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ACETYLCHOLINE RECEPTORS IN THE CILIARY GANGLION AND IN THE IRIS MUSCLE OF THE CHICK: SPECIFIC BINDING AND EFFECT ON THE SYNAPTIC TRANSMISSION OF THE NEUROTOXIN FROM *Naja naja siamensis*

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1 A specific binding of *Naja naja siamensis* neurotoxin was found both in the iris and in the ciliary ganglion of the chick.

2 Naja-toxin (125 nM) caused a complete block of the iris muscle contraction induced by carbamylcholine.

3 Naja-toxin had a different effect on the two neuronal populations present in the ganglion: it blocked the synaptically evoked response of the ciliary cells, while the response of the choroid ones was only slightly reduced. The effects were the same in a wide range of concentrations (125 to 2500 nM).

4 The results obtained in the iris show the existence of an acetylcholine receptor population similar to the nicotinic receptor of the skeletal muscle.

5 In the ciliary ganglion the results confirm the existence of different acetylcholine receptors on the two cell types.

Introduction

Some toxins present in the venoms of the Elapid snakes (*Bungarus multicinctus*, *Naja naja siamensis*, etc.) bind specifically and with high affinity to nicotinic acetylcholine receptors (AChR) of neuromuscular junctions, electric organs of fish and mammalian brains (Fewtrell, 1976). They are valuable tools for studying the pharmacology and the distribution of AChRs, and in some cases for purifying them. Less agreement exists on the possibility that these toxins bind to the nicotinic AChRs at ganglia in the autonomic nervous system. A specific binding of α-bungarotoxin to the AChR of sympathetic ganglia has been found (Matthews, Miledi & Vincent, 1975; Greene, 1976; Fumagalli, De Renzis & Miani, 1976). However, in contrast to the neuromuscular junction, where the binding of toxin blocks synaptic transmission, these toxins do not seem to impair synaptic function in the sympathetic ganglia (Chou & Lee, 1969; Magazanik, Ivanov & Lukomskaya, 1974; Brown & Fumagalli, 1977; Carbonetto, Fambrough & Muller, 1978).

Less attention has been focused on the other branch of the autonomic system, namely the parasympathetic ganglia and their effector organs. Among these, the ciliary ganglion of the birds, and its target organ, the iris, are of special interest because of their structural and physiological peculiarities. In the ciliary ganglion two different populations of cholinergic neurones send axons in two separate sets of nerves: the ciliary nerves and the choroid nerves (Marwitt, Pilar & Weakly, 1971). The 'ciliary' cells are larger than the 'choroid' cells, and their surface is almost completely covered by the calix, a large synaptic terminal surrounding the neurone as a cap. The choroid cells are smaller and have relatively few bouton-like synapses (Hess, 1965).

Although the AChRs of both cell types may be classified as nicotinic, some differences have been demonstrated by pharmacological experiments; the choroid cells are more susceptible to blockade by hexamethonium than are the ciliary cells, while the latter are more sensitive to (+)-tubocurarine (Marwitt *et al.*, 1971). The main target organ of the ciliary ganglion is the iris, which in the bird is composed of striated muscle fibres (Hess, 1966), whose cholinoceptor has nicotinic characteristics, with pharmacological features similar to those of skeletal muscle fibres (Pilar & Vaughan, 1969; 1971).

In the present paper we report results on the properties of the AChRs of the two ganglion populations and of the iris using, as a tool, the neurotoxin of *Naja naja siamensis*.

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Preliminary results of this study have been reported in the poster session of the Fourth EMBO Annual Symposium on 'Molecular Neurobiology' (Heidelberg, 1978).

Methods

Toxin preparation

The neurotoxin of *Naja naja siamensis* was purified according to Ong & Brady (1974) with minor modifications; the crude venom was obtained from Ross Allen Reptile Institute Inc., Silver Springs, Fla. The purified preparation gave a single band on SDS gel electrophoresis according to Maizel (1971).

The biological activity of the purified toxin was tested on the frog neuromuscular junction of the cutaneous pectoris muscle, using conventional intracellular recording. The miniature endplate potentials (m.e.p.ps) and the endplate potentials were completely blocked after 30 min incubation at toxin concentrations of 125 to 2500 nM. The block was not preceded by any detectable changes in the frequency of the m.e.p.ps or in the resting membrane potential of the muscle fibres in this range of concentrations, which was the one used for the physiological experiments on the ganglia and the iris.

The purified toxin was labelled with ^{125}I (Sorin, Saluggia, Italy) by the Chloramine T method (Greenwood & Hunter, 1963), yielding a specific activity of 70 to 80 Ci/mM, or by the solid state lactoperoxidase method (David, 1972), yielding a specific activity of 10 to 12 Ci/mM. These two preparations were used for the ganglia and for the iris, respectively. The labelled toxin was passed through a Sephadex G-25 column every 3 to 4 days, in order to eliminate any trace of free ^{125}I which might have been released from the labelled toxin.

Binding assay

The toxin binding to AChR was assayed according to a modification of the method of Felstoff & Engel (1974). Two intact ganglia (dissected under the stereo microscope from 30 to 40 days old chick and deprived of the connective capsule) or one homogenized iris (1 iris/100 µl of 0.01 M Na phosphate buffer, pH 7.4) were incubated in siliconized glass tubes, with increasing concentrations of ^{125}I Naja-toxin, and 0.01 M Na phosphate buffer pH 7.4 in a volume of 150 µl. The toxin concentrations ranged from 0.22 to 22 nM. Every sample was prepared at least in duplicate and incubated at 24°C for 1 h. To stop the binding reaction, 2.5 ml of the same cold Na phosphate buffer, containing 0.15 M NaCl, was added to each tube. The ganglia were then washed four times at 4°C for 10 min in an excess of this buffer. The samples containing the iris homogenate were filtered under mild vacuum on three layers of Whatman DE 81 filter paper discs, previously equilibrated with the buffer containing 0.15 M NaCl, and then washed with 40 ml of this buffer. The radioactivity was counted in a Packard gamma-counter, with an efficiency of about 45%. The nonspecific binding was determined both in the presence of a 1000 fold excess of unlabelled toxin or in the presence of 10^{-4} M (+)-tubocurarine.

Physiological assays

Ciliary ganglia were dissected from 30 to 40 day old chicks as described by Martin & Pilar (1963a) and the connective capsule was removed. The ganglia were incubated in a solution described by Hess, Pilar & Weakly (1969), the composition of which was based on an analysis of chicken plasma, kept at 23°C and bubbled with a mixture of 95% O_2 and 5% CO_2. The ganglion was mounted in a chamber similar to that described by Larrabee, Klingman & Leicht (1963). Pre- and postganglionic nerve trunks were drawn into suction electrodes. The preganglionic trunk was stimulated supramaximally (0.05 ms pulse duration at 5 stim/s) and the postsynaptic compound action potentials were recorded from the postganglionic trunks. The response to preganglionic stimulation recorded from the ciliary nerves was a double-peaked compound action potential reflecting the dual mode of synaptic transmission through the ciliary cells: the electrically (first peak) and the chemically (second peak) mediated transmission (Martin & Pilar, 1963a).

When the recording was made from all the postganglionic trunks (ciliary and choroid nerves together) a third peak appeared, with a longer latency; this peak corresponds to the chemically mediated action potentials of the cells sending their axons to the choroid nerves (Martin & Pilar, 1963b; Marwitt *et al.*, 1971) which can be seen in isolation when recording from the choroid nerves alone (see Figure 2).

The iris with a portion of the sclera was removed from the eye immediately after decapitation. In this way the pupil retained its normal size. The incubation was carried out in a glass chamber, under the same conditions as used for the ganglion experiments. The contraction of the sphincter muscle of the iris, in response to 100 µg/ml (547 nM) carbamylcholine (Fluka AG CH-9470 Buchs, Schweiz) added to the incubation medium, was evaluated from the variation of the diameter of the pupil.

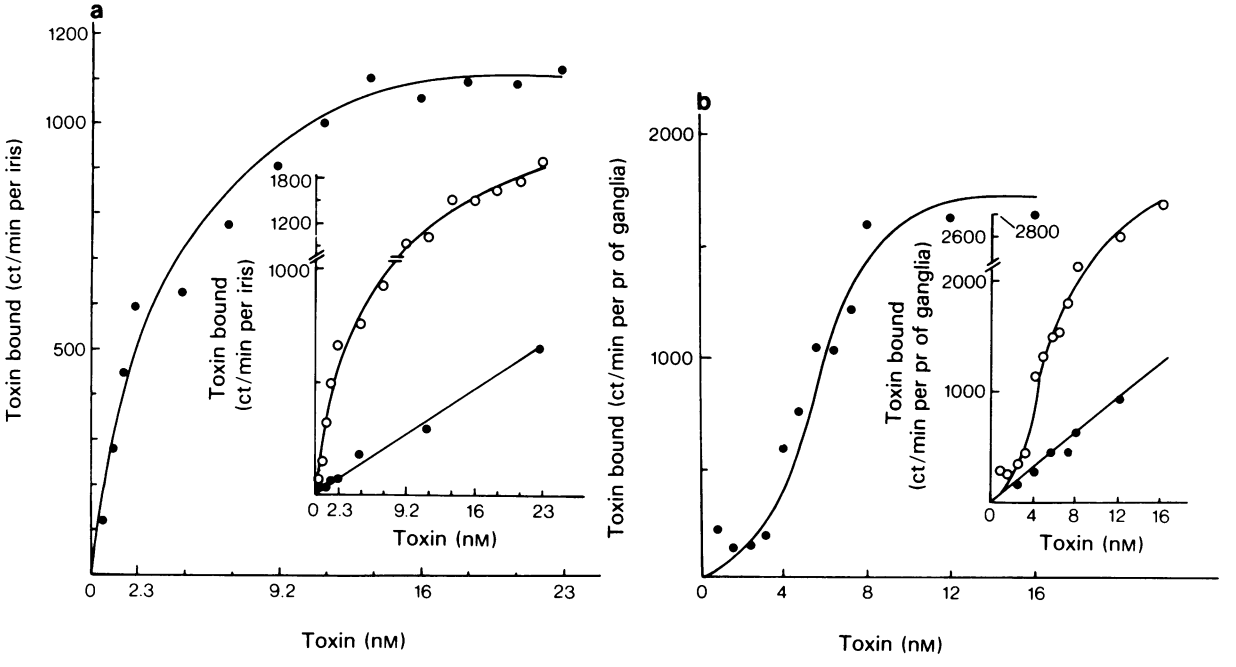

Figure 1 Binding of increasing concentrations of ^{125}I-neurotoxin of Naja naja siamensis to: (a) constant volume of iris homogenate, corresponding to one iris; (b) two intact ganglia. The curves show the specific binding of toxin (total binding minus non displaceable binding). In the inset: (○) show the total amounts of bound radioactivity; (●) show the amounts of bound radioactivity in the presence of a 1000 fold excess of unlabelled toxin.

Results

Iris

In the iris we detected a specific saturable binding of Naja-toxin. The total amount of toxin bound per iris was 333 ± 15.9 fmol. In our experimental conditions, half of the total binding sites was saturated at a concentration of 5.43 ± 1 nM toxin (mean ± s.e. mean; $n = 10$). Since no attempt was made to assess the reversibility of the binding, the latter value should not be taken as an expression of the affinity of the toxin for the receptor. In Figure 1a the curve obtained in a typical binding assay is shown. The inset in Figure 1a shows the ct/min obtained in the absence and in the presence of 1000 fold excess of unlabelled toxin. The amount of nonspecifically bound toxin was always lower than 30% of the total.

Incubation of the iris in the presence of 125 nM toxin (1 μg/ml) completely inhibited the contraction of the sphincter muscle, induced by 100 μg/ml carbamylcholine. The block could not be eliminated after 1 h of repeated washings.

Ciliary ganglion

A specific saturable binding of Naja-toxin was also detected in the ganglion. The total amount of toxin bound by a single ganglion varied from 8.5 to 18.5 fmol, with an average value of 13 ± 0.6 (mean ± s.e. mean; $n = 6$). In our experimental conditions half of the total binding sites was saturated at a concentration of 4.73 ± 0.59 nM toxin (mean ± s.e. mean; $n = 6$).

Figure 1b shows the curve obtained in a typical binding assay with intact ganglia; an S-shaped binding curve was consistently obtained. However, in two experiments carried out with homogenized ganglia, although the total amount of bound toxin per ganglion was unmodified, the shape of the curve approached a hyperbolic pattern.

The inset in Figure 1b shows the ct/min obtained in the presence or in the absence of 1000 fold excess of unlabelled toxin; the amount of nonspecifically bound ^{125}I toxin never exceeded 30% of the total counts. Similar results were obtained with 10^{-4} M (+)-tubocurarine.

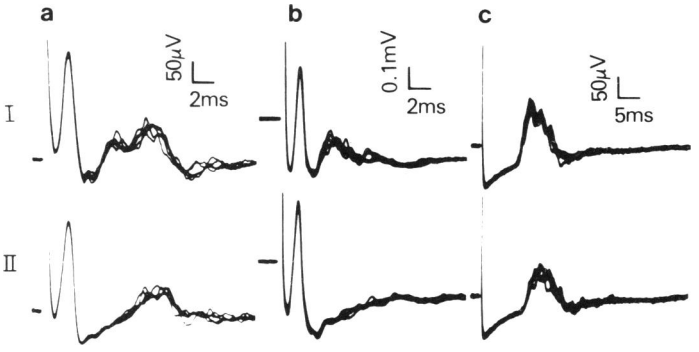

Figure 2 Effect of neurotoxin (125 nM) of *Naja naja siamensis* on synaptic transmission in the chick ciliary ganglion. The responses were elicited by supramaximal stimulation of the preganglionic trunk (oculomotor nerve) and recorded from: (a) ciliary and choroid nerves; (b) ciliary nerves; (c) choroid nerves. The upper set of records (I) was obtained before the addition of the toxin, the lower records (II) were obtained after 30 min incubation in the presence of the toxin. For further details see Methods.

The effects of 125 nM toxin on the compound action potentials evoked by preganglionic stimulation and recorded from the whole postganglionic trunk of an isolated ganglion incubated *in vitro* are shown in Figure 2a. Thirty min after adding the toxin, the first peak (due to the electrical synaptic transmission through the ciliary cells) was unaffected; the second peak (due to the chemical synaptic transmission through the same cells) was almost completely abolished, while the third peak (due to the chemical synaptic transmission through the choroid cells) was only slightly reduced.

In another series of experiments, the postganglionic action potentials were recorded from the ciliary trunks alone (Figure 2b). Again in this case the toxin, at the same final concentrations, abolished the chemically evoked 'short-latency' potentials (Figure 2b). When the postganglionic action potentials were recorded from the choroid nerves alone (Figure 2C), the toxin caused only a slight reduction of their amplitude and a small increase in their latency. These effects persisted for up to 4 h after repeated washings.

It was observed that the onset and the time to block were dependent on the toxin concentration. Thus, while the effect of 125 nM toxin started about 10 to 15 min after its addition and the block was reached 30 min later, in the presence of higher toxin concentrations (1250 to 2500) the effect started after 5 min and the block was reached in 20 min.

Discussion

Iris

Both from the binding assays and from the physiological experiments our results with chick iris give further support to the idea of a close pharmacological similarity between this parasympathetic effector, which in birds has a striated muscular structure (Hess, 1966) and skeletal muscle.

From our binding assays, we obtained a mean figure of 333 fmol of binding sites/iris. Since in the chick the average wet weight of one iris is about 4 mg we can calculate a value of 83 pmol of binding sites/g fresh tissue. This value is from 10 to 80 fold higher than the number of binding sites obtained by different authors in various striated muscles (Berg, Kelly, Sargent, Williamson & Hall, 1972; Fulpius, 1976).

This fact could reflect the particularly rich innervation of the muscle fibres in the chick iris, resulting from the high number of nerve endings per muscle fibre and the large extension of the synaptic contacts (Zenker & Krammer, 1967; Mussini, personal communication).

Ciliary ganglion

A specific binding of Naja-toxin to the ciliary ganglion was demonstrated by its saturability and by its inhibition in the presence of an excess of unlabelled toxin or (+)-tubocurarine. The average total binding capacity (13 fmol of binding sites/ganglion) agrees well with that found for α-bungarotoxin by Chiappinelli & Giacobini (1978) in the same preparation and with that found in the sympathetic ganglia in the chick and in the rat (Greene, 1976; Fumagalli *et al.*, 1976) for α-bungarotoxin. The unusual shape of the binding curve could be ascribed to a limited diffusion of the toxin in the intact ganglion, especially at the lower toxin concentrations, perhaps due to the presence of complex myelin sheaths surrounding the ciliary cells. This hypothesis is supported by the fact

that with homogenized ganglia the curve approaches a hyperbolic shape.

The effects of Naja-toxin on synaptic transmission are most probably explained by the fact that the toxin impairs the interaction between acetylcholine and its receptors. The possibility that the blockade of synaptic transmission is due to a decrease of the electrical excitability of the ganglion neurones or to a block of the impulse propagation into the nerve terminals is ruled out by the lack of effect on the electrical coupling potentials. The fact that the blocking action of the toxin is exerted mainly on the transmission through the ciliary neurones, fits well with the reported higher sensitivity to (+)-tubocurarine of the AChR of these cells (Marwitt *et al.*, 1971). Thus this AChR appears to be similar to the receptor at the neuromuscular junction. The possibility that the different sensitivity of the two types of ganglion cells is due to a different number of the same AChR on their postsynaptic membranes or to a different accessibility of the toxin to their synaptic clefts, can be excluded because even with 20 fold higher toxin concentrations, the same effects were observed.

It is possible that both the concentrations required for saturation (binding assay) and for blockade (physiological experiments) could be reduced by increasing the incubation time, as suggested by the progressive and apparently irreversible effect observed in our physiological experiments.

Besides diffusion problems, the difference found between the blocking concentration and the saturating concentration could also be due to the different ionic strength of the media used in the two experimental procedures. Such behaviour would be similar to that described for α-bungarotoxin, for which the binding rate to the AChRs from *Torpedo* electric organs, pigeon retina and mammalian cortex is reduced in the presence of physiological concentrations of simple cations, such as Na ions (Schmidt & Raftery, 1974; McQuarrie, Salvaterra, De Blas, Routes & Mahler, 1976; Yazulla & Schmidt, 1977).

In conclusion, our results demonstrate the existence of a specific binding of Naja-toxin to the ciliary ganglion, with a parallel impairment of chemical synaptic transmission; moreover, the results of the physiological experiments lend further support for the existence of two different populations of AChR, differently distributed on 'choroid' and 'ciliary cells'.

While this manuscript was in progress, a paper on the effect of α-bungarotoxin on synaptic transmission in avian ciliary ganglia appeared (Chiappinelli & Zigmond, 1978) in which it was shown that α-bungarotoxin also affects ganglionic synaptic transmission.

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References

BERG, D.K., KELLY, R. B., SARGENT, P.B., WILLIAMSON, P. & HALL, Z.W. (1972). Binding of α-bungarotoxin to acetylcholine receptors in mammalian muscle. *Proc. natn. Acad. Sci. U.S.A.*, **69**, 147–151.

BROWN, D.A., & FUMAGALLI, L. (1977). Dissociation of α-bungarotoxin binding and receptor block in the rat superior cervical ganglion. *Brain Res.*, **129**, 165–168.

CARBONETTO, S. T., FAMBROUGH, D.M. & MULLER, J. (1978). Non equivalence of α-bungarotoxin receptors and acetylcholine receptors in chick sympathetic neurons. *Proc. natn. Acad. Sci. U.S.A.*, **75**, 1016–1020.

CHIAPPINELLI, V.A. & GIACOBINI, E. (1978). Rate of appearance of α-bungarotoxin binding sites during development of chick ciliary ganglion and iris. *Neurochem Res.*, **3**, 465–478.

CHIAPPINELLI, V.A. & ZIGMOND R.E. (1978). α-bungarotoxin blocks nicotinic transmission in the avian ciliary ganglion. *Proc. natn. Acad. Sci., U.S.A.*, **75**, 2999–3003.

CHOU, T.C. & LEE, C.Y. (1969). Effect of whole and fractioned cobra venom on sympathetic ganglionic transmission. *Eur. J. Pharmac.*, **8**, 326–330.

DAVID, G.S. (1972). Solid state lactoperoxidase: a highly stable enzyme for simple gentle iodination of protein. *Biochem. biophys. Res. Commun.*, **48**, 464–471.

FELSTOFF, B.W. & ENGEL, W.K. (1974). In vitro analysis of the general properties and junctional receptor characteristics of skeletal muscle membrane. Isolation, purification and partial characterization of sarcolemmal fragments. *Proc. natn. Acad. Sci., U.S.A.*, **71**, 2435–2439.

FEWTRELL, C.N.S. (1976). The labelling and isolation of neuroreceptors. *Neuroscience*, **1**, 249–273.

FULPIUS, B.W. (1976). Characterization, isolation and purification of cholinergic receptors. In *Motor Innervation of Muscle*. ed. Thesleff S., p. 1. New York & London: Academic Press.

FUMAGALLI, L., DE RENZIS, G. & MIANI, N. (1976). Acetylcholine receptors: number and distribution in intact and deafferented superior cervical ganglion of the rat. *J. Neurochem.* **27**, 47–52.

GREENE, L.A. (1976). Binding of α-bungarotoxin to chick sympathetic ganglia: properties of the receptor and its rate of appearance during development. *Brain Res.*, **111**, 135–145.

GREENWOOD, F.C. & HUNTER, W.M. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.*, **89**, 114–117.

HESS, A. (1965). Developmental changes in the structure

of the synapse on the myelinated cell bodies of the chicken ciliary ganglion. *J. cell Biol.*, **25**, 1–19.

HESS, A. (1966). The fine structure of the striated muscle fibres and their nerve terminals in the avian iris: morphological "twitch-slow" fibres. *Anat. Rec.*, **154**, 356–357.

HESS, A., PILAR, G. & WEAKLY, J.N. (1969). Correlation between transmission and structure in avian ciliary ganglion synapses. *J. Physiol.*, **202**, 339–354.

LARRABEE, M.G., KLINGMAN, J.D. & LEICHT, W.S. (1963). Effects of temperature, calcium and activity on phospholipid metabolism in a sympathetic ganglion. *J. Neurochem.*, **10**, 549–570.

MAGAZANIK, L.G., IVANOV, A. YA & LUKOMSKAYA, N. YA. (1974). The effect of snake venom polypeptides on cholinoreceptors in isolate rabbit sympathetic ganglia. *Neurofiziol.* **6**, 652–656 (in Russian).

MAIZEL, J.V. (1971). Polyacrylamide gel electrophoresis of viral proteins. In *Methods in Virology*. ed. Maramorosh K., & Koprowski M., vol. 5. pp. 179–246. New York & London: Academic Press.

Martin, A. R., & Pilar, G. (1963a). Dual mode of synaptic transmission in the avian ciliary ganglion. *J. Physiol.* **168**, 443–463.

MARTIN, A.R. & PILAR, G. (1963b). Transmission through the ciliary ganglion of the chick. *J. Physiol.*, **168**, 464–475.

MARWITT, R., PILAR, G. & WEAKLY, J.N. (1971). Characterization of two ganglion cell populations in avian ciliary ganglia. *Brain Res.*, **25**, 317–334.

MATTHEWS, M.R., MILEDI, R. & VINCENT, A. (1975). Light and electron microscopic localization of ^{125}I-α-bungarotoxin in a sympathetic ganglion. *Proceedings 10th Int. Congr. Anat. Tokyo.* ed. Yamada, E. p. 221. Science Council of Japan.

MCQUARRIE, C.M., SALVATERRA, C.M., DE BLAS, A., ROUTES, J. & MAHLER, M. R. (1976). Studies on nicotinic acetylcholine receptors in mammalian brain. Preliminary characterization of membrane bound-α-bungarotoxin receptors in rat cerebral cortex. *J. biol. Chem.* **251**, 6335–6339.

ONG, D.E. & BRADY, R.N. (1974). Isolation of cholinergic receptor protein from Torpedo nobiliana by affinity chromatography. *Biochemistry*, **13**, 2822–2827.

PILAR, G. & VAUGHAN, P.C. (1969). Electrophysiological investigations of the pigeon iris neuromuscular junctions. *Comp. Biochem. Physiol.* **29**, 51–72.

PILAR, G. & VAUGHAN, P.C. (1971). Ultrastructure and contractures of the pigeon iris striated muscle. *J. Physiol.*, **219**, 253–266.

SCHMIDT, J. & RAFTERY, M.A. (1974). The cation sensitivity of the acetylcholine receptor from Torpedo California. *J. Neurochem.*, **23**, 617–623.

YAZULLA, S. & SCHMIDT, J. (1977). Two types of receptors for α-bungarotoxin in the synaptic layers of the pigeon retina. *Brain Res.*, **138**, 45–57.

ZENKER, A. & KRAMMER, E. (1967). Untersuchungen über Feinstruktur und Innervation der inneren Augenmuskulatur des Huhnes. *Z. Zellforsch. Mikrosk. Anat.*, **83**, 147–168.

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