Br. J. Pharmac. (1982) **77**, 731–739

THE BINDING PROPERTIES OF THE MUSCARINIC RECEPTORS OF THE CYNOMOLGUS MONKEY CILIARY BODY AND THE RESPONSE TO THE INDUCTION OF AGONIST SUBSENSITIVITY

E. BÁRÁNY*, C.P. BERRIE, N.J.M. BIRDSALL, A.S.V. BURGEN & E.C. HULME

Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA and Department of Medical Pharmacology*, University of Uppsala, Sweden.

1 The binding properties of the muscarinic receptors in the ciliary muscle of cynomolgus monkeys have been evaluated.

2 The concentration of receptor binding sites is the highest yet reported. As found in many species and tissues, there are subclasses of agonist binding sites. Agonist binding is not affected by the non-hydrolysable guanosine triphosphate (GTP) analogue, GppNHp, suggesting that these receptors are not linked to adenylate cyclase.

3 Ciliary muscles made subsensitive by treatment with muscarinic agonists have a decreased receptor concentration but no other changes in the binding properties of the receptors could be detected.

Introduction

In the medical treatment of glaucoma, the anterior part of the eye often is chronically exposed to high concentrations of autonomic drugs, such as cholinesterase inhibitors, muscarinic agonists, α- and β-adrenoceptor agonists and β-antagonists. At least part of the therapeutic action of the cholinergic drugs is on the iris sphincter (in angle closure glaucoma) and the ciliary muscle (in chronic simple glaucoma). These smooth muscles are very fast, very densely innervated multiunit muscles (Ishikawa, 1962; Laties & Jacobowitz, 1966; van der Zypen, 1967) strikingly different from those of the intestine (van der Zypen, 1967). Under the conditions of cholinergic glaucoma treatment, they can be expected to develop some cholinergic subsensitivity and this, in fact, has been observed in the iris sphincter of a variety of animal species (Bito, Dawson & Petrinovic, 1971; Bito & Baroody, 1979) and in man (Smith & Smith, 1980). Subsensitivity has also been demonstrated in the ciliary muscle of several species of monkeys (Kaufman & Bárány, 1975; Bárány, 1977). Several explanations for the agonist-induced subsensitivity of the iris sphincter have been put forward. It has been ascribed to receptor loss (Raina & Bito, 1979) in a study of the cat iris, to a loss in affinity in a study on the albino rabbit (Mittag, 1978) and the opposite phenomenon, denervation supersensitivity in the iris of the cat, has been ascribed to post-receptor changes (Sachs, Kloog, Korczyn, Heron & Sokolovsky, 1979). The muscarinic receptors in subsensitivity of the ciliary muscle have not been studied previously. In this paper the ligand binding properties of the muscarinic receptors in the ciliary muscle of cynomolgus monkeys have been defined and any changes that occur in the receptors of muscles made physiologically subsensitive by carbachol or pilocarpine, evaluated.

Methods

Three groups of cynomolgus monkeys (*Macacus irus*) were used. The first group of 6 monkeys had been iridectomized several years earlier to allow accurate determination of refraction even under cholinergic stimulation which normally would have constricted the pupil too much (Table 1). The second and third groups each consisted of 8 intact young monkeys, to be killed as kidney donors. The iridectomized group had been used many times for cholinergic stimulation and subsensitivity experiments over several years. The intact groups had never been treated with drugs and were correspondingly younger. None of the animals was of exactly known age. They were kept on a diet of R3 and A5 pellets from Astra-Ewos, Södertälje, Sweden, with the addition of fresh fruit and raw potatoes.

Production of unilateral cholinergic subsensitivity

In 14 of the intact and 2 of the iridectomized monkeys the procedure was as follows: on day 1 the monkey was anaesthetized with intramuscular ketamine (10–12 mg/kg) and a lid speculum inserted

0007-1188/82/120731-09 $01.00

Table 1 Details of cynomolgus monkeys used in the experiments

Monkey	*In use since*	*Sex, body weight*[a]	*Last unilateral miotic treatment*[b]	*Last systemic test*[c]	*Terminal experiment*
A	1975	M, 4.9	Jan 76	July 15,80	Aug 22,80
B	1976	F, 2.8	Apr 80	July 22,80	Aug 19,80
C	1975	F, 4.3	Nov 79	July 15,80	Aug 28,80
D	1976	M, 5.0	Nov 79	July 16,80	Aug 28,80
E	1976	F, 1.6	Dec 79	July 16,80	Aug 26,80
F	1976	F, 3.3	Aug 79	July 17,80	Aug 26,80

[a] At terminal experiment; [b] different topical direct-acting cholinergics; [c] to test for complete recovery: pilocarpine followed by atropine, same result on both eyes.

on one eye. Then 10 μl of a 1% solution of carbachol chloride was applied to the cornea of that eye in the form of 10 droplets of 1 μl each, delivered centrally on to the cornea at 60 s intervals. After the last droplet a hard plastic contact lens was placed on the cornea and kept there for 30 min. This dose, 100 μg of carbachol, causes an intense contraction of the ciliary muscle (and iris sphincter if present). Both miosis and myopia disappear in the course of a week and leave a subsensitive ciliary muscle at day 8. On this day, the irridectomized group was tested for subsensitivity and the animal killed after the eyes had been excised *in vivo*.

In two iridectomized monkeys subsensitivity was produced not with carbachol but with pilocarpine continuously released at 30 μg/h as described previously (Bárány, 1977). The treatment started on day 1 and finished with removal of the releasing device on day 6. On day 7, subsensitivity was tested and the eyes excised and dissected. At that time, no drug-induced accommodation remained before the infusion of pilocarpine. The rest of the treatment was the same as for the other animals.

There were also two iridectomized and two intact monkeys without any unilateral treatment.

Testing of subsensitivity

The animals were anaesthetized with intramuscular ketamine supplemented with intramuscular pentobarbitone. Contact lenses (optic strength −7D) were placed on the corneae and the animal laid prone in front of a Zeiss-Hartinger coincidence optometer. The adjustment dial of the instrument was coupled to a helical potentiometer connected to a stabilized d.c. supply and a strip chart recorder. The contraction of the ciliary muscle was measured as change in refraction and expressed as diopters of myopia. After a stable baseline refraction had been established, the ciliary muscle of both eyes was exposed to gradually increasing identical concentrations of pilocarpine by the intramuscular infusion into a thigh of pilocarpine HCl. The infusion was stopped when the treated eye showed a few diopters of accommodation, but since the drug was given intramuscularly some further rise in accommodation usually occurred. All the iridectomized animals had been subjected to this procedure repeatedly and only such monkeys were chosen which had shown identical responses on both sides after recovering from the last unilateral treatment.

The coincidence optometer is a subjective instrument in so far as the observer turns a dial to produce coincidence of certain images. The use of the strip chart recorder allowed the observer to set the instrument to coincidence repeatedly without having to read the scale. The record was evaluated only at the end of the experiment. Thus no element of suggestion could enter into the results. Refraction was measured alternately on the two eyes. One measurement consisted of 2–3 adjustments to coincidence (readings) and required about 1 min. Readings always agreed to within 0.5 D. Thus each eye yielded a value once every 1–2 min. Since the refractions were changing gradually, the values of the control eye were plotted against time and the control eye refraction corresponding to the time of the reading of the treated eye was obtained by linear interpolation. Since the pilocarpine level in the circulation could not be reliably measured and the relation between that level and accommodation is unknown, the response of the control eye was taken as a measure of the pilocarpine level and plotted on the abscissa scale while the simultaneous response of the treated eye was plotted on the ordinate scale (Figure 1). In this kind of plot, identical responses from the two eyes yield points on the 45° line.

Dissection

In the iridectomized group, the eyes were excised immediately after and under the same anaesthesia as the measurement of subsensitivity, with the systemic dose of pilocarpine injected for the measurement still circulating. In the 'intact' groups the monkeys were

used as kidney donors on day 8 after unilateral carbachol treatment to 6 of them and the eyes were excised from the cadavers about 1 h after death. The bodies had been at room temperature until then. The excised eyes were immediately placed in ice-cold saline and transported to the laboratory where dissection started about 3 h after death. Dissection was performed at room temperature but on a plastic sheet resting on ice. The eyes were bisected at the equator, the lens removed from the anterior half, and the remaining anterior eye shell cut into quadrants with razor blades. When still present, the iris was torn off. A cut was made at the level of the pars plana, behind the bulging ciliary body. Under the dissection microscope the fibrous membrane separating the ciliary processes from the ciliary muscle was grasped with fine forceps and lifted away from the muscle. This leaves the smooth muscle of the ciliary body easily accessible and after its anterior tendons had been cut behind the scleral spur it could be removed in one piece from each quadrant. The dissected ciliary processes with their underlying fibrous membrane and the pieces of muscle from each eye were separately pooled and frozen in isopentane cooled with dry ice and acetone. They were sent to London packed in dry ice for receptor analysis.

Drug doses refer to those of the free base.

Receptor binding assays

[^{3}H]-N-methylscopolamine (53 Ci/mmol or 83 Ci/mmol) was obtained from New England Nuclear.

Ciliary muscles were stored at −20°C until assayed. The individual muscles were weighed and homogenized at 4°C in 50 ml 100 mM NaCl/10 mM $MgCl_2$/20 mM HEPES-Na^+ pH 7.0 in a Polytron homogenizer (setting 5, 3 × 15 s). This homogenate was stored at 0°C until diluted further for the binding assays which were carried out using a microcentrifuge assay as described by Hulme, Birdsall, Burgen & Mehta (1978). Membranes (0.3–1 mg tissue/ml) were incubated in the same buffer used for homogenization at 30°C for 20 min with [^{3}H]-N-methylscopolamine and, where appropriate, varying concentrations of non-radioactive drugs. Non-

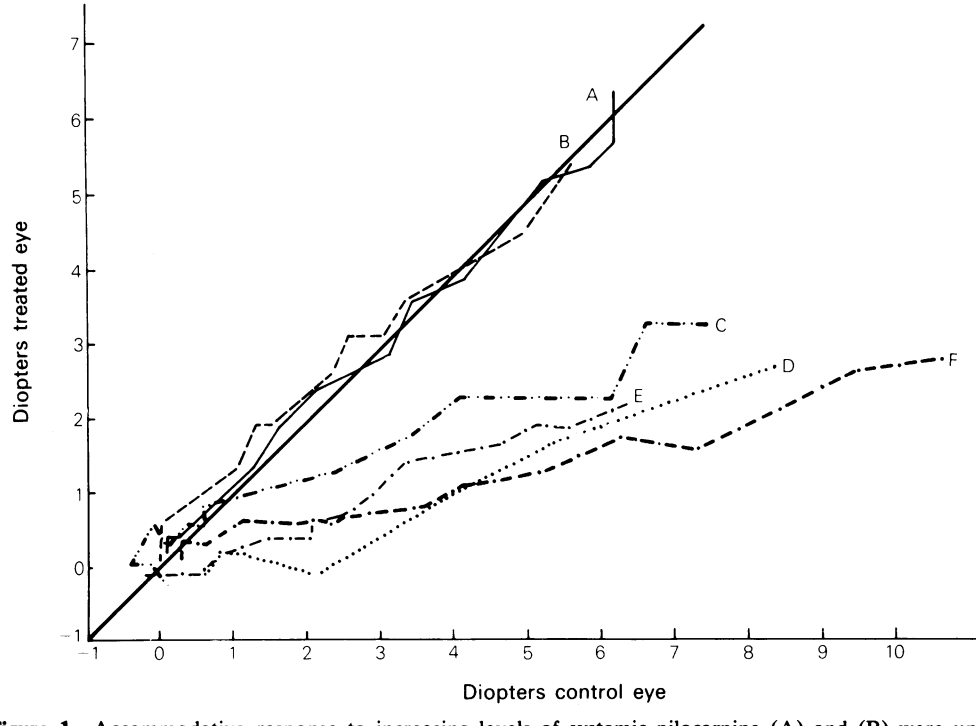

Figure 1 Accommodative response to increasing levels of systemic pilocarpine (A) and (B) were untreated monkeys. In (A) the right eye is plotted as 'treated', in (B) the left one. (A) received 1.72 mg/kg pilocarpine i.m. in an infusion lasting 25 min, (B) received 1.65 mg/kg over 18 min. (C) and (D) were unilaterally treated with carbachol and when tested received 1.16 mg/kg pilocarpine over 20 min and 1.39 mg/kg over 12 min respectively. (E) and (F) were unilaterally treated with pilocarpine and when tested received 2.37 mg/kg pilocarpine over 25 min and 0.85 mg/kg over 20 min respectively. For further details, see Table 1 and the experimental section. The solid line represents the line of equivalence.

specific binding was defined as the binding of [^{3}H]-N-methylscopolamine that was not inhibited by the presence in the incubation of a high concentration (10^{-6} M) of the potent muscarinic antagonist 3-quinuclidinylbenzilate. At 2×10^{-10} M [^{3}H-NMS, the non-specific binding was only 0.3–2% of the specific binding. Because of the limitations on the amount of tissue from the first and second groups, only single or duplicate estimates of [^{3}H]-NMS bound under various conditions was possible. In the studies on tissues from the third group, only the total numbers of specific receptor binding sites were estimated (homogenization volume 10 ml, 3–5 mg tissue/ml, quadruplicate estimates of specific and non-specific binding) using 10^{-8} M [^{3}H]-NMS.

Statistical analyses were carried out by paired, unpaired *t*-tests or analysis of variance, as appropriate, or by regression analysis as detailed in the text.

Results

The degree of subsensitivity produced in the iridectomized monkeys by the pretreatment given is shown in Figure 1. The two control monkeys A and B had very similar responses to systemic pilocarpine on the two eyes, their graphs run close to the diagonal. The two monkeys treated unilaterally with sustained release pilocarpine (E and F), or a single large dose of carbachol (C and D) evidently reacted less on the treated eyes. The degree of subsensitivity happened to be quite similar with both pretreatments but cannot be expressed in the conventional manner. Only in the case of D is there an indication of a rise in threshold on the treated side, in the other cases accommodation starts nearly simultaneously on both sides.

The results of the receptor binding studies are presented in Tables 2 and 3. The ciliary muscles were divided into several groups according to the treatment: (a) untreated control: animals not exposed to exogenous muscarinic agonists; (b) contralateral control: muscles contralateral to eyes receiving direct application of muscarinic agonists; (c) all controls: (a) and (b); (d) carbachol treated; (e) pilocarpine treated; (f) agonist treated: (d) and (e).

N-methylscopolamine binding

The radioligand used in the receptor binding studies was [^{3}H]-NMS, a potent muscarinic antagonist which binds with high affinity to central and peripheral muscarinic receptors (Birdsall, Burgen & Hulme, 1978; Berrie, Birdsall, Burgen & Hulme, 1979; Hammer, Berrie, Birdsall, Burgen & Hulme, 1980; Hulme, Berrie, Birdsall & Burgen, 1981). The extent of binding of [^{3}H]-NMS to the muscarinic receptors in homogenates of the ciliary muscles was measured at four [^{3}H]-NMS concentrations. In all cases the data were compatible with the presence of a uniform population of binding sites for [^{3}H]-NMS. This is illustrated by a Scatchard plot of the binding data for one muscle (Figure 2). The data points fit on a straight line, the intercept on the x-axis giving the concentration of binding sites (0.31 nM). In this muscle the receptor density was 300 fmol/mg tissue. The gradient of the Scatchard plot gives an estimate of the [^{3}H]-NMS affinity constant (2.1×10^9 M^{-1}), which is

Table 2† [^{3}H]-N-methylscopoamine binding by ciliary muscles and its inhibition by pirenzepine, pilocarpine and carbachol

	[^{3}H]-N-methylscopolamine binding		*% inhibition of [^{3}H]-N-methylscopolamine (2×10^{10} M) binding by:*			
	n	Affinity ($\times 10^9$ M^{-1})	*Pirenzepine* (10^{-7} M)	*Pilocarpine* (10^{-5} M)	*Carbachol* (10^{-4} M)	Carbachol (10^{-4} M) + GppNHp (10^{-4} M)
(a) Untreated control	8	1.8±0.5	26± 4	50± 5	37±11	37±13
(b) Contralateral control	10	2.0±0.3	27± 7	56±10	48±17	46±19
(c) All controls	18	1.9±0.3	27± 5	53± 9	43±15	42±16
(d) Carbachol-treated	8	2.1±0.3	28± 6	55± 9	49±14	49±13
(e) Pilocarpine treated	2	1.8, 1.7	29, 28	51,50	33, 31	30, 31
(f) Agonist-treated	10	2.1±0.3	28± 5	54± 8	45± 1	45±14

† The data on individual monkey ciliary muscles are available on request from NJMB.

Table 3 Concentrations of muscarinic receptors in ciliary muscle

	n		*Receptor concentration* (fmol/mg tissue ± s.e.mean)
Untreated control	4	Group I*	503 ± 70
	4	Group II	568 ± 64
Pooled results	8		535 ± 46
Contralateral control	4	Group I	284 ± 55
	6	Group II	395 ± 69
	8	Group III	192 ± 12
Pooled results	18		218 ± 33
Agonist treated	4†	Group I	215 ± 60
	6	Group II	323 ± 33
	8	Group III	136 ± 10
Pooled results	18		216 ± 26

† These data include the results from two animals treated with pilocarpine

* Groups I–III are separate groups of monkey ciliary muscles, dissected and assayed at different times (see also Methods).

comparable to that found in other tissues. In the muscles there was a small range of estimated affinity constants ($1.4–2.3 \times 10^9$ M^{-1}, mean 1.9 ± 0.3 (s.d.) $\times 10^9$ M^{-1}) but a wide range in concentration of binding sites (90–700 fmol/mg tissue, mean 300 ± 167 (s.d.) fmol/mg tissue, $n = 44$) (Table 3). To our knowledge, these are the highest reported concentrations of muscarinic binding sites in any tissue. The paired differences in affinity constant (0.1 ± 0.1 (s.e.mean) $\times 10^9$ M^{-1}) ($n = 10$) between contralateral control and agonist-treated groups were not significant ($P > 0.1$), nor were the paired differences between subgroups of these 10 animals (e.g. (d) vs (b)) significant.

There was a 23% decrease in the receptor concentration on comparing agonist-treated muscles with the paired contralateral control. This difference was found for all three groups of animals (Table 3) significant at the 1% level ($t = 3.08$, $n = 18$, paired t test). An alternative statistical approach is to test whether there is a significant fractional change in concentration of binding sites, the null hypothesis being that there is no change (Figure 3). The regression line shown in Figure 3 which is constrained by

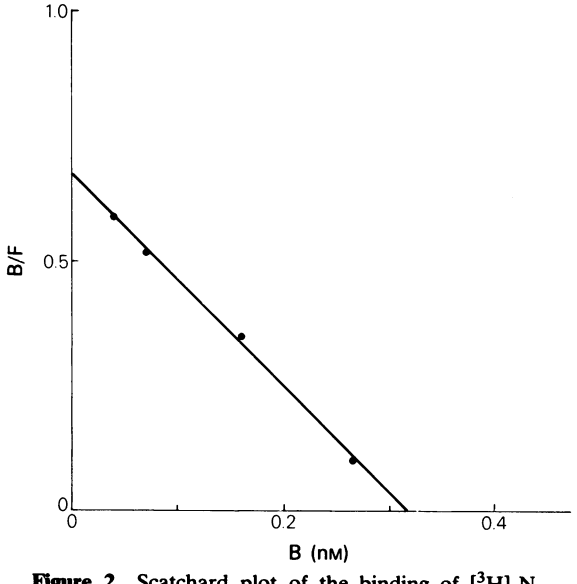

Figure 2 Scatchard plot of the binding of [^{3}H]-N-methylscopolamine to muscarinic receptors on monkey ciliary muscle. The gradient of the line gives the affinity constant 2.1×10^9 M^{-1}. The total concentration of receptor sites is 0.31 nM in the assay. This is equivalent to a receptor density of 300 fmol/mg tissue.

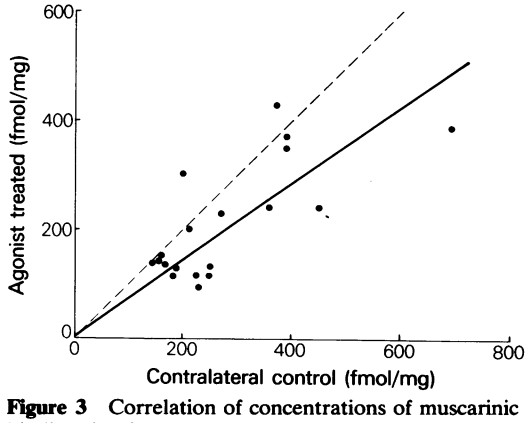

Figure 3 Correlation of concentrations of muscarinic binding sites in control and agonist-treated ciliary muscles. The dashed line represents the line of equivalence. The full line is the least squares fit with the line constrained to go through the origin. The gradient (0.71 ± 0.07), was significantly different from 1 ($P < 0.001$).

the null hypothesis to go through the origin has a gradient of 0.71 ± 0.07 and is significantly different from 1.00 at the 1% level. However, when the contralateral control muscles (group (b)) were compared with untreated control (group (a)), the decrease (47%) was also significant at the 0.1% level, ($t = 4.4$, 24 d.f. t test).

As there was some indication of a change in concentration of muscarinic receptors as assessed by [^{3}H]-NMS binding, we have examined whether we could detect changes in the binding of a selective antagonist pirenzepine, a partial agonist pilocarpine and a potent agonist carbachol. The technique used was to examine the ability of a fixed concentration of the drug to inhibit the binding of a low concentration of [^{3}H]-NMS (2×10^{-10} M) to muscarinic receptors in homogenates of ciliary muscle. The concentrations of drugs, given in Table 2, were chosen to give 20–75% inhibition of [^{3}H]-NMS binding.

Pirenzepine

Pirenzepine is a selective muscarinic antagonist which can discriminate between subclasses of muscarinic receptors (Hammer *et al.*, 1980). At 10^{-7} M it inhibited 27 ± 6% (s.d.) of the [^{3}H]-NMS binding but no significant effect of agonist pretreatment on the inhibition was observed (Table 2, paired differences in percentage inhibition 1 ± 2% (s.e.mean), $n = 10$). The *apparent* affinity constant of pirenzepine calculated from the single point estimate is 5×10^6 M^{-1} which is in between the estimated affinity constants for the highest (5×10^7 M^{-1}) and lowest (1×10^6 M^{-1}) affinity sites found in other tissues (Hammer *et al.*, 1980). Its value is close to the postulated subclass of receptors with an intermediate affinity (ca. 4×10^6 M^{-1}) for pirenzepine, but without further experimentation it is not possible to determine whether there is a uniform population of these sites in ciliary muscle or a mixture of high and low affinity sites, although the considerable variation in the percentage inhibition by pirenzepine found between ciliary muscles might argue for the latter possibility (Figure 4). There was not a significant correlation between NMS affinity and % inhibition by pirenzepine (Figure 4).

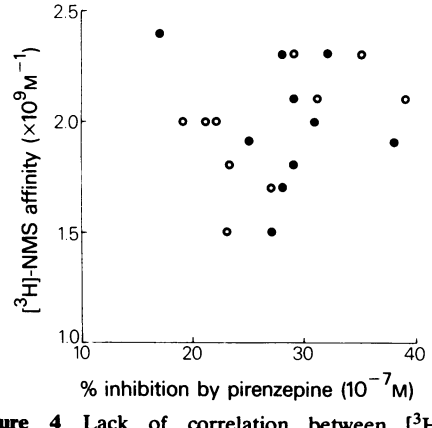

Figure 4 Lack of correlation between [^{3}H]-N-methylscopolamine [^{3}H]-NMS) affinity and pirenzepine potency. (○): Control eyes; (●): treated eyes. ($r = -0.02$, $P > 0.1$).

Agonist binding

The ability of the two agonists pilocarpine (10^{-5} M) and carbachol (10^{-4} M) to inhibit [^{3}H]-NMS binding to muscarinic receptors was examined (Table 2). There was no significant difference between the contralateral control group and agonist-treated groups (paired difference in inhibition 2 ± 4% and 2 ± 6% (s.e.mean) ($n = 10$) for pilocarpine and carbachol respectively). However, this experiment examined only single concentrations of these agonists. A more detailed study of the inhibition of [^{3}H]-NMS binding by 21 different concentrations of carbachol was undertaken. As in the previous experiment, no significant difference in carbachol binding was seen, in the range of carbachol concentrations between 10^{-7} and 10^{-2} M, between the contralateral control group and agonist-treated groups (paired difference in inhibition 1 ± 1.5%, $n = 21$). There was a considerable variation between samples in carbachol inhibition; at 10^{-4} M carbachol, values ranged from 24–73%. The carbachol potency did not appear to be correlated with receptor concentration (Figure 5). A more detailed picture of the variation in carbachol binding is shown in Figure 6 in which pooled data from tissues

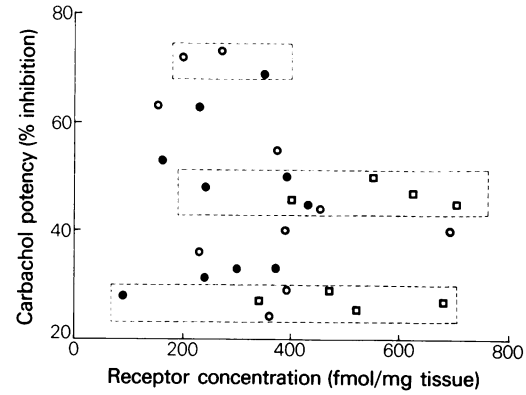

Figure 5 Lack of correlation between receptor concentration and carbachol potency (determined as the % inhibition of 2×10^{-10} M [^{3}H]-N-methylscopolamine binding by 10^{-4} M carbachol). The outlined rectangles enclose samples whose agonist binding properties are similar and are represented in Figure 6. Open symbols: control; closed symbols: agonist-treated; squares: untreated controls. ($r = -0.30$, $P > 0.1$).

exhibiting high ($n = 3$) intermediate ($n = 8$) and low ($n = 7$) carbachol potency (enclosed by the rectangles in Figure 5) are presented. These curves, like the individual binding curves deviated significantly from a simple mass action binding curve. Such deviations from a simple binding curve are well documented for agonist binding to muscarinic receptors in a wide range of tissues (Birdsall & Hulme, 1976; Birdsall *et al.*, 1978; Birdsall *et al.*, 1980) and these 'flat' curves have been analysed in terms of the presence of three populations of agonist sites (SH, H and L) which have differing affinities for a given agonist and are present in varying proportions in different tissues (Birdsall *et al.*, 1980). Analysis of the binding curves in Figure 6 suggests the presence of L sites (affinity 4×10^3 M^{-1}) H sites (affinity 1×10^5 M^{-1}) but no detectable SH sites. However the binding curves showed considerable variation in the proportions of L and H sites (95:5 to 25:75).

The variations in carbachol binding properties are correlated with differences in the pilocarpine binding properties (Figure 7). This correlation and the greater variation in carbachol binding is to be expected in view of the fact that pilocarpine distinguishes between subclasses of agonist binding sites in a similar manner but to a lesser extent than carbachol (Birdsall *et al.*, 1980). There was no correlation between the differences in the pirenzepine and carbachol binding properties of the receptors (Figure 8). Furthermore, we found that the 'non-hydrolysable' analogue of GTP (5'-guanyl-imidodiphosphate (10^{-4} M) had no effect on carbachol (10^{-4} M)) binding (paired difference in inhibition of carbachol = 0.50% ± 0.62 (s.e.mean)) (Figure 9).

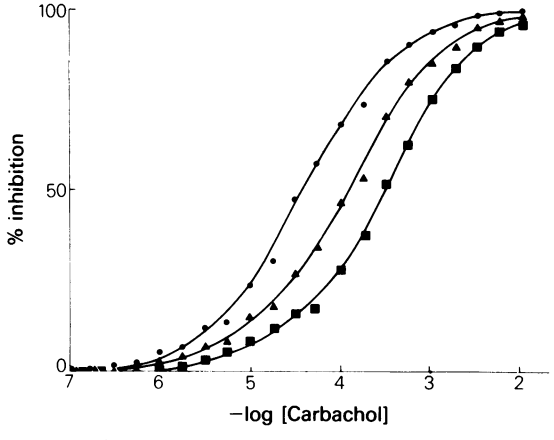

Figure 6 Detailed curves for carbachol inhibition of ([^{3}H]-NMS) (2×10^{-10} M) binding to muscarinic receptors in the three groups of ciliary muscles outlined in Figure 5. The solid lines through the data points are non-linear least squares fit to a two-site model. The relevant parameters, after correction of the affinity constants by a factor of 1.4 for the [^{3}H]-NMS occupancy are: (●): (30 ± 7)% low affinity sites having a log affinity constant, log $K_A = 3.85 \pm 0.14$; 70% high affinity sites, log $K_A = 4.73 \pm 0.05$. (▲): (74 ± 5)% low affinity sites, log $K_A = 3.6 \pm 0.05$; 26% high affinity sites, log $K_A = 4.80 \pm 0.12$. (■): (91 ± 1)% low affinity sites, log $K_A = 3.44 \pm 0.01$; 9% high affinity sites, log $K_A = 5.13 \pm 0.20$.

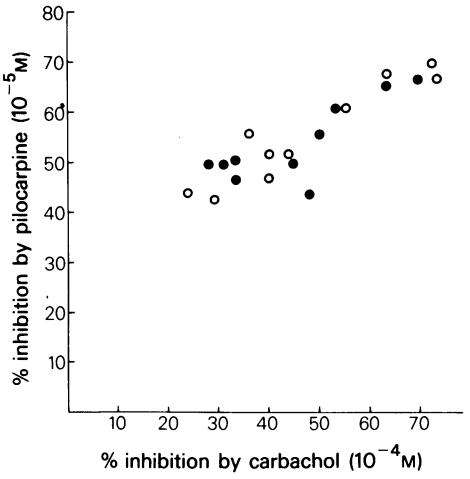

Figure 7 Correlation between pilocarpine and carbachol binding properties of muscarinic receptors in a given ciliary muscle. The lack of change in receptor binding properties in the subsensitive muscles is evident. The two estimates of agonist binding are significantly correlated. ($r = +0.86$, $P < 0.001$). (○): Contralateral control; (●): agonist-treated.

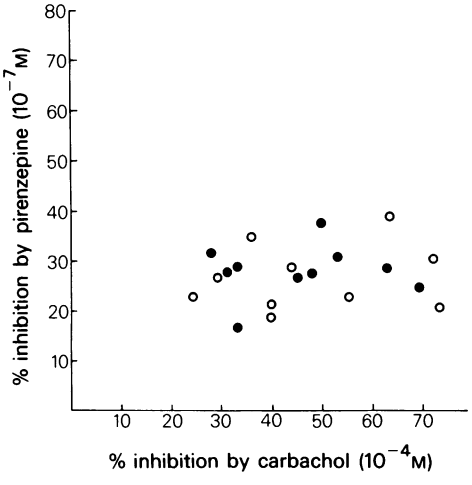

Figure 8 Lack of correlation between the carbachol and pirenzepine binding properties of muscarinic receptors of the ciliary muscle. ($r = 0.05$). (○): Contralateral control; (●): agonist-treated.

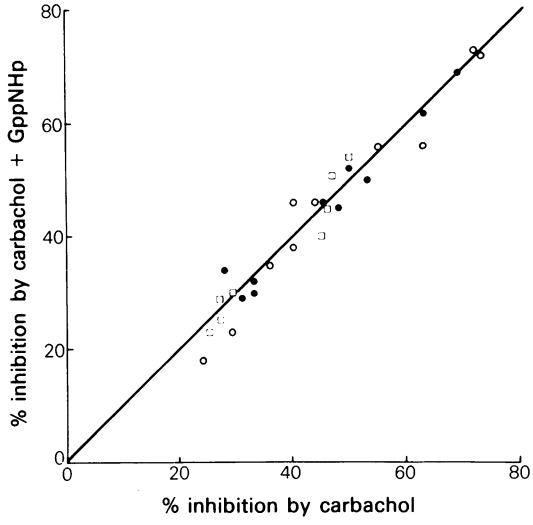

Figure 9 Comparison of the inhibition of [^{3}H]-N-methylscopolamine binding by carbachol (10^{-4} M) in the presence and absence of GppNHp (10^{-4} M). (●): Agonist-treated eyes; (○): contralateral controls; (□): untreated controls. The drawn line is the line of equivalence.

Discussion

The results presented here show that the muscarinic receptor in the ciliary muscle has many of the properties found in other cholinergic structures.

The concentration of receptors is the highest so far encountered and is several times higher than that in the highest area of the central nervous system, the striatum. This may reflect the very dense innervation. There are also large variations in the receptor concentrations between animals, as well as variations in the proportion of subclasses of agonist (and probably pirenzepine) binding sites. The results in the subsensitive ciliary muscle show a small decrease in receptor concentration but no change in the binding parameters for antagonists or agonists. This finding is in agreement with a number of reports concerning other tissues in which the amount of receptor (measured by antagonist binding) was reduced by exposure to agonists or indirectly through the administration of anticholinesterase (see for example, Uchida, Takeyasu, Matsudu & Yoshida, 1979; Ehlert, Kokka & Fairhurst, 1980; Galper & Smith, 1980; Shifrin & Klein, 1980; Ben-Barak, Gazit, Silman & Dudai, 1981; Marks, Artman, Patinkin & Collins, 1981). The reasons why the percentage decrease in receptor binding sites found in this and other equivalent studies is so small in comparison to the magnitude of the change in the physiological response is not known. It may be that only a fraction of the measured receptor binding sites is directly coupled to the response and that this is reduced to a much greater extent than the total receptor pool.

There is considerable interest in the finding that the non-hydrolysable GTP analogue GppNHp was totally without effect on the binding of agonists to the receptor in this muscle. This is in contrast to the effects of GTP (and GppNHp) on the receptor in intestinal smooth muscle, heart, exocrine glands and most areas of the central nervous system where a substantial decrease in agonist affinity is produced (Berrie *et al.*, 1979; Rosenberger, Yamamura & Roeske, 1979; Wei & Sulahke, 1979; Hulme *et al.*, 1980; 1981; Ehlert, Roeske & Yamamura, 1981). On the other hand, no significant effect of GTP has been seen in the conducting tissue of the ox heart, or in the rat hypothalamus (Burgen, Birdsall & Hulme, 1981; Hulme *et al.* unpublished results). Where a pronounced GTP effect is seen, this is presumptive evidence of coupling between the muscarinic receptor and adenylate cyclase. Where this is not present one should suspect that there is a direct coupling of the receptor to an ionic mechanism and this indeed would be physiologically appropriate for such a fast-acting structure as the ciliary muscle.

The work of E.H.B. was supported by the U.S. Public Health Service, Grant EY 00231 and made possible by the technical assistance of Mrs Ingalill Wersäll.

References

BÁRÁNY, E.H. (1977). Pilocarpine-induced subsensitivity to carbachol and pilocarpine of ciliary muscle in vervet and cynomolgus monkeys. *Acta Ophthal.*, **55**, 141–163.

BEN-BARAK, J., GAZIT, H., SILMAN, I., & DUDAI, Y. (1981). *In vivo* modulation of the number of muscarinic receptors in rat brain by cholinergic ligands. *Eur. J. Pharmac.*, **74**, 73–81.

BERRIE, C.P., BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1979). Guanine nucleotides modulate muscarinic receptor binding in the heart. *Biochem. biophys. Res. Commun.*, **87**, 1000–1005.

BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1978). The binding of agonists to brain muscarinic receptors. *Mol. Pharmac.*, **14**, 723–736.

BIRDSALL, N.J.M. & HULME, E.C. (1976). Biochemical studies on muscarinic receptors. *J. Neurochem.*, **27**, 7–6.

BIRDSALL, N.J.M., HULME, E.C. & BURGEN, A.S.V. (1980) The character of the muscarinic receptors in different regions of the rat brain. *Proc. R. Soc. B.*, **207**, 1–12.

BITO, L.Z. & BARODDY, R.A. (1979). Gradual changes in the sensitivity of rhesus monkey eyes to miotics and the dependence of these changes on the regimen of topical

cholinesterase inhibitor treatment. *Invest. Ophthal.*, **18,** 794–801.

BITO, L.Z., DAWSON, M.J. & PETRINOVIC, L. (1971). Cholinergic sensitivity: normal variability as a function of stimulus background. *Science.*, **172,** 583–585.

BURGEN, A.S.V., BIRDSALL, N.J.M. & HULME, E.C. (1981). The nature of muscarinic receptors in the heart. In *Cell Membrane in Function and Dysfunction of Vascular Tissue.* ed. Godfraind, T. & Meyer, P. pp.15–25. Amsterdam: Elsevier.

EHLERT, F.J., KOKKA, N. & FAIRHURST, A.S. (1980). Altered [^{3}H]-quinuclidinyl benzilate binding in the striatum of rats following chronic cholinesterase inhibition with diisopropylfluorophosphate. *Mol. Pharmac.*, **17,** 24–30.

EHLERT, F.J., ROESKE, W.R. & YAMAMURA, H.I. (1981). Muscarinic receptor: regulation by guanine nucleotides, ions and N-ethylmaleimide. *Fedn Proc.*, **40,** 153–159.

GALPER, J.B. & SMITH, J.W. (1980). Agonist and guanine nucleotide modulation of muscarinic cholinergic receptors in cultured heart cells. *J. biol. Chem.*, **255,** 9571–9579.

HAMMER, R., BERRIE, C.P., BIRDSALL, N.J.M., BURGEN, A.S.V., & HULME, E.C. (1980). Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature.*, **283,** 90–92.

HULME, E.C., BERRIE, C.P., BIRDSALL, N.J.M. & BURGEN, A.S.V. (1980). Interactions of muscarinic receptors with guanine nucleotides and adenylate cyclase. In *Drug Receptors and their Effectors* ed. Birdsall, N.J.M. pp.23–34. London: Macmillan.

HULME, E.C., BERRIE, C.P., BIRDSALL, N.J.M. & BURGEN, A.S.V. (1981). Two populations of binding sites for muscarinic antagonists in the rat heart. *Eur. J. Pharmac.*, **73,** 137–142.

HULME, E.C., BIRDSALL, N.J.M., BURGEN, A.S.V. & MEHTA, P. (1978). The binding of antagonists to brain muscarinic receptors. *Mol. Pharmac.*, **14,** 737–750.

ISHIKAWA, T. (1962). Fine structure of the human ciliary muscle. *Invest. Ophthal.*, **1,** 587–608.

KAUFMAN, P.L. & BÁRÁNY, E.H. (1975). Subsensitivity to pilocarpine in primate ciliary muscle following topical anticholinesterase treatment. *Invest. Ophthal.*, **14,** 302–306.

LATIES, A.M. & JACOBOWITZ, D. (1966). A comparative study of the autonomic innervation of the eye in monkey, cat and rabbit. *Anat. Rec.*, **156,** 383–396.

MARKS, M.J., ARTMAN, L.D., PATINKIN, D.M. & COLLINS, A.C. (1981). Cholinergic adaptations to chronic oxotremorine infusion. *J. Pharmac. exp. Ther.*, **218,** 337–343.

MITTAG, T.W. (1978). Acetylcholine receptors in intraocular tissues of the rabbit. *Invest. Ophthal.*, **17,** ARVO Suppl., 189.

RAINA, M.K. & BITO, L.Z. (1979). Correlation between muscarinic receptor concentration, measured by ^{3}H-quinuclidinylbenzilate binding, and in vivo cholinergic sensitivity of cat eyes. *Invest. Ophthal.*, **18,** ARVO Suppl. 189–90.

ROSENBERGER, L.B., YAMAMURA, H.I. & ROESKE, W.R. (1980). Cardiac muscarinic cholinergic receptor binding is regulated by Na^+ and guanyl nucleotides. *J. biol. Chem.*, **255,** 820–823.

SACHS, D.I., KLOOG, Y., KORCZYN, A.D., HERON, D.S. & SOKOLOVOSKY, M. (1979). Denervation, supersensitivity and muscarinic receptors in the cat iris. *Biochem. Pharmac.*, **28,** 1513–1518.

SHIFRIN, G.S. & KLEIN, W.I. (1980). Regulation of muscarinic acetylcholine receptor concentration in cloned neuroblastoma cells. *J. Neurochem.*, **34,** 993–999.

SMITH, S.A. & SMITH, S.E. (1980). Subsensitivity to cholinoceptor stimulation of the human iris spincter *in situ* following acute and chronic administration of cholinomimetic miotic drugs. *Br. J. Pharmac.*, **69,** 513–518.

UCHIDA, S., TAKEYASU, K., MATSUDU, T. & YOSHIDA, H. (1979). Changes in muscarinic acetylcholine receptors of mice by chronic administration of diisopropylfluorophosphate and papaverine. *Life Sci.*, **24,** 1805–1812.

VAN DER ZYPEN, E. (1967). Licht- und elektronenmikroskopische Untersuchungen über den Bau und die Innervation des Ciliarmuskels bei Mensch und Affe (Cercopithecus aethiops) *Albrecht v. Graefes Arch. Klin. exp. Ophthal.*, **174,** 143–168.

WEI, J.W. & SULAKHE, P.V. (1979). Agonist-antagonist interactions with rat atrial muscarinic receptor sites: differential regulation by guanine nucleotides. *Eur. J. Pharmac.*, **58,** 91–92.

(Received June 4, 1982. Revised July 3, 1982.)