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Selective labelling of muscarinic M_1 receptors in calf superior cervical ganglia by [^{3}H](±)-telenzepine

Roland Feifel [1], Jaap F. Rodrigues de Miranda [2], Carsten Strohmann [3], Reinhold Tacke [3], Arne J. Aasen [4], Ernst Mutschler [1] and Günter Lambrecht [1]

[1] *Department of Pharmacology, University of Frankfurt, Theodor-Stern-Kai 7, Gebäude 75A, D-6000 Frankfurt/Main, F.R.G.,* [2] *Department of Pharmacology, University of Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands,* [3] *Institute of Inorganic Chemistry, University of Karlsruhe, Engesserstrasse, Gebäude 30.45, D-7500 Karlsruhe, F.R.G. and* [4] *Department of Pharmacy, University of Oslo, P.O. Box 1068, N-0316 Oslo 3, Norway*

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A method was developed to determine the affinities of antimuscarinic drugs at M_1 receptors. [^{3}H](±)-Telenzepine served as radioligand in crude preparations of calf superior cervical ganglia and showed high affinity for a single receptor population, consisting of M_1 receptors ($K_D = 1.12$ nM). Kinetic experiments showed monophasic association ($k_1 = 0.017$ min^{-1} nM^{-1}) and dissociation ($k_{-1} = 0.017$ min^{-1}) kinetics, the half-life of dissociation being 41 min at 37°C. The kinetic K_D value amounted to 1.00 nM. M_1 affinities for pirenzepine, methoctramine, hexahydro-sila-difenidol and p-fluoro-hexahydro-sila-difenidol determined in competition experiments were similar to those found in functional studies with M_1 receptors in rabbit isolated vas deferens. The binding assay was used to determine the affinities of the (R) and (S) enantiomers of tertiary (trihexyphenidyl, hexahydro-difenidol, hexbutinol, p-fluoro-hexbutinol) and quaternary muscarinic antagonists (trihexyphenidyl methiodide, hexbutinol methiodide). Comparison of results obtained with the rabbit vas deferens suggested that the ionic environment may influence the affinities.

[^{3}H](±)-Telenzepine; Muscarinic M_1 receptors; Superior cervical ganglia (calf); Hexahydro-difenidol analogues; Trihexyphenidyl; Hexbutinol; Stereoselectivity

1. Introduction

At least three subtypes of muscarinic receptors (M_1, M_2 and M_3) have been identified on the basis of the affinities of antagonists determined in functional experiments as well as in radioligand binding studies (for review, see Levine and Birdsall, 1989). There is a candidate M_4 receptor, found in rat striatum (Waelbroeck et al., 1990) and NG108-15 cells (Michel et al., 1989), which is considered to be the pharmacological correlate of the m_4 gene product (Brann et al., 1988). This concept was recently confirmed by studies with cloned, sequenced and expressed complementary DNA encoding these receptors (Brann et al., 1988; Levine and Birdsall, 1989).

Muscarinic M_1 receptors are found in discrete regions of the brain (Hammer et al., 1980; Watson et al., 1983; 1984), in the intramural enteric nervous system (North et al., 1985) and in peripheral autonomic ganglia (Hammer and Giachetti, 1982; Giraldo et al., 1985; Newberry and Priestley, 1987; Eltze et al., 1988).

Binding studies with mammalian sympathetic ganglia have revealed the presence of multiple receptors for muscarinic ligands (Hammer and Giachetti, 1982; Watson et al., 1984; Giraldo et al., 1985; Galvan et al., 1989), which has been confirmed in functional studies (Newberry and Priestley, 1987).

In many investigations pirenzepine (Hammer et al., 1980) and/or [^{3}H]pirenzepine (Watson et al., 1983) have been used as selective antagonists to label M_1 receptors selectively. However, in most of the binding studies in peripheral tissues, the binding of pirenzepine has been measured indirectly in competition experiments, since the ratio receptor binding/non-specific binding is too low to achieve direct specific [^{3}H] pirenzepine binding. More recently, telenzepine has been reported to possess a more than 10-fold higher affinity for muscarinic receptor subtypes than pirenzepine, whilst its receptor selectivity for M_1 muscarinic receptors is the same (Eltze, 1988; Schudt et al., 1988; 1989; Eveleigh et al., 1989; Galvan et al., 1989). In contrast to

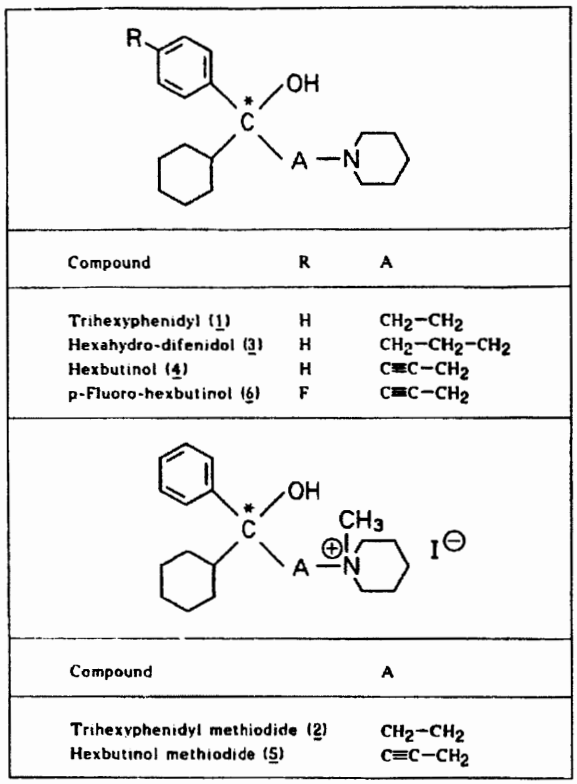
Correspondence to: R. Feifel, Preclinical Research, Endocrinology 386/622, Sandoz Pharma AG, CH-4002 Basel, Switzerland.

pirenzepine, there are two chemically stable enantiomers of telenzepine, the stereoselectivity ratio at M_1 receptors being ca. 400-500 (Eveleigh et al., 1989; Schudt et al., 1989).

The aim of the present study was to design a simple model to determine the affinities of drugs acting at muscarinic M_1 receptors. We therefore investigated the binding behaviour of muscarinic M_1 receptors present in calf superior cervical ganglia by using [^{3}H](±)-telenzepine as a selective radioligand and compared its binding with that of [^{3}H]pirenzepine. Furthermore, the kinetic behaviour of [^{3}H](±)-telenzepine binding was studied in calf ganglia, since it has been reported to dissociate slowly from muscarinic M_1 receptors (Eveleigh et al., 1989; Galvan et al., 1989).

Besides these direct binding experiments, which were intended to elucidate the M_1 receptor binding characteristics of [^{3}H](±)-telenzepine in calf superior cervical ganglia, we performed competition experiments with antimuscarinic drugs. The compounds investigated were pirenzepine (Hammer et al., 1980), methoctramine (Melchiorre et al., 1987; Waelbroeck et al., 1989a), racemic hexahydro-sila-difenidol (Mutschler and Lambrecht, 1984; Lambrecht et al., 1989a) and racemic p-fluoro-hexahydro-sila-difenidol (Lambrecht et al., 1988a; 1989a) as reference drugs. In addition, the M_1 affinities of the enantiomers of trihexyphenidyl (1), trihexyphenidyl methiodide (2), hexahydro-difenidol (3), hexbutinol (4), hexbutinol methiodide (5) and p-fluoro-hexbutinol (6) (fig. 1) (Lambrecht et al., 1988b; Feifel et al., 1990) were determined. The pK_I values of these compounds were compared with previously published pA_2 values obtained in functional experiments with presynaptic M_1 muscarinic heteroreceptors in isolated rabbit vas deferens (Lambrecht et al., 1988a,b; Feifel et al., 1990; Eltze and Figala, 1988; Eltze, 1988), since these neuronal muscarinic receptors in rabbit vas deferens have been shown to be of the ganglionic M_1 type (Eltze et al., 1988). Additionally, stereoselectivity ratios for the affinities of the chiral compounds were calculated because of their relevance in muscarinic receptor subtype identification (Lambrecht et al., 1988b; 1989b; Feifel et al., 1990; Waelbroeck et al., 1989b).

Some of the present results have been reported elsewhere (Feifel et al., 1989; Lambrecht et al., 1989a).



Compound	R	A
Trihexyphenidyl (1)	H	CH_2-CH_2
Hexahydro-difenidol (3)	H	$CH_2-CH_2-CH_2$
Hexbutinol (4)	H	$C\equiv C-CH_2$
p-Fluoro-hexbutinol (6)	F	$C\equiv C-CH_2$

Compound	A
Trihexyphenidyl methiodide (2)	CH_2-CH_2
Hexbutinol methiodide (5)	$C\equiv C-CH_2$

Fig. 1. Chemical structure of trihexyphenidyl (1), trihexyphenidyl methiodide (2), hexahydro-difenidol (3), hexbutinol (4), hexbutinol methiodide (5) and p-fluoro-hexbutinol (6). The asterisk denotes the centre of chirality.

2. Materials and methods

2.1. Membrane preparation

The preparation of crude pellets of calf superior cervical ganglia and the binding experiments were performed in Tris-HCl buffer of the following composition (mM): Tris (tris(hydroxymethyl)aminomethane) 50.0, NaCl 120.0, $MgCl_2$ 5.0, adjusted with HCl to pH 7.4 at 4°C and at 37°C for tissue preparation and binding experiments, respectively.

Calf superior cervical ganglia were obtained from a regional slaughterhouse and brought to the laboratory within 70 min. After separation of surrounding tissue, crude ganglia were minced with scissors and homogenized in ice-cold buffer solution (30 g tissue wet weight/100 ml) by means of an Ultra-Turrax (TP 18/2, Janke + Kunkel KG, Staufen i.Br., FRG) at maximal speed for 2 × 30 s. The resulting suspension was centrifuged (Mikro Rapid/K 1305, Hettich, Tuttlingen, FRG) at 160 × g for 10 min and the supernatant was separated. The pellet was ground a second time and processed as before. The supernatants were combined and centrifuged (B-60 Z ultracentrifuge, IEC, Needham Heights, MA, USA) at 100000 × g for 60 min. The supernatants were removed and the resulting pellets were stored at −20°C until required. The pellets consisted of 20% of the wet weight of the original tissue. No differences in binding parameters were observed after 2 months storage of preparations (data not shown).

Protein concentration was determined according to the BioRad method (Bradford, 1976), using bovine serum albumin as standard. A factor for the conversion of the wet weight of the pellets to g protein was esti-

mated (= 0.062); however, receptor densities are expressed with reference to the wet weight of the pellets.

2.2. Ligand binding assays

Frozen pellets were resuspended in ice-cold buffer solution with a Potter-Elvehjem (type 853202, Braun, Melsungen, FRG; 10 strokes at 1000 rpm). All binding experiments were performed in 6-ml glass vials in a final volume of 0.35 ml per sample, usually with a tissue density of ca. 5 mg pellet wet weight/ml. Different tissue concentrations were used in some experiments to determine the M_1 affinity of [^{3}H](±)-telenzepine and to investigate its binding to M_1 receptors as a function of protein concentration. The incubations for the saturation and competition experiments were carried out in a shaking water bath (37°C, 120 rpm) for 120 min. Specific binding was obtained by subtracting the radioactivity bound in the presence of 10^{-5} M atropine (non-specific binding) from the radioactivity bound in the absence of atropine (total binding). All experiments were carried out in duplicate and were repeated at least twice. Each sample contained 0.05 ml solution of ^{3}H-labelled ligand (for concentrations see below), 0.05 ml of buffer or a solution of atropine (saturation and kinetic experiments) or competitor (competition experiments) and 0.25 ml tissue suspension.

Incubations were terminated by the addition of 2 ml of ice-cold buffer followed by rapid vacuum filtration through Whatman GF/C glass fibre filters (Tamson, Zoetermeer, NL), which were washed twice with 2 ml and twice with 3 ml of 0.9% (w/v) aqueous NaCl solution. The filters were placed into 6-ml polyethylene scintillation vials (Milli-6®, Lumac, Landgraaf, NL), 4 ml of Aqualuma® was added and after 5-10 h β-decay was counted in a Packard Tri-Carb 460 CD liquid scintillation counter at an efficiency of 45%.

Similar experiments carried out in the presence and absence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) revealed no differences in binding parameters (data not shown). Therefore no protease inhibitors were used in this study.

2.3. Saturation experiments

For saturation curves nine different concentrations of the ^{3}H-labelled ligands were used, 0.05-8 nM [^{3}H](±)-telenzepine and 0.13-20 nM [^{3}H]pirenzepine, respectively.

2.4. Kinetic experiments

Fixed concentrations (= $[D_0]$) of 1.1 and 2.2 nM [^{3}H](±)-telenzepine were incubated and association was determined by terminating the reaction after different time intervals (2-150 min). Dissociation of [^{3}H](±)-telenzepine was measured after a 60-min incubation and consecutive addition of 10^{-5} M atropine to the samples; the reaction was stopped after 2-95 min. Five independent experiments were performed.

2.5. Competition experiments

In competition experiments, a fixed concentration of 0.8 nM [^{3}H](±)-telenzepine and 11 increasing concentrations of competitor (0.05-50 times the estimated K_I value) were added to separate samples. In some cases, when Hill coefficients proved to be significantly greater than unity, 0.5-1.0% bovine serum albumin was added to the incubation vials (see below).

2.6. Data analysis

Binding parameters (K_D, K_I and R_T (total receptor density) values) were calculated by fitting model functions for one and for two binding sites using the FARMFIT program on an IBM computer (VM 370) of the Universiteit Rekencentrum Nijmegen, NL. FARMFIT uses essentially the same iterative non-linear least-squares regression procedure as the well-known LIGAND program, originally written by Munson and Rodbard (1980) and described by McPherson (1985). The statistical difference between one-site and two-site receptor models was determined by comparing the residual variance between the predicted and actual data points in an F-test ($P < 0.05$).

The kinetic data were analysed using the KINETIC subroutine of the computer program LIGAND. Dissociation constants (k_{-1}) and observed association constants (k_{obs1}) were fitted by appropriate functions and a kinetic K_D value was calculated according to the following equation: $K_D = k_{-1}/k_1$, where k_1 represents the actual association constant according to $k_1 \times [D_0] + k_{-1} = k_{obs1}$ with $[D_0]$ representing the concentration of [^{3}H](±)-telenzepine. A $t_{1/2}$ value for dissociation was calculated by using the equation $t_{1/2} = 0.693/k_{-1}$.

2.7. Statistics

The data are presented as means ± S.E.M. Linear regression analyses were done with the least-squares method. Student's t-test ($P < 0.05$) was used to determine the statistical significance of the differences between mean values.

2.8. Drugs and chemicals

Racemic [^{3}H]telenzepine (specific activity 85 Ci/mmol, lot 2425-235) was a generous gift from Byk Gulden Lomberg, Konstanz, FRG. [^{3}H]Pirenzepine (specific activity 74.4 Ci/mmol, lot 2604-012) was purchased from NEN Du Pont, 's-Hertogenbosch, NL.

Pirenzepine dihydrochloride (Boehringer Ingelheim, FRG) and methoctramine tetrahydrochloride (Dr. C. Melchiorre, Bologna, Italy) were kindly donated.

Racemic hexahydro-sila-difenidol hydrochloride (Tacke et al., 1985), (R)- and (S)-trihexyphenidyl hydrochloride ((R) – 1 · HCl and (S) – 1 · HCl) (Schjelderup et al., 1987), (R)- and (S)-trihexyphenidyl methiodide ((R) – 2 and (S) – 2) (Schjelderup et al., 1987), (R)- and (S)-hexahydro-difenidol hydrochloride ((R) – 3 · HCl and (S) – 3 · HCl) (Tacke et al., 1989), (R)- and (S)-hexbutinol ((R) – 4 and (S) – 4) (Tacke et al., 1989) and (R)- and (S)-hexbutinol methiodide ((R) – 5 and (S) – 5) (Feifel et al., 1990) were synthesized in our laboratories according to the methods described in the literature. Racemic p-fluoro-hexahydro-sila-difenidol hydrochloride was prepared in a similar way as hexahydro-sila-difenidol (unpublished results). The enantiomers of p-fluoro-hexbutinol ((R) – 6 and (S) – 6) were synthesized in a similar way as (R)- and (S)-hexbutinol ((R) – 4 and (S) – 4) (unpublished results). The enantiomeric excess (ee) of the enantiomers of 1-6 was > 99.8%, determined by calorimetric analysis as described by Tacke et al. (1989).

Aqualuma was purchased from Lumac, Landgraaf, NL. All other chemicals were obtained from commercial sources and were used as purchased.

3. Results

3.1. Saturation studies

[^{3}H]Pirenzepine exhibited an unfavourable specific to non-specific binding ratio in crude pellets of calf superior cervical ganglia (CSCG), the non-specific binding amounting to 50-70% of the total bound radioactivity in the concentration range of 2-20 nM. The binding parameters determined in this preparation ($K_D = 6 \pm 4$ nM, $R_T = 11 \pm 3$ pmol/g wet weight, n = 3) could not be regarded as a reliable basis for competition experiments and therefore no further studies were undertaken with this radioligand.

Specific binding of [^{3}H](±)-telenzepine to M_1 receptors in CSCG was saturable and of high affinity. Non-specific binding accounted for 16% (maximal value) of total binding (fig. 2). Scatchard plot analysis (Scatchard, 1949) (fig. 2, inset) indicated the presence of a homogeneous population of binding sites. A dissociation constant (K_D) of 1.12 ± 0.02 nM and a receptor density (R_T) of 22.6 ± 0.3 pmol/g pellet wet weight, equal to 1.40 pmol/g protein, were obtained (6.2 ± 0.1% of the wet weight of the pellet represents membrane protein). Incubations with various tissue concentrations (5, 12, 17 and 21 mg pellet wet weight/ml, respectively) did not show significant changes in dissociation constant (K_D) and receptor density (R_T). Specific binding at a concentration of 1.1 nM [^{3}H](±)-telenzepine increased linearly with these tissue concentrations (data not shown). The specifically bound radioactivity amounted to 12.1 ± 0.6 pmol/g pellet wet weight.

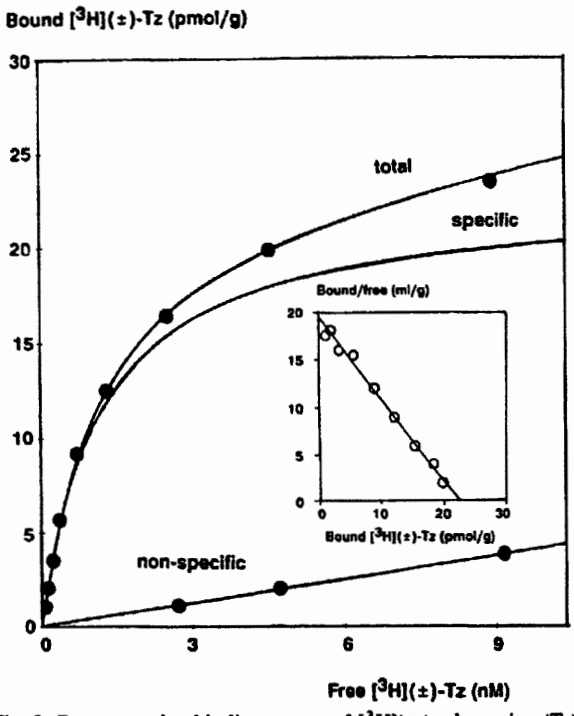


Fig. 2. Representative binding curves of [^{3}H](±)-telenzepine (Tz) at muscarinic M_1 receptors in crude pellets of calf superior cervical ganglia. Specific binding was the difference between total and non-specific binding. The inset shows a Scatchard plot of the saturation isotherm.

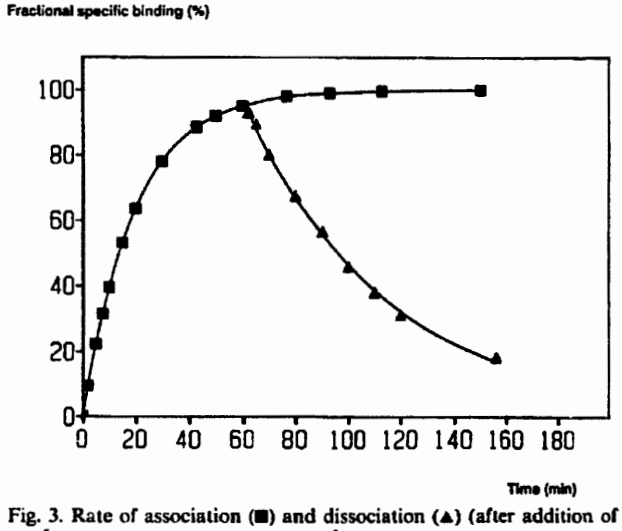


Fig. 3. Rate of association (■) and dissociation (▲) (after addition of 10^{-5} M atropine) of specific [^{3}H](±)-telenzepine binding at muscarinic M_1 receptors in crude pellets of calf superior cervical ganglia (given as percentage of maximal binding) as a function of time. The data represent the means of five separate experiments. S.E.M. values were smaller than 5% in all cases and are therefore not shown.

3.2. Kinetic studies

The kinetics of the binding of [^{3}H](±)-telenzepine to M_1 receptors in calf superior cervical ganglia could be best described by assuming monoexponential functions for association ($k_{obs1} = (5.1 \pm 0.5) \times 10^{-2}$ min^{-1}; $k_1 = (1.7 \pm 0.2) \times 10^{-2}$ min^{-1} nM^{-1}) and dissociation ($k_{-1} = (1.7 \pm 0.1) \times 10^{-2}$ min^{-1}; $t_{1/2} = 41 \pm 3$ min) (fig. 3). Non-specific binding amounted to less than 10% of the total binding at equilibrium and was constant over the time range examined (data not shown). The kinetic K_D value (1.00 ± 0.02 nM), calculated as k_{-1}/k_1, was very similar to that found in saturation experiments (1.12 ± 0.02 nM), thus confirming that there was a simple bimolecular interaction.

3.3. Competition binding studies

Preliminary competition studies with hexahydro-sila-difenidol, p-fluoro-hexahydro-sila-difenidol and compounds 4-6 (fig. 1) showed that the binding data were best fitted by a one-binding site model with Hill coefficients (nH) significantly greater than unity (data not shown). However, if 0.5-1% bovine serum albumin was added to the incubation medium, Hill coefficients not significantly different from unity were obtained. The displacement curve of (R)-trihexyphenidyl methiodide ((R) − 2) had a Hill coefficient smaller than 1 and was adequately described by a two-binding site model. However, the fraction of low-affinity binding sites was very small, and the estimated K_D for the high-affinity binding sites determined in the two-site model did not differ significantly from that found in the one-site analysis (data not shown). All other compounds investigated showed no significant deviation from a concentration-dependent competitive interaction with telenzepine at a single binding site. Some representative competition curves are shown in fig. 4; the mean pK_I values of all compounds are listed in table 1.

In general, the (R) enantiomers of compounds 1-3 exhibited higher affinity than their corresponding (S) antipodes, whereas the (R) and (S) enantiomers of compounds with a triple bond (4-6) showed only slight differences in their binding affinities. The (R) enantiomers of trihexyphenidyl ((R) − 1) and trihexyphenidyl methiodide ((R) − 2) were very potent antimuscarinic compounds at M_1 receptors in CSCG with pK_I values of 9.01 and 9.47, respectively. (R)-hexahydro-difenidol ((R) − 3), pirenzepine, hexahydro-sila-difenidol, as well as both enantiomers of hexbutinol (4) and hexbutinol methiodide (5), showed high and intermediate affinity (pK_I values 8.20-7.35), respectively. p-Fluoro-hexahydro-sila-difenidol, methoctramine, (S) − 1, (S) − 2 and both enantiomers of p-fluoro-hexbutinol (6) exhibited relatively low affinity (pK_I values 7.08-6.36), whereas the (S) enantiomer of hexahydro-difenidol ((S) − 3) was a very poor antimuscarinic agent with a pK_I value of 5.91.

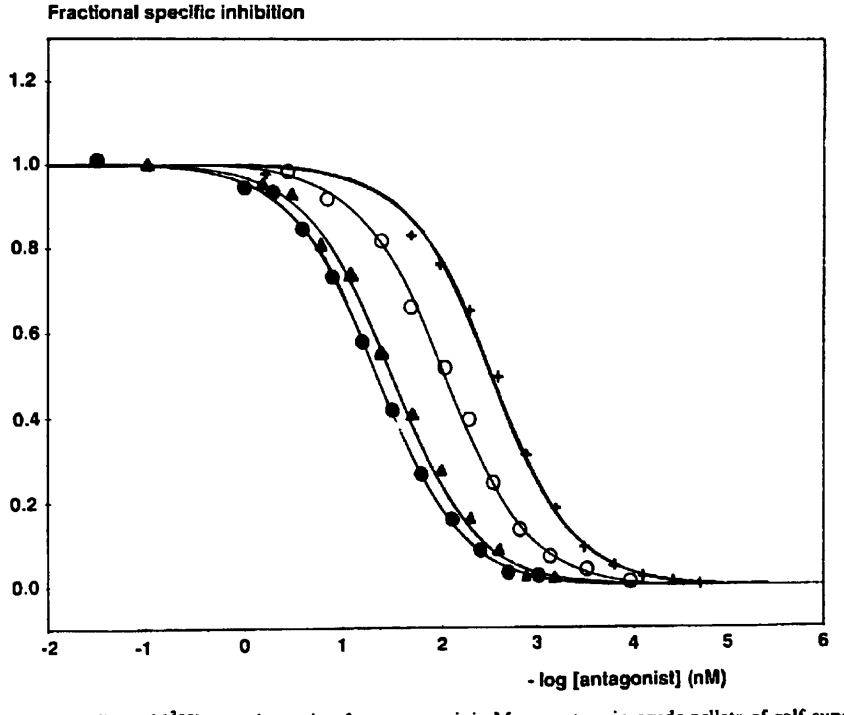


Fig. 4. Displacement of specific binding of [^{3}H](±)-telenzepine from muscarinic M_1 receptors in crude pellets of calf superior cervical ganglia by pirenzepine (●), methoctramine (+), hexahydro-sila-difenidol (▲) and p-fluoro-hexahydro-sila-difenidol (○). The data represent the means of three to eight independent experiments. S.E.M. values were smaller than 5% in all cases and are therefore not shown.

TABLE 1

Affinity values (pK_1 and pA_2) of muscarinic antagonists and stereoselectivity (R)/(S) ratios for chiral compounds obtained in binding studies with [^{3}H](±)-telenzepine labelled M_1 receptors in calf superior cervical ganglia (CSCG, pK_1) as well as in functional experiments with M_1 receptors in rabbit isolated vas deferens (RVD, pA_2). The affinity values represent the means ± S.E.M. For binding experiments, the numbers (n) of independent experiments are given in parentheses. (R)/(S) ratios represent the antilogs of the differences between corresponding pK_1 or pA_2 values, respectively. pA_2 values were taken from [a] Eltze (1988), [b] Eltze and Figala (1988), [c] Lambrecht et al. (1988a), [d] Lambrecht et al. (1988b) and [e] Feifel et al. (1990).

Compound	pK_1 (CSCG) (n)	pA_2 (RVD)	(R)/(S) ratio	
			CSCG	RVD
Pirenzepine	7.85 ± 0.02 (8)	7.79 ± 0.10 [a]		
Methoctramine	6.59 ± 0.02 (3)	6.85 ± 0.07 [b]		
Hexahydro-sila-difenidol	7.62 ± 0.03 (3)	7.92 ± 0.07 [a]		
p-Fluoro-hexahydro-sila-difenidol	6.92 ± 0.03 (3)	6.68 ± 0.03 [c]		
Trihexyphenidyl (1)				
(R)−1	9.01 ± 0.04 (3)	10.11 ± 0.07 [d]	457	1700
(S)−1	6.35 ± 0.06 (4)	6.88 ± 0.09 [d]		
Trihexyphenidyl methiodide (2)				
(R)−2	9.47 ± 0.13 (3)	10.61 ± 0.10 [d]	245	661
(S)−2	7.08 ± 0.01 (3)	7.79 ± 0.04 [d]		
Hexahydro-difenidol (3)				
(R)−3	7.97 ± 0.04 (3)	8.71 ± 0.05 [e]	115	550
(S)−3	5.91 ± 0.02 (3)	5.97 ± 0.04 [e]		
Hexbutinol (4)				
(R)−4	7.75 ± 0.09 (4)	8.78 ± 0.05 [e]	2.5	107
(S)−4	7.35 ± 0.09 (4)	6.75 ± 0.07 [e]		
Hexbutinol methiodide (5)				
(R)−5	8.20 ± 0.02 (3)	9.43 ± 0.06 [e]	4.6	40
(S)−5	7.54 ± 0.10 (4)	7.83 ± 0.05 [e]		
p-Fluoro-hexbutinol (6)				
(R)−6	6.85 ± 0.04 (8)	8.08 ± 0.06 [e]	1.8	34
(S)−6	6.59 ± 0.18 (5)	6.55 ± 0.08 [e]		

3.4. Comparison of affinities for M_1 receptors in calf ganglia and rabbit vas deferens

Binding affinities (pK_1 values) for M_1 receptors in calf superior cervical ganglia were found to be highly correlated with functional potencies (pA_2 values) at M_1 receptors in rabbit isolated vas deferens ($r^2 = 0.86$) (fig. 5). However, the slope of the experimental equality line was not equal to unity (slope = 0.66 ± 0.07).

Pirenzepine, methoctramine, hexahydro-sila-difenidol and p-fluoro-hexahydro-sila-difenidol revealed pK_1 values in calf superior cervical ganglia quite similar to the pA_2 values in rabbit vas deferens (table 1 and fig. 5) and rat ganglia (Eltze et al., 1988; Lambrecht et al., 1989a; Field and Newberry, 1989), thus confirming the selective labelling of M_1 receptors in calf ganglia with [^{3}H](±)-telenzepine. The same holds true for the affinities of the (S) enantiomers of compounds 3, 5 and 6 determined in calf ganglia and rabbit vas deferens. The (S) enantiomers of compounds 1, 2 and 4, as well as the (R) enantiomers of all compounds ((R) − 1-(R) − 6), showed binding affinities in calf ganglia (pK_1 values) different from the pA_2 values observed in rabbit vas deferens, most of them being lower by factors of 3.4-17 (table 1, fig. 5). However, (S)-hexbutinol ((S) − 4), exhibited a pK_1 value (7.35) that exceeded the respective pA_2 value in rabbit vas deferens by a factor of 4.

3.5. Stereoselectivity ratios

The stereoselectivity ratios at M_1 receptors in calf superior cervical ganglia were lower than those obtained in rabbit vas deferens. For compounds without a triple bond in the carbon chain (1-3, fig. 1), stereoselectivity was lower by factors of 2.7-4.8. For the acetylenic enantiomers (4-6), there was no pronounced stereoselectivity at M_1 receptors in calf ganglia.

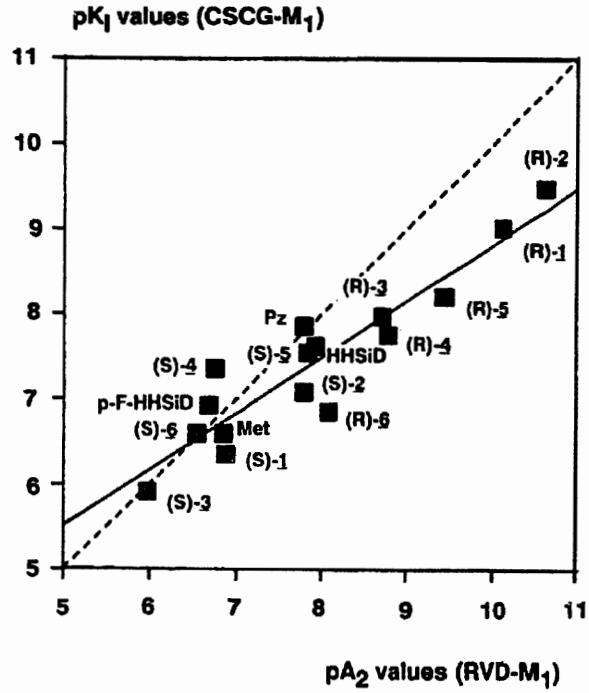


Fig. 5. Relationship between mean pA_2 and pK_1 values, respectively, (see table 1) of pirenzepine (Pz), methoctramine (Met), hexahydro-sila-difenidol (HHSiD), p-fluoro-hexahydro-sila-difenidol (p-F-HHSiD) and the (R) and (S) enantiomers of compounds 1-6 measured in functional studies using rabbit vas deferens and at M_1 binding sites in calf superior cervical ganglia. The dotted line represents the 1:1 relationship. All data were best correlated by the solid line, with a slope different from unity (slope = 0.66 ± 0.07, $r^2 = 0.86$).

4. Discussion

4.1. [^{3}H](±)-Telenzepine as radioligand in crude preparations of calf superior cervical ganglia

The present study was designed to develop a simple method to determine the affinity of antimuscarinic agents for M_1 receptors in calf superior cervical ganglia, using the highly potent novel radioligand [^{3}H](±)-telenzepine. In contrast, [^{3}H]pirenzepine did not label muscarinic M_1 receptors in crude ganglia preparations satisfactorily due to unfavourable specific/non-specific binding ratios. The non-specific binding of [^{3}H](±)-telenzepine was low enough in ganglionic preparations to determine reproducibly M_1 affinities in saturation as well as in competition experiments. The presence of about 30-50% of M_2 receptors in superior cervical ganglia (Giraldo et al., 1985; Galvan et al., 1989) did not seem to influence the selective labelling of M_1 receptors, since Scatchard representation of the data (fig. 2, inset) indicated a homogeneous receptor population.

The racemic structure of telenzepine and the existence of chemically stable enantiomers raised questions about the affinities of these enantiomers at muscarinic M_1 receptors in calf superior cervical ganglia. As shown by Eveleigh et al. (1989), the affinity of (+)- and (−)-telenzepine towards M_1 receptors present in rat cerebral cortex differ by a factor of 510, with (+)-telenzepine being more potent than the respective (−) enantiomer. Therefore, it is reasonable to assume that, in calf superior cervical ganglia, only (+)-telenzepine will be bound to M_1 receptors when the racemate is used. The availability of the pure (+) enantiomer of [^{3}H]telenzepine would aid further progress in muscarinic receptor research.

The results of the kinetic experiments in calf ganglia with [^{3}H](±)-telenzepine ($k_1 = 0.017$ min^{-1} nM^{-1}, $k_{-1} = 0.017$ min^{-1}, $K_D = 1.00$ nM) confirmed both the affinity value estimated from saturation experiments ($K_D = 1.12$ nM) and the monoexponential character of the ligand-receptor interaction. The slow dissociation of [^{3}H](±)-telenzepine from M_1 receptors, with a half-time of about 41 min, is consistent with observations reported previously (Schudt et al., 1988; Eveleigh et al., 1989; Galvan et al., 1989).

It should be noted that in order to achieve reasonable receptor density in the membrane preparation, the procedure for preparing the crude pellets of calf ganglia is critical. High tissue concentrations are required for effective homogenization with an Ultra Turrax, although a great loss of tissue (ca. 80%) has to be accepted, which is due to enrichment of the membrane fraction.

4.2. Binding affinities of antimuscarinic drugs

In general, the binding affinities of the antimuscarinic compounds investigated were consistent with a competitive interaction between these drugs and the [^{3}H](±)-telenzepine-labelled muscarinic M_1 receptor population. The observed deviations (Hill slopes greater than unity for some compounds in preliminary experiments and the significantly better two-binding site fit for the quaternary compound (R) − 2) might be explained by the different structural and physico-chemical properties of the compounds.

Compounds with a triple bond (4-6), as well as those with a central silanol moiety (hexahydro-sila-difenidol and its p-fluoro analogue), seemed to adhere to the glass vials or other materials during the incubations. As a consequence, at low concentrations the actual concentration of competitor in the incubation mixture was lower than the value calculated, thus resulting in an apparently higher amount of radioligand bound. At high concentrations of competitor, however, adsorption was saturated and caused relatively little additional binding of radioligand and therefore the slope of the fitted competition curve was greater than unity. Adsorption was circumvented by using bovine serum albumin in the experiments with these compounds. The Hill slopes were shifted to values not significantly different from unity and no effect on the affinity constants of the compounds was observed.

Previous investigations of the behaviour of antimuscarinic compounds in direct binding and competition experiments have revealed heterogeneity of antagonist binding sites, which has been explained by isomerization of the antagonist-receptor complex (Jarv et al., 1979). For instance, Lee and El-Fakahany (1985) reported on such effects with [^{3}H]quinuclidinyl benzilate as radioligand in competition experiments with quaternary antimuscarinic compounds at muscarinic receptors in rat brain. Since classical quaternary antimuscarinic compounds fail to discriminate between muscarinic receptor subtypes (El-Fakahany et al., 1986), this effect should not be regarded as being caused by the presence of two distinct receptors. In contrast to El-Fakahany et al. (1986), who found different equilibrium dissociation constants when they analysed N-methylscopolamine binding in various [^{3}H]quinuclidinyl benzilate-labelled brain tissues by a one-site or a two-site model, analysis of (R)-trihexyphenidyl methiodide ((R) − 2) binding did not reveal significantly different dissociation constants in the one-site model and the high-affinity site of the two-site model in [^{3}H](±)-telenzepine-labelled M_1 receptors in calf ganglia, possibly due to the small fraction of low-affinity sites.

4.3. Affinities in calf superior cervical ganglia and rabbit vas deferens

Comparison of the affinities for M_1 receptors in calf superior cervical ganglia and the antimuscarinic potencies in rabbit vas deferens revealed some differences in

the absolute values of the compounds investigated (table 1, fig. 5). One reason for these discrepancies could be the different ionic conditions in the binding and functional experiments. Tris-HCl buffer, which is similar to the buffers used in previous binding studies with ganglionic tissues (Hammer and Giachetti, 1982), was used in the binding assays for M_1 receptors in calf ganglia; however, modified Krebs buffer (for composition see Feifel et al., 1990) was used as the bath fluid in functional experiments with M_1 receptors of rabbit vas deferens. Although both buffers are similar to the physiological environment in terms of their ionic strength, distinct influences on receptor-ligand interaction cannot be excluded due to their different ionic composition.

As has been shown recently by Delmendo et al. (1989), the influence of different ionic condition, on the binding affinities of antimuscarinic agents may complicate attempts to correlate binding and functional data. Therefore, any correlation between affinities determined under different ionic conditions should be examined more in terms of their rank order of potency than in terms of their absolute values.

In conclusion, the new radioligand [^{3}H](±)-telenzepine is useful for muscarinic receptor research. It shows high affinity for muscarinic M_1 receptors and labels them selectively even in tissues with unfavourable specific/non-specific binding ratio for [^{3}H]pirenzepine. Crude preparations of calf superior cervical ganglia labelled with [^{3}H]telenzepine can be used as a simple model to investigate the M_1 properties of antimuscarinic drugs.

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