European Journal of Pharmacology - Molecular Pharmacology Section, 189 (1990) 135-142
Elsevier

EJPMOL 90098

Stereoselectivity of procyclidine binding to muscarinic receptor subtypes M_1, M_2 and M_4

Magali Waelbroeck [1], Jean Camus [1], Michèle Tastenoy [1], Günter Lambrecht [2], Ernst Mutschler [2], Reinhold Tacke [3] and Jean Christophe [1]

[1] *Department of Biochemistry and Nutrition, Medical School, Universtité Libre de Bruxelles, Boulevard of Waterloo 115, B-1000 Brussels, Belgium,* [2] *Department of Pharmacology, University of Frankfurt/M, Theodor-Stern-Kai, 7, D-6000 Frankfurt/M, F.R.G.,* and [3] *Institute of Inorganic Chemistry, University of Karlsruhe, Engesserstrasse, Geb. 30.45, D-7500 Karlsruhe, F.R.G.*

Received 24 January 1990, revised MS received 18 April 1990, accepted 7 May 1990

The goals of the present study were: (1) to investigate the binding properties of (R)- and (S)-procyclidine and two achiral derivatives of muscarinic M_1, M_2 and M_4 receptor subtypes and (2) to identify the interactions which allow these receptors to discriminate between the two stereoisomers. (R)-Procyclidine showed a higher affinity for human neuroblastoma NB-OK 1 muscarinic M_1 and rat striatum muscarinic M_4 receptors, as compared to rat cardiac M_2 receptors. (S)-Procyclidine had a 130-fold lower affinity than (R)-procyclidine for M_1 and M_4 receptors, and a 40-fold lower affinity for M_2 receptors. Pyrrinol, the achiral diphenyl derivative with the cyclohexyl group of (S)-procyclidine replaced by a phenyl group, has an eight-fold lower affinity for M_1 and M_4 receptors, as compared to (R)-procyclidine, and a three-fold lower affinity for M_2 receptors. Hexahydro-procyclidine, the corresponding achiral dicyclohexyl compound, had a 10- to 20-fold lower affinity than (R)-procyclidine for the three receptors.

The increase in binding free energy, which is observed when the phenyl and cyclohexyl groups of procyclidine are separately replaced by cyclohexyl and phenyl groups, respectively, was additive in the case of M_1, M_2 and M_4 receptors. This indicates that the muscarinic receptor stereoselectivity was based on the coexistence of two binding sites, one preferring a phenyl rather than cyclohexyl group and the second preferring a cyclohexyl rather than a phenyl group. In addition, there were also binding sites for the hydroxy moiety and the protonated amino group of the ligands. The greater affinity and stereoselectivity of M_1 and M_4 muscarinic receptors for (R)-procyclidine reflected the better fit of the cyclohexyl group of (R)-procyclidine to the subsite of M_1 and M_4 as compared to M_2 receptors.

Muscarinic M_1 receptors; Muscarinic M_2 receptors; Muscarinic M_4 receptors; (S)-Procyclidine; (R)-Procyclidine; Pyrrinol; Hexahydro-procyclidine; Muscarinic receptors (stereoselectivity)

1. Introduction

At least four pharmacologically and biochemically distinct muscarinic receptors coexist in mammalian tissues (for review: see Mitchelson, 1988; Levine and Birdsall, 1989): (a) M_1 receptors, with a high affinity for pirenzepine, are typically found in neuronal tissues (Hammer et al., 1980). These receptors also have a high affinity for 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) and HHSiD (hexahydro-sila-difenidol) but a low affinity for AF-DX 116 ([11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido-(2,3-b) (1,4)-benzodiazepin-6-one) (Waelbroeck et al., 1987b; 1988; 1989); (b) M_2 receptors, with a high affinity for AF-DX 116 and a low affinity for pirenzepine are especially

Correspondence to: J. Christophe, Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, Boulevard of Waterloo 115, B-1000 Brussels, Belgium.

0922-4106/90/$03.50 © 1990 Elsevier Science Publishers B.V. (Biomedical Division)

present in cardiac tissue (Hammer et al., 1986). They also show a low affinity for 4-DAMP and HHSiD (Waelbroeck et al., 1987b; 1988; 1989); (c) M_3 receptors have high affinities for 4-DAMP (Barlow et al., 1976) and HHSiD (Mutschler and Lambrecht, 1984) and low affinities for pirenzepine and AF-DX 116. They are typically detected in secretory glands and smooth muscle (Waelbroeck et al., 1987a; Korc et al., 1987); (d) M_4 receptors are typically found in NG 108-15 cells (Michel et al., 1989) and rat striatum (Waelbroeck et al., 1990). They have low affinities for pirenzepine and AF-DX 116 but high affinities for methoctramine and HHSiD.

We previously demonstrated that receptors labeled by [^{3}H]-N-methylscopolamine ([^{3}H]NMS) in NB-OK 1 cells (a human neuroblastoma cell line), rat heart, and rat striatum (those receptors showing slow [^{3}H]NMS dissociation) display M_1, M_2 and M_4 selectivities, respectively (Waelbroeck et al., 1986; 1987a,b; 1988; 1989; 1990). We decided to compare these three systems to analyze the structure-affinity/selectivity relationships of muscarinic antagonists related to procyclidine.

A majority of previous studies comparing the binding or functional properties of chiral muscarinic antagonists and agonists used the drugs as racemates. While this is sometimes unavoidable (for example if the drug racemizes quickly in solution), there are important drawbacks in utilizing a racemate rather than the individual enantiomers (see for example: Lambrecht and Mutschler, 1986; Lambrecht et al., 1988; Tacke et al., 1986; 1987; 1989; and the Series on Chirality (published in Trends Pharmacol. Sci. 7, 1986, 20-24, 60-65, 112-115, 155-158, 200-205, 227-230, 281-301). Receptors are indeed asymmetrical macromolecules. When studying the binding or functional properties of a racemic mixture of compounds, the information bears at best on the eutomer (high-affinity enantiomer) but the properties are in some cases affected by the presence of the distomer (low-affinity enantiomer). If the absolute configuration of the eutomer is not known, it is, for example, impossible to map the relative positions of receptor 'subsites' recognizing the protonated amino group and the hydroxyl group of antimuscarinics of the procyclidine type family.

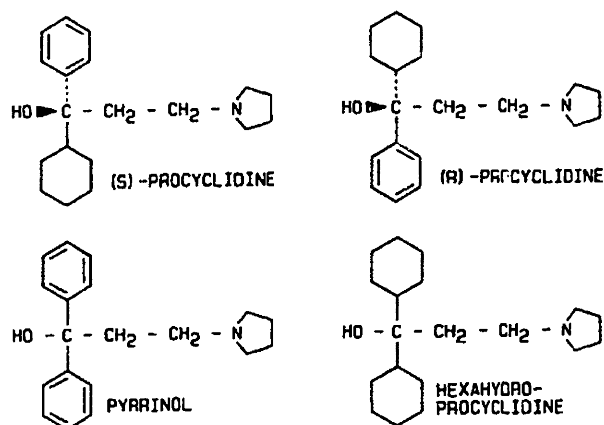

Fig. 1. Chemical structure of (S)-procyclidine, (R)-procyclidine, pyrrinol and hexahydro-procyclidine. In the case of (S)- and (R)-procyclidine, the carbinol carbon atom is a center of chirality.

The first aim of the present study was to compare the binding properties of (R)- and (S)-procyclidine to the three reasonably pure muscarinic receptor systems at hand. The affinity and stereoselectivity of M_1, M_2 and M_4 receptors for procyclidine enantiomers proved to be different in our binding experiments. In order to identify the interactions responsible for muscarinic receptor stereoselectivity, we extended the binding analysis to two achiral compounds structurally related to (R)- and (S)-procyclidine: pyrrinol (the diphenyl derivative) and hexahydro-procyclidine (the dicyclohexyl derivative). The structures of these compounds are shown in fig. 1.

2. Materials and methods

2.1. Human NB-OK 1 neuroblastoma cells

The NB-OK 1 cells were cultured as previously described (Waelbroeck et al., 1988) in RPMI-1640 medium enriched with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. For 1-[N-methyl-^{3}H]scopolomine methyl chloride ([^{3}H]NMS) binding experiments, the cells were rinsed, detached 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 until use.

2.2. Rat tissue homogenate preparations

Male Wistar albino rats (200-250 g) were decapited and the heart and striatum immediately removed. All following operations were performed at 4°C.

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, seven up and down strokes with a glass-Teflon homogenizer and filtration on two layers of medical gauze. The resulting homogenate was used immediately or stored in liquid nitrogen until use.

The striatum was homogenized in 2 ml of 20 mM Tris/HCl buffer (pH 7.5) enriched with 250 mM sucrose, using a glass-Teflon homogenizer (seven up and down strokes). The resulting homogenate was stored in liquid nitrogen until use and diluted 20-fold with the same buffer immediately before the experiment.

The protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin as standard.

2.3. Binding studies

All binding studies were performed at 25°C, at equilibrium, in a 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$, [^{3}H]NMS, and the indicated unlabeled drug concentrations, in a total volume of 1.2 ml.

To measure [^{3}H]NMS binding to human NB-OK 1 cell homogenates, we used 80 μl of homogenate, corresponding to about 200 μg protein per assay. The incubation period was 2 h in the presence of 0.25 nM [^{3}H]NMS (this concentration was equivalent to two-fold the tracers' K_D value to M_1 receptors).

For incubation with rat heart homogenates, we used 80 μl of the homogenate, corresponding to 400–500 μg protein per assay. The 2 h incubation period was sufficient to allow equilibrium binding. The [^{3}H]NMS concentration used was 1.0 nM, i.e. two-fold the tracers' K_D value to M_2 binding sites.

In rat striatum homogenates, [^{3}H]NMS labels M_1 and M_4 sites but dissociates faster from M_1 receptors (Waelbroeck et al., 1986, 1987b, 1988). We preincubated 80 μl of the homogenate (equivalent to about 30 μg protein) in a total volume of 1.2 ml, in the presence of [^{3}H]NMS and unlabeled drugs. A 2 h preincubation period allowed equilibrium binding. We then added 1 μM atropine and allowed tracer dissociation for 35 min before filtration. This procedure allowed us to investigate tracer binding to striatum M_4 receptors only (Waelbroeck et al., 1987b; 1988; 1990). The tracer concentration used in these experiments (0.25 nM) was equivalent to five-fold the tracers' K_D value to striatum M_4 receptors (Waelbroeck et al., 1988).

All incubations were terminated by addition of 2 ml of ice-cold filtration buffer (50 mM sodium phosphate buffer pH 7.4). Bound and free tracer were immediately separated by filtration on GF/C glass-fiber filters presoaked overnight in 0.05% polyethyleneimine. The samples were rinsed three times with filtration buffer. The filters were then dried and the bound radioactivity counted by liquid scintillation. Nonspecific [^{3}H]NMS binding was defined as tracer bound in the presence of 1 μM atropine.

2.4. Analysis of binding data

All competition curves were repeated in duplicate, on at least three different preparations. IC_{50} values were determined by a computer-aided procedure described by Richardson and Humrich (1984), assuming the existence of only one receptor subtype. Indeed, experimental data points were within 3% of expected values, assuming that the molecules investigated competed with [^{3}H]NMS for binding to a single site.

K_i values were calculated from IC_{50} values using the Cheng and Prusoff equation (Cheng and Prusoff, 1973) which assumes competitive inhibition of tracer binding to a single receptor subtype. The [^{3}H]NMS K_D value for the three systems investigated was determined in separate experiments, as described by Waelbroeck et al. (1987a,b;

1988). The pK_i values, mentioned in table 1, corresponded to $-\log K_i$ values.

The standard deviation of pIC_{50} ($-\log IC_{50}$) determinations was always equal to or below 0.1 log unit. Repeated determinations of [^{3}H]NMS K_D values were within 10% of each other. This error should be added to errors in IC_{50} determinations, since [^{3}H]NMS K_D values were used to calculate pK_i values. We therefore estimated the standard deviation of pK_i values as being of approximately 0.15 log unit (40% of K_i value).

The binding free energy (ΔG) for the formation of a ligand-receptor complex is related to its affinity constant K_a by equation (1):

$$\Delta G = -RT \ln K_a \quad (1)$$

ΔG values were therefore calculated according to equation (2), using experimentally determined K_i values ($K_a = K_i^{-1}$):

$$\Delta G = -RT \ln 1/K_i \quad (2)$$

2.5. Materials

[^{3}H]NMS (80 to 85 Ci/mmol) was obtained from Amersham International (Bucks, England). Atropine sulfate and polyethyleneimine were from Sigma Chemical Co. (St Louis, MO, U.S.A.), and GF/C glass-fiber filters from Whatman (Maidstone, England). All the others reagents were of the highest grade available. All antagonists tested were synthesized in our laboratories: the procyclidine enantiomers were prepared as previously published (Tacke et al., 1986), pyrrinol was synthesized according to the literature (Adamson, 1949) and hexahydro-procyclidine was obtained by catalytic hydrogenation of pyrrinol.

3. Results

As shown in fig. 2, the four compounds investigated in this study inhibited [^{3}H]NMS binding to the three muscarinic receptors in a manner consistent with competition for a single binding site (Hill coefficients were not significantly different from 1).

The affinity of the procyclidine eutomer, (R)-procyclidine, for M_1 and M_4 receptors was greater than its affinity for M_2 receptors (table 1 and fig. 2). The procyclidine distomer, (S)-procyclidine, had a similar affinity for the three subtypes (table 1 and fig. 2). As a result, the eudismic index (pK_i (eutomer) $-$ pK_i (distomer)) at M_1 and M_4 receptors was greater than that at M_2 receptors (table 1).

Pyrrinol and hexahydro-procyclidine had lower affinities than (R)-procyclidine, and higher affinities than (S)-procyclidine, at the three subtypes (table 1 and fig. 2).

Hexahydro-procyclidine had the same receptor selectivity pattern as (R)-procyclidine. In contrast, pyrrinol was almost nonselective (table 1 and fig. 2), as observed for (S)-procyclidine.

The binding free energies of the compounds studied in this work and their differences are

TABLE 1

Comparison of pK_i values [a] and free energies of binding (ΔG) [a] (in $kJ \cdot mol^{-1}$) of (R)-procyclidine, (S)-procyclidine, pyrrinol and hexahydro-procyclidine for muscarinic receptor subtypes M_1, M_2 and M_4.

Muscarinic antagonist	M_1 (NB-OK 1)		M_2 (heart)		M_4 (striatum)	
	pK_i;	ΔG	pK_i;	ΔG	pK_i;	ΔG
1) (R)-Procyclidine	8.4	47.95	7.3	41.68	8.1	46.24
2) Pyrrinol	7.5	42.81	6.9	39.37	7.2	41.09
3) Hexahydro-procyclidine	7.1	40.55	6.1	34.81	7.0	39.96
4) (S)-Procyclidine	6.3	35.94	5.8	33.10	6.0	34.27
1-4 [b]	2.1	12.01	1.5	8.58	2.1	11.97

[a] The pK_i and ΔG values were calculated as explained in Materials and methods (2.4, analysis of binding data). The standard deviation of pK_i values was estimated at ± 0.15 log units.

[b] Eudismic index (difference of the pK_i values) and differences between the free energies of binding of (R)- and (S)-procyclidine at each receptor subtype.

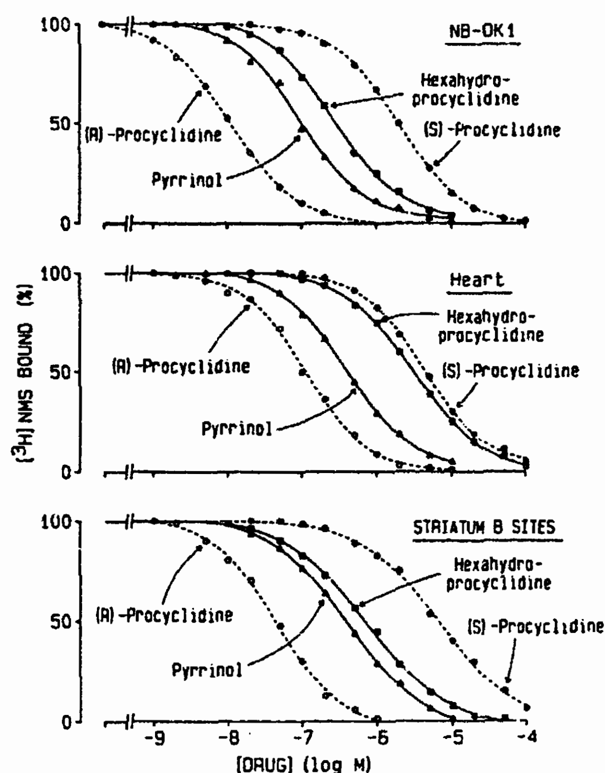

Fig. 2. [^{3}H]NMS competition curves in membranes from human neuroblastoma NB-OK 1 cells (upper panel), rat heart (middle panel) and rat striatum (lower panel). [^{3}H]NMS binding was measured in the absence or presence of (R)-procyclidine (O), (S)-procyclidine (●), pyrrinol (▲) and hexahydroprocyclidine (■), as described in Materials and methods. Average of three experiments performed in duplicate.

quoted in tables 1 and 2. The difference between the binding free energies of (R)-procyclidine and hexahydro-procyclidine (about 7 $kJ \cdot mol^{-1}$) was

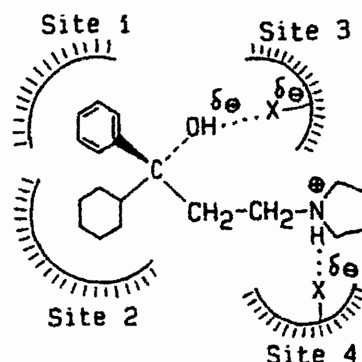

Fig. 3. Interaction pharmacophores of (R)-procyclidine (eutomer) binding to four subsites of muscarinic receptors.

very similar for the three receptors. In contrast, the difference between the binding free energies of (R)-procyclidine and pyrrinol was smaller at cardiac M_2 than at M_1 or M_4 receptors. The difference observed between the free binding energies of (R)- and (S) procyclidine corresponded to the sum of the differences between free binding energies of (R)-procyclidine and pyrrinol, and between (R)-procyclidine and hexahydro-procyclidine, at M_1, M_2 and M_4 receptors.

4. Discussion

The fact that procyclidine binding was highly stereoselective indicates that at least three groups surrounding the asymmetrically substituted carbon atom contributed to overall drug binding affinity (fig. 3). The free energy of (R)-procyclidine binding can therefore be described by equation (3):

$$\Delta G = \alpha\Delta G_1 + \beta\Delta G_2 + \gamma\Delta G_3 + \delta\Delta G_4 \quad (3)$$

TABLE 2

Differences in free energies ($kJ \cdot mol^{-1}$) for binding of (R)-procyclidine, (S)-procyclidine, pyrrinol and hexahydro-procyclidine to muscarinic receptor subtypes M_1, M_2 and M_4.

Muscarinic antagonist	M_1 (NB-OK 1)	M_2 (heart)	M_4 (striatum)
(R)-Procyclidine/pyrrinol	+5.14	+2.31	+5.15
(R)-Procyclidine/hexahydro-procyclidine	+7.40	+6.87	+6.28
(R)-Procyclidine/(S)-procyclidine			
observed [a]	+12.01	+8.58	+11.97
expected [b]	+12.54	+9.18	+11.43

[a] Difference between the free energies of the binding of (R)-procyclidine and (S)-procyclidine at each receptor subtype.
[b] Sum of the differences of the free energies of binding of (R)-procyclidine and pyrrinol as well as of (R)-procyclidine and hexahydro-procyclidine.

where ΔG_1, ΔG_2, ΔG_3 and ΔG_4 represent the free energy achievable by an optimal interaction of, respectively, the hydrophobic phenyl ring of the ligand with receptor site 1, the cyclohexyl group with receptor site 2, the hydroxy group with receptor site 3 and the protonated amino group with receptor site 4. The value of ΔG should be as negative as possible to obtain high-affinity binding. Factors α, β, γ and δ in equation 3 take into account the fact that all four groups are not necessarily simultaneously in optimal position to interact with receptor sites 1 to 4 (α, β, γ and δ values probably vary between 0 and 1, provided that the corresponding group does not obstruct binding by steric hindrance).

The protonated amino group of procyclidine (fig. 3) might conceivably contribute different interactions with the fourth receptor subsite: an ion-ion interaction (ligand$^+$ ------ $^-$receptor), an ion-dipole interaction (ligand$^+$ ------ receptor) and a hydrogen bond (N——H------X-receptor). The average intrinsic binding energy of protonated nitrogens was estimated at 11.5 kcal · mol^{-1} (i.e. 48.1 kJ · mol^{-1}; Andrews, 1986). This very important contribution to drug binding is compatible with the observation that all muscarinic antagonists possess a cationic group. Ionic interactions per se probably made an important contribution to binding, since the two enantiomers of the quaternary ammonium derivative tricyclamol (with a permanent charge and no N-H group), show higher affinities than procyclidine for the three receptors (unpublished results).

The hydroxy group of (R)-procyclidine (fig. 3) probably forms a hydrogen bond with the third receptor subsite: desoxyprocyclidine (without an hydroxy group) showed the same low potency as (S)-procyclidine (cited by Lambrecht and Mutschler, 1986). Misplacing the hydroxy group of, for example, (S)-procyclidine might be even more unfavorable for binding than replacing it with a hydrogen atom, if the hydrogen bonds formed with the solvent (water) must be broken to allow the drug-receptor interaction.

The binding energy of ionic and hydrogen bonds depends strongly on the distance between the two atoms considered; furthermore, the orientation of the O-H bond respective to the electron-rich acceptor atom also affects the hydrogen bond energy. Parameters γ and δ in equation 3 are therefore strongly dependent on the relative positions of the nitrogen, oxygen and OH-hydrogen atoms of the drug considered, relative to subsites 3 and 4 of the receptor.

The phenyl and cyclohexyl groups probably contribute to the binding energy by two other types of interactions: (a) hydrophobic interactions, when a nonpolar surface is removed from water and (b) van der Waals interactions (dipole-dipole, dipole-induced dipole and induced dipole-induced dipole interactions, brought about by the close contact between nonbonded atoms or molecules). The hydrophobic interactions of the phenyl and cyclohexyl groups with receptor sites 1 and 2, respectively, are somewhat more independent than van der Waals interactions on the exact position of the two ring systems, relative to sites 1 and 2. Therefore, substituting the cyclohexyl and phenyl groups of the muscarinic antagonist in hydrophobic receptor sites 1 and 2 might be less unfavorable than suppressing the interaction of the hydroxy or ammonium groups of the antagonist with their respective receptor subsites 3 and 4. To test this hypothesis, we investigated the binding properties of two achiral molecules, in which the phenyl or cyclohexyl groups of (R)-procyclidine were replaced by a cyclohexyl or phenyl group. We assumed that increases in binding free energy, due to the loss of van der Waals interactions with receptor sites 1 and 2, should be additive provided that the ammonium and hydroxy groups of the 4 ligands retain their normal binding position (fig. 3). This was indeed observed experimentally: the differences of binding free energies of (R)-procyclidine → pyrrinol and (R)-procyclidine → hexahydro-procyclidine were small, suggesting that steric hindrance did not prevent the interaction of the (larger) cyclohexyl group with the phenyl-preferring subsite (site 1). They were additive at M_1, M_2 and M_4 receptors (table 2). The stereoselectivity of these three receptors for procyclidine binding apparently reflected poor interactions of the phenyl group at the cyclohexyl binding site and vice versa.

Our results also gave valuable information concerning the preferential binding of (R)-procycli-

dine to M_1 and M_4 receptors: the lower affinity of (R)-procyclidine for M_2 sites was apparently due to a poorer fit of the cyclohexyl group in receptor subsite 2. This would indeed explain the following observations:

(1) (R)-Procyclidine and the dicyclohexyl derivative hexahydro-procyclidine were M_1, $M_4 > M_2$ selective as a cyclohexyl group was in contact with the 'cyclohexyl receptor site 2'.

(2) (S)-Procyclidine and pyrrinol, the diphenyl derivative, were not selective as the cyclohexyl receptor site 2 was occupied by a phenyl group.

(3) The affinity loss when replacing the cyclohexyl group of (R)-procyclidine by a phenyl group was much smaller at M_2 (2.31 $kJ \cdot mol^{-1}$) than at M_1 and M_4 receptors (5.15 $kJ \cdot mol^{-1}$).

In conclusion, muscarinic M_1, M_2 and M_4 receptors clearly discriminated between the two procyclidine enantiomers, and preferred (R)-procyclidine. This is in line with functional studies on guinea-pig ileum (Tacke et al., 1986). The enantioselectivity of cardiac M_2 receptors was lower than that of neuroblastoma M_1 and striatum M_4 receptors. A systematic comparison of the binding properties of the two procyclidine enantiomers and of the related achiral compounds pyrrinol and hexahydro-procyclidine suggested that the receptors' stereoselectivity reflected the loss of van der Waals interactions of the hydrophobic receptor subsites recognizing the phenyl and cyclohexyl groups of the ligand. The lower affinity and eudismic index of muscarinic M_2 receptors were due to the poorer interaction of their subsites with the cyclohexyl group (as compared to the cyclohexyl subsite of M_1 or M_4 receptors).

Acknowledgements

M.W., J.C., M.T. and J.C. thank the Fund for Medical Scientific Research (Belgium) for grant 3.4571.85. R.T. thanks the Deutsche Forschungsgemeinschaft, and G.L., E.M. and R.T. thank the Fonds der Chemischen Industrie for financial support.

References

Adamson, D.W., 1949, Aminoalkyl tertiary carbinols and derived products. Part 1. 3-Amino-1 : 1-diphenylpropan-1-ols, J. Chem. Soc. Suppl., S144.

Andrews, P., 1986, Functional groups, drug-receptor interactions and drug design, Trends Pharmacol. Sci. 7, 148.

Barlow, R.B., K.J. Berry, P.A.M. Glenton, N.M. Nikolaou and K.S. Soh, 1976, A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells at 29°C and in ileum at 29°C and 37°C, Br. J. Pharmacol. 58, 613.

Cheng, Y. and W.H. Prusoff, 1973, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes a 50 percent inhibition (I_{50}) of an enzymatic reaction, Biochem. Pharmacol. 22, 3099.

Hammer, R., C.P. Berrie, N.J.M. Birdsall, A.S.V. Burgen and E.C. Hulme, 1980, Pirenzepine distinguishes between different subclasses of muscarinic receptors, Nature 283, 90.

Hammer, R., E. Giraldo, G.B. Schiavi, E. Monferini and H. Ladinsky, 1986, Binding profile of a novel cardioselective muscarine receptor antagonist, AF-DX 116, to membranes of peripheral tissues and brain in the rat, Life Sci. 38, 1653.

Korc, M., M.S. Ackerman and W.R. Roeske, 1987, A cholinergic antagonist identifies a subclass of muscarinic receptors in isolated rat pancreatic acini, J. Pharmacol. Exp. Ther. 240, 118.

Lambrecht, G., R. Feifel, U. Moser, A.J. Aasen, M. Waelbroeck, J. Christophe and E. Mutschler, 1988, Stereoselectivity of the enantiomers of trihexyphenidyl and its methiodide at muscarinic receptor subtypes, European J. Pharmacol. 155, 167.

Lambrecht, G. and E. Mutschler, 1986, Chirality as a tool for subclassification of receptors, in: Innovative Approaches in Drug Research, ed. A.F. Harms (Elsevier Science Publishers, Amsterdam) p. 353.

Levine, R.R. and N.J.M Birdsall (eds.), 1989, Subtypes of muscarinic receptors. IV, Trends Pharmacol. Sci. 10 (Suppl.).

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

Michel, A.D., R. Delmendo, E. Stefanich and R.L. Whiting, 1989, Binding characteristics of the muscarinic receptor subtype of the NG108-15 cell line, Naunyn-Schmiedeb. Arch. Pharmacol. 340, 62.

Mitchelson, F., 1988, Muscarinic receptor differentiation, Pharmacol. Ther. 37, 357.

Mutschler, E. and G. Lambrecht, 1984, Selective muscarinic agonists and antagonists in functional tests, Trends Pharmacol. Sci. 5, Suppl. 39.

Richardson, A. and A. Humrich, 1984, A microcomputer program for the analysis of radioligand binding curves and other dose-response data, Trends Pharmacol. Sci. 5, 47.

Tacke, R., H. Linoh, L. Ernst, U. Moser, E. Mutschler, S. Sarge, H.K. Cammenga and G. Lambrecht, 1987, Preparation and properties of the enantiomers of the antimuscarinic agents sila-procyclidine and sila-tricyclamol iodide: optically active silanols with silicon as the center of chirality, Chem. Berat. 120, 1229.

Tacke, R., H. Linoh, D. Schomburg, L. Ernst, U. Moser, E. Mutschler and G. Lambrecht, 1986, On the absolute configuration of the enantiomers of the antimuscarinic agents procyclidine and tricyclamol iodide: X-ray structural analy-

sis of (R)-1-[3-cyclohexyl-3-hydroxy-3-phenylpropyl]-1-methylpyrrolidinium iodide, Liebigs Ann. Chem., 242.

Tacke, R., C. Strohmann, S. Sarse, H.K. Cammenga, D. Schomburg, E. Mutschler and G. Lambrecht, 1989, Preparation and properties of the enantiomers of the selective antimuscarinic agent 1-cyclohexyl-1-phenyl-4-piperidino-1-butanol (hexahydro-difenidol), Liebigs Ann. Chem., 137.

Waelbroeck, M., J. Camus, M. Tastenoy and J. Christophe, 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 M_1 receptors, FEBS Lett. 226, 287.

Waelbroeck, M., J. Camus, M. Tastenoy and J. Christophe, 1990, Identification of the striatum 'B' sites as belonging to the M_4 muscarinic receptor subtype, Naunyn-Schmiedeb. Arch. Pharmacol. 341, Suppl. R80.

Waelbroeck, M., J. Camus, J. Winand and J. Christophe, 1987a, Different antagonist binding properties of rat pancreatic and cardiac muscarinic receptors, Life Sci. 41, 2235.

Waelbroeck, M., M. Gillard, P. Robberecht and J. Christophe, 1986, Kinetic studies of [^{3}H]N-methylscopolamine binding to muscarinic receptors in the rat central nervous system: evidence for the existence of three classes of binding sites, Mol. Pharmacol. 30, 305.

Waelbroeck, M., M. Gillard, P. Robbcrecht and J. Christophe, 1987b, Muscarinic receptor heterogeneity in the rat central nervous system: I. Binding of four selective antagonists to three muscarinic receptor subclasses: a comparison with M_2 cardiac muscarinic receptors of the C type, Mol. Pharmacol. 32, 91.

Waelbroeck, M., M. Tastenoy, J. Camus, J. Christophe, C. Strohmann, H. Linoh, H. Zilch, R. Tacke, E. Mutschler and G. Lambrecht, 1989, Binding and functional properties of antimuscarinics of the hexocyclium/sila-hexocyclium and hexahydro-diphenidol/hexahydro-sila-diphenidol type to muscarinic receptor subtypes,Br. J. Pharmacol. 98, 197.