

κ -BUNGAROTOXIN BLOCKS NICOTINIC TRANSMISSION AT AN IDENTIFIED INVERTEBRATE CENTRAL SYNAPSE

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Accepted 4 July 1988

Summary

A comparison was made between the effects of κ -bungarotoxin and α -bungarotoxin upon nicotinic cholinergic transmission at an identified synapse (the cercal afferent, giant interneurone 2 synapse) in the central nervous system of the cockroach (*Periplaneta americana*). κ -Bungarotoxin, a snake venom κ -neurotoxin, completely blocked nicotinic unitary excitatory postsynaptic potentials (EPSPs) and evoked composite EPSPs when applied at a concentration of $1.0 \times 10^{-7} \text{ mol l}^{-1}$. No recovery was observed after a 2 h wash in normal saline. κ -Bungarotoxin produced a decrease in acetylcholine-induced nicotinic responses which paralleled decreases in nicotinic synaptic potentials and currents, indicating that κ -bungarotoxin blocked postsynaptic nicotinic receptors. This blockade appeared to be specific as resting membrane potential, input resistance and the ability to elicit an action potential in response to direct stimulation of giant interneurone 2 were unchanged following prolonged toxin exposures. Samples of α -bungarotoxin which were free from κ -neurotoxin contamination were also found to be potent antagonists of cockroach neuronal nicotinic receptors. It is concluded that the cockroach receptor is the first reported example of a neuronal nicotinic receptor which is sensitive to blockade by both κ -neurotoxins and α -neurotoxins.

Introduction

A new class of snake venom polypeptide neurotoxins, referred to as κ -neurotoxins, has recently been described (Chiappinelli, 1985). κ -Bungarotoxin (Chiappinelli, 1983; Grant & Chiappinelli, 1985) and κ -flavitoxin (Chiappinelli *et al.* 1987) exhibit considerable (approximately 50%) sequence homology with

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Key words: κ -Bungarotoxin (= kappa-Bungarotoxin), nicotinic acetylcholine receptor, excitatory postsynaptic potentials, synaptic current.

the curaremimetic snake venom α -neurotoxins but have pharmacological properties that are quite distinct from those of the α -neurotoxins. κ -Neurotoxins (3.0×10^{-8} – 1.0×10^{-6} mol l⁻¹) block nicotinic cholinergic transmission in avian and murine autonomic ganglia (Chiappinelli & Dryer, 1984) whereas 1.0×10^{-5} mol l⁻¹ α -bungarotoxin has no effect on such transmission (Brown & Fumagalli, 1977; Chiappinelli *et al.* 1981). Conversely the κ -neurotoxins are weak antagonists at muscle nicotinic receptors, being several orders of magnitude less potent in binding to these receptors than α -bungarotoxin (Chiappinelli *et al.* 1985). Thus, κ -neurotoxins have been used as selective probes for vertebrate neuronal nicotinic receptors (Chiappinelli, 1985). The effects of κ -neurotoxins on invertebrate neuronal nicotinic receptors have not previously been examined.

Invertebrate neuronal nicotinic receptors have been well characterized in the cockroach central nervous system (Sattelle *et al.* 1983, 1986; Harrow & Sattelle, 1983; Sattelle & Breer, 1985). These receptors exhibit a pharmacological profile which is more closely related to that of vertebrate neuronal receptors than to that of vertebrate muscle receptors (David & Sattelle, 1984; Lummis & Sattelle, 1985). Nevertheless, the cockroach nicotinic receptor is quite sensitive to blockade by α -bungarotoxin (Sattelle *et al.* 1980, 1983; David & Sattelle, 1984), a property which distinguishes this receptor from most vertebrate neuronal nicotinic receptors. To further characterize the properties of both κ -neurotoxins and invertebrate nicotinic receptors, the effects of κ -bungarotoxin have now been examined at an identified nicotinic cholinergic synapse in the cockroach central nervous system.

Materials and methods

Preparation

Adult male cockroaches *Periplaneta americana* (L.) reared at 24°C were used for all experiments. Cercal afferent, giant interneurone synapses are located in the neuropile of the terminal (A6) abdominal ganglion. A preparation consisting of the abdominal nerve cord (including ganglia A1–A6), cercal nerves XI and the cerci was isolated and transferred to a drop of saline on a glass slide. Using a binocular microscope ($\times 120$) and fine dissecting needles, the large-diameter (50 μ m) axon of giant interneurone 2 (GI2) was isolated from one of the paired connectives linking the fifth and sixth abdominal ganglia. The preparation was transferred to a recording chamber (Callec, 1974), illustrated in schematic form in Fig. 1A. The contralateral connective was completely severed leaving the single isolated axon surrounded by oil, and only the ipsilateral cercal nerve XI (Roeder *et al.* 1960) was retained for presynaptic stimulation. This permitted electrophysiological recording of postsynaptic membrane properties of GI2 across the oil gap (Fig. 1B). The desheathed sixth abdominal ganglion was continuously superfused (0.1 ml min⁻¹) with a physiological saline of the following composition (in mmol l⁻¹): NaCl, 210; KCl, 3.1; CaCl₂, 5.4; NaHCO₃, 2.0; Na₂HPO₄, 0.1; sucrose, 26.0 (pH 7.2). Experiments were performed at 21–23°C.

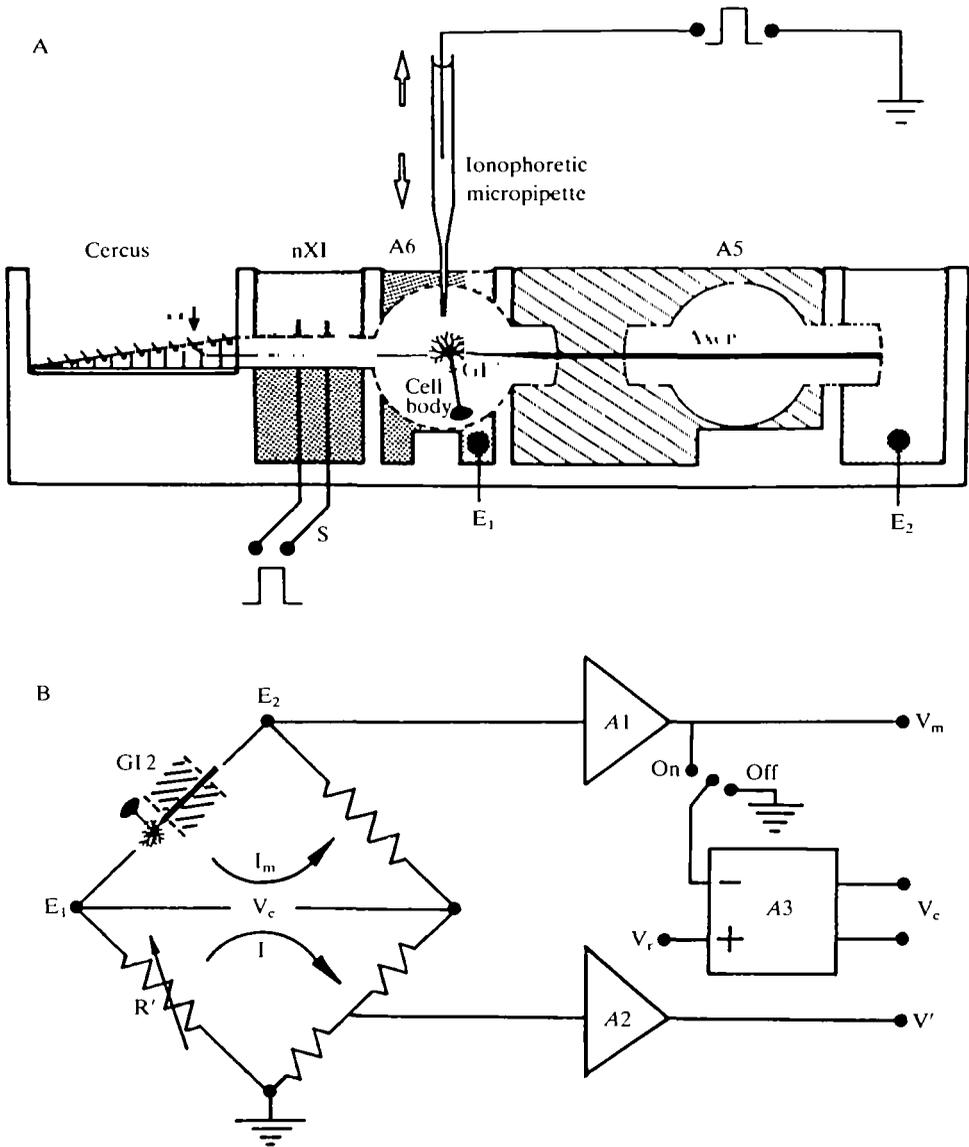


Fig. 1. (A) Schematic representation of an oil-gap, single-fibre experiment in which synaptic potentials and responses to ionophoretically applied acetylcholine are recorded from an identified cockroach giant interneurone (GI2). The preparation is mounted under saline apart from the isolated axon which is bathed in mineral oil and the cercus which is in air. (B) The electronic circuit consists of a Wheatstone bridge balanced with a resistance R' . Two high-impedance amplifiers (A1 and A2) allow continuous records of V_m (membrane potential) and V' which is proportional to membrane current (I_m). A switch to turn on the voltage-clamp is shown, and under voltage-clamp the optically isolated amplifier A3 compares the voltage reference (V_r) with V_m . The control voltage is denoted by V_c . A5, A6, abdominal ganglia 5 and 6; nXI, cercal nerve XI; E1, E2 recording electrodes; M, mechanical stimulation; S, electrical stimulation.

Electrophysiology

A high-input-impedance amplifier was used to record postsynaptic potentials from GI2. Excitatory postsynaptic potentials (EPSPs) were generated either by mechanical stimulation of a single cercal mechanoreceptor (unitary EPSPs) or by electrical stimulation of nerve XI (composite EPSPs). Changes in resting potential of GI2 were continuously displayed on a pen recorder. Acetylcholine (ACh) was delivered ionophoretically *via* a micropipette (containing 1.0 mol l^{-1} ACh) inserted into the neuropile of the sixth abdominal ganglion in the region containing the dendrites of GI2 (Harrow *et al.* 1980). The ionophoretic micropipette was positioned by a stepping microdrive unit (Callec *et al.* 1982). Control experiments showed that stable EPSPs and ionophoretically induced potentials could be recorded from GI2 for more than 300 min (see also Callec, 1974).

Voltage-clamp experiments were performed using a single-electrode voltage-clamp method adapted for use with the oil-gap, single-fibre recording technique (L. Mony, B. Hue & J. C. Tessier, in preparation). By this means postsynaptic currents were recorded in response to electrical stimulation of presynaptic mechanosensory neurones.

Purified toxins

κ -Bungarotoxin and α -bungarotoxin (containing no detectable traces of κ -bungarotoxin) were prepared as described elsewhere (Chiappinelli, 1983) and purity was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, isoelectric focusing and analytical HPLC. The purified α -bungarotoxin used in these experiments had no effect on nicotinic transmission in chick ciliary ganglia at a concentration of $1.0 \times 10^{-5} \text{ mol l}^{-1}$, whereas the purified κ -bungarotoxin completely blocked chick ganglia nicotinic responses at $5.0 \times 10^{-8} \text{ mol l}^{-1}$. Stock solutions of neurotoxins were kept at -20°C , and were diluted in physiological saline to give the appropriate final concentration immediately prior to each experiment.

Results

Actions of κ -bungarotoxin on an identified nicotinic synapse

Synaptic transmission between cercal afferent neurones and giant interneurone 2 (GI2) is mediated entirely by nicotinic acetylcholine receptors, and is unaffected by muscarinic antagonists (Sattelle *et al.* 1983). κ -Bungarotoxin ($1.0 \times 10^{-6} \text{ mol l}^{-1}$) was found to reduce progressively the unitary and composite EPSPs recorded postsynaptically from GI2 over a period of 120–150 min (Fig. 2). There was complete blockade of synaptic transmission without any modification of the resting membrane potential of the giant interneurone. Action potentials evoked by passing depolarizing pulses through the axon of GI2 were also unaffected by exposure to κ -bungarotoxin (Fig. 2). The blockade produced by κ -bungarotoxin was not reversed by prolonged washing with normal saline (up to 2.5 h in some experiments). κ -Bungarotoxin also blocked synaptic transmission at

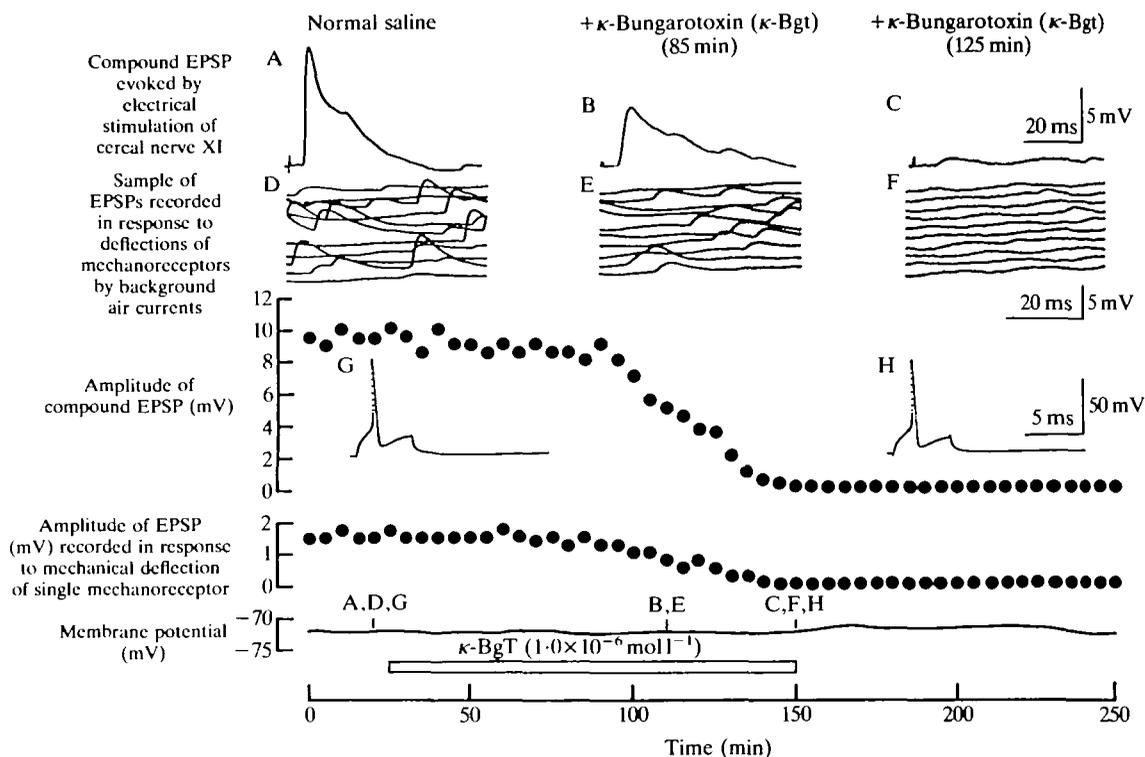


Fig. 2. Effects of $1.0 \times 10^{-6} \text{ mol l}^{-1}$ κ -bungarotoxin (κ -BgT) on synaptic transmission between cercal nerve XI afferents and giant interneurone 2 (GI2). Excitatory postsynaptic potentials (EPSPs) were observed in response to mechanical stimulation of a single cercal mechanoreceptor, and in response to deflection of mechanoreceptors by background air currents. Compound EPSPs were observed in response to the activity of many afferents evoked by electrical stimulation of nerve XI. Blockade of EPSPs by κ -bungarotoxin, which was not reversible on washing with saline for 100 min, was not accompanied by any significant changes in polarization of the postsynaptic membrane. Action potentials of normal amplitude and duration were recorded after complete blockade of EPSPs by κ -bungarotoxin. Data shown in insets A–C (compound EPSPs), D–F (continuously recorded EPSPs due to deflection of cercal mechanoreceptors by background air currents), G and H (action potentials recorded in the ganglion following application of depolarizing pulses to the axon of GI2) were all recorded at the points indicated on the membrane potential trace.

$1.0 \times 10^{-7} \text{ mol l}^{-1}$, but the times required to reduce EPSP amplitude by 50% and 100% were increased (Table 1). In control preparations, perfusion with normal saline for up to 8 h produced no significant changes in synaptic potentials or resting membrane potential. The results demonstrate a specific synaptic blocking action for κ -bungarotoxin at an identified invertebrate nicotinic synapse.

Actions of α -bungarotoxin on nicotinic synaptic transmission

In agreement with earlier observations (Sattelle *et al.* 1983), α -bungarotoxin

Table 1. Comparison of times taken to block nicotinic transmission at cercal afferent, GI2 synapses by purified κ -bungarotoxin and α -bungarotoxin

Toxin	Concentration (mol l ⁻¹)	N	Time (min) to block EPSPs	
			50% block	100% block
κ -Bungarotoxin	1.0×10^{-7}	6	172 ± 11*	227 ± 20
	1.0×10^{-6}	3	133†	157 ± 33
α -Bungarotoxin	1.0×10^{-7}	7	135 ± 19	171 ± 21
	1.0×10^{-6}	1	105	120

* Mean ± s.e.m. for the number of experiments indicated by N.
† Mean of two experiments.

(1.0×10^{-6} mol l⁻¹) blocked synapses between cercal afferents and giant interneurone 2. In the present experiments the α -bungarotoxin samples used were demonstrated to be free of κ -neurotoxin contamination. Synaptic blockade was also observed at 1.0×10^{-7} mol l⁻¹ α -bungarotoxin, and a comparison of the time courses of action (Table 1) of the toxins at various concentrations showed that α -bungarotoxin produced a more rapid block than κ -bungarotoxin. Nevertheless, both neurotoxins emerged as extremely potent blocking agents at this insect central nervous system synapse.

Actions of neurotoxins on nicotinic EPSPs and the response to ionophoretically applied acetylcholine

In a separate series of experiments the effects of κ -bungarotoxin (1.0×10^{-7} mol l⁻¹) and α -bungarotoxin (1.0×10^{-7} mol l⁻¹) were examined on both the composite EPSP recorded from GI2 and the depolarizing response of this interneurone to ionophoretically applied ACh. Both responses were blocked (Fig. 3). The parallel decline of the EPSP and the ACh-induced potential indicated that the site of action of both κ -bungarotoxin and α -bungarotoxin was on the postsynaptic neurone (GI2).

Synaptic currents recorded from giant interneurone 2 are blocked by κ -bungarotoxin

The synaptic membrane of giant interneurone 2 (GI2) was clamped at resting potential, allowing direct measurement of synaptic currents in response to presynaptic stimulation. Exposure to κ -bungarotoxin (1.0×10^{-6} mol l⁻¹) resulted in a progressive reduction of the synaptic current, with a complete blockade observed after 140 min (Fig. 4).

Discussion

There is considerable evidence for a functional postsynaptic role for nicotinic

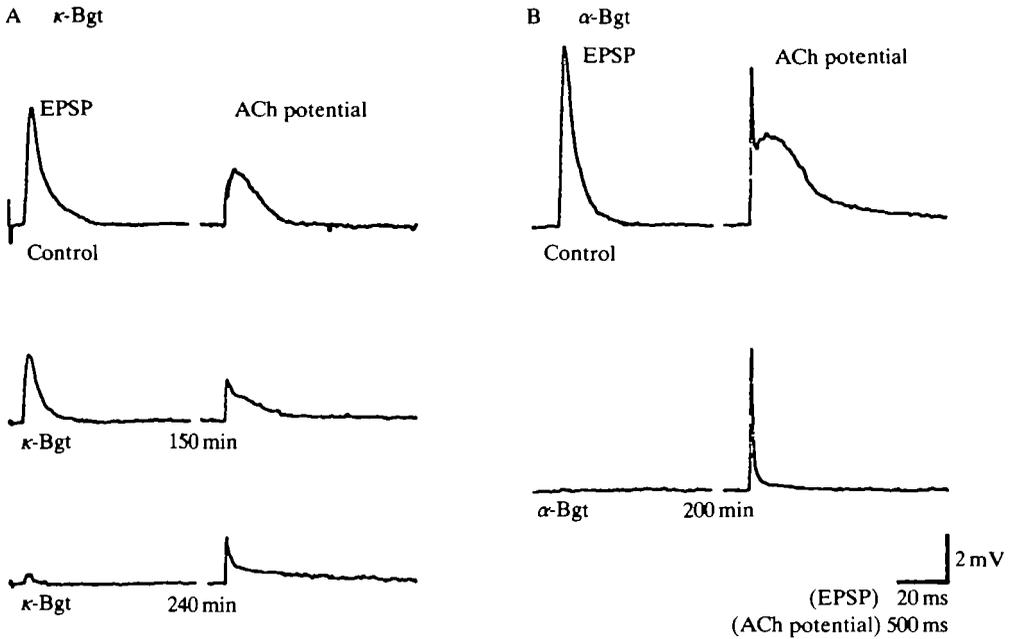


Fig. 3. Block of both EPSP and acetylcholine (ACh)-induced potentials recorded from GI2 by (A) κ -bungarotoxin (κ -Bgt) ($1.0 \times 10^{-7} \text{ mol l}^{-1}$) and (B) α -bungarotoxin (α -Bgt) ($1.0 \times 10^{-7} \text{ mol l}^{-1}$). The toxins progressively blocked the composite EPSP recorded in response to electrical stimulation of cercal nerve XI. A parallel decline was observed in the response of GI2 to ACh applied ionophoretically into the neuropile of ganglion A6 in the vicinity of the dendritic branches of this interneurone. The traces in the right-hand column exhibit a larger stimulus artefact, and the ACh-induced potential is fully blocked by α -bungarotoxin. These observations indicate a postsynaptic blocking action of both κ -bungarotoxin and α -bungarotoxin.

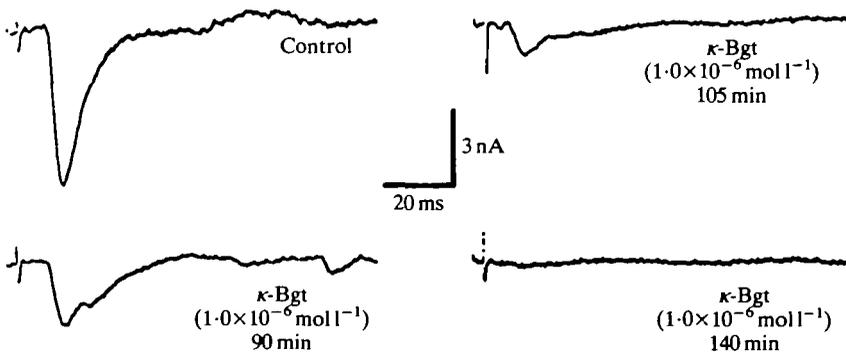


Fig. 4. Synaptic currents recorded from giant interneurone 2 (GI2) in response to electrical stimulation of cercal nerve XI. Postsynaptic, inwardly directed current is progressively blocked in the presence of $1.0 \times 10^{-6} \text{ mol l}^{-1}$ κ -bungarotoxin. The membrane potential at which this interneurone was clamped was -80 mV .

receptors at synapses between cercal mechanosensory neurones and giant interneurons in the cockroach *Periplaneta americana* (Sattelle, 1980, 1986). The present results demonstrate that κ -bungarotoxin is a potent antagonist at this nicotinic cholinergic synapse. Excitatory postsynaptic potentials and currents are completely blocked by $1.0 \times 10^{-7} \text{ mol l}^{-1}$ κ -bungarotoxin. In addition, a parallel blockade of ACh-induced nicotinic responses is observed, indicating that κ -bungarotoxin blocks postsynaptic nicotinic receptors. The blockade is specific, as neurones continue to display normal-looking action potentials, resting membrane potentials and input resistances during prolonged exposure to the toxin.

Earlier findings demonstrated that two α -neurotoxins, α -bungarotoxin and α -cobratoxin, were potent antagonists of cockroach neuronal nicotinic receptors (Sattelle *et al.* 1983; David & Sattelle, 1984). SDS-polyacrylamide gel electrophoresis indicated that these α -neurotoxin samples were not contaminated by traces of other toxin fractions (Sattelle *et al.* 1983; David & Sattelle, 1984). However, in some vertebrate preparations α -bungarotoxin samples contaminated with small amounts of κ -neurotoxins blocked neuronal nicotinic transmission, whereas uncontaminated α -bungarotoxin was ineffective even at very high concentrations (Chiappinelli *et al.* 1981). In the present experiments, α -bungarotoxin uncontaminated with κ -neurotoxins was found to be as potent in blocking cockroach nicotinic receptors as were the previously tested samples of α -neurotoxins. We therefore conclude that both α -neurotoxins and κ -neurotoxins are potent antagonists at this invertebrate neuronal nicotinic receptor. Hitherto, κ -neurotoxins (5.0×10^{-8} – $1.0 \times 10^{-6} \text{ mol l}^{-1}$) have been shown to block nicotinic responses in all neuronal preparations examined, but in each case α -neurotoxins were without effect at concentrations up to $1.0 \times 10^{-5} \text{ mol l}^{-1}$. The preparations tested were avian ciliary and sympathetic ganglia (Chiappinelli, 1983; Chiappinelli & Dryer, 1984), the rat superior cervical ganglion (Chiappinelli & Dryer, 1984) and cultured bovine chromaffin cells (V. A. Chiappinelli, J. J. Lambert & J. M. Nooney, unpublished observations). The cockroach receptor is thus the first reported example of a functional neuronal nicotinic receptor that is sensitive to blockade by both κ -neurotoxins and α -neurotoxins.

In chick ciliary ganglia, κ -neurotoxins bind to two distinct nicotinic sites. κ -Bungarotoxin (also known as Bgt 3.1 and Toxin F; Loring *et al.* 1986) and κ -flavitoxin bind with high affinity ($K_d = 5.0 \times 10^{-9} \text{ mol l}^{-1}$) to a synaptic nicotinic site not detected by $1.0 \times 10^{-5} \text{ mol l}^{-1}$ α -bungarotoxin (Chiappinelli, 1983; Halvorsen & Berg, 1986; Chiappinelli *et al.* 1987; Loring & Zigmond, 1987). This synaptic site is presumed to represent the functional nicotinic receptor in chick ganglia. Both κ -neurotoxins and α -neurotoxins bind to a second, extrasynaptically located nicotinic site in chick ciliary ganglia, which has no known function (Chiappinelli *et al.* 1981; Chiappinelli, 1983; Loring *et al.* 1985). In contrast, the functional nicotinic receptor in cockroach nervous tissue is identified by α -bungarotoxin ($K_i = 1.2 \times 10^{-9} \text{ mol l}^{-1}$, Lummis & Sattelle, 1985) as well as κ -bungarotoxin ($K_i = 2.7 \times 10^{-8} \text{ mol l}^{-1}$; D. B. Sattelle, R. D. Pinnock, S. C. R. Lummis & V. A. Chiappinelli, unpublished observations). The higher affinity of α -bungarotoxin for

the cockroach nicotinic receptor is consistent with the more rapid blockade of nicotinic transmission by α -bungarotoxin observed in the present study.

A number of nicotinic sites have been described in vertebrate central nervous tissue. In chick optic lobe, at least two snake neurotoxin sites have been detected (Chiappinelli *et al.* 1985) and a nicotinic 'agonist-site' – identified by high-affinity binding of [³H]nicotine or [³H]acetylcholine – has a different pharmacology and is not recognized by α -neurotoxins (Schneider *et al.* 1985; Whiting & Lindstrom, 1986). Whiting & Lindstrom (1987) report different subunit structures for vertebrate central α -neurotoxin sites (four subunits, presumably arranged $\alpha_2\beta_2$) and vertebrate agonist-sites (two subunits, presumably arranged $\alpha_2\beta_2$ or $\alpha_3\beta_2$). In contrast, nicotinic receptors from locust (Breer *et al.* 1985) and cockroach (Sattelle & Breer, 1985) nervous tissue may contain only a single subunit (M_r 65 000), presumably arranged α_4 or α_5 . The possibility that other subunits are present in cockroach and locust cannot be completely eliminated but, if present, they must be of almost identical relative molecular mass. In this context it is of interest to note that the M_r 65 000 component can be reconstituted into an artificial bilayer yielding channel-like activity (Breer *et al.* 1985). Antibodies prepared to this material result in immunocytochemical staining in a region of neuropile known to possess functional nicotinic receptors (Breer & Sattelle, 1987).

All these nicotinic receptors are structurally related, since some antibodies raised against vertebrate nicotinic receptors cross-react with invertebrate receptors (Breer *et al.* 1985). As invertebrates diverged from vertebrates at least 600 million years ago, it is possible that the invertebrate nicotinic receptor represents a primordial form, and the two-subunit and four-subunit nicotinic sites of vertebrates represent the end products of various gene duplications and mutations (see Breer & Sattelle, 1987). κ -Neurotoxins bind with high affinity to a remarkably wide range of neuronal nicotinic receptors found in cockroach, chick, rat and cow but bind only weakly to chick muscle and *Torpedo* electric organ nicotinic receptors (Chiappinelli *et al.* 1985; Loring *et al.* 1986). Thus, the κ -neurotoxins appear to be selective neuronal nicotinic receptor antagonists and can be classified with drugs such as trimethaphan, mecamylamine and hexamethonium, which consistently show higher potency in blocking neuronal nicotinic receptors than in inhibiting neuromuscular transmission. In contrast, it is not possible to generalize about the selectivity of α -bungarotoxin, since it blocks most, but not all, neuromuscular junction nicotinic receptors (Burden *et al.* 1975) and also some neuronal nicotinic receptors, notably those of the cockroach (Sattelle *et al.* 1983; present study) and the frog (Marshall, 1981).

κ -Neurotoxins exhibit approximately 50% sequence homology with long-type α -neurotoxins such as α -bungarotoxin. There are, however, several unusual amino acid substitutions which distinguish κ -neurotoxins from all the known α -neurotoxins. For example, an invariant tryptophanyl residue near the active site of α -neurotoxins is non-conservatively substituted in both κ -bungarotoxin and κ -flavitoxin. It thus appears that κ -neurotoxins evolved and have been retained in snake venom because of their high-affinity binding to neuronal nicotinic

receptors. The present results demonstrate that the κ -neurotoxins are useful probes for a variety of neuronal nicotinic receptors, and indicate that the acetylcholine recognition sites on invertebrate neuronal receptors are structurally related to those found on vertebrate neurones.

VAC was supported by NIH grant NS17574 and by an NIH Fogarty Center Senior International Fellowship (TW01254). BH was supported by INSERM grant no. 866009 and an EMBO short-term grant. LM was supported by INSERM grant no. 866009.

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