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DENERVATION, SUPERSENSITIVITY AND MUSCARINIC RECEPTORS IN THE CAT IRIS

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Abstract—An attempt was made to establish the molecular basis for denervation supersensitivity in the muscarinic system of the cat iris. Several components of that system, namely cholineacetyltransferase (CAT), acetylcholinesterase (AcChE) and the muscarinic acetylcholine receptor were determined following unilateral extirpation of the ciliary ganglion in cats that resulted in supersensitivity to pilocarpine. Marked decreases in CAT activity (80–90 per cent) as well in AcChE activity (25–60 per cent) were noted, starting on the 4th day following ciliary ganglionectomy. No change occurred in the concentration of the muscarinic receptors, nor in the dissociation constant of the muscarinic antagonist *N*-methyl-4-piperidyl benzilate (4-NMPB), on days 4, 7, and 14 following denervation. It is concluded that the development of denervation supersensitivity is not underlain by quantitative or qualitative changes in the muscarinic receptors. These and other results suggest, rather, that the mechanism responsible for parasympathetic denervation supersensitivity at the smooth muscle of the cat iris sphincter, must be connected with post-receptorial events.

It has been shown in many systems that supersensitivity in target organs can be induced by prevention of neuronal outflow to this organ or, alternatively, but reduction of long-term receptor occupancy by agonists (see review by Fleming *et al.* [1]).

The first tissue which a mechanism for supersensitivity was delineated was skeletal muscle (see review by Thesleff [2]). Since then it has been shown by several authors that the molecular basis for the supersensitivity induced following surgical denervation in that system was the proliferation of nicotinic acetylcholine receptors at the post-junctional membrane and their spreading from the narrow locality of the end-plate to cover the whole muscle membrane [3–5]. However, it was shown later that in addition to the spread of receptors, the denervated muscles undergo changes in permeability and electrical properties of the membrane, as well as changes in excitation–contraction coupling mechanisms [6 7]. These events may explain the supersensitivity to noncholinergic agents such as potassium ions [1].

Based on those findings, it was reasonable to anticipate such changes in denervated muscarinic systems. Bito and Dawson [8] have proposed that postjunctional changes in sensitivity in the denervated smooth muscle of the cat iris-sphincter, are the result of a proliferation of muscarinic receptors. However, this assumption has never been tested experimentally, and was the subject of the present study. In addition, we have studied AcChE and CAT activities following ciliary ganglionectomy.

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MATERIALS AND METHODS

Materials

The method of *N*-methyl-4-piperidyl benzilate (4-NMPB) preparation and tests for purity are as described previously [9, 10]. [^{14}C]acetyl coenzyme-A (59.5 mCi/m-mole) and [^{3}H]acetylcholine (250 mCi/m-mole), were purchased from Amersham. Acetylthiocholine-iodide, dithio-bis-(2-nitrobenzoic acid) (DTNB), acetylcholine chloride, physostigmine salicylate and atropine sulfate hydrate were obtained from Sigma. All other compounds were of the best grade available.

Methods

Animal treatment. Cats of either sex, weighing 1.8–3 kg were anesthetized with sodium pentobarbital (30–35 mg/kg i.v.), and the procedure for ciliary ganglionectomy was followed as described by Bito and Dawson [2]. The sensitivity of the iris was tested by instilling pilocarpine drops (0.2%) to both denervated and innervated eyes at various periods following the operation. This test was performed only once, on the day before the animals were killed. Animals showing pupillary light response in the ganglionectomized eye and/or incomplete constriction (pupils which were not slit-shaped) in response to pilocarpine were eliminated from the study. About 10 per cent of the animals had these apparently incomplete ganglionectomies and may represent aberrant pupillary innervation.

Tissue preparation was carried out as described in detail in the accompanying manuscript [10]. Pooled denervated and control irides, were subjected to the following procedures: (i) Irides were scissor-minced

and homogenized in 0.32 M ice-cold sucrose (1 iris/ml) as described [10]. The pellet (P) was resuspended in 0.32 M sucrose and used for binding assays. (ii) Irides were scissor-minced and homogenized as in (i), but in 1 M sucrose (1 iris/0.75 ml). The whole homogenate was centrifuged at 9750 *g* for 30 min (Sorval RC-2B, SS-34) and the supernatant collected. The pellet was rehomogenized in 1 M sucrose (0.25 ml/original iris), then recentrifuged and the supernatant added to the former. This fraction ($S_{1,2}$) was used for binding assays. The pellet (P_2) was resuspended and used for enzymatic assays.

Binding assays were performed as described in detail in previous reports [10, 11, 13]. Samples were assayed for radioactivity by liquid scintillation spectrometry (Packard Prias Model PL). Each vial was counted for 10 min, and corrections for quenching were made by using quench curve based on the external ratio method, using standard tritiated water and toluene (Packard). Specific binding is defined as the total binding minus binding in the presence of 5×10^{-5} M unlabeled 4-NMPB or atropine.

Acetylcholinesterase (AcChE) activity. (a) Acetylthiocholine hydrolysis was determined in whole homogenates and fractions (as specified), according to Ellman *et al.* [14], using a Varian Techtron spectrophotometer, model 635. The reaction mixture contained: 3 ml of 0.1 M phosphate buffer (pH 8.0), 25 μl of 0.075 M acetylthiocholine, 100 μl of 0.01 M DTNB and 50–100 μl of diluted homogenate. All reactions were performed at 25°.

(b) In several cases AcChE activity was determined by the radioactive method described by Johnson and Russel [15]. The reaction mixture contained: 50 μl of 0.05 M potassium phosphate buffer (pH 7.0), 20 μl of the homogenate, 2×10^{-3} M acetylcholine (final concentration) and [^{3}H]acetylcholine (0.1 μCi). The volume was completed to 0.1 ml with distilled water. The reaction was terminated by adding 0.1 ml of the stopping mixture (1 M chloracetic acid, 2 M NaCl and 0.5 M NaOH), followed by the addition of scintillation liquid (0.5% PPO, 0.03% POPOP (Packard), 10% isoamyl alcohol in toluene). The vials were counted one hour later in a scintallation spectrometer (Packard Prias Model PL) at 45 per cent efficiency.

Cholineacetyltransferase (CAT) Activity. CAT activity was measured by incubation of 10 μl homogenate with the incubation mixture described by Fonnum [16], which consisted of (final concentration): 0.035 mM[1-^{14}C]acetyl-CoA (8.25×10^4 c.p.m.), 300 mM NaCl, 50 mM sodium phosphate buffer (pH 7.4), 8 mM choline bromide, 20 mM EDTA and 0.1 mM physostigmine. The reaction mixture was incubated for 30 min at 37°. At the end of the incubation period, 50 μl of a solution (according to Massarelli *et al.* [17]) of tetraphenylboron in diisobutylketone (15 mg/ml) were added, and after mixing and centrifuging (2 minutes at 20,000 *g*), 10 μl of the organic phase were transferred to counting vials containing 4 ml omnifluor in toluene. Vials were counted in a scintillation spectrometer (Packard Prias Model PL) at 85 per cent efficiency.

Protein content. Protein was determined by the method of Lowry [18] using bovine serum albumin as standard.

RESULTS

Immediately following the ablation of the ciliary ganglion, mydriasis appeared and the pupil stopped reacting to light. Supersensitivity was tested in a group of unilaterally denervated cats daily, following the operation to check the course of its development. These animals were not used for other purposes. It is noted from Table 1 that the induced supersensitivity to pilocarpine developed relatively slowly and was demonstrated in all animals after the 4th post-operative day.

The weight and protein content of the innervated and denervated irides in a representative group were similar. For example, the weight and protein content of irides in a group of one week after denervation were 69 ± 8 mg and 4.55 ± 0.51 mg for denervated irides and 67 ± 10 and 4.57 ± 0.7 mg for innervated irides respectively. In all other experimental groups the irides were weighed in concert in the two separate pools, and there was never a difference greater than 10 per cent between them.

A marked decrease (77–91 per cent) in CAT activity was noted since the 4th day following denervation. By the fourteenth day there seemed to be an almost total disappearance of CAT activity in the denervated irides

Table 1. Course of supersensitivity development in a control group of 7 cats with unilaterally denervated irides*

Time after denervation (days)	Number of denervated eyes showing the specified reaction to pilocarpine			
	absent	minimal	submaximal	maximal
1	7	—	—	—
2	4	2	1	—
3	1	2	4	—
4	—	1	2	4
5	—	—	1	6
6	—	—	1	6
7	—	—	1	6
8–14	—	—	1	6

*Sensitivity to 0.2% pilocarpine was measured. Normally innervated pupils did not respond to this pilocarpine concentration.

Table 2. Effect of ciliary ganglionectomy on the cat iris cholineacetyltransferase (CAT) activity

Time after denervation (days)	nmole/hour/iris		nmole/hour/mg protein	
	denervated	innervated	denervated	innervated
Naive*	—	11.8	—	2.56
4	2.1 (12%)	18.0	0.40 (13%)	3.1
4	2.0 (23%)	8.6	0.58 (28%)	2.1
7	1.5 (15%)	10.0	0.20 (11%)	1.8
7	0.8 (9%)	8.8	0.16 (9%)	1.79
14	1.0 (10%)	10.0	0.20 (6%)	3.6

* Mean value from 6 separate experiments (7 cats in each). Each line represents a separate experiment. Each value represents the mean of duplicate samples (the values of which did not differ by more than 10 per cent) of the pooled homogenates from 7 irides. Values in brackets represent per cent of activity of denervated irides from that of contralateral control.

as is shown in Table 2. AcChE activity (Table 3) was also reduced in the denervated compared to the control side on the 4th day and thereafter, but the reduction was less drastic than that of CAT.

Muscarinic receptor level was assayed by [^{3}H]-4-NMPSB binding in two factions prepared from the whole homogenate but not in the original one (the reason for this will be discussed latter). Figures 1a and 1b show a binding curve of [^{3}H]-4-NMPB to P_2 fraction (see Methods) that was prepared from denervated and control cat irides, 2 weeks following denervation. In both cases the curves are hyperbolic, while the non-specific binding is low and varies linearly with ligand concentration. There seems to be practically no difference in the specific binding (total minus non-specific binding), between the operated and control eyes. Replotting the data according to Scatchard [19] also failed to demonstrate a significant effect of denervation on the maximum binding capacity (Fig. 2a and Table 3). In these Scatchard plots there was noted a binding which cannot be related to the muscarinic receptor (probably binding to the melanin pigment) that caused concavity of the curve in higher concentration of the ligand (Fig. 2a). Nevertheless, the higher affinity site, defined as a muscarinic receptor site [10] seems to be identical in both denervated and innervated irides. Similar assays were performed in the $S_{1,2}$ fraction (see Methods) as is shown in Figs. 1c and 1d and 2b. The

Table 3. Effect of ciliary ganglionectomy on the acetylcholinesterase activity and muscarinic binding capacity in the cat iris

Time after denervation (days)	μ mole/min/iris*		μ mole/min/mg prot*		[^{3}H]-4-NMPB maximal binding (B_{max}) fmole/mg tissue‡	
	denervated	innervated	denervated	innervated	denervated	innervated
Naive+	—	0.19	—	0.071	—	4.50 ± 1.2
4	0.17 (74%)	0.23	0.037 (93%)	0.040	3.40	4.15
4	0.08§ (42%)	0.19§	0.022§ (49%)	0.045	2.20	2.30
7	0.09 (110%)	0.09	0.011 (69%)	0.016	3.30	3.66
7	0.17§ (38%)	0.45§	0.033§ (37%)	0.090§	3.02	4.04
14	0.21 (72%)	0.29	0.030 (30%)	0.100	3.06	2.88

* Each line represents a separate experiment. Each value represents the mean of triplicate samples (the values of which did not differ by more than 10 per cent) of the pooled homogenate from 7 irides determined by the Ellman method. Values in brackets represent percent of activity of denervated compared to innervated irdes. § Mean value of duplicate samples determined by the alternative radioactive method.

+ Mean value from 6 separate experiments (7 cats in each).

‡ Each number represents the value calculated by extrapolation from Scatchard plots, for $S_{1,2}$ fraction.

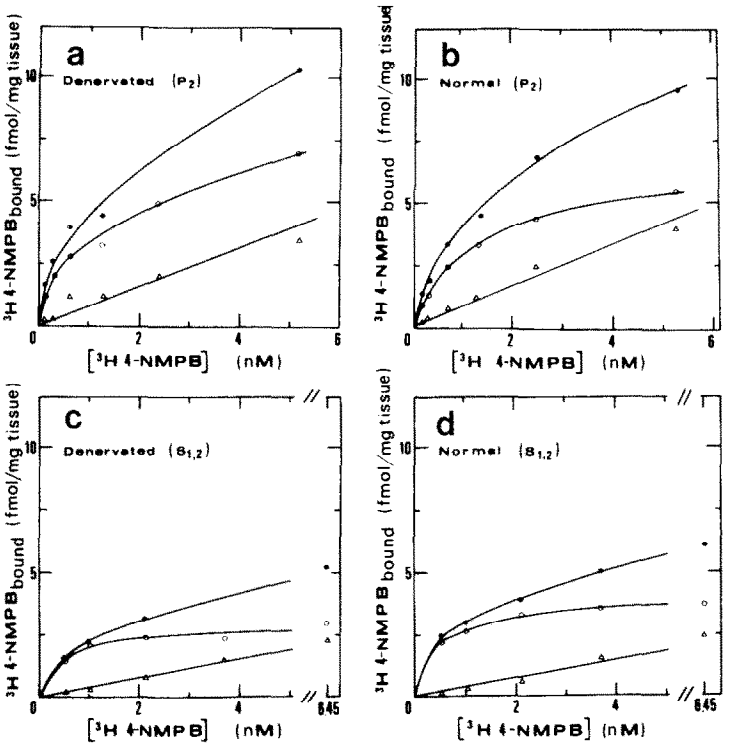

Fig. 1. Binding of [^{3}H]-4-NMPB at 25° as a function of concentration. 0.2 ml samples from the specified fraction were incubated with varying concentrations of [^{3}H]-4-NMPB for 30 min at 25° in 2 ml modified Krebs solution (pH 7.4). ●—●, total binding. △ △, non-specific binding (in the presence of 5×10^{-5} M 4-NMPB). ○—○, specific binding. Each experimental point represents the mean of duplicate samples. (a, b) Binding to the P_2 fraction (see Methods) from denervated and normal irides (respectively) of the same animals. (c,d) Binding to the $S_{1,2}$ fraction (see Methods) from denervated and normal irides (respectively) of the same animals.

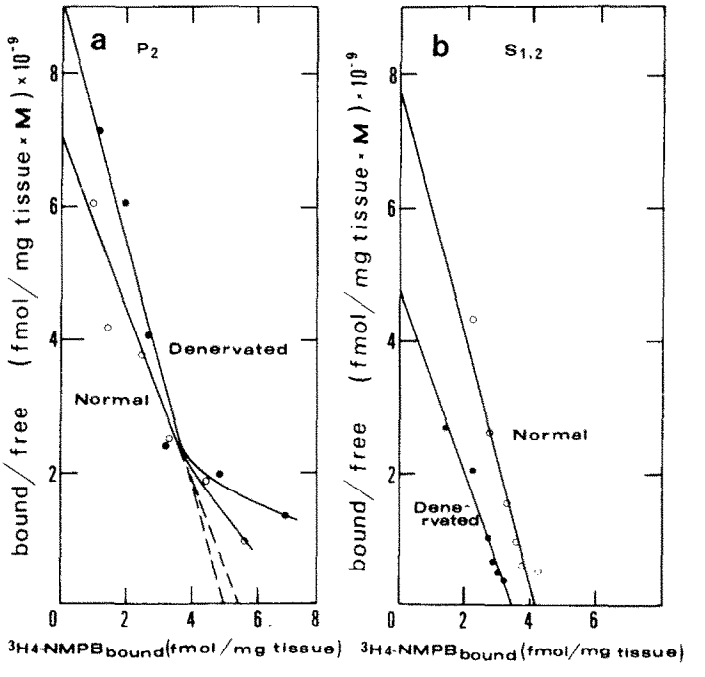

Fig. 2. Same data as in Fig. 1 replotted according to Scatchard. (a) Data from the P_2 fraction of denervated, (●—●) and normal (○—○) irides. (b) Data from the $S_{1,2}$ fraction of denervated (●—●) and normal (○—○) irides.

results for the fractions tested on the 4th, 7th and 14th day after denervation are summarized in Table 3. The mean values of K_d for denervated and normal irides from 4 separate experiments were 0.63 ± 0.14 and 0.60 ± 0.14 nM respectively.

DISCUSSION

It is clear from the data of the present experiments that the procedure we followed for the ciliary ganglionectomy, as described by Bito and Dawson [8], proved to be highly successful and induced supersensitivity to pilocarpine, which developed relatively slowly and reached a plateau between the 4th and 7th day after-denervation (Table 1). It should be emphasized again that only apparently maximally super-sensitized irides were used for the biochemical assays and compared to the normal irides of the same animals.

No degeneration of the iris sphincter occurred one week after denervation, as judged by tissued weight and protein content of the denervated irides. This is in agreement with the report of Armaly [20], that ciliary ganglionectomy failed to produce degeneration of ocular smooth muscles in the cat. This fact enabled the comparison of the cholinergic compenents tested (AcChE, CAT and the muscarinic receptor) in the denervated and normal irides, with the exclusion of the possibility of postoperative non-specific changes in the tissue. Data are usually presented both as per whole tissue and per mg protein. In our opinion, expressing data as per whole tissue is superior, because calculated in this way, they were not subject to contamination errors from adherent vitreous protein (although irides were washed immediately after removal).

In spite of the great variability of CAT in ocular tissues in different species, concordance of CAT activities in the tissues of the two eyes of the same animal has been reported [21]. Thus, one is justified to assume that the changes of CAT activity in the denervated as compared to the inervated eyes, stem from the specific manipulation and not from the natural CAT variability. The dramatic decrease of CAT activity in the denervated iris, following ablation of the ciliary ganglion (Table 2) is consistent with other reports [10, 22]. This decrease in CAT activity may serve as a supplementary proof that the ciliary ganglionectomy and the resultant nerve terminal degeneration were virtually complete.

The marked decrease in AcChE in the denervated irides (Table 3) is in agreement with other reports [23]. It should be mentioned that we have measured AcChE activity without inhibiting butyrylcholinesterase, so that our results might reflect a certain contribution from the enzyme. However, a greater contribution of AcChE is expected, since we have used acetylthiocholine (which is a better substrate for AcChE) for the assay. Some authors have argued that decreased AcChE activity in denervated skeletal muscle, can serve as a possible way to reconcile the differences between denervation-induced increase in nicotinic receptor (20-fold) and supersensitivity to acetylcholine (1000-fold increase) [5]. Others reported that in the smooth muscle of the vas deferens, the increase in sensitivity to acetylcholine after denervation has, in addition to the post-junctional component, a prejunctional one (decrease in AcChE activity), but showed that the contribution of this component to the total increase in sensitivity to acetylcholine is small [24]. This, of course, is feasible in the case of acetylcholine and other cholinomimetics acted upon by AcChE. However, when one considers supersensitivity to agents not affected by AcChE, such as potassium salts and pilocarpine (as in our case), it seems difficult to implicate the AcChE even as partially responsible for the development of supersensitivity. Furthermore, a decrease of 60 per cent and even more in the AcChE activity, cannot explain such changes in tissue sensitivity, since AcChE is known to be present (at least in skeletal muscle) in great excess [25]. Based on those arguments and our present data, it is concluded that the decrease in AcChE activity is secondary to the degeneration of ciliary nerves and is not in itself causative of the supersensitivity to pilocarpine.

After the findings of Thesleff [2], receptors were naturally regarded as prime candidates involved in the mechanisms underlying development of postjunctional supersensitivity. Thus Bito and Dawson's proposal [8] that postjunctional changes in sensitivity in the smooth muscle of the cat iris are the result of an increase in the concentration of receptors, was justified and reasonable. Retrospectively, however, the important differences in morphology and physiology, as well as in the specificity and degree of supersensitivity between smooth and skeletal muscles [1] makes it unnecessary to assume in the first place, that the mechanism of supersensitivity must be identical in all respects in the two tissues.

The results of our study show that no change occurred in muscarinic receptor capacity at the supersensitized cat iris (Table 3), nor in its characteristic 4-NMPB affinity constant.

As mentioned above, the binding of [^{3}H]-4-NMPB was assayed in two fractions prepared from the whole homogenate but not in the original one. The reason for this was that 4-NMPB, as well as other muscarinic antagonists, bind to the abundant melanin pigment present in cat irides [26]. This non-receptorial binding is much higher than that to the muscarinic receptors, even when using concentrations of ligand close to the dissociation constant of 4-NMPB from the receptor. This fact imposed on us two requirements: (a) to have complete binding curves, and (b) to work with fractions in which the non-receptorial binding was relatively low. The purest available fractions were P_2 and $S_{1,2}$ (see Methods), which we have characterized in normal cat irides [10], and shown to contain at least 60 per cent of the muscarinic receptors. However, since we followed the same procedures in the homogenates and fractions from the innervated and denervated irides, our results were reasonably controlled and probably reflect the true picture. These results and the preliminary observation that there was also no change in the affinity of pilocarpine to the receptor (as measured by competition binding studies) indicate, therefore, that the mechanism responsible fo this supersensitivity is post receptorial. These findings are in agreement with an increasing body of evidence indicating that post-receptorial ionic and/or membrane mechanisms are connected with post-junctional supersensitivity in smooth muscle following preganglionic sympathectomy [27] and in skeletal muscle. Such mechanisms may involve changes in permeability of the membrane accompanied with reduction of the resting membrane potential which is associated with changes in calcium availability [1, 7, 8]. Thus, it seems

that mechanisms underlying supersensitivity in effector organs may have reverberations in all spheres of cellular functions, and as long as post-receptorial events are not fully elucidated, other factors involved in those events (e.g. cGMP), might be considered as candidates.

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