SNAKE α -TOXIN EFFECTS ON CHOLINERGIC AND NONCHOLINERGIC RESPONSES OF *APLYSIA CALIFORNICA* NEURONS¹

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Abstract

The effects of α -toxins from *Bungarus multicinctus* (α BuTX) and *Naja naja siamensis* (α NTX) were studied on synaptic responses and on extrasynaptic responses to focally applied acetylcholine (ACh), histamine (Hm), γ -aminobutyric acid (GABA), and glutamate (glu) in neurons of the marine mollusc, *Aplysia californica*. Binding of ¹²⁵I- α BuTX to *Aplysia* ganglia homogenates was pharmacologically characterized and compared with the pharmacological sensitivity of ¹²⁵I- α BuTX binding to rat brain membrane preparations. Both α BuTX and α NTX at 10^{-5} M reversibly inhibited the ACh-induced increase in Cl⁻ conductance ($\uparrow G_{Cl}$) without affecting ACh responses mediated by an increase in Na⁺ conductance ($\uparrow G_{Na}$) or an increase in K⁺ conductance ($\uparrow G_K$). In addition, both α -toxins reversibly inhibited the $\uparrow G_{Cl}$ responses induced by Hm and, in some cases, glu. GABA-induced $\uparrow G_{Cl}$ responses were not affected by either toxin even at concentrations as high as 10^{-3} M. Both toxins also inhibited synaptic cholinergic $\uparrow G_{Cl}$ responses as well as the $\uparrow G_{Cl}$ component of postsynaptic potentials mediated by noncholinergic presynaptic neurons.

Studies of ¹²⁵I- α BuTX binding to *Aplysia* ganglia homogenates demonstrated the presence of a single saturable, high affinity site with a dissociation constant (K_D) of 3.6×10^{-9} M. The pharmacological profile of agents which inhibit ¹²⁵I- α BuTX binding to *Aplysia* ganglia was similar to the profile of vertebrate ganglionic and brain preparations; *d*-tubocurarine, nicotine, and carbamylcholine effectively and competitively inhibit binding, whereas hexamethonium and decamethonium are less effective and noncompetitive. Strychnine and bicuculline, agents known to block responses to a variety of agonists in *Aplysia*, were effective competitive inhibitors of toxin binding in *Aplysia* and in rat brain. Strychnine is not as potent in inhibiting toxin binding to electric organ preparations. Chloride channel blockers, such as penicillin and picrotoxin, and noncholinergic agonists, such as Hm and glu, had no effect on toxin binding.

The α -toxins appear to inhibit noncholinergic $\uparrow G_{Cl}$ responses through a cholinergic receptor associated with the Cl⁻ conductance mechanism for the noncholinergic agonist.

The long chain postsynaptic neurotoxins, such as α -bungarotoxin (α BuTX), isolated from *Bungarus multicinctus* venom, and α -*Naja* toxin (α NTX), isolated from *Naja naja siamensis* venom, bind specifically to skeletal muscle and electric organ nicotinic acetylcholine (ACh) receptors (see references in Lee, 1972). In addition, both toxins inactivate the postsynaptic conductance changes induced by ACh at concentrations comparable to the toxin concentrations necessary to saturate binding. Phar-

macological agents which antagonize or activate the neuromuscular ACh receptor also inhibit toxin binding at comparably effective concentrations (Fambrough, 1979).

The qualitative and quantitative agreement between binding studies and physiological experiments observed with vertebrate neuromuscular preparations and electric organs is lacking in comparable studies of neuronal preparations. There appears to be saturable, high affinity, specific binding of iodinated α BuTX in a wide variety of neuronal preparations from vertebrate, invertebrate, and tissue culture sources (reviewed by Morley et al., 1979; Brown, 1979). Toxin binding in these neuronal preparations appears to be nicotinic, based on the effectiveness of nicotine and *d*-tubocurarine in inhibiting binding, but differs from the neuromuscular and electric organ binding by a relative insensitivity to blockade by decamethonium and by hexamethonium. However, the α -neurotoxins do not antagonize reliably cholinergic responses when ap-

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plied in concentrations required to saturate toxin binding sites (Carbonetto et al., 1978). Although there have been reports of α -neurotoxins blocking cholinergic responses in chick ciliary ganglia (Chiappinelli and Zigmond, 1978; Conti-Tronconi et al., 1979) and in cat cochlea (Fex and Adams, 1978), 1 to 10 μ M toxin was required to block the cholinergic responses. These concentrations of toxins are 100 times greater than those required to saturate binding in most neuronal preparations, and the possibility of contamination has been raised (Ravdin and Berg, 1979). Moreover, in sympathetic ganglia, hexamethonium antagonizes the nicotinic ACh response but is ineffective in inhibiting toxin binding (Brown, 1979). Thus, in contrast to toxin effects on skeletal muscle and electric organ preparations, there is a disparity between toxin binding results and physiological effects of toxin on neuronal preparations.

The central nervous system (CNS) of the mollusc, *Aplysia californica*, is especially suited for determining the specificity and mechanism of action of various pharmacological agents. The large neurons in *Aplysia* can be characterized and identified by their responses to ACh and to other agonists applied to the soma. It is possible, therefore, to study the specificity of a drug in terms of its effects on different conductance mechanisms and its effects on responses to particular agonists. In addition, synaptic connections mediated by identified pre- and postsynaptic neurons are available to test further the specificity of pharmacological agents on junctional receptors.

Responses to ACh in neurons of *Aplysia* are mediated by three pharmacologically distinct ACh receptors: (1) an excitatory response mediated by an increase in Na^+ conductance ($\uparrow G_{\text{Na}}$) which is blocked by hexamethonium and curare and, therefore, is similar to the vertebrate C-6 or ganglionic type of nicotinic ACh receptor; (2) an inhibitory response mediated by an increase in Cl^- conductance ($\uparrow G_{\text{Cl}}$) which is insensitive to hexamethonium, is blocked by curare, and appears to be pharmacologically most like the skeletal muscle type of nicotinic ACh receptor; and (3) a slow inhibitory response mediated by an increase in K^+ conductance ($\uparrow G_{\text{K}}$) which is insensitive to hexamethonium and curare but is inhibited by arecoline or low concentrations of tetraethylammonium (TEA) and, as yet, has no vertebrate counterpart (Kehoe, 1972a, b).

Previous studies using various snake venom α -neurotoxins have produced conflicting observations on their ability to antagonize ACh-induced responses in invertebrate neurons, including *Aplysia*. Szczepaniak (1974) found that 10^{-5} M α BuTX did not block ACh responses in *Helix*, whereas long chain α -neurotoxins from *Dendroaspis viridis* blocked only the ACh response mediated by an $\uparrow G_{\text{Cl}}$. Shain et al. (1974) found that 10^{-8} M α BuTX reversibly blocked all three types of ACh-induced responses in *Aplysia*. However, Kehoe et al. (1976) demonstrated that α BuTX selectively and reversibly blocked only the ACh response mediated by an $\uparrow G_{\text{Cl}}$ without affecting the Na^+ -dependent or K^+ -dependent responses.

In the present study, we investigated the pharmacological specificity of α BuTX and of α NTX on responses of *Aplysia* neurons to ACh as well as to several noncholin-

ergic agonists. During the course of our study, we discovered that the α -neurotoxins were selectively inhibiting chloride-mediated responses to cholinergic as well as noncholinergic agonists and transmitters. In order to elucidate further the nature of these toxin effects, we also examined the pharmacological specificity of ^{125}I - α BuTX binding to *Aplysia* ganglia homogenates. The results of these experiments indicate that the toxin binding site in *Aplysia* tissues is pharmacologically similar to that in vertebrate CNS and is characteristic of nicotinic ACh receptors.

Materials and Methods

Experimental preparation

Cerebral, buccal, or pleural ganglia from 300 to 500 gm *Aplysia californica* (Pacific Bio-Marine, Venice, CA, and animals collected from the wild) were isolated and pinned out on the Silastic (Sylgard, Dow-Corning, Midland, MI) base of a 1.9-ml capacity chamber. Pleural ganglion neurons previously demonstrated to respond to ACh with an $\uparrow G_{\text{Na}}$ (anterior pleural cells) and dual inhibitory response mediated by an $\uparrow G_{\text{Cl}}$ and $\uparrow G_{\text{K}}$ (medial pleural cells) (Kehoe, 1972b) were used to test the specificity of α BuTX and α NTX on the various types of ACh responses. Neurons used to test the specificity of α BuTX and α NTX on responses induced by various agonists were the A and B cluster neurons (Fredman and Jahan-Parwar, 1975) of the cerebral ganglion and several of the B type cells (Fiore and Meunier, 1975) of the buccal ganglia. These neurons respond to ACh with an $\uparrow G_{\text{Cl}}$ and, in addition, responded to γ -aminobutyric acid (GABA), histamine (Hm), and/or glutamate (glu) with an $\uparrow G_{\text{Cl}}$.

The effects of the neurotoxins on synaptically activated receptors involving an $\uparrow G_{\text{Cl}}$ component were studied in (1) the M cluster neurons (Ono and McCaman, 1980) which are identifiable followers of the histaminergic neurons (Weinreich, 1977; McCaman and McKenna, 1978) in the cerebral ganglion and (2) the B type neurons (Fiore and Meunier, 1975) of the buccal ganglia which receive convergent input from the identifiable cholinergic B4 and B5 neurons (Gardner and Kandel, 1977) and from the S_1 neurons (Fiore and Meunier, 1975). The transmitter utilized by the buccal S_1 neurons is not known. Pharmacological studies suggest that the S_1 transmitter is not ACh, and the S_1 neurons do not contain measurable levels of ACh, GABA, Hm, 5-hydroxytryptamine (5HT), dopamine, or octopamine (J. K. Ono, R. E. McCaman, and M. W. McCaman, unpublished).

Electrodes and physiological measurements

Neurons were impaled with double-barreled microelectrodes (10 to 30 megohms) filled with 0.5 M K_2SO_4 . One barrel was used to record membrane potential and the other to inject current in order to control membrane voltage. Membrane input resistance was periodically monitored by passing a pulse of constant current (0.2 to 1.0 nA) through the current barrel. Responses of neurons to agonists or synaptic input were monitored at several membrane potentials to assure that alterations in response amplitude were not due to changes in reversal potential. When toxin effects were studied on synapti-

cally activated responses, the presynaptic neurons were also impaled with double-barreled microelectrodes and activated by passing depolarizing current. Certain neurons in our study were injected with tetraethylammonium (TEA) to augment transmitter release by prolonging the presynaptic action potential or to block $\uparrow G_K$ induced by iontophoresis of agonists. TEA injection was accomplished by interbarrel iontophoresis (McCaman and McKenna, 1978). Records were photographed from storage oscilloscope traces or displayed on a chart recorder.

Iontophoretic application of agonists

Acetylcholine bromide, histamine hydrochloride, and monosodium glutamate were obtained from Sigma Chemical Co., St. Louis, MO and GABA from Calbiochem-Behring Corp., La Jolla, CA. Iontophoretic pipettes were filled with agonists at a concentration of 0.5 M, and the pH was adjusted with HCl or NaOH. A constant current system was used to deliver pulses of 30 to 900 nA for periods of 20 to 200 msec. The magnitude of membrane-polarizing currents, as well as iontophoretic currents, was assessed with a bath current monitor through a virtual ground circuit. When studying the specificity of toxin effects, GABA, Hm, or glu was applied to the same neuron paired with ACh through either two separate micropipettes or a double-barreled micropipette. Multibarrel iontophoretic studies utilized a stimulus distribution system that permitted independent control of the phoretic and braking currents in each barrel. Responses to ACh could be elicited almost anywhere on the neuron soma, whereas responses to the other agonists were localized to discrete regions.

Responses mediated by an $\uparrow G_{Cl}$ were identified on the basis of: (a) a "rapid" hyperpolarizing response at membrane potentials between -40 to -60 mV which reversed at a membrane potential of about -55 to -70 mV (Ascher et al., 1976) and (b) a shift in the reversal potential of the response to a more depolarized level following perfusion with saline containing half the normal Cl^- concentration or by intracellular iontophoresis of Cl^- ions.

Perfusion media

Ganglia were normally bathed in artificial sea water (ASW) containing double the normal Mg^{2+} concentration. This ASW had the following composition (in millimolar concentrations): Na^+ , 494; K^+ , 11; Mg^{2+} , 100; Ca^{2+} , 10; Cl^- , 565; SO_4^{2-} , 30; acetate, 50. The elevated divalent cation concentration diminished the amount of spontaneous activity in the ganglia by increasing spike threshold. In experiments aimed at ascertaining whether a response was mediated by $\uparrow G_{Cl}$, the normal Cl^- concentration was decreased to half (250 mM) by substituting NaCl with sodium methyl sulfate.

The amount of toxin needed for these studies was minimized by utilizing a perfusion system that could be switched from an open to a closed (recirculating) mode. This system consisted of a peristaltic pump and a four-way Teflon valve. The total volume of recirculating perfusate was 3.9 ml. The perfusion rate throughout the experiments was 1 ml/min. Control responses were taken while the bath was recirculating to assure that any dim-

inution of responses was not attributable to desensitization. Control saline consisted of double strength Mg^{2+} ASW containing 0.1 mg/ml of bovine serum albumin (BSA) serving as a protein carrier for the toxin. A stock solution (usually 10^{-3} M) of α BuTX or α NTX was made in 50 mM Tris, 50 mM NaCl, 0.02% NaN_3 buffer, pH 7.4, containing 1 mg/ml of BSA and diluted with control saline just prior to use. The bath concentration of Tris from the toxin stock solution was always kept considerably less than 1 mM, since Tris (10 mM) has been shown to antagonize ACh responses in *Aplysia* (Wilson et al., 1977; Ascher et al., 1978). Toxin was added to the bath by first withdrawing an aliquot of the bath equal to the volume of toxin stock to be added ($\sim 10\%$ of the total volume) and then adding 300 μ l of a toxin solution 10 times more concentrated than the final bath concentration.

Purification of α BuTX and α NTX

Crude lyophilized *Bungarus multicinctus* and *Naja naja siamensis* venoms were obtained from Miami Serpenterium (Miami, FL). α -Toxins were purified by two CM-Sephadex ion exchange columns followed by gel filtration (Sephadex G-50) chromatography (Mebs et al., 1972). Purified toxins showed only a single component when examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate or cellulose acetate electrophoresis indicating a purity of greater than 99%.

^{125}I -labeled α -bungarotoxin binding assay

Tissue preparation. Fresh or frozen *Aplysia* ganglia were homogenized in 10 volumes (w/v) of ice cold 50 mM Tris, 50 mM NaCl, 0.02% NaN_3 buffer, pH 7.4, with a Polytron. The homogenate was sedimented at $106,000 \times g$ for 30 min at $4^\circ C$ in a Beckman Ti-50 rotor. The resulting pellet was resuspended in Tris/NaCl/ NaN_3 buffer and used for all binding assays. The resuspended homogenate was used immediately or resuspended by polytroning a once frozen and thawed sample. Rat brain membranes were prepared as described (Salvaterra and Matthews, 1980).

Binding assay. ^{125}I - α BuTX binding was determined essentially as described (Salvaterra and Fodors, 1979) with slight modifications. Samples (0.2 mg of protein) were incubated in duplicate in a total volume of 0.2 ml of Tris/NaCl/ NaN_3 buffer for 2 hr at room temperature with an appropriate concentration of ^{125}I - α BuTX (New England Nuclear, Boston, MA; 95% mono-iodo; specific activity, 80 to 160 Ci/mmol). Free and bound toxin were separated by centrifugation for 5 min at $12,000 \times g$ in an Eppendorf microfuge with two washes of the pellet. "Nonspecific" binding was defined as the binding not blocked by a 1000-fold excess of unlabeled α BuTX in the incubation mixture and was subtracted from the total ^{125}I - α BuTX bound. "Nonspecific" binding ranged from 2 to 20% of the total ^{125}I - α BuTX bound. In a few cases, binding was studied in ASW.

For initial pharmacological characterization of the ^{125}I - α BuTX binding, ligands at various concentrations were preincubated 30 min before addition of ^{125}I - α BuTX to a final concentration of 1×10^{-9} M. Inhibition constants (K_i) and the type of inhibition were determined by

preincubating triplicate membrane samples with or without ligands for 30 min at room temperature. ^{125}I - αBuTX was added to final concentrations of 1, 2, 4, 8, and 16 nM, followed by further incubation for 2 hr. Double reciprocal plots (1/bound versus 1/toxin concentration) were analyzed by the method of least squares. Competitive inhibition was indicated by intersecting intercepts of the control plot and the sample plus ligand plot on the 1/bound axis. Noncompetitive inhibition was indicated if the intercepts and slopes of the control and experimental plots were significantly different (Student's *t* test, $p < 0.05$).

The various ligands tested for their ability to inhibit ^{125}I - αBuTX binding were nicotine, *d*-tubocurarine chloride, carbamylcholine chloride, decamethonium bromide, hexamethonium bromide, neostigmine bromide, picrotoxin, atropine sulfate, histamine dihydrochloride, strychnine hydrochloride, and penicillin G (all from Sigma Chemical Co., St. Louis, MO), and L-(+)-glutamic acid (Eastman, Rochester, NY), ouabain (strophanthin G; Calbiochem, La Jolla, CA), and bicuculline methiodide (Pierce, Rockford, IL).

Protein was determined by the method of Lowry et al. (1951) using BSA as a standard.

Results

Effects of αBuTX and αNTX

ACh-induced responses. Both αBuTX and αNTX specifically antagonized the $\uparrow G_{\text{Cl}}$ responses elicited by focal application of ACh. In Figure 1A, the $\uparrow G_{\text{Na}}$ response of an anterior pleural neuron (Kehoe, 1972b) appears to be

unaffected by 8×10^{-6} M αNTX . αBuTX at 1×10^{-5} M also had no effect on this response. In contrast, the two-component inhibitory response of a medial pleural neuron is affected as shown in Figure 1B. The fast component mediated by $\uparrow G_{\text{Cl}}$ is completely blocked by αNTX (and αBuTX , data not shown) while the slower component mediated by $\uparrow G_{\text{K}}$ is unaffected. The toxin block of the $\uparrow G_{\text{Cl}}$ component occurs at a membrane potential of either -50 or -70 mV and is slowly reversible. These results are similar to those of Kehoe and co-workers (1976) for the effects of αBuTX on ACh responses.

Noncholinergic agonist-induced responses. αBuTX and αNTX will also antagonize $\uparrow G_{\text{Cl}}$ -mediated responses initiated by focal application of noncholinergic agonists. Figure 2 illustrates the effect of αNTX on ACh- and Hm-induced $\uparrow G_{\text{Cl}}$ responses in a cerebral A neuron. This neuron has been shown previously to respond to both ACh and Hm with a two-component inhibitory response mediated by an $\uparrow G_{\text{Cl}}$ and an $\uparrow G_{\text{K}}$ (Gruol and Weinreich, 1979). The $\uparrow G_{\text{K}}$ component has been diminished by intracellular injection of TEA ions since this component is very large relative to the $\uparrow G_{\text{Cl}}$ component. Furthermore, responses to ACh and Hm were collected at the K^+ reversal potential (-85 mV) for this neuron and represent an outward flow of Cl^- .

Both the ACh- and Hm-induced $\uparrow G_{\text{Cl}}$ responses were blocked within 5 min after bath application of 1×10^{-5} M αNTX . This inhibition of the $\uparrow G_{\text{Cl}}$ response could be reversed after washing for 1.5 hr. αBuTX and αNTX were also observed to block $\uparrow G_{\text{Cl}}$ responses initiated by iontophoresis of glu. This effect was variable, however, since some glu-initiated $\uparrow G_{\text{Cl}}$ responses were completely

Figure 1. Effect of α -neurotoxins on ACh responses involving different ionic conductances. **A**, Anterior cell from the left pleural ganglion previously shown by Kehoe (1972b) to respond to ACh with an $\uparrow G_{\text{Na}}$. The $\uparrow G_{\text{Na}}$ ACh response is not affected by a 1-hr perfusion of 8×10^{-6} M αNTX . The input resistance test pulse is 1.0 nA. ACh was applied by a pressure pulse of 40 psi for 7 msec, ejecting a 4-pl droplet of 10^{-4} M ACh. Calibration: 10 mV; 10 sec. **B**, Medial pleural neuron shown by Kehoe (1972a, b) to respond to ACh with a two-component inhibition involving an $\uparrow G_{\text{Cl}}$ (arrow) and an $\uparrow G_{\text{K}}$. At a membrane potential (E_m) of -50 mV, the Cl^- component is hyperpolarizing; at $E_m = -70$ mV, the Cl^- component is reversed and depolarizing. Only the Cl^- component is blocked after 1 hr in 8×10^{-6} M αNTX with no change in cell input resistance (0.2 nA current pulse). This inhibition is reversed after 1 hr washing with toxin-free saline. ACh iontophoresed with a current pulse of 600 nA for 50 msec. Calibration: 4 mV; 10 sec. Data for both anterior and medial pleural neurons were collected simultaneously from the same ganglion.

Figure 2. Effect of α -neurotoxins on $\uparrow G_{Cl}$ responses induced by ACh and by Hm. *Upper traces*, Bath current monitor trace of iontophoretic pulses. *Lower traces*, Responses to iontophoretic ejections of ACh and Hm. *Control figure* depicts the reversed $\uparrow G_{Cl}$ responses to iontophoretically applied ACh and Hm in a cerebral A cell. After 20 min in 1×10^{-5} M α NTX, both ACh and Hm $\uparrow G_{Cl}$ responses are eliminated. The responses return after 1.5 hr of washing with toxin-free saline. ACh was applied with a 30 nA, 50 msec pulse of current; Hm with 840 nA of current for 120 msec. E_m was held at -85 mV (see text). Calibration: 1 μ A, 4 mV; 2 sec.

blocked by 1×10^{-5} M toxin while others were only partially affected. In all cases, the same neurons responded to ACh application with an $\uparrow G_{Cl}$ which was completely blocked by either toxin.

In contrast to the effects of α -toxins on ACh and Hm responses, GABA-induced $\uparrow G_{Cl}$ responses were unaffected by either toxin at concentrations up to 1×10^{-3} M even though the same neurons had ACh-initiated $\uparrow G_{Cl}$ responses that were completely blocked (also observed by Yarowsky and Carpenter, 1978).

Synaptic cholinergic $\uparrow G_{Cl}$ responses. Synaptically evoked cholinergic responses mediated by an $\uparrow G_{Cl}$ were also blocked by α NTX or α BuTX. In Figure 3A, the identifiable cholinergic neuron, B4, in the buccal ganglion mediates an inhibitory postsynaptic potential (IPSP) involving an $\uparrow G_{Cl}$ in an unidentified buccal follower (B?). The cholinergic IPSP was blocked after a 1-hr exposure to α NTX, and the block was partially reversed after washing for 4.5 hr.

Noncholinergic synaptic responses. This same buccal follower, B? (Fig. 3B), also receives convergent synaptic input from several buccal neurons called the S_1 's (Fiore and Meunier, 1975). As shown in the neuronal circuit depicted in Figure 3, the cholinergic neuron (B4) also receives synaptic input from the S_1 's. Figure 3B illustrates the excitatory PSP (EPSP) and the inhibitory-excitatory PSP (IEPSP) evoked in B4 and B?, respectively, by action potentials in the S_1 neuron. Only the fast inhibitory component of the $S_1 \rightarrow B?$ IEPSP is blocked by 5×10^{-6} M α NTX. Toxin did not affect the input resistance of any of the neurons studied, the $S_1 \rightarrow B4$ EPSP, or the excitatory component in the $S_1 \rightarrow B?$ IEPSP. The fast inhibitory response appears to be mediated by $\uparrow G_{Cl}$ since its reversal potential is approximately -60 mV, and this reversal potential can be raised to a more depolarized level by decreasing the external Cl^- concentration or by intracellular injection of Cl^- ions. The transmitter released by the S_1 's is not ACh since (a) B4 responds to ACh with an $\uparrow G_{Cl}$ (Gardner, 1977), whereas the S_1 -mediated EPSP appears to involve an $\uparrow G_{Na}$ which is not antagonized by hexamethonium (J. K. Ono and R. E. McCaman, unpublished) and (b) ACh is

not detectable in the S_1 's (J. K. Ono and R. E. McCaman, unpublished).

Another synaptically mediated $\uparrow G_{Cl}$ response blocked by toxin is demonstrated in Figure 4. The IEPSP in the M-1 neuron of the cerebral ganglion is mediated by a monosynaptic connection from an identified histamine-containing neuron (McCaman and McKenna, 1978). α BuTX blocked the inhibitory component which has been shown previously to involve an $\uparrow G_{Cl}$ (McCaman and McKenna, 1978). This inhibition of the $\uparrow G_{Cl}$ component by toxin was reversible after 1 hr of washing.

The α -neurotoxins do not block all synaptic $\uparrow G_{Cl}$ responses induced by the histamine-containing neuron, HN. Figure 5 demonstrates that another one of the HN followers, M-2, responds to action potentials in HN with an IEPSP. This response appears to be similar to the IEPSP observed in M-1 (Fig. 4). The $\uparrow G_{Cl}$ component of M-2, however, is not blocked by toxin, even after 5 hr of constant perfusion with 2×10^{-5} M α NTX. The two HN followers, M-1 and M-2, are adjacent to one another on the ventromedial surface of the anterior tentacular nerve. Figure 5 demonstrates that the $\uparrow G_{Cl}$ component is blocked only in M-1, whereas the $\uparrow G_{Cl}$ component of M-2 is not affected. In some M-1 followers, a fast excitatory component is unmasked (Fig. 5) by blocking the $\uparrow G_{Cl}$ response with toxin or with *d*-tubocurarine. Both M-1 and M-2 respond to ACh iontophoresed onto their soma. However, M-1 responds with an $\uparrow G_{Cl}$, which is blocked by toxin (*right panel* in Fig. 5), whereas M-2 responds to ACh with an $\uparrow G_{Na}$ inducing action potentials in M-2 (as evidenced by Fig. 5), and this excitatory ACh response is not affected by the α -toxins.

¹²⁵I- α BuTX binding studies

Levels and affinity of ¹²⁵I- α BuTX binding. ¹²⁵I- α BuTX binding to *Aplysia* homogenates indicated the presence of a single, saturable high affinity site. Figure 6 shows a representative binding isotherm using fresh whole nervous system. The *inset* is a Scatchard plot of the binding data. In three separate experiments using fresh or frozen ganglia, we have obtained linear Scatchard plots with maximal binding (B_{max}) of 117.3 ± 29.1 fmol/mg of pro-

Figure 3. Effect of α -neurotoxins on synaptically evoked cholinergic and noncholinergic postsynaptic responses. A diagram of the neuronal circuit in the buccal ganglion is depicted on the left. Data for this figure were collected in the same preparation. The S_1 and B4 neurons were injected with TEA to prolong the presynaptic action potential and, therefore, augment the postsynaptic responses. A, 5×10^{-6} M α NTX blocks the cholinergic IPSP involving an $\uparrow G_{Cl}$ in an unidentified buccal follower (B?). Weak electrotonic coupling between B4 and B? is revealed after elimination of the $\uparrow G_{Cl}$ response. Elimination of the cholinergic IPSP's required 1 hr of perfusion with 5×10^{-6} M α NTX. E_m of B? = -54 mV. Calibration: 200 mV for B4; 4 mV for B?; 0.4 sec. B, α NTX blocks the $\uparrow G_{Cl}$ component mediated by the noncholinergic S_1 neurons on B? (same cell in A) without affecting other types of PSP's. The $\uparrow G_{Cl}$ component in B? was eliminated after a 0.5-hr perfusion of 5×10^{-6} M toxin and returned after 3 hr of washing. No alteration of input resistance occurred during exposure to toxin. E_m of B4 = -60 mV; B? = -54 mV. Input resistance pulse for B4 = 0.4 nA; for B? = 0.2 nA. Calibration: 20 mV for B4, 4 mV for B?, and 200 mV for S_1 ; 0.4 sec.

Figure 4. Inhibition of the Cl^- component of a multicomponent PSP mediated by an identified histaminergic neuron (HN). The fast IPSP's in the follower neuron, M-1, are blocked after a 1-hr perfusion with 8×10^{-6} M α BuTX. This inhibition is reversed after 3 hr washing (not shown). Only the $\uparrow G_{Cl}$ component of this response is affected, with no change in M-1 input resistance. E_m of M-1 = -52 mV. Calibration: 4 mV for M-1; 40 mV for HN; 0.4 sec.

tein and an apparent dissociation constant (K_D) of $3.6 \pm 1.3 \times 10^{-9}$ M (mean $\pm$ SEM).

Pharmacological characterization of ^{125}I - α BuTX binding. Figure 7, A to C shows the concentration-dependent ability of various ligands to inhibit ^{125}I - α BuTX binding to *Aplysia* membranes. The data from these graphs were used to select an appropriate concentration of inhibitory ligand for double reciprocal analysis. Classical cholinergic agonists and antagonists were found to be most effective in inhibiting ^{125}I - α BuTX binding. The noncholinergic agonists, Hm and glu, as well as the Cl^- channel blockers, penicillin G and picrotoxin, showed no effect on toxin binding at concentrations up to 1 mM. In contrast to the results of Shain et al. (1974), we find no effect of the (Na^+, K^+) -ATPase inhibitor, ouabain, at concentrations up to 1 mM.

The K_i 's of the various ligands were calculated from double reciprocal plots, as described under "Materials and Methods," and are summarized in Table I for *Aplysia* and rat brain membranes. The results for both of these preparations are strikingly similar. All ligands were competitive inhibitors of ^{125}I - α BuTX binding except decamethonium and hexamethonium which were noncompetitive. Both unlabeled α BuTX and α NTX have affinities in the nanomolar range. The nicotinic agonists, nicotine and carbamylcholine, as well as the classical nicotinic antagonist, *d*-tubocurarine, have micromolar affin-

Figure 5. Demonstration that not all $\uparrow G_{Cl}$ responses mediated by the histaminergic neuron, HN, are blocked by α -neurotoxins. Experimental paradigm is depicted to the left of the figure. The control figure demonstrates that both M-1 (see also Fig. 4) and M-2 respond to action potentials in the HN with an IEPSP (open arrows indicate $\uparrow G_{Cl}$ component of the synaptic response). However, M-1 responds to ACh applied to the soma (solid arrow) with an $\uparrow G_{Cl}$, whereas M-2 responds to ACh with an $\uparrow G_{Na}$ (tops of M-2 action potentials cut off by oscilloscope screen). After a 0.5-hr exposure to 1×10^{-5} M α -NTX, the $\uparrow G_{Cl}$ component of the M-1 synaptic response and the response to ACh are blocked, whereas the $\uparrow G_{Cl}$ component in M-2 is not affected. Note that elimination of the $\uparrow G_{Cl}$ in this particular M-1 reveals an underlying EPSP; no change in E_m occurs during perfusion with toxin. No change in input resistance (current pulse of 0.2 nA for both cells) occurs during toxin perfusion. E_m of M-1 = -50 mV; E_m of M-2 = -40 mV. ACh was applied by iontophoretic pulses of 600 nA for 20 msec. Calibration: 10 mV for M-1 and M-2; 200 mV for HN; 2 sec.

Figure 6. Representative binding isotherm of ^{125}I - α -BuTX to an *Aplysia* ganglia homogenate. The curve represents specific binding (total binding minus binding not displaced by a 100-fold excess of unlabeled α -BuTX) for fresh whole nervous system homogenates. "Nonspecific" binding ranged from 2 to 20% of the total binding. The inset is a Scatchard plot of this binding curve. See "Materials and Methods" for experimental details.

ities. A surprising finding was the competitive nature, as well as relatively high affinity, of strychnine and bicuculline binding. These agents are usually considered as antagonists of glycine- and GABA-induced responses, respectively. Atropine, a muscarinic antagonist, and neostigmine, usually considered an acetylcholinesterase inhibitor, also displayed some affinity for the toxin binding site.

While the pharmacological profiles of the ^{125}I - α -BuTX binding sites are similar for *Aplysia* and rat brain, several distinct differences were noted when comparing these results to results obtained with peripheral electric organ (see Table I). Nicotine and carbamylcholine appear to bind to neuronal membranes with higher affinity. Strychnine is approximately 10-fold more effective in inhibiting neuronal ^{125}I - α -BuTX binding. Perhaps one of the most striking pharmacological differences between peripheral electric organ and neuronal binding sites is the higher affinity of the former for decamethonium and hexamethonium.

Discussion

Our results demonstrating the specificity not only of α -BuTX but also of α -NTX for ACh responses mediated by an $\uparrow G_{Cl}$ confirm and support the results obtained by Kehoe et al. (1976) with α -BuTX. These results contradict the earlier observations of Shain et al. (1974), which

TABLE I
Ability of various ligands to prevent ^{125}I - αBuTX binding to *Aplysia* and rat membranes

Ligand	Concentration ^a	Inhibition Constant, K_i ^a		Electric Organ ^b
		<i>Aplysia</i> Ganglia	Rat Brain	
	M	μM		
αBuTX	2.5×10^{-9}	0.0015 ± 0.0009	0.0023 ± 0.0007	C ^c
αNTX	2.5×10^{-9}	0.0016 ± 0.0006		C
Nicotine	5×10^{-6}	2.14 ± 1.38	0.15 ± 0.75	C
Carbamylcholine	2×10^{-5}	3.47 ± 1.33	11.2 ± 2.1	C
<i>d</i> -Tubocurarine	2×10^{-5}	4.12 ± 3.98	2.56 ± 1.49	C
Strychnine	1×10^{-5}	6.80 ± 2.09	2.22 ± 1.8	C
Bicuculline	1×10^{-4}	10.9 ± 6.4	33.1 ± 8.5	C
Atropine	1×10^{-4}	51.8 ± 36.6		C
Decamethonium	2×10^{-4}	151 ± 77.6	604 ± 356	NC
Neostigmine	2.5×10^{-3}	484 ± 42	773 ± 281	C
Hexamethonium	1×10^{-3}	824 ± 46	699 ± 176	NC
Histamine		$>1000^e$		
Glutamate		$>1000^e$		
Penicillin G		$>1000^e$	$>1000^e$	
Picrotoxin		$>1000^e$	$>1000^e$	
Ouabain		$>1000^e$		

^a Inhibition constant (K_i) and type of inhibition for *Aplysia* ganglion and rat brain were determined by least squares analysis of double reciprocal plots of ^{125}I - αBuTX binding as a function of increasing ^{125}I - αBuTX concentration in the presence of the indicated concentration of ligand. See "Materials and Methods" for additional details. Results are expressed as the mean $\pm$ SD for at least two independent experiments.

^b Data taken from Weber and Changeux (1974).

^c Type of inhibition for *Aplysia* and rat brain. C, competitive; NC, noncompetitive. See "Materials and Methods" for details.

^d Determined by inhibition of the initial rate of toxin binding as described by Schmidt and Raferty (1974).

^e No inhibition of ^{125}I - αBuTX binding was observed for these ligands at concentrations up to 1 mM.

indicated that αBuTX blocked *all* ACh responses in *Aplysia*, and of Szczepaniak (1974), who found that αBuTX did not affect any ACh responses in the snail, *Helix aspersa*. The basis for these discrepancies, however, remains unclear and could be due to species differences or the source and purification of αBuTX .

The major finding in our study is the demonstration that the α -neurotoxins antagonize $\uparrow\text{G}_{\text{Cl}}$ responses induced by certain *noncholinergic* agonists (Hm, some glu, but not GABA). The specificity of the α -toxins for Cl^- conductances is further demonstrated in the specific antagonism of $\uparrow\text{G}_{\text{Cl}}$ components of monosynaptic PSP's in several synaptic connections. Moreover, there appears to be a correlation between the presence of an ACh receptor mediating an $\uparrow\text{G}_{\text{Cl}}$ response and the ability of toxin to block a noncholinergic $\uparrow\text{G}_{\text{Cl}}$ response (Fig. 5). However, to further substantiate this correlation, other neurons must be found which respond to noncholinergic agonists and transmitters with an $\uparrow\text{G}_{\text{Cl}}$ but lack an $\uparrow\text{G}_{\text{Cl}}$ response to ACh to see if the α -toxins fail to antagonize these noncholinergic responses. The antagonism of the $\uparrow\text{G}_{\text{Cl}}$ component of the PSP mediated by the S_1 neurons, whose transmitter does not appear to be any of the transmitters identified in *Aplysia*, suggests that the $\uparrow\text{G}_{\text{Cl}}$

response to compounds other than Hm and glu may also be antagonized by the α -toxins.

The ^{125}I - αBuTX binding data for *Aplysia* ganglia homogenates are remarkably similar to that for vertebrate CNS preparations (see Table I) and indicate a specific binding site with a pharmacological profile characteristic of a nicotinic cholinergic site. The nicotinic cholinergic antagonists, αBuTX , αNTX , and *d*-tubocurarine, as well as the agonists, nicotine and carbamylcholine, are potent competitive inhibitors of toxin binding in both *Aplysia* and in rat brain. In addition, three compounds, neostigmine, bicuculline, and strychnine, were found to be competitive inhibitors of ^{125}I - αBuTX binding in both *Aplysia* and in rat brain. These ligands were tested to see if they affected toxin binding because they have been shown to inhibit $\uparrow\text{G}_{\text{Cl}}$ responses induced by cholinergic and noncholinergic agonists in *Aplysia* (Kehoe, 1972b; Gerschenfeld and Paupardin-Tritsch, 1974; Faber and Klee, 1974; Yarowsky and Carpenter, 1978).

Strychnine proved to be an especially potent competitive inhibitor of toxin binding in *Aplysia* and in rat brain and may be a useful ligand in differentiating the CNS toxin binding site from the toxin binding site in skeletal neuromuscular junctions. Our rationale for testing

Figure 7. Concentration-dependent ability of various ligands to inhibit specific ^{125}I - αBuTX binding to *Aplysia* ganglia homogenates. *A*, Effects of various concentrations of unlabeled αBuTX , αNTX , nicotine, carbamylcholine, *d*-tubocurarine, and strychnine in inhibiting ^{125}I - αBuTX binding. These ligands were the most potent inhibitors of toxin binding. *B*, Effects of atropine, bicuculline, decamethonium, hexamethonium, and neostigmine on ^{125}I - αBuTX binding. These compounds were less effective in inhibiting toxin binding. *C*, Lack of effects of noncholinergic ligands and Cl^- channel blockers on ^{125}I - αBuTX binding. Millimolar concentrations of glu and Hm had no effect on toxin binding even though toxin blocks glu- and Hm-induced $\uparrow\text{G}_{\text{Cl}}$ responses. The Cl^- channel blockers, penicillin and picrotoxin, had no effect on toxin binding at millimolar levels. The $(\text{Na}^+, \text{K}^+)$ -ATPase inhibitor, ouabain, also had no effect on ^{125}I - αBuTX binding. See "Materials and Methods" for details.

strychnine effects on toxin binding were based on earlier observations that strychnine and *d*-tubocurarine had similar pharmacological effects on *Aplysia* neurons. Both compounds antagonize $\uparrow G_{Na}$ and $\uparrow G_{Cl}$ responses to ACh (Kehoe, 1972b), as well as to other agonists (Faber and Klee, 1974; Carpenter et al., 1977), and are ineffective on GABA-induced $\uparrow G_{Cl}$ responses (Carpenter et al., 1977; Yarowsky and Carpenter, 1978). Strychnine is often classified as a glycine antagonist in the vertebrate CNS (Curtis et al., 1971), but it also has antagonist actions at several cholinergic junctions (Lanari and Luco, 1939; Alving, 1961). Although *d*-tubocurarine and strychnine appear to share many pharmacological similarities, there is an important difference in their effects on ^{125}I - α BuTX binding to neuronal membranes and to *Torpedo* electric organ. *d*-Tubocurarine is a potent competitive inhibitor of ^{125}I - α BuTX binding in *Torpedo* electric organ as well as neuronal membranes. However, strychnine is less effective in *Torpedo* than in *Aplysia* or rat brain. Strychnine may, therefore, serve as a useful ligand in differentiating neuronal ^{125}I - α BuTX binding sites from peripheral electric organ or skeletal muscle sites.

The efficacy of other ligands in antagonizing ^{125}I - α BuTX binding in *Aplysia* was tested in an attempt to gain some insight into the mechanism of toxin antagonism of noncholinergic $\uparrow G_{Cl}$ responses. Hm and glu do not antagonize toxin binding, which suggests that the inhibition of Hm and glu $\uparrow G_{Cl}$ responses by the α -neurotoxins is not mediated by binding to the recognition sites for these agonists. In addition, penicillin G and picrotoxin, two compounds which have been designated Cl^- channel blockers, were without effect on toxin binding. Penicillin G has been shown to block $\uparrow G_{Cl}$ responses to ACh, GABA, 5HT, and dopamine in *Aplysia* (Pellmar and Wilson, 1977). Similarly, picrotoxin blocks the $\uparrow G_{Cl}$ response induced by ACh and GABA in *Aplysia* (Yarowsky and Carpenter, 1978) and has been shown by Takeuchi and Takeuchi (1969) to block Cl^- channels at the crayfish inhibitory neuromuscular junction. It thus appears that the α -neurotoxins do not inhibit noncholinergic $\uparrow G_{Cl}$ responses by acting as Cl^- channel blockers since none of the compounds which are known to inhibit $\uparrow G_{Cl}$ responses, including GABA $\uparrow G_{Cl}$ responses, has a significant effect on ^{125}I - α BuTX binding.

The effective toxin concentration (10^{-6} to 10^{-5} M) for blocking physiological responses in *Aplysia* is several orders of magnitude higher than that necessary to saturate α BuTX binding sites in membrane preparations. High concentrations of toxin are also required to block ACh responses in the chick ciliary ganglion (Chiappinelli and Zigmond, 1978; Conti-Tronconi et al., 1979) and in cat cochlea (Fex and Adams, 1978). This quantitative difference between binding and physiological effectiveness may be due to several factors inherent in the different experimental conditions used for each study. Binding studies are done with tissue homogenates, whereas physiological studies are conducted with practically intact preparations. Physiological experiments with *Aplysia* are conducted in a saline (ASW) containing high concentrations of salts. In a few preliminary binding studies conducted in ASW, we have observed a significantly reduced ^{125}I - α BuTX binding to *Aplysia* membranes.

An alternative explanation for the absence of a close quantitative correlation between binding studies and physiological effectiveness of toxins could be that two unrelated domains of the receptor-ionophore complex are being observed with the separate experimental approaches. This seems unlikely, however, in view of the close qualitative correspondence between the pharmacological characteristics of ^{125}I - α BuTX binding and the pharmacology of ACh responses in *Aplysia*. Of the three types of ACh-induced conductances, the α -toxins block only the $\uparrow G_{Cl}$, while hexamethonium antagonizes only the $\uparrow G_{Na}$ ACh response. Toxin binding in *Aplysia* is relatively insensitive to hexamethonium, and the inhibition is noncompetitive in nature, suggesting an indirect mechanism. In contrast, *d*-tubocurarine blocks both $\uparrow G_{Na}$ and $\uparrow G_{Cl}$ responses (Kehoe, 1972b) and is a potent competitive inhibitor of ^{125}I - α BuTX binding. The mammalian ganglionic nicotinic acetylcholine response appears to be pharmacologically similar to the $\uparrow G_{Na}$ response in *Aplysia* in its sensitivity to blockade by hexamethonium and insensitivity to blockade by α BuTX (Brown, 1979). Perhaps the toxin binding sites observed in mammalian neuronal preparations are related to an ACh receptor mediating an $\uparrow G_{Cl}$ rather than $\uparrow G_{Na}$ response. In fact, one case of α BuTX-induced blockade of a mammalian neuronal nicotinic cholinergic response is thought to be mediated by $\uparrow G_{Cl}$ (Fex and Adams, 1978).

In summary, the ^{125}I - α BuTX binding studies characterize the toxin binding site as a nicotinic cholinergic site even though the α -neurotoxins antagonize $\uparrow G_{Cl}$ responses induced by certain noncholinergic agonists and transmitters. The ineffectiveness of Cl^- channel blockers (penicillin and picrotoxin) in inhibiting toxin binding suggests that the toxins are not, themselves, Cl^- channel blockers. Moreover, toxin does not inhibit all Cl^- -mediated responses (e.g., GABA-, certain glu-, and Hm-induced $\uparrow G_{Cl}$ responses) and the antagonism of noncholinergic $\uparrow G_{Cl}$ responses by toxin appears to be related to the presence of an $\uparrow G_{Cl}$ ACh response. A tenable conclusion based on these results is that the toxin inhibition of noncholinergic $\uparrow G_{Cl}$ responses may be mediated through a cholinergic receptor associated with the Cl^- conductance mechanism for the noncholinergic agonist.

A more specific working hypothesis which would explain our observations is the possibility that the α -neurotoxins block noncholinergic $\uparrow G_{Cl}$ responses because of multireceptor sharing of Cl^- channels. When the α -neurotoxins bind to the ACh receptors, the response of noncholinergic receptors sharing the Cl^- channel are also blocked. This hypothesis predicts that, wherever toxin blocks a noncholinergic $\uparrow G_{Cl}$ response, an ACh receptor is also present and shares the Cl^- response mechanism. However, not all Cl^- channels may be coupled to multireceptors. This is suggested by the observation that responses to ACh were readily elicited from all areas of the cell soma, whereas responses to noncholinergic agonists were restricted to certain locations, and by the variability of toxin inhibition of $\uparrow G_{Cl}$ glu responses on cells where the $\uparrow G_{Cl}$ ACh response was completely blocked by toxin. The hypothesis suggested by our observation is testable and is currently under investigation.

The generality of our observations on α BuTX and

α NTX effects in *Aplysia* remains to be tested in other neuronal systems. Whether another class of nicotinic ACh receptors mediating an $\uparrow G_{Cl}$ response exists in the vertebrate central nervous system is an intriguing possibility, especially in light of the observations of Fex and Adams (1978) that α BuTX reversibly blocks an $\uparrow G_{Cl}$ cholinergic response in the cat cochlea. Moreover, our hypothesis on the mechanism by which the α -neurotoxins block noncholinergic $\uparrow G_{Cl}$ responses, i.e., because of multireceptor sharing of ion channels, is testable and would add an interesting dimension to our concepts of receptor-ionophore organization. In addition, the existence of these proposed multireceptor-ionophore mosaics at extra- and subsynaptic sites may help to explain the often confusing classification of pharmacological agents.

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