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Research Article

Different Behavior Toward Muscarinic Receptor Binding Between Quaternary Anticholinergics and Their Tertiary Analogues

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A number of corresponding tertiary and quaternary anticholinergic analogues were examined for their ability to inhibit specific ^{3}H-dexetimide binding to calf brain muscarinic receptors. In all cases the tertiary antagonists (except pirenzepine) showed steep and monophasic inhibition curves, whereas those of the quaternary derivatives were shallow (thiazinamium, methylbenactyzine) or even biphasic (oxyphenonium, methylatropine, methylscopolamine). These observations show that the addition of a methyl group to the nitrogen atom changes the mode of interaction of the anticholinergics to muscarinic receptor binding sites. Whether there are separate binding sites present or differences in interaction mode for only the quaternary moiety is discussed.

KEY WORDS: Muscarinic receptors; calf brain; anticholinergics; heterogeneity; receptor interaction.

INTRODUCTION

The chemical structure of acetylcholine and those of various muscarinic agonists, such as methylfurthretonium (1), muscarine, muscarone, dioxolanes and other pentacyclic compounds, aceclidine and oxotremorine (2), and piperidine, quinuclidine, and thiacyclohexane derivatives (3), are remarkably similar. The presence of the ester function (or functionally comparable groups) and the amino group at a constant distance in acetylcholine and in these agonists allows several muscarinic conformations that could accommodate different interaction modes with the receptor.

However, such structural resemblances are often absent between acetylcholine and the antimuscarinic drugs. Furthermore, only a few antagonists, with a quaternary moiety appear to have been studied. The antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide had a 10-fold difference in binding potency between the heart and the intestine of the rat (4), and *N*-methylscopolamine discriminates between two binding sites in the rat heart (5).

Figure 1 shows inhibition curves of the enantiomers of oxyphenonium (OX) at muscarinic receptors from calf brains, with the pure anticholinergic drug dexetimide as the labeled ligand (6). The biphasic shape of these curves indicates the presence of different muscarinic receptor subtypes, binding sites, or receptor conformations and suggests OX as a suitable probe for further identification of the muscarinic receptor binding sites. However, *N*-desmethyl oxyphenonium, the tertiary analogue of OX, gave an inhibition curve comparable to that of dexetimide and following the law of mass action of interaction with a single binding site. This suggested that the quaternary moiety is involved in the bimodal interaction with the muscarinic binding sites.

The aim of the present study was to investigate in more detail whether the above receptor heterogeneity is linked to the quaternary character of the N atoms. Therefore, a number of corresponding tertiary and quaternary anticholinergic analogues were tested in the radioreceptor assay (RRA) over a wide concentration range and compared with the tertiary antagonist pirenzepine (M_1 selective) and the tertiary agonist oxotremorine. The influence of the nonhydrolyzable guanine nucleotide, 5′-guanylylimidodiphosphate (GppNHp), on the latter two compounds was studied as well. The receptor heterogeneity described here was already mentioned by Ensing (6); however, no computer modeling of the obtained inhibition curves was carried out at that time, which is reported here.

MATERIALS AND METHODS

Chemicals

^{3}H-Dexetimide (^{3}H-DEX; 15 Ci/mmol) was supplied by IRE (Utrecht, The Netherlands). Unlabeled dexetimide was kindly donated by Janssen Pharmaceutica (Beerse, Belgium). Oxyphenonium bromide was a gift from Ciba–Geigy (Basel, Switzerland), while pirenzepine dihydrochloride was a gift from Boehringer (Ingelheim, FRG). Atropine, methylatropine bromide, scopolamine hydrobromide, methylscopolamine bromide, promethazine hydrochloride, thiazinamium methylsulfate, benactyzine hydrochloride, benzilonium bromide, and oxotremorine were commercially available. *N*-Desmethyloxyphenonium and methylbenacty-

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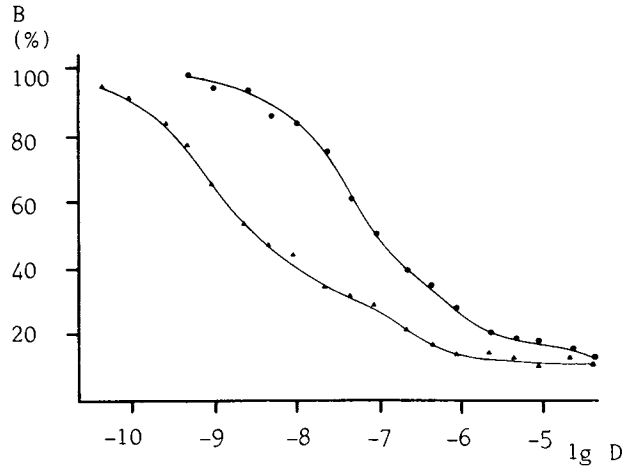

Fig. 1. Inhibition of ^{3}H-DEX binding by *d*-oxyphenonium (▲) and *l*-oxyphenonium (●). D is the concentration of the enantiomer (M); B is bound radiolabeled ligand.

zine iodide were synthesized at our laboratory. 5'-Guanylylimidodiphosphate was obtained as its sodium salt from Sigma (St. Louis, Mo., USA). All other chemicals of analytical grade were obtained from Merck (Amsterdam, The Netherlands). Polyethylene tubes were from Greiner (Alphen a/d Rijn, The Netherlands). GF/B glass-fiber filters were obtained from Whatman (Maidstone, UK). The 50 mM sodium phosphate buffer (pH = 7.4) was composed of 4 vol 50 m*M* sodium dihydrogen phosphate and 1 vol 50 m*M* disodium hydrogen phosphate. Plasmasol as the scintillation liquid was obtained from Packard Instruments (Groningen, The Netherlands).

Preparation of Receptor Material

Freshly prepared calf brains without the cerebellum were homogenized in 12 vol 0.32 *M* sucrose using a Teflon–glass Potter–Elvejehem homogenizer at 1200 rpm (R.W. 18, Janke & Kunkel, Staufen i. Breisgau, FRG). The homogenate was centrifuged for 10 min at 1000*g*, the pellet was discarded, and the supernatant was centrifuged for 60 min at 100,000*g*. The pellet (P_2) was resuspended in buffer and used

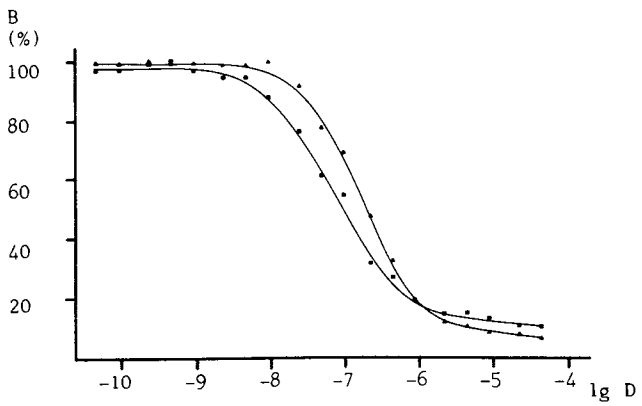

Fig. 2. Inhibition of ^{3}H-DEX binding by promethazine hydrochloride (▲) or thiazinamium methylsulfate (■). B is bound ^{3}H-DEX—total binding is 100%; D is the concentration of drug (*M*).

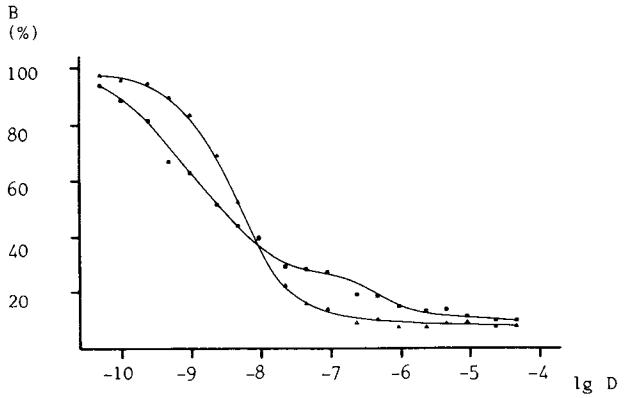

Fig. 3. Inhibition of ^{3}H-DEX binding by desmethyloxyphenonium hydrochloride (▲) or oxyphenonium bromide (■). B is bound ^{3}H-DEX—total binding is 100%; D is the concentration of drug (*M*).

immediately or stored at −80°C. The receptor concentration of the prepared membrane suspension was determined as follows. The receptor suspension was incubated with increasing concentrations of ^{3}H-DEX, with or without 2×10^{-6} *M* unlabeled dexetimide. The difference in binding is specific receptor-bound radiolabeled ligand, and by means of Scatchard analysis the receptor concentration was calculated. Subsequently, the receptor suspension was diluted to a final concentration of 1.15×10^{-8} *M*.

Radioreceptor Assay

To duplicate tubes was added 25 µl of aqueous solutions of anticholinergics, to give final concentrations in the assay varying from 5×10^{-12} to 5×10^{-4} *M*. Then 25 µl of a solution of the radiolabeled ligand ^{3}H-dexetimide was added to give a final concentration of 2×10^{-9} *M*, and finally 500 µl of a freshly thawed receptor preparation was added to give a receptor concentration of approximately 5×10^{-10} *M*. Polyethylene tubes were used because of its low nonspecific binding of ^{3}H-dexetimide (6). After mixing, the tubes were incubated for 10 min at 37°C. Then they were mixed

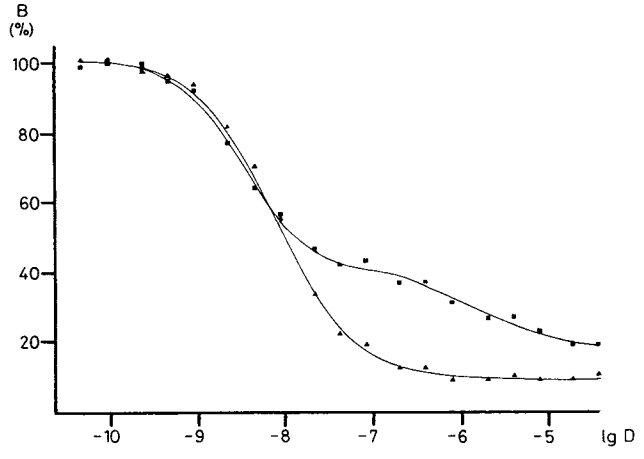

Fig. 4. Inhibition of ^{3}H-DEX binding by atropine (▲) or methylatropine bromide (■). B is bound ^{3}H-DEX—total binding is 100%; D is the concentration of drug (*M*).

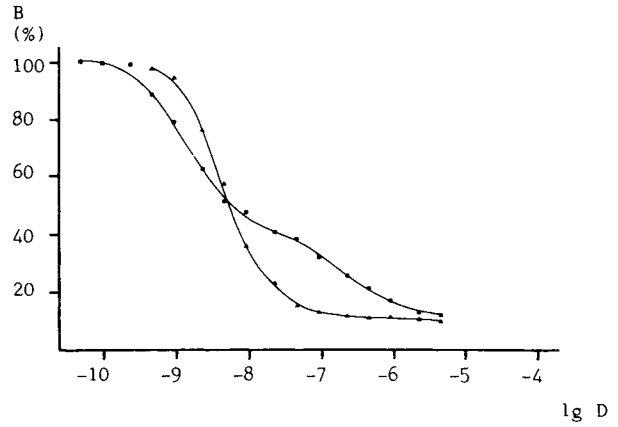

Fig. 5. Inhibition of ^{3}H-DEX binding by scopolamine hydrobromide (▲) or methylscopolamine (■). B is bound ^{3}H-DEX—total binding is 100%; D is the concentration of drug (*M*).

again after the addition of 4 ml ice-cold phosphate buffer. The samples were immediately filtered through Whatman GF/B glass-fiber filters under vacuum, using a filtration apparatus (Multividor 40 S, Janssen Scientific Instruments, Beerse, Belgium). The tubes were rinsed with 4 ml ice-cold buffer, which was also filtered. The filters were washed with 4 ml ice-cold buffer and dispersed in 6 ml Plasmasol by shaking for 60 min. The total filtration, rinsing, and washing process, taking place in approximately 15 sec, was carried out on each tube separately. The vials were counted for 10 min or to 40,000 counts in a liquid scintillation counter (Isocap 300, Nuclear Chicago Division, Searle, Chicago, Ill., USA). All binding values were expressed as disintegrations per second (dps) and converted from counts per second (cps). The counting efficiency of these samples ranged between 47 and 50%. The inhibition curves were corrected for small variations in the maximum binding values set at 100%. No corrections for nonspecific binding were made. The inhibition curves were fitted with the ligand method of Munson and Rodbard (14). The best fits for a one- and a two-site model were compared with an *F* test; a two-site model was accepted when $P < 0.05$.

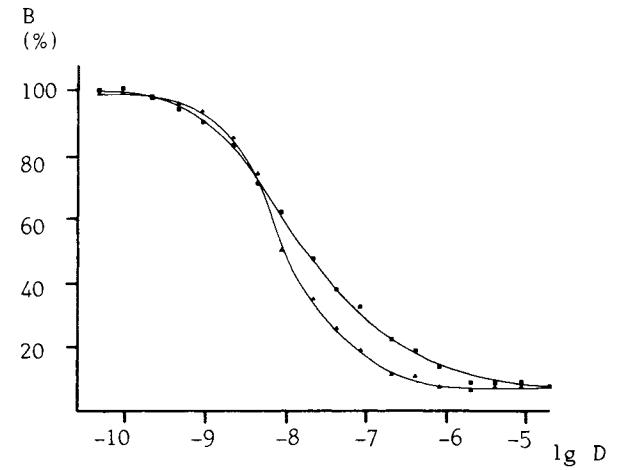

Fig. 6. Inhibition of ^{3}H-DEX binding by benactyzine hydrochloride (▲) or methylbenactyzine iodide (■). B is bound ^{3}H-DEX—total binding is 100%; D is the concentration of drug (*M*).

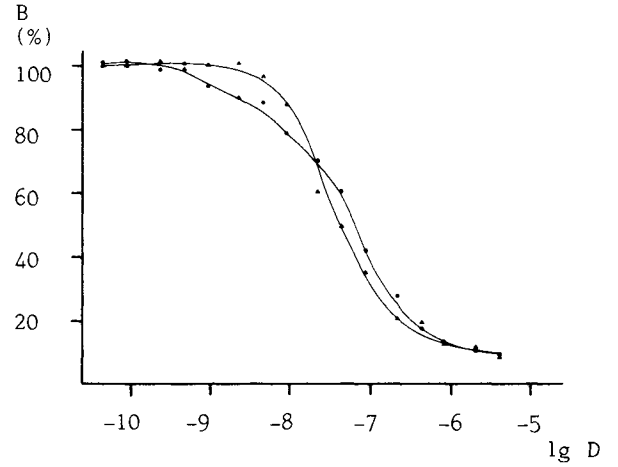

Fig. 7. Inhibition of ^{3}H-DEX binding by oxotremorine in the presence (▲) or absence (●) of GppNHp. B is bound ^{3}H-DEX—total binding is 100%; D is the concentration of drug (*M*).

RESULTS AND DISCUSSION

The inhibition curves for the tertiary and quaternary anticholinergic analogues were determined in paired experiments using the same batch of membranes and radiolabeled ligand (Figs. 2–9). Figure 10 shows the compounds that were compared in this study.

When the inhibition curves of each pair of analogues are compared, again differences were noted between tertiary and quaternary compounds. In all cases the tertiary antagonists (except pirenzepine) showed steep and monophasic inhibition curves, whereas those of the quaternary derivatives were shallow (thiazinamium, methylbenactyzine) or clearly biphasic (remaining compounds). Similar results with rat brain muscarinic receptors and methylatropine or methylscopolamine as competitive or radiolabeled ligand were obtained by Ellis and Lenox (7) and Lee and El-Fakahany (8).

All displacement curves were analyzed with the ligand curve-fitting program, and nonspecific binding could be determined adequately because the competition curves were sufficiently detailed. A two-site model was accepted over a

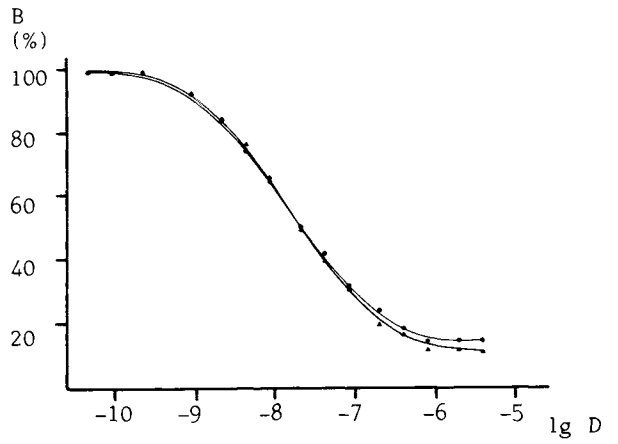

Fig. 8. Inhibition of ^{3}H-DEX binding by pirenzepine dihydrochloride in the presence (▲) or absence of GppNHp (●). B is bound ^{3}H-DEX—total binding is 100%; D is the concentration of drug (*M*).

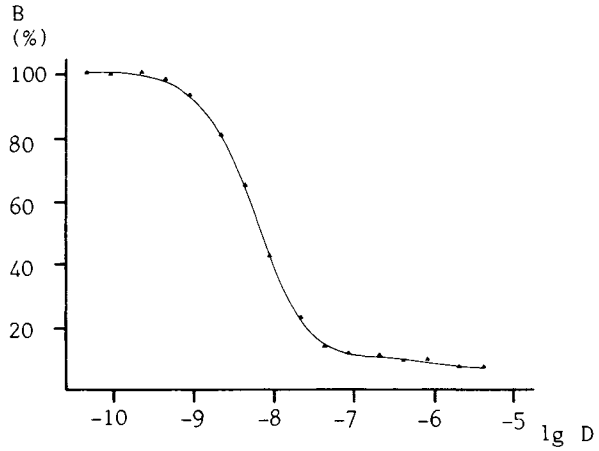

Fig. 9. Inhibition of ^{3}H-DEX binding by dexetimide (▲). B is bound ^{3}H-DEX—total binding is 100%; D is the concentration of drug (M).

one-site model when $P < 0.05$ in the F test. The data shown in Table I indicate that, except for pirenzepine, the interaction of the tertiary anticholinergics with the muscarinic receptor can be described by the law of mass action for a single site. However, except for thiazinamium, the interaction of the quaternary anticholinergics with the receptor can be described more adequately by a two-binding site model. The relative distribution of high- and low-affinity sites shows some variation with the different antagonists. Nevertheless, this variation, or the heterogeneity as such, is difficult to explain by the variation in lipophilicity of the drugs.

With pirenzepine a two-component displacement was also found, in agreement with previous data with cortical membranes (9). These authors also found that the addition of GppNHp did not influence the displacement curve (Fig. 8). Moreover, with the tertiary agonist oxotremorine the expected steepening of the displacement curve with the addition of GppNHp was found (Fig. 7) (10), confirming the transition of high (and superhigh) states into a low-affinity state. However, no statistically ($P < 0.05$) better fit for a two-site model could be made for the experiment without GppNHp.

The above observations show that the addition of a methyl group to the nitrogen atom to produce quaternary compounds changes the interaction mode of the anticholinergics to muscarinic binding sites. The difference in binding properties between the quaternary and the tertiary anticholinergics is remarkable, on the one hand, because the tertiary compounds are largely protonated at physiological pH,

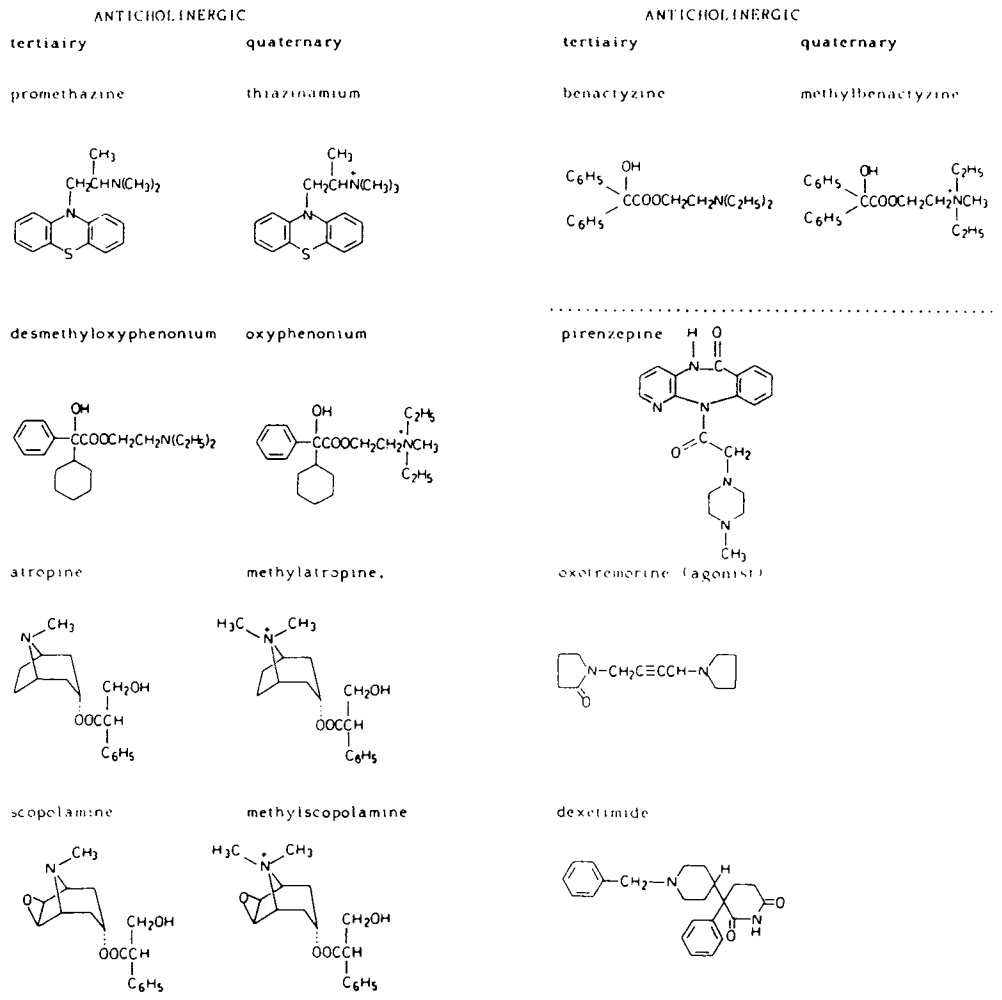

Fig. 10. Structures of tested tertiary and quaternary anticholinergic analogues.

Table I. Results of the Ligand Curve Fitting for the Displacement of ^{3}H-DEX from Calf Brain Muscarinic Receptors by a Series of Tertiary and Quaternary Anticholinergic Analogues

	K_{D1} (*M*)	K_{D2} (*M*)	R1 (%)	R2 (%)
Promethazine	3.17 E-8		100	
Thiazinamium	1.46 E-8		100	
Desmethyloxyphenonium	8.47 E-10		100	
Oxyphenonium	2.65 E-10	4.50 E-8	75.2	24.8
Atropine	4.52 E-10		100	
Methylatropine	6.49 E-10	4.98 E-7	76.0	24.0
Scopolamine	4.29 E-10		100	
Methylscopolamine	1.64 E-10	2.86 E-8	68.8	31.2
Benactyzine	1.90 E-9		100	
Methylbenactyzine	1.20 E-9	4.17 E-8	71.6	28.4
Oxotremorine	1.01 E-6		100	
Oxotremorine + GppNHp	6.37 E-7		100	
Pirenzepine	5.03 E-9	7.41 E-8	40.6	59.4
Pirenzepine + GppNHp	1.48 E-8	1.08 E-7	60.8	39.2
Dexetimide	3.75 E-10		100	

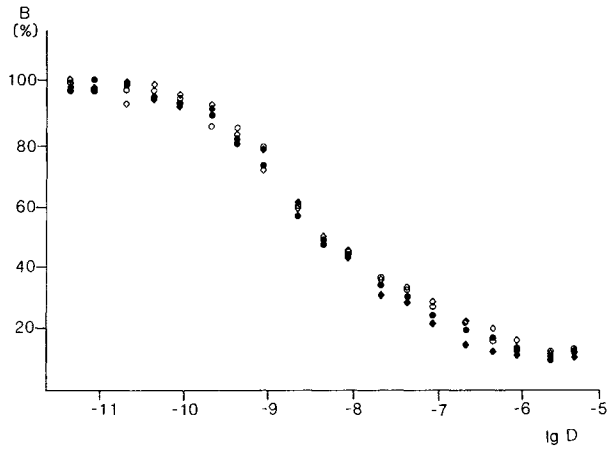

Fig. 12. Effect of incubation time [10 (◇), 30 (●), 60 (○), and 120 min (◆)] on the inhibition of ^{3}H-DEX by *N*-methylscopolamine. B is bound ^{3}H-DEX; D is the concentration of drug (*M*).

with the binding associated with the charged species according to Barlow and Winter (11). On the other hand, the fast association and dissociation of the proton to the N atom avoid the formation of two conformational states that remain stable during the association process with the receptor. In the case of pirenzepine, which also shows a biphasic interaction with muscarinic receptors, two relatively stable conformations coexist (12) in aqueous solutions, with the protonated form of pirenzepine having a higher affinity for the receptor than the uncharged drug (13).

Whether there are separate binding sites present or differences in the interaction mode for the quaternary moiety remains to be established. In separate experiments using the d- and l-enantiomers of oxyphenonium, it was found that GppNHp did not influence the biphasic displacement curves seen with both isomers (data not shown). These results indicate that GppNHp-sensitive affinity states cannot account for the biphasic nature of the interaction.

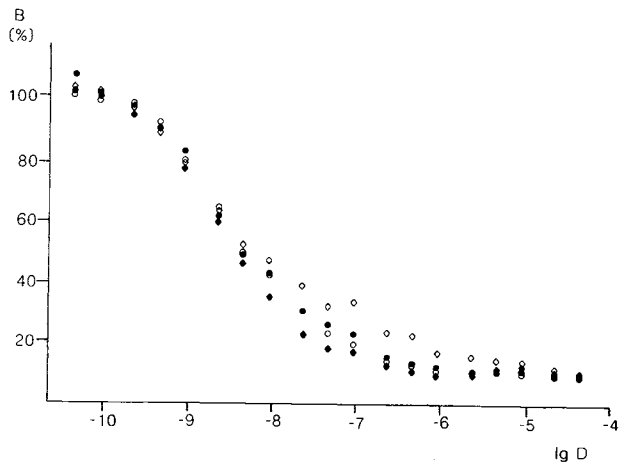

Fig. 11. Effect of incubation time [10 (◇), 30 (●), 60 (○), and 120 min (◆)] on the inhibition of ^{3}H-DEX by oxyphenonium. B is bound ^{3}H-DEX—total binding is 100%; D is the concentration of drug (*M*).

In order to exclude inadequate equilibration as a cause of the observed differences, the inhibition experiments with *N*-methylscopolamine and oxyphenonium were repeated with incubation times of 10, 30, 60, and 120 min at 37°C. Despite small shifts in the second phase of the curves of the quaternaries, the biphasic character of these curves was still present after 120 min, as can be seen from Figs. 11 and 12 and Table II. No statistically significant differences were found for the equilibrium dissociation constants and receptor concentrations of oxyphenonium and methylscopolamine for the different times of incubation. Therefore, one can rule out the following alternative explanations of the biphasic receptor interaction, i.e., the presence of membrane vesicles with inside receptors or the existence of structurally identical receptors inside the lipophilic part of the membrane. In these circumstances the lipophilic tertiary ligands may easily reach the receptor, whereas for the more polar quaternaries the diffusion rate into and through the membrane is so slow that equilibrium cannot be obtained within 10 min. In that case, a 12-fold increase in incubation time should result in a steepening of the curve and a concomitant 12-fold decrease in K_{D2}, which was not detected. Moreover the variation in lipophilicity of the quaternary anticholinergic analogues did not result in any systematic differences in the ratio of high- and low-affinity binding sites. The lipo-

Table II. Results of the Ligand Curve Fitting for the Displacement of ^{3}H-DEX from Calf Brain Muscarinic Receptors by Oxyphenonium and *N*-Methylscopolamine; Influence of Incubation Time

	K_{D1} (*M*)	K_{D2} (*M*)	R1 (%)	R2 (%)
Methylscopolamine				
10 min	1.64 E-10	2.86 E-8	68.8	31.2
30 min	3.42 E-10	3.42 E-8	72.0	28.0
60 min	2.75 E-10	2.42 E-8	71.7	28.3
120 min	2.97 E-10	1.41 E-8	73.4	26.6
Oxyphenonium				
10 min	2.65 E-10	4.50 E-8	75.2	24.8
30 min	2.73 E-10	1.67 E-8	78.5	21.5
60 min	2.61 E-10	6.41 E-9	72.4	27.6
120 min	3.94 E-10	1.57 E-8	90.3	9.7

philicity of the quaternaries is based on the extraction constants of their ion pair with picrate into dichloroethane (unpublished data).

The clinical observations of Van Bork *et al.* (15) with oxyphenonium and thiazinamium in the treatment of chronic obstructive lung diseases indicate that these two anticholinergics are equipotent bronchodilators. However, the anticholinergic side effect of thiazinamium (tachycardia) is more pronounced than with oxyphenonium. Presently, no proper relation between this organ selectivity and the heterogeneity of muscarinic receptor binding sites of the quaternary anticholinergics can be given. Further investigations on receptor populations in other organs (such as heart, lung, ileum) using selected quaternary anticholinergics may clarify the therapeutic action of these compounds.

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