Br. J. Pharmac. (1985), **84**, 193–198

Muscarinic subsensitivity without receptor change in monkey ciliary muscle

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1 Intense muscarinic stimulation of the monkey ciliary muscle causes long-lasting muscarinic subsensitivity. This could be due to changes in number or affinity of muscarinic receptors which would cause a threshold elevation detectable *in vivo*.

2 Since plasma levels of the agonists causing contraction *in vivo* were not available, the accommodation response to systemic muscarinic agents in subsensitized eyes was compared with that in the normal fellow eyes, usually seven days after a single subsensitizing dose of 100 μg carbachol to cornea.

3 Subsensitivity was present whether the agonist tested was pilocarpine, carbachol or bethanechol but no evidence for threshold elevation was found.

4 The conclusion is that changes in the muscarinic receptors are of minor importance for the kind of subsensitivity studied.

Introcution

The ciliary muscle of several monkey species can be at least partly desensitized ('subsensitized') by strong or long muscarinic stimulation so that the accomodative response to pilocarpine especially but also other muscarinics is reduced for weeks or months (Kaufman & Bárány, 1975; Bárány, 1977). The present paper deals with subsensitivity on this time scale of weeks, late subsensitivity.

One possible explanation for late subsensitivity is downregulation of the number or the affinity of muscarinic receptors on the muscle. A transnational collaboration was therefore established and the number and affinity of the receptors binding [^{3}H]-N-methylscopolamine (NMS) in individual subsensitized and control ciliary muscles of cynomolgus monkeys were determined (Bárány *et al.*, 1982). The change in receptor density found was too small to explain the degree of subsensitivity and the affinity to NMS was not changed. Nor was the proportion between high and low affinity receptor populations (Birdsall *et al.* 1978), changed by subsensitization. (No superhigh affinity receptors were found in the muscle.) There remained the possibility that a small population of strategic receptors, changes in which are swamped in binding experiments, could be responsible for the contraction and the subsensitivity.

The remote possibility also exists that the *in vitro* situation allows receptors to recover from the kind of subsensitization studied. Therefore an attempt was made to detect a change in receptors *in vivo*. Since no method yet exists for the estimation of plasma concentrations of pilocarpine in the range of interest, which would allow a horizontal displacement of the dose-response curve to be detected, advantage was taken of the fact that the eye is a paired organ and the level of agonist was monitored by its effect on the untreated control eye.

Basic principles

The hypothesis under test is that a change in the number or affinity of the muscarinic receptors is the main cause of late subsensitivity.

The experiments consist of measurements of the accommodation (in diopters, D) of monkey eyes during gradually increasing concentrations of a muscarinic stimulant given systemically. One eye is subsensitized, the other is untreated control. Accomodation increases more slowly on the subsensitized side. When plotted (the 'accomodation plot') with the accommodation of the control eye on the abscissa (x) and that of the subsensitized eye on the ordinate (y), points representing simultaneous accommodations in the two eyes fall below the 45° line.

Assume only one population of contraction-causing receptors and that some of them are changed

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or lost through subsensitization. Since every muscle has a threshold and needs a certain number of occupied receptors to begin contraction, the response in the subsensitized eye would start later than in the control eye, its threshold would be elevated. Linear extrapolation of the accommodation plot would yield a negative intercept on the ordinate. This would also happen if affinity had been reduced.

The above also holds if there are several populations of non-silent receptors, which can be subsensitized, since one will have the highest affinity and thus determine the threshold. If only a low affinity population were affected by subsensitization, the slope of the accommodation plot would decrease markedly at higher agonist concentrations and the plot would be concave towards the abscissa.

The reasoning up to this point deals with the (primate) ciliary muscle as a unit, excited evenly by blood-borne agonist. But embryologically it has two portions, the meridional and the circular which appear at different stages of development (see Mann, 1964). The two portions could have different receptor populations. Moreover, the subsensitivity was produced by application of carbachol to the cornea and accommodation persisted for at least one day during which most of the drug in the aqueous humour is lost. Therefore, since contraction of the anterior part of the muscle closes tissue spaces leading to the posterior parts and contraction in the monkey ciliary muscle is not propagated (Figure 18 of Bárány, 1966) the degree of subsensitivity of the anterior, circular part probably exceeds that of the posterior part. These complications are reduced in importance, however, by the indirect way in which accommodation measures muscle contraction. Accommodation is caused by relaxation of a complex fibre system which (grossly speaking) suspends the lens fror$_0$ the ciliary muscle belly (see Rohen, 1979). The eye accommodates because the muscle belly becomes thicker, the tension in the suspending fibres relaxes. The posterior and meridional parts of the muscle are of little importance in the connection.

The relation between fibre relaxation and accommodation is governed by the properties of the lens capsule. Over the range studied the two lenses were essentially identical since the animals were selected to give symmetrical accommodations.

In view of the above it seems permissible to use the accommodation plot in the present search for a threshold elevation on the subsensitized side.

Methods

The methods used in the production and testing of subsensitivity have been fully described (Bárány *et al.*, 1982).

Cynomolgus monkeys (*Macaca irus*) of either sex and unknown but different ages were iridectomized under ketamine (Kaufman & Lütjen-Drecoll, 1975). After the eyes had healed completely the monkeys were repeatedly tested for accommodation during intramuscular infusion of pilocarpine. Only those lacking appreciable asymmetry were used for the present purpose.

Data from two groups of monkeys were utilized. Group I (5 monkeys) was part of larger experiment where all the animals were tested with both pilocarpine and carbachol and where the time schedule was rigidly standardized and symmetrical. Group II (9 monkeys) was not symmetrically arranged, not all animals received all agonists or were tested on the same day of subsensitization. Four were used only once.

In both groups, one eye was subsensitized on day 0 by corneal application of 100 μg carbachol. On day 7 (as a rule), ketamine and if necessary pentobarbitone was given, followed by determination of several resting refractions. The accommodation values are counted from these prevalues. The group I monkeys then received intramuscular infusions of either pilocarpine hydrochloride 1 mg kg^{-1} (1.5 mg in one case) over 18 min or carbachol chloride 25 μg kg^{-1} over 18 min (except one case 25 min). In some cases carbachol was given first and in other cases pilocarpine but all animals received both infusions with an interval of at least 2 months.

In group II, testing was carried out in the same way but besides pilocarpine and carbachol, bethanechol chloride at 0.5–1 mg kg^{-1} was used. There was always a long resting period ($>$ 6 weeks) after each subsensitization but the dose and total time of infusion was not the same in all experiments even with the same agonist. Most were tested on day 7 but some a few days earlier or later.

In all cases for both groups, accommodation was followed in both eyes as described (Bárány *et al.*, 1982) and all data obtained during the rising phase of accommodation of the control eye were used. The time interval between points was 1–2 min.

The accommodation values from the two eyes were plotted against time on the same graph paper. For each subsensitized eye observation, the simultaneous control accommodation was read by linear interpolation. Thus, accommodations in the two eyes belonging to the same concentration of circulating agonist could be plotted, the 'accommodation plot'. The control eye accomodation was always the abscissa. Usually the points fell on an approximately straight line with slope lower than 1.0. Figure 1 shows results from an experiment (not used here) selected to explain the further procedure.

At the start of the i.m. infusion, it has not yet caused any measurable accommodation. After a

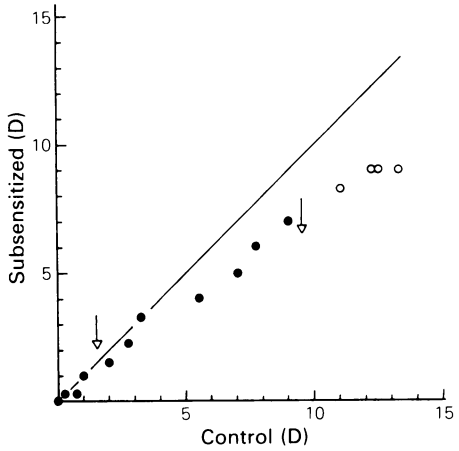

Figure 1 Accommodation plot to show sites of truncation. Abscissa scale: accommodation in diopters (D) of control eye; ordinate scale: same of subsensitized eye. (●) During carbachol infusion; (○) after cessation of infusion. The left arrow shows where lower truncation would have been made, the upper indicates the same for upper truncation. *Cynomolgus* monkey, day 11 after subsensitization, tested with carbachol chloride 25 $\mu g\,kg^{-1}$.

number of minutes, accommodation begins to change. Since there is no clearcut border line between the subthreshold points at the beginning and the first points of the clearly suprathreshold phase, one cannot simply discard the first points because they are close to zero. Accepting them, however, tends to pull the accommodation plot through the origin and to reduce the apparent size of a threshold change. Therefore calculations were performed in three ways:

(1) Using all points obtained, 'All'.
(2) Using no readings with a control eye accommodation less than 1.5 D. Data truncated in this way (left arrow in Figure 1) are called 'Not lower end'.
(3) At higher degrees of ciliary muscle contraction and accommodation, one eye sometimes reached an accommodation plateau before the other and the plot then turned either down or up. Fitting a straight line to this plot would give misleading values of the threshold, the intercept. Therefore, truncation both at the lower and if necessary the upper end was used. The upper truncation consisted in discarding everything above the last but one point before the first visual or numerical indication of appreciable curvature at the end. If there was no curvature, no truncation was done. Figure 1 shows where such truncation would have been used (right hand arrow). All experiments were candidates for upper truncation. This whole group is listed under 'Middle only'.

With carbachol as the infused agonist, fall in blood pressure limits the amount that can be used (Törnqvist, 1967). For this and similar reasons the number of evaluated points was not identical in the different experiments. Weighted as well as unweighted pooling of the data was therefore used.

Statistics

The treatment was as follows: a least squares straight line $y = a_0 + a_1x$ was fitted to the points of each experiment and the y-intercept a_0 and slope a_1 with their variances calculated. The control eye readings were always taken as x. For unweighted means of a_0 and a_1 the usual s.e. was taken. When calculating the weighted means of the a-values, their inverse variances were used as their weights. The weight of a weighted mean is the sum of all the entering weights, if the weights are known beforehand. A measure of the dispersion of the weighted mean analogous to the standard error is then obtained by inverting the square root of the sum of weights (w) entering that mean, $\sqrt{(1/w)}$. In the present case, however, the individual weights were calculated from the n experiments. Therefore the variance of the weighted mean is larger than 1/w and the number of degrees of freedom smaller than the conventional $n-1$.

Taking $\sqrt{(1/w)}$ as if it were s.e. when applying the t test with $n-1$, exaggerates the significance of any deviation from 0.

Introduction of a second order term, $y = a_0 + a_1x + a_2x^2$ was also attempted. An arbitrary minimum of 6 pairs of data was stipulated, in a few cases truncated sets of data therefore had to be discarded. No weighting was used. Despite the requirement for 6 pairs, sometimes the value of a_1 obtained was quite unreasonable, < 0. Nonetheless the experiment was not excluded.

Results

Straight line fitting: $y = a_0 + a_1x$

Table 1 shows the results of the group I experiments. Besides the results, the table also shows the range of diopters used for calculating the slopes and intercepts. The size of the interval on the accommodation scale used for the calculation differed between experiments and between truncated and non-truncated. It is listed as median range. Thus, median is the median size of the ranges and the listed (range) here is not the range of accommodation of the single experiment, it is the range of ranges.

The monkeys tested with carbachol and with pilocarpine in group I are identical. One monkey was tested twice with pilocarpine, the experiments were

Table 1 Results of fitting $y^{\P} = a_0 + a_1x$ to the accommmodation plots of monkeys group I

Test Drug	Points included (no. expts)	Accommodation utilized* Median and (range) Control	Subsensitized	Weighted means ±√(1/w)§ Intercept a_0*	Slope a_1	Unweighted means ±s.e. Intercept a_0*	Slope a_1
Pilocarpine	All	12.9	5.75	0.00	0.45	−0.01	0.49
	(6)	(8.75–14.4)	(4.5–9.0)	±0.06	±0.01	±0.09	±0.06
	Not lower end	10.5	5.0	−0.28	0.51	−0.26	0.52
	(6)	(6.75–12.0)	(4.5–7.75)	±0.12	±0.01	±0.18	±0.07
	Middle only	6.1	3.0	0.12	0.47	−0.10	0.49
	(6)	(2.75–10.0)	(1.25–3.5)	±0.06	±0.01	±0.22	±0.07
Carbachol	All	6.0	4.75	0.03	0.70	0.06	0.69
	(5)	(4.5–9.25)	(2.0–6.5)	±0.03	±0.01	±0.09	±0.04
	Not lower end	4.5	3.75	−0.04	0.70	0.12	0.67
	(5)	(2.75–7.25)	(0.75–5.25)	±0.07	±0.01	±0.14	±0.06
	Middle only	4.5	2.5	−0.06	0.69	0.05	0.69
	(5)	(2.75–6.75)	(0.75–4.75)	±0.08	±0.02	±0.16	±0.07

* Values in diopters; §w = weight of mean; ¶accommodation of subsensitized eye

far apart and the results sufficiently dissimilar to allow a second use. (The reason for the duplication was that a subsequent unrelated experiment failed the first time.) With pilocarpine the slopes are around 0.5; with carbachol around 0.7. Thus subsensitivity was less when tested with a choline ester than with pilocarpine, in accord with previous findings (Bito *et al.*, 1971; Bárány, 1977). The y-intercepts are all quite small, the line passes very close to the origin. The largest negative value, −0.28 + 0.12 (not significant) combined with the slope of ~0.5 D corresponds to an x-intercept of ~0.5 D which is quite a small threshold elevation. The different kinds of truncation made little difference and there is little

Table 2 Results of fitting $y^{\P} = a_0 + a_1x$ to the accommodation plots of monkeys group II

Test drug	Points included (no. expts)	Accommodation utilized* Median and (range) Control	Subsensitized	Weighted means ±√(1/w)§ Intercept a_0*	Slope a_1	Unweighted means ±s.e. Intercept a_0*	Slope a_1
Pilocarpine	All	12.5	5.0	0.17	0.45	0.18	0.46
	(9)	(8.7–18.25)	(2.75–12.0)	±0.04	±0.01	±0.06	±0.07
	Not lower end	11.5	5.25	0.42	0.36	0.35	0.45
	(9)	(7.5–16.5)	(2.25–11.5)	±0.05	±0.01	±0.11	±0.08
	Middle only	5.75	2.0	0.29	0.48	0.32	0.44
	(9)	(3.25–8.25)	(1.0–6.0)	±0.04	±0.01	±0.13	±0.07
Carbachol	All	7.63	4.37	0.09	0.59	0.09	0.58
	(4)	(6.75–10.0)	(4.0–6.75)	±0.03	±0.01	±0.08	±0.03
	Not lower end	6.13	3.37	0.26	0.53	0.19	0.56
	(4)	(5.25–8.0)	(2.75–5.25)	±0.06	±0.01	±0.13	±0.05
	Middle only	2.75	1.50	0.20	0.55	0.24	0.55
	(4)	(2.25–3.75)	(1.25–2.0)	±0.07	±0.02	±0.16	±0.04
Bethanechol	All	14.25	11.5	0.18	0.72	−0.10	0.72
	(7)	(8.75–17.5)	(6.25–14.0)	±0.05	±0.01	±0.20	±0.04
	Not lower end	11.0	9.25	0.20	0.72	−0.49	0.75
	(7)	(6.5–15.0)	(3.75–12.0)	±0.08	±0.01	±0.46	±0.05
	Middle only	6.75	4.5	0.22	0.63	0.15	0.64
	(7)	(5.75–9.5)	(3.5–7.0)	±0.06	±0.01	±0.14	±0.04

*Values in diopters; §w = weight of mean; ¶accommodation of subsensitized eye

difference between weighted and unweighted means. In these group I animals, straight line analysis thus gave no support for the existence of any appreciable receptor loss or change on the subsensitized side, no negative intercept was significant.

Table 2 shows results from 20 other experiments with the stable muscarinic choline ester, bethanechol, as well as pilocarpine and carbachol, No evidence for a negative a_0 was found.

If all 31 experiments are pooled, regardless of the testing agonist, the a_0-values of the weighted data were for 'all': $+0.1 \pm 0.02$; 'not lower': $+0.24 \pm 0.03$ and 'middle only': $+0.19 \pm 0.03$. Similar pooling of unweighted data yielded for 'all': $+0.03 \pm 0.05$; 'not lower end': -0.02 ± 0.13 and 'middle only': $+0.15 \pm 0.07$. Thus there is no hint of a threshold elevation on the subsensitized side.

Second order fitting: $y = a_0 + a_1x + a_2x^2$

In group I, all the intercepts a_0 were small and positive with one exception: a value -0.13 ± 0.52 was obtained as the mean of 4 carbachol experiments and truncation only at the lower end. All means were statistically insignificant. The median corresponding to the mentioned value was -0.36, much more negative than any other. In group II, there were no negative a_0. Thus, there was no evidence for a threshold elevation on the subsensitized side.

As mentioned (p. 194) if subsensitivity only acted on low-affinity receptors but there were non-silent high-affinity ones, one would expect an accommodation plot with a concavity towards the abscissa, a negative a_2. The only negative a_2 in group I was -0.013 ± 0.018 with 5 carbachol 'All' and in group II -0.0063 ± 0.0070, with 4 carbachol 'All'. Thus there is no evidence for a selective loss of low-affinity receptors on the subsensitized side. There was no statistically significant a_2 anywhere.

Discussion

Statistics cannot prove the absence of an effect. However, the results show that receptor change or loss can play at most a minor role in the type of subsensitivity studied in the present experiments. Taken as a whole, the accommodation plots run close to the origin, most of them with a slightly positive y-intercept and have little or no upwards convexity in the interval studied. The findings hold for all three agonists used for testing sensitivity: pilocarpine, carbachol and bethanechol.

Despite the fact that straight lines usually fit the accommodation plots rather well, exceptions do exist. One is illustrated in Figure 1 of Bárány *et al.* (1982), where monkey D shows evidence of a threshold elevation of the order of 2 diopters, while monkeys C, E and F do not. However, the converse has also been seen; Figure 12 of Bárány (1979) is a case in which subsensitivity is very low at the start and becomes more marked at accommodations above 3–4 D.

The term 'subsensitization' used here is equivalent to 'partial desensitization'. It is not known if full desensitization can ever be reached *in vivo* in the ciliary muscle.

Subsensitivity may or may not be specific. There is no way of testing this in the primate ciliary muscle *in vivo*, since, as far as is known, it contracts only on muscarinic stimulation.

The kind of subsensitivity dealt with in the present experiments is of a much slower kind than that usually studied *in vitro*, e.g. by Morgenstern & Bluth (1976) who also studied the recovery rate. Their desensitizing period was 10 min and the recovery of the order of 1 h. In the rat iris, studied by the method of Bito *et al.* (1971), some subsensitization of the sphincter by continuous light was seen after 6 h, but full steady state needed more than one week. Recovery may be a little faster (Claesson & Bárány, 1978). It is therefore possible that the mechanism of the subsensitivity studied here in the monkey ciliary muscle is related to that in the rat iris sphincter. In the cynomolgus ciliary muscle, marked subsensitivity was seen after continuous pilocarpine treatment with the low dose of 30 $\mu g\,h^{-1}$ for 5–6 days, as reflected in the effect of the muscle on the trabecular meshwork outflow resistance (Figure 2a, Bárány, 1977). Subsensitivity must have started earlier than that.

The duration of the late subsensitivity phenomenon differs. After 8 weeks treatment of *Cercopithecus ethiops* monkey eyes with echothiophate, it was 4–5 months after the end of treatment before sensitivity became normal (Kaufman & Bárány, 1975). In cynomolgus monkeys only incomplete recovery (of response of outflow resistance) was seen 6 weeks after the end of such treatment (Kaufman & Bárány, 1976). In contrast, after the single dose carbachol used in the present experiments, subsensitivity usually disappears in at most 3 weeks. It is not certain that the mechanism behind these two long-lasting subsensitivities is the same and it is highly unlikely that it is even related to the rapidly appearing and disappearing subsensitivity studied by e.g. Morgenstern & Bluth (1976).

It is hard to imagine a physiological function for the long-lasting subsensitivities. The suggestion that subsensitization is an important adaptive process (Bito *et al.*, 1971; Hurwitz & McGuffee, 1979) could well be relevant for the fast and early muscarinic subsensitivities conventionally studied *in vitro*. The late and long-lasting subsensitivities probably are of more interest as causes of failures of long-term agonist treatment, such as is used in glaucoma.

Supported by grant EY 00231 from the US Public Health Service. The author is indebted to Mrs Ingalill Wersäll for expert technical assistance.

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(*Received July 2, 1984.*
Revised September 5, 1984.
Accepted September 10, 1984.)