]*N*-methylscopolamine binding to homogenates from NB-OK-1 neuroblastoma cells (M_1) and rat heart (M_2) was measured after 2 h of incubation at 25°C in a 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$ by a filtration method. Under the same conditions, [^{3}H]*N*-methylscopolamine labels mainly M_1 and M_4 receptors in rat striatum homogenates. If atropine (1 μM) is added before filtration, the tracer dissociates faster from the M_1 sites than from the M_4 sites. After 35 min, most (> 85%) of the residual tracer labels M_4 receptors (Waelbroeck et al., 1990). CHO cells expressing recombinant Hm3 receptors (a generous gift from Dr N. Buckley, London, UK) were maintained in Dulbecco's Modified Essential Medium enriched with 10% foetal calf serum and 200 μg/ml genetecin (G418). For binding studies, the cells were harvested, homogenized in a 20 mM Tris-HCl buffer (pH 7.5) enriched with 250 mM sucrose, and frozen in liquid nitrogen. The homogenates were incubated 4 h at 25°C in the same sodium phosphate $MgCl_2$ buffer as for the M_1, M_2 and M_4 receptor binding assays and in the presence of 0.2 nM [^{3}H]*N*-methylscopolamine. Binding was measured by filtration.

The competition curves (Fig. 2 and results not shown) were analysed by non-linear curve-fitting, and the pK_i values calculated according to Cheng and Prusoff (1973), using the previously determined [^{3}H]*N*-methylscopolamine K_d values. All pK_i values and Hill coefficients are presented as means ± S.E.M. of three experiments.

2.2. Pharmacological studies

Functional pharmacological experiments were performed as previously described (Waelbroeck et al., 1994). Briefly, experiments were performed with segments of vasa deferentia from male New Zealand white rabbits and with left atria as well as with strips of ileal longitudinal muscle from guinea-pigs. Isolated tissues were suspended in a modified Krebs buffer (vasa deferentia; 31°C; 750 mg basal tension; Eltze, 1994; Waelbroeck et al., 1994) or in Tyrode solution (atria and ileum; 32°C; 500 mg basal tension). The medium was aerated with 95% O_2-5% CO_2. A 30-min equilibration period was followed by a reduction in contractility of paced atria (2 Hz, 3 ms duration, 10 V) or contraction of ileal smooth muscle induced by cumulative addition of arecaidine propargyl ester. Twitch contractions in vasa deferentia were elicited by electrical field stimulation (0.05 Hz, 0.5 ms, 30 V). These effects were concentration dependently inhibited by cumulative addition of the M_1 receptor agonists 4-(4-chlorophenyl-

carbamoyloxy)-2-butynyl trimethylammonium iodide (4-Cl-McN-A-343) and 4-(4-fluorophenylcarbamoyloxy)-2-butynyl-*N*-methyl-pyrrolidinium tosylate (4-F-PyMcN$^+$) (Lambrecht et al., 1993).

Antimuscarinic potencies (pA_2 values) were determined by obtaining concentration-response curves for the agonists in the absence and in the presence of antagonists. Each concentration of antagonist was tested 3–6 times (see Table 1 and Fig. 2) in the three tissues. The antagonists were allowed to equilibrate for 15–30 min (ileum) and 30 min (atrium) in guinea-pig preparations and for 30–60 min in rabbit vas deferens. Preliminary experiments indicated that these intervals were sufficient for equilibration of the antagonist concentrations used. pA_2 values were estimated by Schild analysis (Arunlakshana and Schild, 1959) or from individual dose ratios (Tallarida et al., 1979). When the slopes of the Arunlakshana and Schild plots were not significantly different from unity, pA_2 values were calculated as the intercept of the abscissa scale by fitting the data to the best straight line with a slope of unity (Tallarida et al., 1979). Linear regression analyses were carried out by the method of least squares and the significance of a deviation from linearity was quantified by analysis of variance (Kenakin, 1987). $P < 0.05$ was accepted as being significant. All data are presented as means ± S.E.M., and the total number of data points (n) is given in Table 1 and in the legend of Fig. 3.

2.3. Drugs

Arecaidine propargyl ester (Mutschler and Hultzsch, 1973), 4-Cl-McN-A-343 (Nelson et al., 1976), 4-F-PyMcN$^+$ (Lambrecht et al., 1993) and BS-6181 (Funcke et al., 1959) were synthesized in our laboratories according to methods described in the literature. [^{3}H]*N*-Methylscopolamine methyl chloride, 80–85 Ci/mmol, was obtained from Amersham International (Bucks, UK). All other chemicals were of reagent grade and used as purchased.

The enantiomers of cyclohexyl derivatives of BS-6181, (R)- and (S)-BS-7826 were prepared by alkylation of the potassium salt of (*R*)-cyclohexylphenylglycolic acid and (*S*)-cyclohexylphenylglycolic acid (Schjelderup and Aasen, 1986) with 1-iodo-3,3-dimethylbutane in dimethyl formamide at 40°C and distilled under reduced pressure (160°C at 0.1 mm Hg). (R)-BS-7826: $[\alpha]_D^{20} = +1.2$ (c = 1.0, ethanol); (S)-BS-7826: $[\alpha]_D^{20} = -1.3$ (c = 1.3, ethanol).

Stock solutions (0.1–1.0 mM) of BS-6181, (R)-BS-7826 and (S)-BS-7826 were prepared in ethanol or dimethylsulfoxide and further diluted with water. In all assays, it was verified in control experiments that the final concentrations of the solvents (≤ 1% ethanol or dimethylsulfoxide) had no effect per se on the binding or pharmacological parameters.

3. Results

BS-6181 and the (*R*)- and (*S*)-enantiomers of BS-7826 concentration dependently inhibited tracer binding to muscarinic M_1, M_2, Hm3 and M_4 receptors. The competition curves (Fig. 2) did not deviate significantly from the results expected for competitive inhibition of [^{3}H]*N*-methylscopolamine binding to a single receptor (Hill coefficients not significantly different from unity; concentration-dependent parallel shift to the right with increasing tracer concentrations). The pK_i values obtained with the three carbocholine esters are summarized in Table 1.

BS-6181, (R)-BS-7826 and (S)-BS-7826 (up to 30 μM) did not elicit an agonist response themselves (data not shown), but all three compounds antagonized the inhibition of neurogenic contractions of rabbit vas deferens elicited by 4-Cl-McN-A-343 ($-\log EC_{50} = 6.44 \pm 0.05$, $n = 20$; BS-6181 as antagonist) or 4-F-PyMcN$^+$ [$-\log EC_{50} = 6.24 \pm 0.07$, $n = 19$; (R)- and (S)-BS-7826 as antagonists] as well as the negative inotropic effects in paced guinea-pig atria and the contractions of the guinea-pig ileum elicited by arecaidine propargyl ester ($-\log EC_{50} = 7.98 \pm 0.03$, $n = 35$ and 7.35 ± 0.06, $n = 42$, respectively). This is shown for BS-6181 in Fig. 3. For BS-6181 and (R)-BS-7826, there was a concentration-dependent parallel shift to the right of agonist concentration-response curves without either the basal tension or the maximal responses being affected, and the Schild plots were linear ($P > 0.05$, analysis of variance with test for linearity) throughout the antagonist concentration range studied (Fig. 3). Furthermore, the slopes of the dose-response curves for rabbit vas deferens and guinea-pig ileum were not significantly dif-

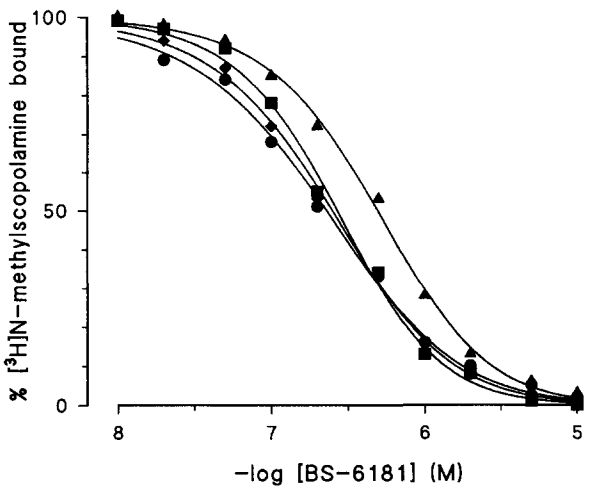

Fig. 2. [^{3}H]*N*-Methylscopolamine BS-6181 competition curves, obtained at muscarinic M_1 (■; NB-OK-1 cells), M_2 (▲; rat heart), Hm3 (●) and M_4 receptors (◆; rat striatum). [^{3}H]*N*-Methylscopolamine binding was measured in the absence and presence of BS-6181, as described in Materials and methods. The data points represent means of three experiments performed in duplicate.

ferent from unity ($P > 0.05$). Thus, BS-6181 and (R)-BS-7826 behaved as normal competitive antagonists at M_1 and M_3 receptors (Table 1). However, at M_2 receptors in guinea-pig atria, the slopes of the Schild plots for BS-6181 and (R)-BS-7826 were significantly different from unity ($P < 0.05$; Table 1). Thus, the pA_2 values of these two compounds in guinea-pig atria (6.67 and 6.74, respectively) might be regarded as purely experimental quantities. The weaker (*S*)-enantiomer of BS-7826 exhibited unspecific effects at higher concentrations in rabbit vas deferens (> 3.0 μM) as well as in guinea-pig ileum (> 10.0 μM), and it showed no antagonism (up to 30 μM) in guinea-pig atria (Table 1). In this respect, (S)-BS-7826 is not the only compound for which the binding affinity at M_2 receptors in rat heart ($pK_i = 5.9$) does not correspond with its affinity measured in the functional assay at M_2 receptors in guinea-pig atria ($pA_2 < 4.5$). This discrepancy has also been observed for *N*-iminomethyl-*N'*-[(2-hydroxy-2-phenyl-2-cyclohexyl)-ethyl]piperazine (DAC 5945; $pK_i = 7.52$, $pA_2 = 6.42$) (Doods et al., 1993).

The achiral ester BS-6181, being nearly equipotent with (R)-BS-7826 at M_1, M_3 (Hm3)-M_4 receptors, displayed highest affinity for M_1, M_3 (Hm3) and M_4 receptors (pA_2 or $pK_i = 7.2–7.6$) and lower affinity for M_2 receptors (pA_2 or $pK_i = 6.7$ and 6.8). The four muscarinic receptor subtypes were able to distinguish between the two enantiomers of BS-7826, with a an up to 79-fold preference for the (*R*)-isomer (the eutomer) (Table 1). Interestingly, the (*S*)-enantiomer of BS-7826 (the distomer) was found to be an M_4-preferring muscarinic receptor antagonist ($pK_i/M_4 = 6.9$; pA_2 or pK_i/M_1-M_3 (Hm3) = 5.6–6.2).

4. Discussion

Coulombic forces between charged groups in the ligand and in the receptors are assumed to make a major contribution to drug-receptor interactions (Albert, 1951; Andrews, 1986). A positive charge is therefore thought to be one of the essential structural features of ligands that interact with

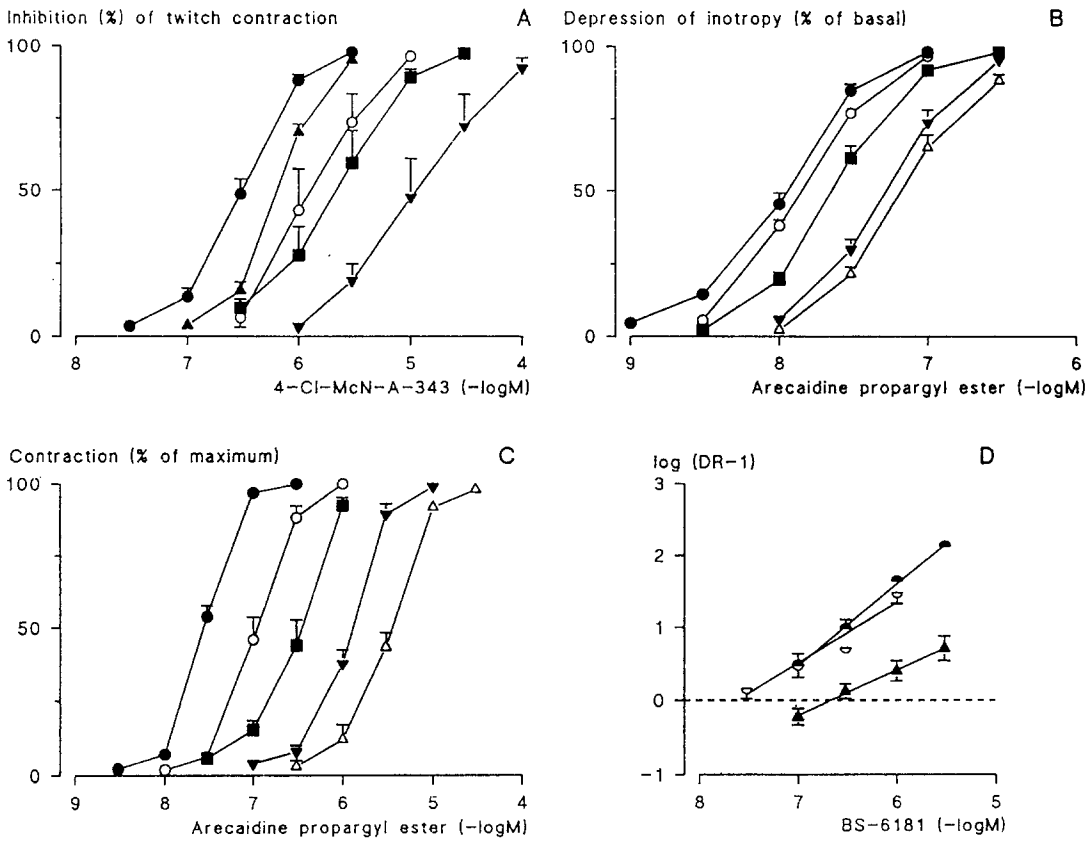

Fig. 3. Antagonism of responses to 4-Cl-McN-A-343 ($-\log EC_{50} = 6.44 \pm 0.05$; $n = 20$) in rabbit vas deferens and to arecaidine propargyl ester in guinea-pig paced left atria ($-\log EC_{50} = 7.71 \pm 0.13$; $n = 19$) and ileum ($-\log EC_{50} = 7.53 \pm 0.04$; $n = 18$) by different concentrations of BS-6181. Data are means ± S.E.M. Errors bars falling within the area covered by a 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 (●, $n = 20$) and presence ($n = 5$) of 0.03 (▲), 0.1 (○), 0.3 (■) and 1.0 (▲) μM BS-6181. (B) Concentration-response curves for arecaidine propargyl ester-induced negative inotropy in guinea-pig atria in the absence (●; $n = 19$) and presence of 0.1 (○; $n = 4$) 0.3 (■; $n = 5$), 1.0 (▲; $n = 5$) and 3.0 (△; $n = 5$) μM BS-6181. (C) Concentration-response curves for arecaidine propargyl ester-induced contractions in guinea-pig isolated ileum longitudinal smooth muscle in the absence (●; $n = 18$) and presence of 0.1 (○; $n = 3$), 0.3 (■; $n = 4$) 1.0 (▲; $n = 6$) and 3.0 (△; $n = 5$) μM BS-6181. (D) Schild regression plots for data from experiments in rabbit vas deferens (○), guinea-pig atria (▲) and guinea-pig ileum (●).

Table 1
Binding affinities (pK_i and Hill coefficients, n_H values [a]) and antimuscarinic potencies (pA_2 values [b]) of BS-6181, (R)-BS-7826 and (S)-BS-7826

(A)	pK_i			
	M_1	M_2	(Hm3)	M_4
BS-6181	7.15±0.08	6.78±0.07	7.20±0.15	7.31±0.06
	(1.00±0.06)	(1.00±0.09)	(1.00±0.10)	(0.92±0.08)
(R)-BS-7826	7.20±0.07	6.91±0.06	7.70±0.16	7.31±0.06
	(0.95±0.07)	(0.95±0.06)	(1.05±0.09)	(0.92±0.08)
(S)-BS-7826	6.23±0.10	5.88±0.10	6.07±0.18	6.92±0.08
	(1.05±0.10)	(1.05±0.10)	(1.15±0.10)	(1.10±0.12)

(B)	pA_2		
	M_1	M_2	M_3
BS-6181	7.47±0.07	6.67±0.13	7.55±0.06
	(0.84±0.13) [c]	(0.62±0.12) [d]	(1.13±0.11) [c]
(R)-BS-7826	7.55±0.18 [e]	6.74±0.66 [f]	7.46±0.10 [g]
	(1.09±0.35) [c]	(0.48±0.25) [d]	(0.84±0.17) [c]
(S)-BS-7826	5.91±0.12 [h]	– [i]	5.56±0.10 [k]

(A) pK_i values for muscarinic receptors in NB-OK-1 cells (M_1), rat heart (M_2), recombinant Hm3 and rat striatum (M_4). (B) pA_2 values for the muscarinic receptors present in rabbit vas deferens (M_1-like) and guinea-pig atria (M_2) and ileum (M_3). [a] pK_i values and Hill coefficients (in parentheses) are given as means±S.E.M. Hill coefficients were not significantly different from unity. [b] pA_2 values and slopes of Schild plots (in parentheses) are presented as means±S.E.M. (for actual numbers of data points for BS-6181, see legend of Fig. 1; these numbers for (R)- and (S)-BS-7826 are given below). [c] Not significantly different from unity: $P > 0.05$. [d] Significantly different from unity: $P < 0.05$. [e] Tested concentrations: 0.03–3.0 μM; log interval = 0.48; $n = 15$. [f] Tested concentrations: 0.03–10 μM; log interval = 0.48; $n = 13$. [g] Tested concentrations: 0.03–1.0 μM; log interval = 0.48; $n = 12$. [h,k] Only one or two concentrations of (S)-BS-7826 were investigated due to unspecific effects of the antagonist itself at higher concentrations: vas deferens = 3.0 μM ($n = 6$); ileum = 3.0 ($n = 4$) and 10.0 ($n = 3$) μM. The pA_2 values were therefore determined from the individual dose ratios (Tallarida et al., 1979). [i] No antagonism (up to 30 μM).

muscarinic receptors. The aziridinium ion of propylbenzilylcholine mustard indeed covalently labels an aspartate residue of muscarinic receptors (Spalding et al., 1994).

In this context, the results of Funcke et al. (1959) and Barlow and Tubby (1974), demonstrating that BS-6181 is a relatively potent muscarinic receptor antagonist in guinea-pig ileum, are quite surprising since this compound is uncharged. In the present study we chose to compare the binding affinities and antimuscarinic potencies of the esters of 3,3-dimethylbutan-1-ol (the carbon analogue of choline) with the achiral diphenylglycolic acid (BS-6181) and with (*R*)- and (*S*)-cyclohexylphenylglycolic acid [(R)- and (S)-BS-7826]. We assumed that if these compounds altered non-specifically the muscarinic receptors, their interactions would be non-stereoselective.

In binding studies, the three compounds behaved as competitive inhibitors of [^{3}H]*N*-methylscopolamine binding to M_1, M_2, Hm3 and M_4 receptors. The four muscarinic receptor subtypes were capable of distinguishing between the (R)- and (S)-enantiomers of BS-7826. As for the chiral choline esters (Brimblecombe et al., 1971), the (*R*)-enantiomers had a greater affinity than the (*S*)-enantiomers for muscarinic receptors. All these results support the hypothesis that the carbon compounds BS-6181, (R)-BS-7826 and (S)-BS-7826 do interact with the muscarinic binding site of M_1 to M_4 receptors. Interestingly, the (*S*)-enantiomer of BS-7826, although being the distomer, was found to be an M_4-preferring muscarinic receptor antagonist.

The utility of these compounds for the functional study of muscarinic receptors is, however, limited. Indeed, in one of the three assay systems studied (guinea-pig atria), the three compounds did not behave as competitive antagonists. This result suggests that, in addition to muscarinic receptors, these compounds might be able to recognize other (unidentified) targets in this tissue.

The charged esters of choline with diphenylglycolic acid, (*R*)-, and (*S*)-cyclohexylphenylglycolic acid, had pA_2 values in guinea-pig ileum of 8.51 (Barlow and Tubby, 1974), 9.66 and 7.38 (Brimblecombe et al., 1971), respectively. These values are only 1–2.2 log units higher than the pA_2 values of the corresponding carbo-analogues (Table 1). This corresponds to differences in the binding free energy of only 6–13 kJ/mol. This is quite similar to the difference in the binding free energy of 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) and its carbo-analogue at M_3 receptors in guinea-pig ileum (Barlow et al., 1992).

Other methods have also been used to evaluate the importance of an ionic bond for drug binding to muscarinic receptors. One method is to study the effect of the pH on the activity (affinity) of compounds which are bases and may lose their positive charge in the pH range tested (Burgen, 1965; Asselin et al., 1983; Ehlert and Delen, 1990; Barlow et al., 1992). Another method is to compare the affinity (activity) of muscarinic receptor agonists and/or antagonists for wild-type receptors and for receptors in which the binding site aspartic acid is replaced by a non-ionic residue asparagine (Fraser et al., 1989; Hulme et al., 1995; Spencer et al., 1995).

In all these studies, the contribution of an ionic charge to binding is highly variable, but – as a rule – below 18 kJ/mol. This is much lower than the contribution expected for an ionic bond: according to Andrews (1986), charged pharmacophores contribute on average 34–38 kJ/mol (about 6 log units) to drug binding. The poor charge contribution to muscarinic binding suggests that the cationic head group of the muscarinic ligands does not come in very close contact with the aspartate residue of transmembrane region 3. If charged ligands and their carbo-analogues form ion-dipole and hydrophobic interactions, respectively, with aromatic amino acids surrounding the aspartate residue, this might explain the small difference between their affinities for the muscarinic receptor.

In conclusion, our results support the hypothesis that uncharged muscarinic ligands, such as BS-6181 and the enantiomers of BS-7826, are able to recognize (stereoselectively) the binding site of muscarinic receptor subtypes. Moreover, this suggests that formation of an ionic bond between the ligand and the receptor facilitates, but is not essential for, receptor recognition.

Acknowledgements

This work was supported by Grant 1.5.011.94 F from the F.N.R.S. (Brussels, Belgium), by the Deutsche Forschungsgemeinschaft (G.L.) and by the Fonds der Chemischen Industrie, Germany (G.L.).

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