Muscarinic Acetylcholine Receptors in the Rabbit Ciliary Body Smooth Muscle: Spare Receptors and Threshold Phenomenon

Fukio KONNO and Issei TAKAYANAGI*

Department of Chemical Pharmacology, Toho University School of Pharmaceutical Sciences,
Miyama, Funabashi, Chiba 274, Japan

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Abstract—Interactions of several muscarinic drugs with their receptors were studied in the ciliary body smooth muscles of the rabbit. The ciliary body smooth muscles responded to carbachol, a muscarinic full agonist, with concentration-dependent contractions, and the pD_2 value of carbachol was 5.16 ± 0.05 . Atropine, a competitive antagonist of muscarinic receptors, produced a parallel shift to the right in the concentration-response curves for carbachol. Pilocarpine which is a well-known partial agonist on muscarinic receptors in most smooth muscles did not cause any contraction in this tissue. The drug, however, behaved as a competitive antagonist on the muscarinic receptors in the ciliary body smooth muscles. The pA_2 values of atropine and pilocarpine versus carbachol obtained from the Schild plot are 8.97 ± 0.25 and 5.17 ± 0.09 , respectively. On the other hand, arecoline and oxotremorine acted as a partial agonist in this tissue. The intrinsic activity, and pD_2 and pA_2 values were 0.41 ± 0.02 , 4.93 ± 0.05 and 5.32 ± 0.05 for arecoline respectively, and were 0.26 ± 0.02 , 5.64 ± 0.08 and 6.12 ± 0.16 for oxotremorine, respectively. The pA_2 values of these drugs were significantly larger than the corresponding pD_2 values of the drugs. The values of the negative log molar dissociation constant of carbachol, arecoline and oxotremorine estimated by the method of partial irreversible blockade of spare receptors with 3×10^{-6} M phenoxybenzamine were 4.53 ± 0.08 , 5.20 ± 0.09 and 6.02 ± 0.06 , respectively. These values were practically equal to those obtained from other peripheral tissues, suggesting that the muscarinic receptors in rabbit ciliary body smooth muscles were qualitatively the same as those of other smooth muscles. However, the density of muscarinic receptors in this tissue is lower than that in other peripheral tissues, and the threshold phenomenon lay between the occupation of muscarinic receptors and the tissue responses. Therefore, the pA_2 values of partial agonists were different from their pD_2 values, and pilocarpine, a partial agonist with lower intrinsic efficacy, behaved as a competitive antagonist in this tissue. These results suggest the importance of the receptor density and the threshold in the tissue as well as the affinity and the intrinsic efficacy of the drug as a determinant of agonist potency.

Iris-ciliary body smooth muscles are innervated by parasympathetic nerve fibers in many mammalian species (1–3) and respond to several muscarinic agonists and antagonists in a characteristic manner. Muscarinic agonists cause miosis and ocular hypotensive responses, while muscarinic antagonists cause mydriasis and elevation of intraocular

pressure (4, 5).

Recently, muscarinic acetylcholine receptors in the iris sphincter smooth muscles have been characterized by biochemical and pharmacological procedures (6–9). However, despite the significance of the muscarinic acetylcholine receptors in ciliary body smooth muscles, very few studies have been made to characterize these receptors. Therefore, we examined the interaction of muscarinic drugs

* To whom reprint requests should be addressed.

with their receptors on rabbit ciliary body smooth muscles using pharmacological procedures.

Materials and Methods

Male albino rabbits weighing 2.0 to 3.0 kg were killed by bleeding from the neck. Pieces (about 2×10 mm) of ciliary body were carefully dissected in a plane parallel to the iris (circular strip) from the enucleated eyeball, and they were mounted in a 20 ml of organ bath containing Krebs solution (118 mM NaCl, 1.2 mM $MgCl_2$, 1.19 mM KH_2PO_4 , 25 mM $NaHCO_3$, 2.54 mM and 11 mM glucose) at $32^\circ C$ and gassed with 5% CO_2 -95% O_2 . The preparations were suspended under 20 to 30 mg tension, and responses were recorded via isometric transducers. The preparations were allowed to equilibrate for at least 60 min before addition of any drugs. After the equilibration, the preparations were exposed to a sub-maximum dose of carbachol, then washed thoroughly. After an additional 30 min of equilibration, three cumulative concentration-response curves to carbachol were determined. In each preparation, when the last two concentration-response curves were similar, all subsequent results were compared with the last of those curves. Responses were expressed as a percentage of the maximum contractile response to carbachol. The agonistic activities of full and partial agonists were expressed as pD_2 values, the negative log concentration (M) which produced 50% of the maximum contractions obtained from individual concentration-response curves. The pD_2 values of full and partial agonists were calculated by the table of van Rossum (10). Antagonistic activity of each antagonist was measured against carbachol. The preparation was allowed to equilibrate with each concentration of antagonist for 5 min before concentration-response curves of carbachol were obtained, and pA_2 values, the dissociation constant of the antagonist, were calculated according to the method of Arunlakshana and Schild (11). The intrinsic activities (i.a.) of partial agonists were expressed as the ratio between the maximum response to a test drug and that to carbachol. The pA_2 values of partial agonists were calculated from the

estimated difference between the curves from the full agonist (carbachol) alone and with the partial agonists according to the method of van Rossum (10).

Since pD_2 values may not be a reliable measure of the actual affinities of full agonists, the dissociation constant of carbachol was determined according to the method of Furchgott and Bursztyn (12), using the irreversible antagonist phenoxybenzamine to occlude a fraction of muscarinic receptors. After the determination of the control concentration-response curves of carbachol, the preparations were treated with 3×10^{-6} M of phenoxybenzamine for 10 min. The preparations were then allowed to re-equilibrate for 60 min, with repeated washing every 10 min, and second cumulative concentration-response curves of carbachol were determined. The dissociation constant of carbachol was calculated from the following equation:

$$\frac{1}{[A]} = \frac{1-q}{qK_A} + \frac{1}{q[A']} \quad (1)$$

where $[A]$ and $[A']$ are corresponding equi-effective concentrations of carbachol before and after irreversible blockade of a fraction of receptors with phenoxybenzamine, respectively, and q is the remaining fraction of active receptors after phenoxybenzamine treatment. $1/[A]$ was plotted versus $1/[A']$, and a straight line was fitted to the data by linear regression analysis. The dissociation constant (K_A) was obtained by the following equation:

$$K_A = \frac{(\text{Slope}-1)}{\text{intercept}} \quad (2)$$

The dissociation constant of the partial agonist was also obtained by the method of Furchgott and Bursztyn (12), in which the partial agonist serves as a competitive antagonist to carbachol (full agonist) after partial irreversible blockade of muscarinic receptors with phenoxybenzamine. This treatment reduces the response to the partial agonist to a greater extent than that to the full agonist, thereby the response to the partial agonist is insignificant with respect to that to the full agonist. Under these circumstances, the partial agonist may be used as a competitive antagonist, and pA_2 values of

partial agonists were estimated by the method of van Rossum (10) using carbachol as the agonist. The pretreatment period of the partial agonist was 5 min in these experiments.

Fractional receptor occupancy for agonist and partial agonist at each concentration $[A]$ was calculated from the following equation:

$$\frac{[RA]}{[RT]} = \frac{[A]}{K_A + [A]} \quad (3)$$

where $[RA]$ is the concentration of receptor-agonist complex, $[RT]$ is the total receptor concentration and K_A is the dissociation constant of the agonist and partial agonist determined by the procedures described previously.

Drugs used: carbachol chloride, arecoline hydrochloride, pilocarpine hydrochloride, atropine sulfate and oxotremorine sesquifumarate were from Sigma; phenoxybenzamine hydrochloride and hexamethonium dibromide was from Tokyo-Kasei; and nicotine bitartrate was from Nakarai, all in powder form. All drugs were used as solutions in distilled water. Other chemicals used were of analytical grade.

Results

The ciliary body smooth muscles responded to carbachol with concentration-dependent contractions. The pD_2 value of carbachol

calculated from their concentration-response curves was 5.16 ± 0.05 ($N=23$), and the maximum tension induced by carbachol was 26.7 ± 1.1 mg ($N=23$). Atropine parallelly shifted the concentration-response curves for carbachol to the right. A Schild plot of these results gave a straight line with a slope of one. The pA_2 value obtained by the Schild plot analysis of the data was 8.97 ± 0.25 (Fig. 1 and Table 1).

Pilocarpine did not contract this tissue. The drug, however, antagonized the contractile effect of carbachol. The Schild plots of these results yielded a straight line with a slope of one, indicating competitive antagonism. The pA_2 value of pilocarpine calculated by the Schild plot analysis was 5.17 ± 0.09 (Fig. 2 and Table 1).

The contractions induced by arecoline and oxotremorine were abolished by the 5 min pretreatment of ciliary body preparations with 10^{-6} M atropine and not influenced by the 5 min pretreatment with 10^{-6} M hexamethonium, which antagonized the contraction of ciliary body smooth muscles induced by 10^{-4} M nicotine (data not shown). These results suggest that the contractile responses of arecoline and oxotremorine are mediated through muscarinic receptors. Each maximum contraction induced by arecoline and oxotremorine was less than that by carbachol,

Fig. 1. Antagonism between carbachol and atropine in rabbit ciliary body preparations (left). Ordinate, fraction of maximal response to carbachol; abscissa, negative log concentration of carbachol. Carbachol alone ($\circ$) and with atropine, 10^{-8} M ($\bullet$), 3×10^{-8} M ($\blacktriangle$) and 10^{-7} M ($\blacksquare$). Each point represents a mean with S.E. of 6 experiments. Schild plots for an antagonism between atropine and carbachol (right). Ordinate, logarithms of equieffective concentration ratios of carbachol minus 1; abscissa, negative log concentrations of atropine. The slope of the line is 1.08 ± 0.15 .

Table 1. The pD_2 , pA_2 and pK_A values and intrinsic activities of drugs in the ciliary body preparations non-treated and treated with phenoxybenzamine, 3×10^{-6} M

	Non-treated preparation			PBZ-treated preparation [#]
	pD_2	pA_2	i.a.	pK_A or pA_2
Carbachol	5.16 ± 0.05	—	1.0	4.53 ± 0.08
Arecoline	4.93 ± 0.05	$5.32 \pm 0.05^*$	0.41 ± 0.02	$5.20 \pm 0.09^*$
Oxotremorine	5.64 ± 0.08	$6.12 \pm 0.16^*$	0.26 ± 0.02	$6.02 \pm 0.06^*$
Pilocarpine	—	5.17 ± 0.09	—	—
Atropine	—	8.97 ± 0.25	—	—

[#]: the preparations were treated with 3×10^{-6} M of phenoxybenzamine (PBZ) for 10 min. The preparations were then allowed to re-equilibrate for 60 min, with repeated washing every 10 min. i.a.: intrinsic activity. *: Significantly different from the corresponding pD_2 values at $P < 0.05$. The relative efficacies of carbachol, arecoline and oxotremorine, which were determined by the method of Furchgott and Bursztyn (12), were 1.0, 0.1 and 0.06, respectively.

Fig. 2. Antagonism between carbachol and pilocarpine in rabbit ciliary body preparations (left). Ordinate and abscissa as for Fig. 1-left. Carbachol alone (○) and with pilocarpine, 3×10^{-5} M (●), 10^{-4} M (▲) and 3×10^{-4} M (■). Each point represents a mean with S.E. of 4 experiments. Schild plots for antagonism between pilocarpine and carbachol (right). Ordinate and abscissa, as for Fig. 1-right. The slope of the line is 1.23 ± 0.17 .

suggesting their intrinsic activities were intermediate. Furthermore, they shifted the concentration-response curves for carbachol towards higher concentrations (Figs. 3 and 4). These results suggest that they are partial agonists on the muscarinic receptors. The intrinsic activities and pD_2 and pA_2 values estimated in this tissue are shown in Table 1. The pA_2 values of the partial agonists were significantly larger than the corresponding pD_2 values.

Figure 5-left illustrates that the addition of 3×10^{-6} M phenoxybenzamine causes a time-dependent loss of the contractile response to carbachol. The ciliary body preparations lost about 50% of its response to carbachol after 10 min incubation with

phenoxybenzamine, and an additional 10 min incubation further attenuated its contractile response. The negative log dissociation constant (pK_A) of carbachol, calculated according to the method of Furchgott and Bursztyn (12), was 4.53 ± 0.08 ($N=13$, Table 1). Pretreatment with 3×10^{-4} M carbachol protected the muscarinic receptor from an irreversible blockade by 3×10^{-6} M phenoxybenzamine (Fig. 5-right). Pretreatment of pilocarpine (3×10^{-4} M) also protected against the loss of contractile response to carbachol (data not shown). Therefore, the decrease of response of the ciliary body preparations to carbachol should be due to the irreversible blockade of the muscarinic receptors.

Fig. 3. Concentration-response curves for carbachol (○) and arecoline (●) in rabbit ciliary body preparations (left). Ordinate, fraction of maximal response to carbachol; abscissa, negative log concentrations of drugs. Each point represents a mean with S.E. of 6 experiments. Antagonism of responses to carbachol by arecoline in rabbit ciliary body preparations (right). ○: carbachol alone and ●: with arecoline, 10^{-4} M. Each point represents a mean with S.E. of 6 experiments. Ordinate and abscissa, as for Fig. 1-left.

Fig. 4. Concentration-response curves for carbachol (○) and oxotremorine (●) in rabbit ciliary body preparations (left). Ordinate and abscissa, as for Fig. 3-left. Each point represents a mean with S.E. of 7 experiments. Antagonism of responses to carbachol by oxotremorine in rabbit ciliary body preparations (right). ○: carbachol alone and ●: with oxotremorine, 10^{-5} M. Each point represents a mean with S.E. of 7 experiments. Ordinate and abscissa, as for Fig. 1-left.

Fig. 5. Concentration-response curves for carbachol before and after an irreversible blockade of receptor with phenoxybenzamine, 3×10^{-6} M (left). The tissue contact times of phenoxybenzamine are 0 min (○), 10 min (●) and 20 min (■), respectively. Each point represents a mean with S.E. of 8 experiments. Ordinate, fraction of maximal response to carbachol before the irreversible blockade of receptor with phenoxybenzamine; abscissa, negative log concentrations of carbachol. Protection of contractile responses to carbachol against a 10 min exposure to phenoxybenzamine, 3×10^{-6} M (right). Non-treated with phenoxybenzamine (○), a 10 min treatment with phenoxybenzamine (▲) and a 10 min treatment with both phenoxybenzamine and carbachol, 10^{-4} M (●). Each point represents a mean with S.E. of 4 experiments. Ordinate and abscissa, as for Fig. 5-left.

By partial irreversible blockade of the muscarinic receptors in the ciliary body preparations with phenoxybenzamine (3×10^{-6} M for 10 min), the agonistic responses to oxotremorine or arecoline were abolished, while the preparations were still contracted appreciably by carbachol. Under these circumstances, oxotremorine and

arecoline were used as a competitive antagonist to carbachol (Figs. 6 and 7), and their pA_2 values were estimated. The pA_2 values of oxotremorine and arecoline under these circumstances were practically equal to the corresponding pA_2 values obtained in the intact preparations. These results are also summarized in Table 1.

Discussion

It is generally accepted that the iris-ciliary body smooth muscles respond to several muscarinic agonists in a characteristic manner (3–5). In the present study, the rabbit ciliary body smooth muscles also responded to carbachol, a muscarinic full agonist, with concentration-dependent contractions. However, oxotremorine and arecoline, which act as full agonists in other peripheral tissues such as guinea pig ileum (13) and rat intestine (14), behaved as partial agonists on the muscarinic receptors in this tissue; and pilocarpine, which is well-known as a partial agonist on muscarinic receptors (12, 14), behaved as a competitive antagonist on the muscarinic receptors of the ciliary body smooth muscles. However, the dissociation constant value (negative log $K_A=4.53$) of carbachol obtained herein by the partial irreversible blockade of muscarinic receptors with 3×10^{-6} M phenoxybenzamine is very similar to the values obtained in rabbit fundus strip (negative log $K_A=4.8$) which reported by Furchgott and Bursztyn (12), in rat ileum (negative log $K_A=4.65$) by Morgenstern (15) and in rabbit iris sphincter (negative log $K_A=4.65$) by Akesson et al. (8). Furthermore, the dissociation constants of arecoline and oxotremorine which are represented as pA_2 values versus carbachol estimated by the partial irreversible blockade of muscarinic receptors were also practically equal to those obtained in other tissues (8, 13, 16). Moreover, the pA_2 value of 5.17 for pilocarpine obtained as a competitive antagonist versus carbachol in the ciliary body smooth muscle was nearly equal to that in the rabbit fundus strip (12) (negative log $K_A=5.2$) where the drug behaved as an agonist, and the pA_2 value of atropine versus carbachol obtained with the Schild plot analysis was practically equal to that in the rat intestine

Fig. 6. Antagonism between carbachol and arecoline, 10^{-4} M, in ciliary body preparations pretreated with phenoxybenzamine, 3×10^{-6} M, at 10 min. Open mark: in the non-treated ciliary body preparations. $\circ$: carbachol alone. Closed mark: in the preparations treated with phenoxybenzamine, $\bullet$: carbachol alone and $\blacktriangle$: with arecoline, 10^{-4} M. Each point represents a mean with S.E. of 5 experiments. Ordinate and abscissa, as for Fig. 5-left.

Fig. 7. Antagonism between carbachol and oxotremorine, 10^{-5} M, in ciliary body preparations pretreated with phenoxybenzamine, 3×10^{-6} M, at 10 min. Open mark: in the nontreated ciliary body preparations. $\circ$: carbachol alone. Closed mark: in the preparations treated with phenoxybenzamine, $\bullet$: carbachol alone and $\blacktriangle$: with oxotremorine, 10^{-5} M. Each point represents a mean with S.E. of 5 experiments. Ordinate and abscissa, as for Fig. 5-left.

($pA_2=8.9$) reported by van Rossum (10). When the respective affinities of several agonists and antagonists are the same in different tissues, it is assumed that the drugs are interacting with the same receptor population (11). Conversely, differences in affinities imply interaction with different receptor populations. Therefore, all these results suggest that in these tissues, the muscarinic receptors are of the same type.

Stephenson introduced the concept of the efficacy (e) which determines the magnitude of stimulus produced by the agonist for any given fraction of receptor occupancy (17). Furchgott and Bursztyn modified this model to differentiate the drug and tissue factors of efficacy by defining intrinsic efficacy (ϵ). The relationship between the efficacy and the intrinsic efficacy was expressed by the following equation:

$$e = \epsilon \cdot [RT]$$

The intrinsic efficacy was strictly a drug-related parameter which should be constant for the same type of receptor across species and tissues. Therefore, it is assumed that the difference between the efficacies (e) of the same drug which acts on the same type receptor population among the different tissues is the result of a different $[RT]$. In the rabbit ciliary body smooth muscles, the difference between the pD_2 values and the negative log K_A for carbachol was small, that is, efficacy (e) of carbachol in this tissue was 5.59 ± 0.86 , indicating a smaller muscarinic receptor reserve for carbachol than those other peripheral tissues, because the efficacies (e) of carbachol in the rabbit fundus strip and in the reserpinized left atrium of guinea pig were 190 to 266 and 50 to 87, respectively (12). In actuality, it was estimated that for carbachol, about 90% receptor occupancy was required to elicit a 100% response (Fig. 8), suggesting that there are fewer spare muscarinic receptors in rabbit ciliary body smooth muscles than in other peripheral tissues. The fact that arecoline and oxotremorine act as a partial agonist in this tissue suggests that these drugs have lower intrinsic efficacy than carbachol. By partial irreversible blockade of the muscarinic receptors in the rabbit ciliary body smooth muscles with phenoxybenzamine, the agonist

Fig. 8. Receptor occupancy-response curves. Ordinate, fraction of receptors occupied: $RA/RT \times 100$. Each mark represents carbachol ($\circ$), arecoline (Δ) and oxotremorine ($\square$), respectively. Note that pilocarpine (---) and atropine (---) can not reach the threshold level, y axis=0, even if these drugs occupy all muscarinic receptors.

responses of arecoline and oxotremorine were reduced to a greater extent than that of carbachol, a full agonist. These results emphasize the importance of the receptor density in the tissue and the intrinsic efficacy of agonists as a determinant of agonist potency.

In this study, the pA_2 values of arecoline and oxotremorine were significantly larger than the corresponding pD_2 values of the drugs. Furthermore, pilocarpine did not cause any contractile response in this tissue. Figure 8 shows the relationship between the contractile responses of the ciliary body preparations to carbachol, oxotremorine and arecoline and the percentage of muscarinic receptors occupied, which was calculated from equation 3 using their dissociation constants. The receptor occupancy-response curve for carbachol became hyperbolic. The ED_{50} value for carbachol was achieved with about 18% receptor occupancy. On the other hand, the occupancy-response curves of oxotremorine and arecoline were flattened, and the fractional receptor-occupancy which produced 50% of the maximum contractions for oxotremorine and arecoline were about 70% and 63%, respectively. Furthermore, the occupation of muscarinic receptors by oxotremorine or arecoline did not induce an

immediate contractile response, that is, a substantial fraction of receptors was occupied before an effect became visible. This view is similar to the "threshold phenomenon" which was first introduced by Ariens et al. (18, 19). Therefore, the stimuli induced by a partial agonist with lower intrinsic efficacy such as pilocarpine can not reach the threshold level, even if the drug occupies all the muscarinic receptors in the rabbit ciliary body smooth muscles; that is, pilocarpine behaved as a competitive antagonist in this tissue, and the pA_2 values of oxotremorine and arecoline differed from their pD_2 values. In order to determine the threshold to induce contraction, occupancy-response curves for oxotremorine and arecoline were extrapolated toward the y-axis. The y-intercept of the occupancy-response curve for oxotremorine was about minus 17%, and this value was virtually equal to that for arecoline (Fig. 8). The ED₅₀ values represented as negative log concentrations for carbachol, arecoline and oxotremorine from their occupancy-response curves were 5.19, 4.97 and 5.65, respectively. These values coincided with the pD_2 values obtained from their concentration-response curves.

In conclusion, the present findings suggest that the muscarinic receptors in rabbit ciliary body smooth muscles are qualitatively the same as those in other peripheral tissues. However, the density of muscarinic receptors in this tissue is lower than in other peripheral tissues, and the threshold phenomenon lay between the occupation of muscarinic receptors and tissue responses. Therefore, the pA_2 values of partial agonists were different from their pD_2 values, and pilocarpine, a partial agonist with lower intrinsic efficacy, behaved as a competitive antagonist in this tissue. The density of the drug receptor and threshold phenomena in the tissues used are also important factors in determining the pharmacological properties of the drugs.

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