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α-Bungarotoxin blocks nicotinic transmission in the avian ciliary ganglion

(nicotinic antagonist/ganglionic nicotinic receptor/parasympathetic ganglia)

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ABSTRACT **α-Bungarotoxin binds to nicotinic receptors in skeletal muscle, blocking neuromuscular transmission. Because this toxin has recently been shown to bind to chicken ciliary ganglia, an attempt has been made to determine whether it also blocks nicotinic transmission in this ganglion. α-Bungarotoxin (1 μM) completely blocked nicotinic transmission in both the ciliary and choroid neurons of chicken and pigeon ciliary ganglia. The effect of the toxin could be partially reversed by prolonged washing (2–8 hr). Incubation of ganglia with *d*-tubocurarine (0.1 mM) prior to the addition of α-bungarotoxin significantly decreased the duration of the washout period necessary to restore transmission. These results suggest that *d*-tubocurarine and α-bungarotoxin are interacting with the same receptor. Under similar conditions, α-bungarotoxin did not block nicotinic transmission in the rat superior cervical ganglion, in agreement with previous reports. The avian ciliary ganglion is the only vertebrate autonomic ganglion in which both α-bungarotoxin binding and α-bungarotoxin blockade of transmission have been shown to occur. This ganglion therefore provides a model system for using α-bungarotoxin to study neuronal nicotinic receptors.**

In the 1960s, a group of polypeptides isolated from various snake venoms was found to block transmission at the skeletal neuromuscular junction and to bind with a high affinity to nicotinic receptors in vertebrate skeletal muscle and electric organs of certain fish and eels (1, 2). One of these toxins, α-bungarotoxin, isolated from the venom of *Bungarus multicinctus*, was shown to bind essentially irreversibly to these receptors. It has since been used to quantitate the number of nicotinic receptors in skeletal muscle under various experimental conditions, to localize the receptors at both the light and electron microscopic levels, and to identify the receptor during its purification (1–5).

Many attempts have been made to use α-bungarotoxin to study the "nicotinic" receptors on neurons, both in the central nervous system and in peripheral autonomic ganglia (6–11). α-Bungarotoxin binding, which is inhibited by nicotinic agonists and antagonists and which shows a dissociation constant in the nanomolar range, has been reported in sympathetic ganglia (8, 9), in adrenal medulla (10), and in a clonal cell line derived from a pheochromocytoma (11). However, in no case has α-bungarotoxin blocked synaptic transmission in these tissues. Thus, α-bungarotoxin blocks neither the electrophysiological response recorded from the surface of the rat superior cervical ganglion following preganglionic stimulation or carbachol superfusion (12) nor the response to acetylcholine of these ganglion cells in culture (13, 14). In addition, the toxin does not block the nicotine-induced secretion of catecholamines in the adrenal medulla (10) or the carbachol-stimulated influx of sodium in the pheochromocytoma cell line (15). A further dissociation of α-bungarotoxin binding sites and acetylcholine receptors in this cell line has been made by Patrick and Stallcup (15). They suggested, on immunological grounds, that the toxin does not bind to the physiologically active receptor sites and concluded that toxin binding has limited usefulness in studying nicotinic receptors on neurons.

Recently, α-bungarotoxin binding sites have also been found in a parasympathetic ganglion in the chicken, the ciliary ganglion (16, 17). We report here that, in contrast to its effects on other vertebrate ganglia, α-bungarotoxin does block nicotinic neurotransmission in the avian ciliary ganglion and that the effect of the toxin can be decreased by pretreating the ganglion with *d*-tubocurarine.

MATERIALS AND METHODS

Chickens of various ages were obtained from SPAFAS (Norwich, CT); adult pigeons were from Palmetto Pigeon Plant (Sumter, SC). The birds were decapitated and their ciliary ganglia were removed with attached oculomotor nerves (containing preganglionic fibers) and ciliary and choroid nerves (containing postganglionic fibers of the ciliary and choroid cells). Ganglia were placed in freshly prepared chicken (18) or pigeon (19) Tyrode's solution. Sprague–Dawley male rats (approximate body weight, 125 g) were anesthetized with ether, and the superior cervical ganglia were removed along with their pre- and postganglionic trunks. All ganglia were desheathed prior to use.

The electrophysiological experiments were performed in organ culture dishes coated with Sylgard. The ganglia were placed in the central chamber of the dish and the preganglionic and postganglionic trunks were drawn into suction electrodes with chloridized silver leads for preganglionic stimulation and postganglionic differential recording. The effects of different drug treatments on synaptic transmission in these ganglia were assessed during brief periods (1–10 sec) of low-frequency stimulation (2–10 Hz). During drug exposures, the inner chamber of the dish (volume, 4 ml) was filled with Tyrode's solution containing the drug and the solution was constantly gassed with 95% O_2/5% CO_2. During washout periods, the entire culture dish (volume, 25 ml) was perfused with Tyrode's solution at a rate of approximately 10 ml/min. All experiments were performed at room temperature (22°).

d-Tubocurarine chloride was obtained from Calbiochem, and α-bungarotoxin, from the Miami Serpentarium and Boehringer Mannheim.

RESULTS

The avian ciliary ganglion contains two discrete types of neurons, ciliary and choroid, which can be distinguished anatomically and electrophysiologically (19). Transmission between preganglionic nerves and the ciliary neurons can be mediated

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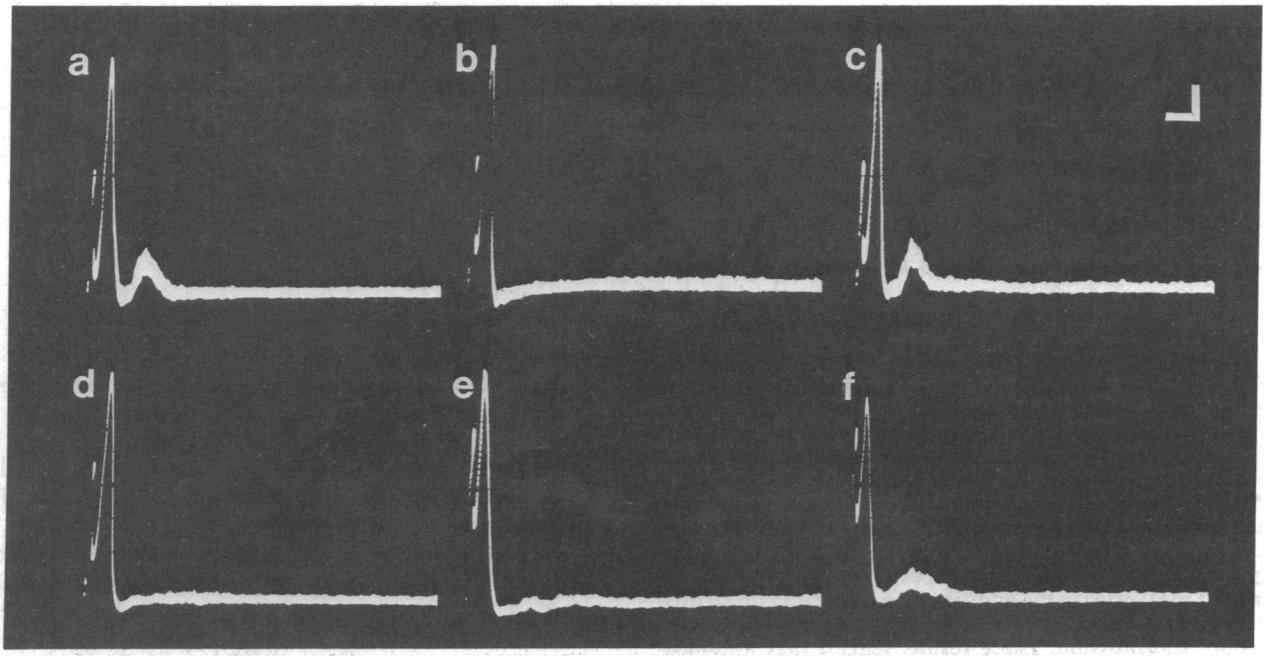

FIG. 1. Effect of α-bungarotoxin on the response of chicken ciliary neurons to preganglionic nerve stimulation. A ciliary ganglion from a 21-day-old chicken was stimulated preganglionically for brief (1–10 sec) periods at 10 Hz and the responses of ciliary neurons were recorded. (*a*) Control response in Tyrode's solution. The large compound action potential immediately after the stimulus artifact is due to ciliary neurons that are electrically excited by preganglionic nerves. The smaller response with a longer latency is mediated by chemical transmission. (*b*) At 10 min after addition of a solution containing 10 mM Mg^{2+} and 0.2 mM Ca^{2+}, the chemical response is blocked but the electrical response is not affected. (*c*) Recovery after washing with Tyrode's solution for 10 min. (*d*) At 30 min after addition of 1 μM α-bungarotoxin, the chemical response is blocked. (*e* and *f*) At 90 and 120 min after washing out the toxin. Bars = 0.1 mV and 5 msec.

either electrically or chemically whereas the choroid neurons are excited only chemically (18, 19). Both types of neurons are innervated by the same preganglionic trunk, but the neurons project into different postganglionic trunks and therefore can be studied separately.

Fig. 1*a* shows the response to preganglionic nerve stimulation of a chicken ciliary ganglion recorded from the postganglionic ciliary nerve. The response shows an early and a late component. Blockade of transmitter release by exposure to a solution containing 10 mM Mg^{2+} and 0.2 mM Ca^{2+} abolished the late component of the response but had no effect on the early component (Fig. 1*b*). This block was readily reversed (Fig. 1*c*), which indicates that the first component is due to electrical transmission and the second component to chemical transmission as previously shown by Martin and Pilar (20).

When this ganglion was exposed to α-bungarotoxin (1 μM) for 30 min, chemical transmission was abolished but electrical transmission was unchanged (Fig. 1*d*). Washing the ganglion for 90 min with normal Tyrode's solution after exposure to the toxin produced no recovery of the chemically stimulated response (Fig. 1*e*). After washing for 2 hr. the response was partially restored (Fig. 1*f*).

α-Bungarotoxin blocked ciliary neuron chemical transmission in all eight chicken ganglia studied. The mean (±SEM) latency to total blockade was 35 ± 4 min. In seven of the eight ganglia, transmission was only partly restored when the washout was stopped after 2–5 hr; in one case, transmission was fully restored after 3 hr.

In another experiment, *d*-tubocurarine (0.1 mM) blocked ciliary neuron chemical transmission after an exposure of 11 min (Fig. 2 *a* and *b*). This blockade was more readily reversible than that produced by α-bungarotoxin. Complete recovery of the chemical response after exposure to *d*-tubocurarine was seen after a washout period of 44 min (Fig. 2*c*).

To determine if both compounds blocked transmission by acting on the same receptor, a protection experiment involving a preincubation with *d*-tubocurarine prior to exposure to α-bungarotoxin was performed. *d*-Tubocurarine (0.1 mM) was added to the bathing medium and, when chemical transmission had been blocked, this solution was replaced with one containing both *d*-tubocurarine (0.1 mM) and α-bungarotoxin (1 μM). The ganglion was exposed to both compounds for 30 min and then washed with fresh Tyrode's solution. The time required for the ganglion response to return to the magnitude of the original response was then determined (Fig. 2 *d–f*). The response of the ciliary neurons recovered more quickly and to a greater extent when the ganglion was exposed to α-bungarotoxin after a preincubation with *d*-tubocurarine than when it was exposed to α-bungarotoxin alone. In the example shown in Fig. 2 the response had totally recovered after 87 min of washing. When this ganglion was subsequently exposed to α-bungarotoxin alone, the response was still completely blocked after 80 min of washing (Fig. 2*i*), and less than 15% of the response had recovered after 2 hr of washing. In a second protection experiment, the time to complete recovery after exposure to both *d*-tubocurarine and α-bungarotoxin was almost identical to that after *d*-tubocurarine alone. After exposure to α-bungarotoxin alone, washing for twice as long produced less than 35% recovery of the response.

The relative amount of chemical transmission in the chicken ciliary nerve varied from preparation to preparation and in some ganglia only an electrical component was recorded as has been reported (20). Because in the adult pigeon a greater proportion of the ciliary nerve response is chemically mediated than is the case in the chicken (19), the effect of α-bungarotoxin on the ciliary neurons of the pigeon was examined. The ciliary ganglion was stimulated via its preganglionic trunk and responses were recorded from both ciliary and choroid nerves

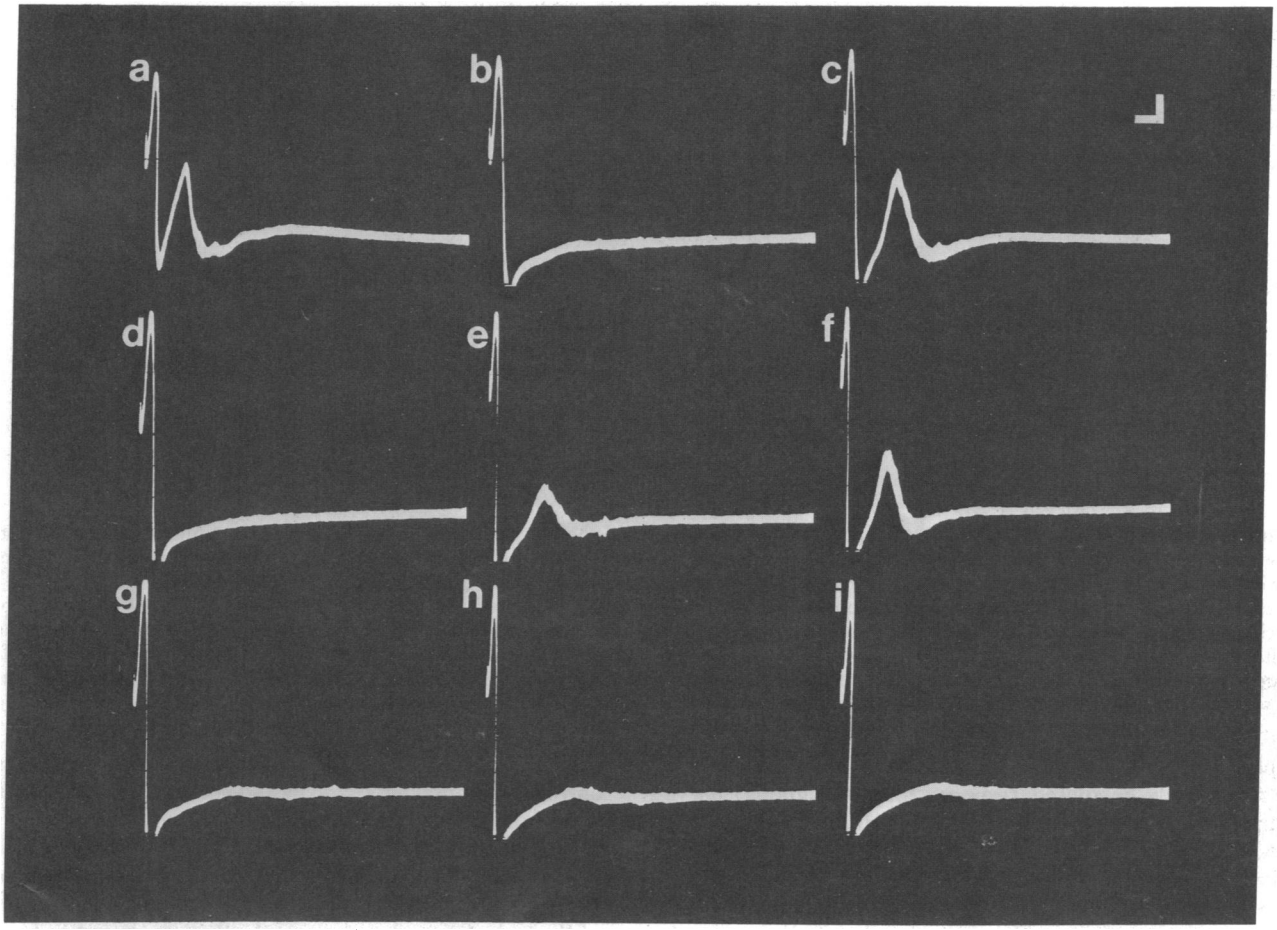

FIG. 2. Effect of α-bungarotoxin on chicken ciliary neurons after pretreatment with *d*-tubocurarine. (*a*) Control ciliary nerve response recorded from ciliary ganglion of a 64-day-old chicken. Distinct electrical and chemical responses are evident. (*b*) Eleven min after exposure to 0.1 mM *d*-tubocurarine, the chemical response was abolished. (*c*) Recovery after 44 min of washout. (*d*) Response of the same ganglion 17 min after a second incubation with 0.1 mM *d*-tubocurarine. At this point the ganglion was exposed to a solution containing both *d*-tubocurarine (0.1 mM) and α-bungarotoxin (1 μM) for 30 min. (*e* and *f*) Recovery after 45 and 87 min of washout respectively. (*g*) Thirty min after the subsequent addition of α-bungarotoxin (1 μM) alone. (*h* and *i*) Forty and 80 min, respectively, after the washout began. Partial return of chemical transmission first became apparent after 2 hr of perfusion with Tyrode's solution (not shown). Bars = 0.1 mV and 5 msec.

simultaneously with two separate electrodes. In the example shown in Fig. 3, the ciliary response was almost totally chemically mediated as judged by its latency (early peak in Fig. 3*a*). As reported (19), the response from the choroid nerve occurred later than the response from the ciliary nerve (late peak in Fig. 3*a*).

After 30 min of exposure to 1 μM α-bungarotoxin, the ciliary nerve response was almost completely blocked and the choroid nerve response was increased in latency but was still considerable in size (Fig. 3*b*). (In both ciliary and choroid nerves, increases in latency and spreading out of responses were seen during initiation of blockade and recovery from exposure to both α-bungarotoxin and *d*-tubocurarine.) After 75 min of exposure to 1 μM α-bungarotoxin, both ciliary and choroid nerve responses were blocked (Fig. 3*c*) and remained blocked after 2 hr of washing (Fig. 3*d*). Washing for 5 hr resulted in a considerable return of choroid nerve response, but the ciliary nerve response was still blocked (Fig. 3*e*). After 9 hr of washing, the ciliary nerve response had partially recovered (Fig. 3*f*, small, early peak), and the choroid nerve response was further increased in magnitude from that seen at 5 hr of washing (Fig. 5*f*, large, late peak).

α-Bungarotoxin also blocked transmission in chicken choroid neurons. In the chicken, as in the pigeon (Fig. 3), α-bungarotoxin blockade was more reversible in choroid than in ciliary neurons. In all three chicken ganglia tested, 100% of the choroid nerve response recovered in 3 hr of washing. As reported above, ciliary neuron transmission was completely recovered in only one of eight ganglia at this time. The choroid nerve response, like the ciliary nerve response (Fig. 2), was protected from α-bungarotoxin blockade by previous exposure to 0.1 mM *d*-tubocurarine.

Because previous reports had indicated that in another autonomic ganglion, the rat superior cervical ganglion, α-bungarotoxin showed specific binding but did not block nicotinic transmission (12), the effects of the toxin on this ganglion were examined under the identical conditions that had produced blockade of transmission in the ciliary ganglion. In Fig. 4, the response to stimulation of the preganglionic cervical sympathetic trunk recorded from the postganglionic internal carotid nerve is shown. Most of the response was reversibly blocked by *d*-tubocurarine (0.1 mM) after 30 min (Fig. 4 *b* and *c*). In contrast, long exposure to 1 μM α-bungarotoxin (90 min) had no effect on the evoked response (Fig. 4*d*). In a second experiment, a 3-hr exposure of another ganglion to 1 μM α-bungarotoxin had no effect on transmission.

DISCUSSION

Our results demonstrate that α-bungarotoxin blocks nicotinic transmission in the avian ciliary ganglion. Exposure of both

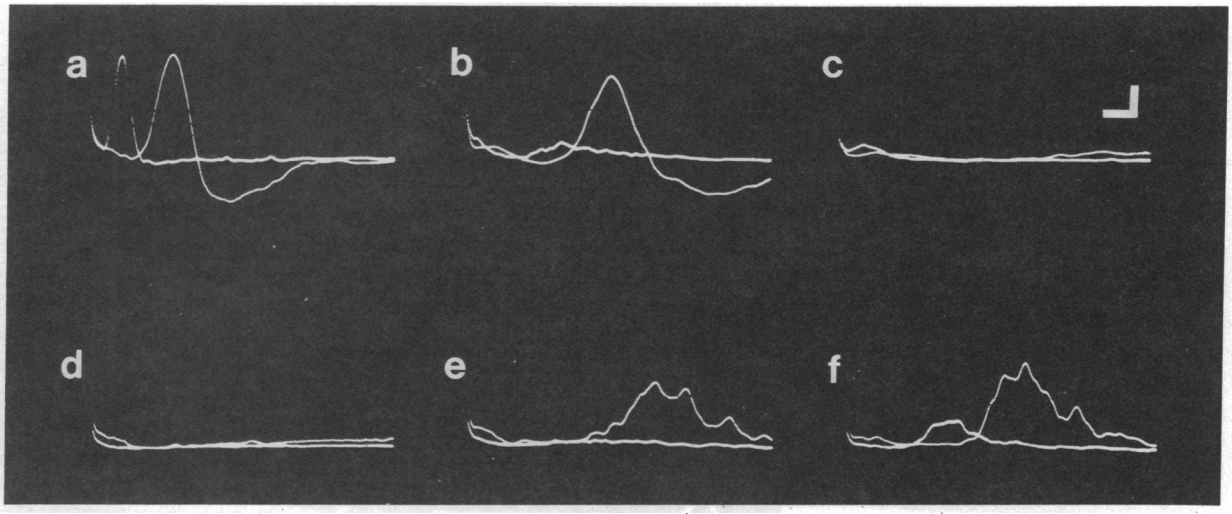

FIG. 3. Effect of α-bungarotoxin on the responses of pigeon ciliary and choroid neurons to preganglionic nerve stimulation. Postganglionic responses were recorded simultaneously from both the ciliary and choroid nerves of an adult pigeon ciliary ganglion. (*a*) Control responses. The ciliary nerve record shows the earlier response, which is chemically mediated. Only a very small electrical component was present in this ganglion. The choroid record shows the later response, which is also chemically mediated. (*b*) At 30 min after exposure to 1 μM α-bungarotoxin, the ciliary response had almost disappeared and the choroid response had increased in latency. (*c*) At 75 min after exposure to 1 μM α-bungarotoxin, both responses had been abolished. (*d*) Two hours of washing did not reverse the blockade. (*e*) After 5 hr of washout, partial recovery of the choroid response was observed, but the ciliary response remained blocked. (*f*) After 9 hr of washing, both responses had partially recovered, the smaller response with the shorter latency being the ciliary response. Bars = 0.2 mV and 2 msec.

chicken and pigeon ganglia to 1 μM α-bungarotoxin blocked the chemically mediated response to preganglionic nerve stimulation recorded from both the ciliary and choroid nerves.

Preincubation of ganglia with 0.1 mM *d*-tubocurarine was successful in protecting against α-bungarotoxin, suggesting that both agents are acting on the same receptor. In these experiments, six different batches of α-bungarotoxin were tried and two of these failed to block nicotinic transmission in the chicken ciliary ganglion.* All of the batches were capable of blocking neuromuscular transmission in cocultures of embryonic nerve and muscle. Although we have referred to the principle that was active in the ciliary ganglion as α-bungarotoxin, it is conceivable that a second component other than this 8000 molecular weight peptide was present and was responsible for the blockade of transmission. If such a component is present, it resembles α-bungarotoxin in that its effects can be prevented by *d*-tubocurarine.

Although α-bungarotoxin binding has been found in various autonomic neurons, the chicken ciliary ganglion is the only one of these tissues in which a blockade of transmission by the toxin has also been found. As already noted, the chicken ciliary ganglion is an unusual autonomic ganglion in several respects. The ciliary neurons can be activated both electrically and chemically. In addition, these neurons innervate skeletal muscle, unlike other postganglionic neurons (21). One might therefore argue that the ciliary neurons represent a special case and might be the only autonomic neurons whose nicotinic receptors are blocked by α-bungarotoxin. Our experiments with the choroid cells, however, argue that this is not true. Choroid cells exhibit only chemical transmission and innervate smooth muscle (21). They are therefore similar to typical postganglionic parasympathetic neurons. Perhaps α-bungarotoxin blocks transmission only in parasympathetic ganglia and not in sympathetic ganglia. The results of Bursztajn and Gershon (22), however, suggest that this generalization does not hold. These workers studied the effect of α-bungarotoxin on transmission in parasympathetic ganglia innervating the guinea pig intestine and found no effect of the toxin. Somewhat surprisingly, they also found no binding of the toxin by histochemical techniques.

Thus, although the nicotinic receptors in the chicken ciliary ganglion are blocked by α-bungarotoxin, the generality of these findings to other ganglia remains to be determined. There are only two other neuronal systems in which both binding and receptor blockade by the toxin have been found. Two groups

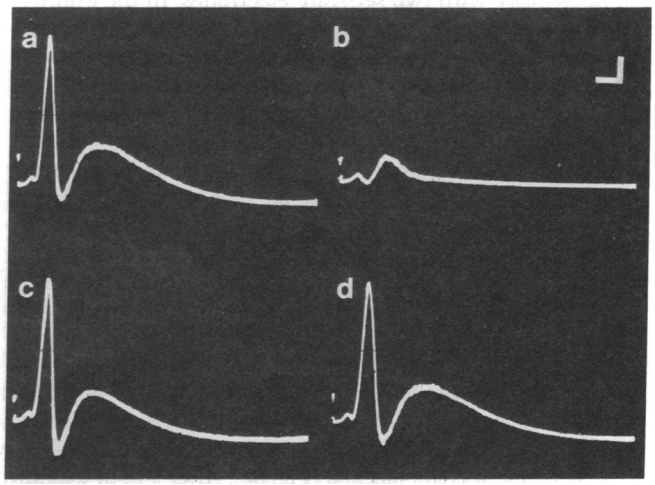

FIG. 4. Failure of α-bungarotoxin to block transmission in the rat superior cervical ganglion. A rat superior cervical ganglion was stimulated preganglionically and the response of neurons projecting into the internal carotid nerve was recorded. (*a*) Normal respose. (*b*) At 34 min after addition of 0.1 mM *d*-tubocurarine, most of the response was blocked. (*c*) At 22 min after washing out the *d*-tubocurarine. (*d*) At 72 min after exposing ganglion to 1 μM α-bungarotoxin, the response was unchanged. Bars = 0.25 mV and 20 msec.

* Blockade of nicotinic transmission in avian ciliary ganglia was seen with lots BMα 7A-1, BMα 7B-1, BMα 7C-1, and BMα 8-1Z purchased from the Miami Serpentarium. However, lot BMα 7D-1Z, also from the Miami Serpentarium, and lot 1117304, from Boehringer Mannheim, produced no effect on transmission in the chicken ciliary ganglion when tested under comparable conditions.

have reported that α-bungarotoxin blocks the Cl^--dependent cholinergic response in *Aplysia*, although there are a number of points of disagreement in their findings (23, 24). Complete blockade of this response required 10 μM α-bungarotoxin (24). In the frog optic tectum, α-bungarotoxin applied topically produces a long-lasting blockade of retinotectal transmission that is thought to be cholinergically mediated (25).

The effects of α-bungarotoxin in the avian ciliary ganglion differ in one respect from those found in skeletal muscle and electric organ—that is, in their reversibility (1, 2). Binding of the toxin and blockade of nicotinic responses in the latter tissues are extremely long lasting and, in fact, can be considered to be irreversible in the context of most experiments. In the ciliary ganglion the effects of the toxin were partially reversible on ciliary neurons and largely reversible on choroid neurons within 5 hr of washing. Nevertheless, α-bungarotoxin binding in ciliary ganglia should prove useful for various studies on the properties of neuronal nicotinic receptors.

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