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Structure-Activity Relationships for the Irreversible Blockade of Nicotinic Receptor Agonist Sites by Lophotoxin and Congeneric Diterpene Lactones

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SUMMARY

Lophotoxin, a diterpene lactone paralytic toxin from gorgonian corals of the genus *Lophogorgia*, inhibits [^{125}I]-α-toxin binding to surface nicotinic receptors of BC3H-1 cells by irreversible occupation of the primary agonist sites. In contrast, receptor-bearing membrane fragments or detergent-solubilized receptors prepared from BC3H-1 cells are not susceptible to lophotoxin block. Thus, lophotoxin inhibition requires intact cells. However, when intact cells were incubated with lophotoxin, subsequent membrane-fragment preparation or detergent solubilization of the receptors did not diminish lophotoxin occupation of [^{125}I]-α-toxin-binding sites, indicating that lophotoxin binds very tightly to nicotinic receptors. These studies further demonstrate that both surface and nonsurface nicotinic receptors of BC3H-1 cells are susceptible to irreversible occupation by lophotoxin, indicating that the lipophilic toxin freely permeates intact cells. We also examined several structural analogs of lophotoxin, one of which was equipotent with lophotoxin for inhibition of [^{125}I]-α-toxin binding to intact cells and, notably, also blocked α-toxin binding to detergent-extracted receptor. Furthermore, this active analog inhibited [^{125}I]-α-toxin binding to receptor-rich membrane fragments prepared from *Torpedo* electric organ, a preparation in which lophotoxin was inactive. Structure-activity relationships exhibited by the lophotoxin congeners suggest mechanisms for covalent bonding to the receptor by way of a Michael addition or by Schiff base formation.

INTRODUCTION

Much of our knowledge of the structure and function of the neuromuscular nicotinic receptor has relied on the utilization of paralytic toxins and other agents which bind with high affinity to critical domains on the receptor and thereby inhibit its functional response to agonists. In this regard, snake venom α-toxins have occupied a prominent role because their high affinity and specificity for agonist/antagonist-binding sites have permitted their use as probes to direct the purification of nicotinic receptor from a variety of sources, including *Electrophorus* and *Torpedo* electric organs (reviewed in Ref. 1), bovine (2) and human (3) muscle, and the BC3H-1 muscle cell line (4). With the availability of purified nicotinic receptor it has become possible to characterize the structural features of this integral membrane protein, so that we now know that the receptor consists of four subunits of partially homologous amino acid sequence, designated α, β, γ, δ. The α-subunit is expressed as two copies giving an overall stoichiometry of $\alpha_2\beta\gamma\delta$ for the functional pentameric receptor. Additionally, the α-subunits each bear a site at which agonists, antagonists, and α-toxins bind in a mutually exclusive manner (1, 5, 6).

The elucidation of the functional response of the nicotinic receptor to agonists has also been facilitated by the use of snake α-toxins. By examining the capacity of agonists to elicit an ion flux response following irreversible inactivation of specified numbers of binding sites by α-toxin, it has been possible to delineate the coupling between agonist occupation of binding sites and the ensuing functional response. Such studies have demonstrated that irreversible α-toxin occupation of one of the two primary agonist sites present on each receptor macromolecule is sufficient to render the receptor inactive in both BC3H-1 cells in culture (7) and in *Torpedo* (8, 9). Activation of the receptor-associated cation channel thus requires that both sites be unblocked and available for interaction with agonist.

The occupation of primary sites by agonists and antagonists has been examined in BC3H-1 cells by way of

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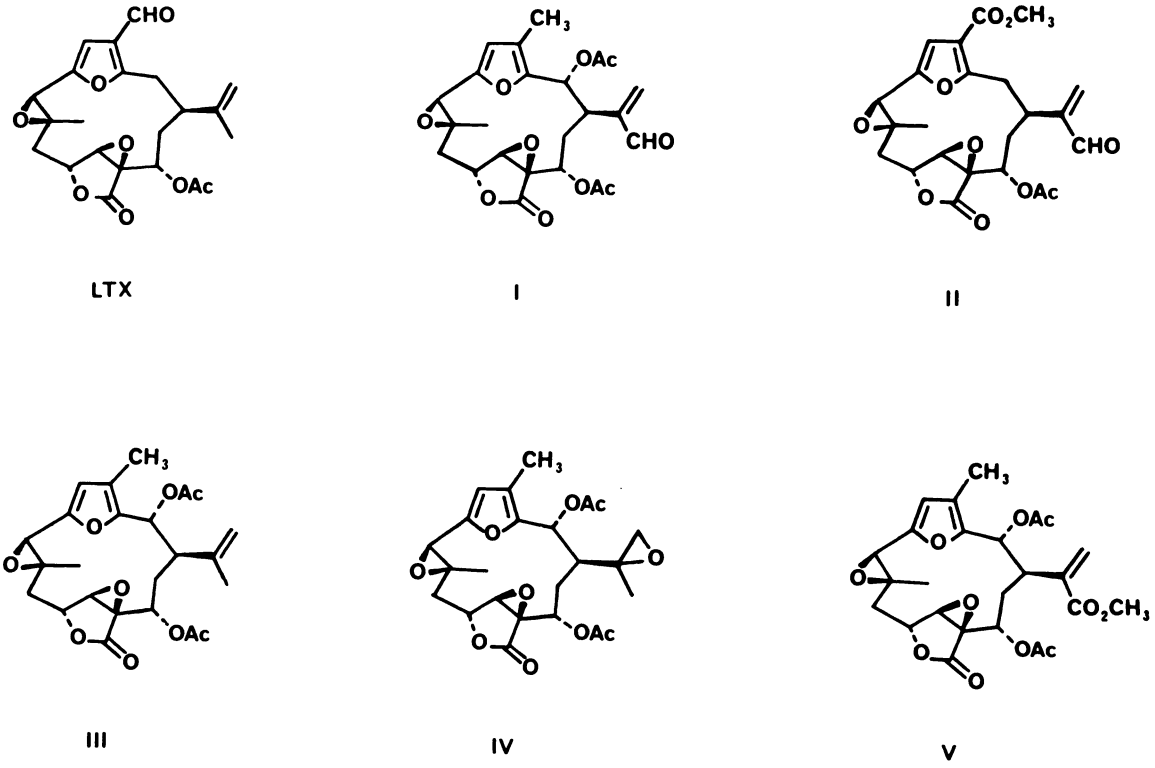


FIG. 1. *Chemical structures for lophotoxin (LTX) and the analogs (I to V).*

competition binding studies using radiolabeled α-toxin (10, 11). Agonists exhibit positive cooperativity in their binding to the two sites present on the receptor complex, whereas antagonist occupation exhibits a Hill coefficient less than unity, which is due to an intrinsic inequivalence in the affinity of antagonists for the two binding sites.

The resolution with which ligand-binding studies can elucidate agonist site topography is constrained by the extent of spatial ambiguity inherent in the structure of the ligand used. With the α-toxins such ambiguity is considerable, owing to the relatively large size (molecular weight ~8000) and potential conformational flexibility of these peptides. Finer detailing of the binding site through structure-activity analysis and peptide mapping will be facilitated by the development of low molecular weight irreversible ligands of the receptor. The affinity labeling agents maleimidobenzyl-tetramethylammonium and bromoacetylcholine do satisfy these criteria but require prior chemical modification of the receptor for covalent labeling to occur (12, 13).

We have been examining the molecular mechanism for the inhibition of neuromuscular transmission by lophotoxin (Fig. 1), a diterpene lactone obtained from Pacific gorgonian corals of the genus *Lophogorgia* (14). Previous characterizations of the toxin's inhibitory action in isolated neuromuscular preparations (15–17) have revealed a postsynaptic site of action which is not reversed with prolonged washout. Studies utilizing the BC3H-1 muscle cell line (18) demonstrated that lophotoxin inactivates nicotinic receptors by irreversible occupation of the two primary agonist/antagonist-binding sites on the α-subunits. Of these two sites, lophotoxin preferentially occupied the site at which classical antagonists exhibit lower binding affinity.

Because of its capacity to irreversibly label receptors in their native state and discriminate between the two primary agonist/antagonist-binding sites present on the receptor pentamer, lophotoxin represents a potentially important low molecular weight probe for studies of nicotinic receptor structure and function. As a prelude to binding studies with a tritiated derivative of lophotoxin which has recently become available, we have conducted an evaluation of lophotoxin competition with [^{125}I]-α-toxin binding to intact versus disrupted BC3H-1 cells and to receptor-rich membrane fragments from *Torpedo* electric organs. Also, several naturally occurring structural analogs of lophotoxin have recently been isolated from Caribbean gorgonians of the related genus *Pseudopterogorgia*, and we have similarly examined their capacity to inhibit [^{125}I]-α-toxin binding. An analysis of the structure-activity relationships exhibited by lophotoxin and the analogs has provided us with new insights regarding the chemical requirements for occupation of the agonist/antagonist-binding sites by these compounds. An abstract of portions of this work has been published (19).

MATERIALS AND METHODS

Toxins and chemicals. Pure, crystalline lophotoxin was prepared as previously described (14), except that we now obtain the toxin from a local gorgonian coral, *Lophogorgia chilensis*. Details of the isolation and structure determination of the lophotoxin analogs from *Pseudopterogorgia sp.* will be published elsewhere.[1] Stock solutions of lophotoxin and the analogs were prepared at a concentration of 10 mM in DMSO,[2] a solvent in which they are freely soluble and can be stored for several months without loss of activity. Dilution from stock solutions into

[1] M. Burch and W. Fenical, manuscript in preparation.

[2] The abbreviation used is: DMSO dimethylsulfoxide.

physiological saline yielded a final DMSO concentration of 1%, which is without effect on our assay systems. Nevertheless, all of the experiments reported here included determinations on control samples which contained 1% DMSO. Lophotoxin and the analogs remained soluble when diluted into buffered saline at final concentrations up to 100 μM.

Pure cobra α-toxin was isolated from *Naja naja siamensis* venom, from which radioactive monoiodo-α-toxin was prepared and separated from noniodinated and diiodo species by isoelectric focusing (20).

Sources of radionuclides, drugs, chemicals, and cell culture supplies used in this study were the same as those previously published (10).

BC3H-1 cell cultures. The BC3H-1 cell line was the generous gift of Drs. J. Patrick and J. Boulter of the Salk Institute and has been carried in the laboratory for 8 years. Stock cultures were maintained as previously described (7), from which experimental cultures were seeded into 35-mm dishes and maintained as detailed elsewhere (18). The resulting monolayers of differentiated cells were used in experiments 13–15 days after plating. Specific [^{125}I]-α-toxin binding capacity ranged from 1.5 to 2.5 pmol/35-mm dish.

Membrane fragment preparations. Receptor-rich membrane fragments were isolated from *Torpedo californica* electric organ by published procedures (21, 22), and ranged from 1 to 2 nmol of α-toxin-binding sites/mg of protein.

BC3H-1 membrane fragments were prepared by harvesting cells from 35-mm tissue culture dishes with a rubber policeman into physiological saline, which contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 5.5 mM glucose, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 0.06 mg/ml bovine serum albumin, adjusted to pH 7.4 with 1 N NaOH. The cells were sedimented by centrifugation, then resuspended in a hypotonic solution containing 5 mM Tris-HCl, 1 mM $MgCl_2$ (pH 7.6) at 4°. The cell suspension was then homogenized with a Kontes glass homogenizer and Teflon pestle (10–15 passes at 4°). The homogenate was centrifuged at 500 × *g* for 10 min to remove cellular debris, and the resulting supernatant was recentrifuged at 39,000 × *g* for 20 min. The final pellet was resuspended in a volume of physiological saline sufficient for a final concentration of 1–2 pmol of α-toxin sites/20 μl. The saturation binding of [^{125}I]-α-toxin to the fragments thus derived was highly specific; nonspecific binding, as determined by prior saturation of receptor sites with unlabeled α-toxin, was < 5% of total binding. However, the recovery of α-toxin-binding sites in the final pellet was 35–50% of the initial value in the monolayer and was not improved by increasing the final centrifugation to 80,000 × *g* for 60 min.

Detergent-solubilized preparations. Triton X-100 detergent solubilization of BC3H-1 cells followed published methods with slight modification (23). Briefly, cells were harvested from 35-mm dishes and sedimented by centrifugation. The cell pellet was extracted at 4° for 60 min in a 3% (w/v) Triton X-100 solution prepared in the physiological saline described above. Extraction volume was 100 μl/35-mm dish. The extract was spun on a Microfuge for 10 min, and the resulting pellet of insoluble material was discarded. The level of nonspecific [^{125}I]-α-toxin binding to solubilized receptor thus prepared was consistently < 10% of total binding.

Measurement of [^{125}I]-α-toxin binding to Torpedo membrane fragment preparations. Determinations of iodinated α-toxin binding to *Torpedo* membrane fragments were conducted in 12 × 75 mm glass test tubes in a 10 mM phosphate buffer containing 100 mM NaCl and 1 mg/ml bovine serum albumin (pH 7.4). Membranes (1.5–2.0 pmol of α-toxin sites) were first incubated with the various experimental treatments in a volume of 230 μl, then a molar excess of [^{125}I]-α-toxin was added in a volume of 20 μl to give a final volume of 250 μl. Following 60 min of incubation with the iodinated toxin to achieve equilibrium, 50-μl aliquots were withdrawn and spotted in duplicate on Whatman DE81 and No. 1 filter papers for the determination of receptor-bound and total (bound + free) radioactivity, respectively, as described elsewhere (24). Nonspecific binding of [^{125}I]-α-toxin was determined from incubations with membranes previously exposed to unlabeled α-toxin under saturating conditions.

Measurement of [^{125}I]-α-toxin binding to membrane fragment or detergent-solubilized preparations from BC3H-1 cells. Determinations of the specific binding of [^{125}I]-α-toxin to these two disrupted cell preparations were conducted as described above for *Torpedo* membrane fragments, with the exception that binding assays were performed in physiological saline instead of NaCl-phosphate buffer. Incubations with lophotoxin or analogs were typically conducted in a volume of 230 μl which contained an aliquot of membrane fragments or detergent extract representing 1–2 pmol of α-toxin-binding sites. Subsequent to the intitial incubation period, a molar excess of [^{125}I]-α-toxin was added in a volume of 20 μl, bringing the final incubation volume to 250 μl. Quantitation of receptor-bound versus free radioactivity and the determination of nonspecific binding were identical to those described above for the *Torpedo* membrane fragments.

Measurement of [^{125}I]-α-toxin binding to intact BC3H-1 cells. Determinations were conducted in duplicate culture dishes at 21° by the protocol previously described (7). Briefly, sister culture dishes were removed from the incubator and allowed to equilibrate for 30 min, after which the monolayers were rinsed free of growth media with 2 ml of physiological saline and allowed to equilibrate with a second 2-ml wash of saline for 20 min. Following the various experimental treatments, which were performed in 0.75 ml of physiological saline, initial rates of [^{125}I]-α-toxin binding were determined by replacing the solution covering the monolayer with 0.75 ml of saline containing 20–40 nM [^{125}I]-α-toxin for 30 sec. The reaction was stopped by aspirating the radioactive solution and immediately rinsing the monolayer with four successive 3-ml portions of physiological saline. Cells were collected from the dishes and bound radioactivity was assessed as described previously (10). Nonspecific binding was determined under identical conditions, but with cells previously saturated by unlabeled α-toxin. Maximal binding capacity of the cells was determined by exposure to [^{125}I]-α-toxin under saturating conditions. The initial rate of α-toxin association, k_T, was calculated from the bimolecular rate equation as previously described (10).

RESULTS

Lophotoxin inhibition of α-toxin binding requires an intact cell. Incubation of intact BC3H-1 cells with lophotoxin results in a progressive, irreversible inhibition of [^{125}I]-α-toxin binding to surface nicotinic receptors, and the extent of the inhibition is both concentration and time dependent (18). When the duration of exposure to lophotoxin was fixed at 2 hr, incubation concentrations of 0.1–100 μM produced a complete range of nicotinic receptor antagonism, as measured by the decrement in initial rates of [^{125}I]-α-toxin binding (Fig. 2A). Under these conditions, half-maximal inhibition of [^{125}I]-α-toxin binding (IC_{50}) typically occurred at lophotoxin concentrations of 1–2 μM.

When membrane fragments prepared from BC3H-1 cells were exposed to the same range of lophotoxin concentrations, lophotoxin's capacity to inhibit [^{125}I]-α-toxin binding was markedly diminished (Fig. 2B). Similar findings were obtained with Triton extracts of BC3H-1 cells, where lophotoxin again exhibited only marginal inhibition of [^{125}I]-α-toxin binding (Fig. 2C).

However, Fig. 2 shows that when intact BC3H-1 monolayers were exposed to lophotoxin, rinsed with toxin-free buffer, then harvested and subjected to either membrane fragment preparation or Triton X-100 solubilization, subsequent determinations of [^{125}I]-α-toxin binding revealed substantial inhibition by lophotoxin. Furthermore, the concentration dependence of the inhibition was in close agreement with that which was observed for

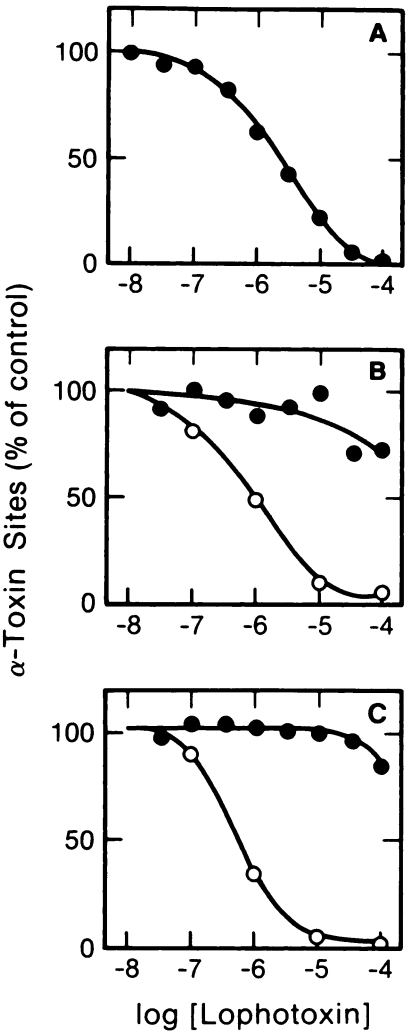


FIG. 2. *Lophotoxin inhibition of [^{125}I]-α-toxin binding to nicotinic receptor of BC3H-1 cells*

A: Duplicate monolayer cultures of BC3H-1 cells were exposed to the indicated concentrations of lophotoxin for 2 hr and then rinsed with four successive 3-ml changes of physiological saline. Initial rates of [^{125}I]-α-toxin binding were then determined. B: Membrane fragments were prepared from BC3H-1 cells and exposed to the indicated concentrations of lophotoxin for 2 hr. Equilibrium binding of [^{125}I]-α-toxin was subsequently determined, and the results (●) are given as a percentage of that observed following 2 hr exposure to 1% DMSO. In another experiment (○), BC3H-1 monolayers were exposed to the indicated lophotoxin concentrations for 2 hr, washed with four changes of saline, then processed separately for the preparation of membrane fragments. Equilibrium binding of [^{125}I]-α-toxin to the membrane fragments was then determined, the results being expressed as a percentage of that obtained from plates treated with 1% DMSO. C: The experiment was performed as described for B, but with Triton X-100 extracts of BC3H-1 cells. The data points give the results of equilibrium [^{125}I]-α-toxin binding determinations in lophotoxin-treated extracts (●) and extracts of lophotoxin-treated monolayers of intact cells (○).

lophotoxin in intact monolayers. We therefore concluded that substantial inhibition of the nicotinic receptor by lophotoxin can only occur when intact BC3H-1 cells are exposed to the toxin. With this requirement satisfied, subsequent disruption of the cells, either by homogenization and membrane fragment isolation or by Triton X-100 solubilization, does not diminish the occupation of [^{125}I]-α-toxin-binding sites by lophotoxin. These findings further attest to the irreversibility of lophotoxin action, since substantial dilution in toxin-free buffer is achieved during the preparation of membrane fragments or detergent extracts without a concomitant loss of lophotoxin inhibition.

Lophotoxin inhibits α-toxin binding to surface and nonsurface nicotinic receptors. We have determined that lophotoxin gains access to and irreversibly inhibits additional nicotinic receptors which are not exposed to the extracellular surface of BC3H-1 cells. These determinations are summarized in Table 1. When BC3H-1 monolayers were exposed to native α-toxin under saturating conditions, washed in toxin-free buffer, and then subjected to Triton X-100 solubilization, subsequent equilibrium [^{125}I]-α-toxin binding assays revealed a residual component of binding sites which were not accessible to the externally applied native α-toxin. These "hidden" or nonsurface α-toxin-binding sites comprised approximately 32% of the total Triton-extractable sites. This determination is in close agreement with assessments of surface and total cellular receptors using α-bungarotoxin binding to intact cells and Triton-solubilized cellular constituents (23). However, when BC3H-1 monolayers were exposed to lophotoxin under saturating conditions, washed, and then subjected to identical extraction and iodinated α-toxin binding procedures, one observed complete inhibition of [^{125}I]-α-toxin binding. Thus, externally applied lophotoxin permeates to an occult receptor pool which is not accessible to externally applied α-toxin.

Inhibition of α-toxin binding to the nicotinic receptors of BC3H-1 cells by structural analogs of lophotoxin. We have examined the capacity of various structural analogs of lophotoxin (Fig. 1) to block irreversibly the [^{125}I]-α-toxin-binding sites of BC3H-1 cells, and the results of these studies are given in Figs. 3 and 4. In order to facilitate comparison of activities among lophotoxin and the analogs, we have maintained the experimental format of Fig. 2, in which the concentration of analog was varied but duration of exposure was fixed at 2 hr. For reference, the IC_{50} for lophotoxin under these conditions of incubation was 1–2 μM. Inspection of Figs. 3 and 4 reveals inhibition of [^{125}I]-α-toxin binding by analogs I, IV, and V, but not by the remaining analogs. Of the three active analogs, only analog I (IC_{50} = 5 μM) exhibited inhibition

TABLE 1

Lophotoxin and native α-toxin inhibition of surface and nonsurface [^{125}I]-α-toxin-binding sites of BC3H-1 cells

Monolayers of cells were exposed to saline (control) or saline containing native α-toxin or lophotoxin under conditions sufficient for receptor saturation. The monolayers were then washed with four changes of toxin-free buffer, extracted with 3% Triton solutions. Equilibrium binding of [^{125}I]-α-toxin was then determined for each extract. Values are given as percentage of [^{125}I]-α-toxin binding obtained from control plates, averaged from four separate determinations.

Treatment	Percentage of [^{125}I]-α-toxin sites remaining
	mean ± SE
None	100%
α-Toxin (100–500 nM)	32.2 ± 1.4
Lophotoxin (10–100 μM)	2.2 ± 1.3

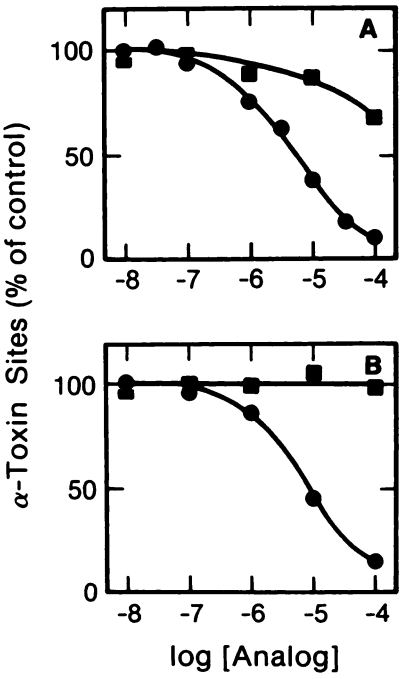


FIG. 3. *Effects of analogs I and II on [^{125}I]-α-toxin binding to BC3H-1 monolayers (A) or to Triton X-100-solubilized receptor from BC3H-1 cells (B)*

Experiments were performed as described in the legend for Fig. 2, A and C. ●, analog I; ■, analog II.

of potency comparable to that of lophotoxin. Inhibition by analogs IV and V required incubation concentrations in excess of 10 μM, these analogs being 30- to 100-fold less potent than lophotoxin. Notably, analog II bears remarkable structural similarity to analog I, yet it is completely devoid of [^{125}I]-α-toxin blocking activity.

Incubation of detergent-solubilized receptor from BC3H-1 cells with analog I produced substantial inhibition of [^{125}I]-α-toxin binding (IC_{50} = 10 μM), whereas analog II was inactive in this preparation (Fig. 3).

Thus, analog I and lophotoxin are comparable in their potencies for inhibition of [^{125}I]-α-toxin binding to BC3H-1 cell monolayers, but they differ significantly in their capacity to alter α-toxin binding to the detergent-solubilized receptor preparation. In the latter case, only analog I exhibits inhibition of [^{125}I]-α-toxin binding, and it does so in a concentration range which is comparable to that observed for the intact BC3H-1 cells in culture. In contrast, analog II is inactive in both intact and detergent-solubilized BC3H-1 cells.

The effect of lophotoxin and analog I on α-toxin binding to the nicotinic receptors of Torpedo membrane fragment preparations. For these studies, we employed an experimental format identical to that utilized for the detergent-solubilized receptor from BC3H-1 cells, whereby *Torpedo* membranes were first incubated with various concentrations of lophotoxin or analog I for 2 hr, followed by the determination of equilibrium [^{125}I]-α-toxin binding to remaining unblocked sites. The results of this study are summarized in Fig. 5, which demonstrates significant inhibition of [^{125}I]-α-toxin binding by analog I. The concentration dependence for analog I inhibition in *Torpedo* membranes indicates a somewhat greater inhibitory

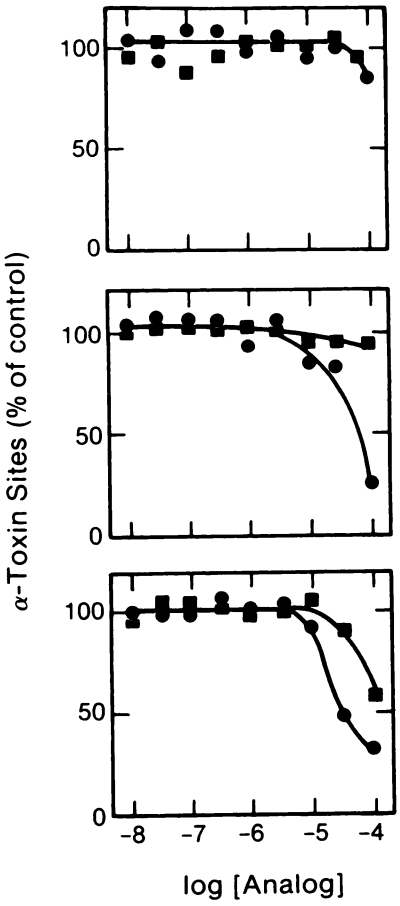


FIG. 4. *Effect of analogs III (top), IV (middle), and V (bottom) on [^{125}I]-α-toxin binding to BC3H-1 monolayers of cells (●) or to Triton X-100-solubilized receptor from BC3H-1 cells (●)*

Experiments were performed as described in the legend for Fig. 2, A and C.

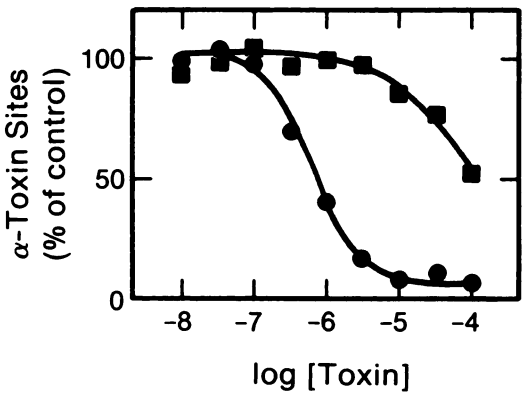


FIG. 5. *Effect of lophotoxin (■) and analog I (●) on the equilibrium binding to [^{125}I]-α-toxin to Torpedo membrane fragments*

Receptor-rich membranes (6.5 nM in α-toxin sites) were incubated with the indicated concentrations of lophotoxin or analog for a duration of 2 hr, followed by the addition of [^{125}I]-α-toxin (20 nM) for the determination of equilibrium binding. Results are expressed as a percentage of the value obtained for [^{125}I]-α-toxin binding following 2 hr exposure to 1% DMSO.

potency in this preparation (IC_{50} = 0.7 μM) than was observed for intact or detergent-solubilized BC3H-1 cells. Furthermore, an identical concentration dependence for inhibition of α-toxin binding by analog I was obtained from experiments in which a 100-fold dilution was interposed between the initial incubation of membranes with analog I and the subsequent determination of [^{125}I]-α-toxin binding (data not shown). Thus, blockade by analog I was not reversed by substantial dilution. In contrast, lophotoxin produced a partial inhibition of *Torpedo* nicotinic receptor, but only at concentrations two orders of magnitude greater than those required for comparable inhibition of receptor on intact BC3H-1 cells.

Protection studies. If the inhibition of [^{125}I]-α-toxin binding by analog I is due to mutually exclusive association of the analog and α-toxin at the primary agonist sites of the nicotinic receptor, then the presence of reversible receptor agonists and antagonists should afford protection against the inhibitory effect of the irreversible analog. Occupation of an increasing fraction of receptor binding sites by reversible ligands results in a directly proportional decrease in the apparent rate at which irreversible ligands bind to these sites. In order to quantitate reversible agonist or antagonist protection against [^{125}I]-α-toxin binding, one simply examines the concentration dependency for the capacity of these agents to reduce k_T, the initial rate of toxin binding (10). However, for a nonradioactive irreversible ligand, the apparent rate of binding, k_{app}, must be assessed indirectly by examining the time course for the disappearance of [^{125}I]-α-toxin-binding sites during exposure to the ligand. We have conducted such studies with lophotoxin and have shown that the time course for the decreases in [^{125}I]-α-toxin binding at any given lophotoxin concentration may be described as a first order process (18). Semilogarithmic plots of [^{125}I]-α-toxin binding as a function of the duration of exposure to lophotoxin approximate linear relationships, the slopes of which give k_{app}. The pseudo-first order treatment also applies to inhibition of [^{125}I]-α-toxin binding by analog I (data not shown).

We have examined the influence of reversible nicotinic receptor ligands on the k_{app} for analog I inhibition of [^{125}I]-α-toxin binding (Fig. 6). Coincubation with the agonist carbamylcholine and the antagonists pancuronium and metocurine (dimethyl-*d*-tubocurarine) diminished analog I binding with K_p values and Hill coefficients approximating those previously obtained for protection against lophotoxin and [^{125}I]-α-toxin binding (Table 2). Analog I inhibition of [^{125}I]-α-toxin binding apparently does not involve interaction with the allosteric local anesthetic binding site of the receptor, since dibucaine does not alter k_{app} for analog I. Thus, we concluded that inhibition of [^{125}I]-α-toxin binding by analog I results from occupation of the agonist/antagonist sites of nicotinic receptors.

Influence of analog I on the occupation function for antagonist. Previous studies of the occupation of [^{125}I]-α-toxin binding sites on BC3H-1 cells by various reversible nicotinic receptor ligands revealed a marked distinction between agonist and antagonist agents. Agonists such as suberyldicholine and carbamylcholine typically exhibit Hill coefficients exceeding unity, suggesting that agonists bind with positive cooperativity to multiple sites on the receptor macromolecule (10). In contrast, the binding of antagonist agents revealed Hill coefficients significantly less than unity, the values of which ranged from a low of 0.51 for metocurine to a high of 0.87 for alcuronium (11). These findings were interpreted in terms of a minimal model in which antagonists exhibit differential binding affinities at two sites, α_A and α_B, present on the two α-subunits of each pentameric receptor complex. For the antagonist alcuronium, the binding affinities at each site were found to be nearly equivalent ($K_A \simeq K_B$), so that the Hill coefficient for occupation of sites by this antagonist approaches the unity value indicative of a single class of binding sites. However, antagonists such as metocurine and gallamine bind to the α_A and α_B sites with markedly differing affinities, so that a much broader range of antagonist concentration is required to demonstrate the complete occupation function for these ligands. From the Hill coefficient value for metocurine occupation and the assumption of the two populations of binding site affinities, one calculates a K_A/K_B ratio of 89 (11).

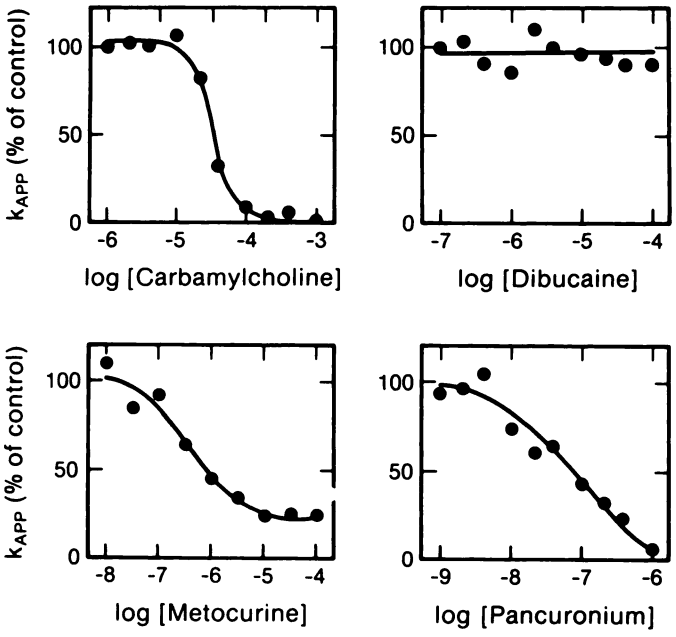


FIG. 6. *Reversible agonist and antagonist protection against irreversible inhibition of [^{125}I]-α-toxin binding by analog I*

Monolayer cultures of BC3H-1 cells were first equilibrated for 20–30 min with solutions containing the indicated concentrations of reversible ligands. Each solution was then replaced with one containing an identical concentration of reversible ligand and 10 μM analog I, in which cells were incubated for 2 hr. The monolayers were then washed extensively (six times 3 ml) with saline to remove reversible ligand and free or reversibly bound analog I and then were subjected to initial rate determinations of [^{125}I]-α-toxin binding. The apparent rate (k_{app}) for the development of analog I inhibition of radiolabeled α-toxin binding was then calculated for each treatment as detailed in the text. The apparent inhibition rates are expressed on the *ordinates* as a percentage of that obtained from control plates which were exposed to 10 μM analog I for 2 hr in the absence of competing ligand.

TABLE 2

Parameters for agonist/antagonist competition with analog I, lophotoxin, and α-toxin

Ligand	Antagonism of analog I binding[a]		Antagonism of lophotoxin binding[b]		Antagonism of α-toxin binding[b]	
	K_p	n_H	K_p	n_H	K_p	n_H
	μM		*μM*		*μM*	
Carbamylcholine	38	2.56 ± 0.31	59	1.21 ± 0.10	23	1.48 ± 0.15
Metocurine	0.81	0.49 ± 0.03	3.6	0.44 ± 0.03	4.2	0.51 ± 0.03
Pancuronium	0.081	0.75 ± 0.11	0.066	0.88 ± 0.08	0.023	0.86 ± 0.02

[a] Compiled from the competition data shown in Fig. 6. The dependence of the k_{app} for analog I on the concentration of reversible ligands was analyzed by the method of Hill (26), yielding a Hill coefficient, n_H, and the concentration of competing ligand that reduces k_{app} by 50%, K_p. Regression analyses with the linear Hill equation provided the values for K_p, n_H, and standard error.

[b] Previously published data are shown here for comparison (11, 18).

Preferential binding to one of the α-subunits by metocurine enables one to determine whether irreversible ligands such as α-toxin or lophotoxin exhibit selectivity for one of the two populations of binding sites. This may be accomplished by first occupying a fraction of the sites with the irreversible ligand, and then assessing the occupation function for metocurine at the remaining unblocked sites. If the irreversible ligand binds randomly to the two populations of sites, then the subsequent occupation function for metocurine should be identical to that obtained in the absence of prior block by an irreversible ligand. Such is the case for prior occupation by native α-toxin (7). However, if the irreversible ligand binds preferentially to the α_A site, then prior occupation with that ligand will generate a situation in which the α_B site will predominate as the unblocked species. Subsequent analysis of metocurine occupation at the unblocked sites should then reveal an increase in the Hill coefficient toward a limiting value of unity, the magnitude of the increase being proportional to the degree of site preference inherent in the binding of the irreversible ligand. In addition, the dissociation constant for metocurine binding would either increase or decrease to reflect the predominance of either low or high affinity sites, respectively. Following various extents of fractional irreversible occupation of binding sites by lophotoxin, one observes Hill coefficients for metocurine approaching unity and a decrease in the apparent dissociation constant, revealing that lophotoxin preferentially occupies the binding site at which metocurine exhibits low affinity (18).

Fig. 7 gives the results of such fractional occupation experiments conducted with analog I. Following prior fractional occupation by analog I, the parameters for metocurine binding were not significantly altered from their control values (Table 3). These findings indicate that analog I does not distinguish between α_A and α_B sites but, instead, binds randomly to the two sites present on the receptor complex. In this regard, analog I more closely resembles cobra α-toxin than lophotoxin.

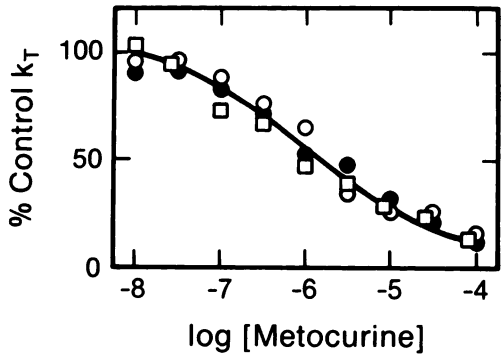


FIG. 7. *The influence of prior occupation of receptor sites by analog I on metocurine competition with [^{125}I]-α-toxin binding*

Cells were exposed to saline containing either 1% DMSO (●) or analog I at concentrations of 10μM (□) or 20μM (○) for a duration of 2 hr, washed with four 3-ml changes of saline, and then assayed for metocurine inhibition of the initial rate of [^{125}I]-α-toxin binding. For this, cells were first equilibrated with the indicated concentrations of metocurine for 30 min, then replaced with solutions containing an identical concentration of metocurine and 35 nM [^{125}I]-α-toxin for 30 sec. Intitial rates of [^{125}I]-α-toxin binding are given on the *ordinate* as a percentage of the rate observed in the absence of metocurine. Thus, in the cases of prior occupation by analog I, the control k_T values actually represented 36 and 27% of that observed in the 1% DMSO pretreatment, since 64% (□) and 73% (○) of the receptor sites were irreversibly occupied by analog I.

TABLE 3

Parameters for metocurine competition with [^{125}I]-α-toxin following partial irreversible occupation of sites by analog I

Competition data were obtained from Fig. 7. Values of K_p and n_H for the concentration dependency of metocurine reduction of k_T for [^{125}I]-α-toxin binding were determined from the Hill equation (see Table 2).

Occupation by analog I	K_p	n_H
%	*M*	
0	1.4×10^{-6}	0.42 ± 0.06
64	9.2×10^{-7}	0.41 ± 0.05
73	2.5×10^{-6}	0.53 ± 0.06

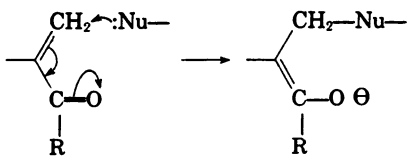
DISCUSSION

The experimental findings presented here show that lophotoxin's capacity to irreversibly inhibit [^{125}I]-α-toxin binding to BC3H-1 cells is shared to various extents by three naturally occurring congeners, analogs I, IV, and V. Of these, only analog I exhibited an inhibitory potency comparable to that of lophotoxin. Since coincubation with classical reversible nicotinic agonists and antagonists abolished the inhibitory effect of analog I, it is reasonable to conclude that its capacity to diminish [^{125}I]-α-toxin binding is due to irreversible occupation of the primary agonist/antagonist sites α_A and α_B of the

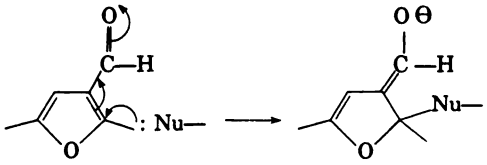
nicotinic receptor, as was previously demonstrated for lophotoxin. However, analog I does not share lophotoxin's capacity for preferential occupation of the low affinity metocurine binding site, α_A. Rather, analog I binds randomly to the two sites α_A and α_B present per receptor macromolecule, as does cobra α-toxin.

Although lophotoxin and analog I exhibited comparable effects on α-toxin binding to intact cells, striking differences were discerned in their capacity to inhibit α-toxin binding to disrupted cell preparations. Analog I inhibited [^{125}I]-α-toxin binding to Triton X-100-solubilized receptor from BC3H-1 cells and to receptor-rich membrane fragments from *Torpedo* electric organ at concentrations comparable to those shown to be effective in the intact BC3H-1 monolayers. In contrast, lophotoxin inhibition required intact BC3H-1 cells. However, membrane fragments or detergent extracts prepared from lophotoxin-treated cells do not exhibit reversal of the inhibitory effect of the toxin on the receptor. The persistence of lophotoxin blockade throughout the repetitive washes and substantial dilution encountered in these procedures suggests that the toxin binds with apparent irreversibility to nicotinic receptors.

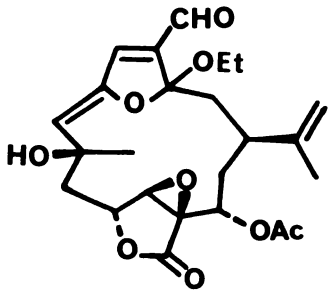
We are not yet able to deduce the absolute structural requirements for nicotinic receptor blockade by the diterpene lactones, in that the congeneric series currently available is incomplete. Nevertheless, data obtained for the analogs do suggest certain hypotheses regarding their reactivity with the receptor that are amenable to future experimental evaluation. Our interpretation of the profile of activity exhibited by the analogs is that blockade results through interaction of two critical domains of the diterpene lactone ligand with the receptor binding site: 1) an acetoxy group, which mimics that of acetylcholine in providing primary recognition between ligand and binding site, and 2) a chemically reactive moiety, capable of covalent bonding to a suitably reactive group on the receptor. Contrary to our initial expectations, the data suggest that the two epoxides common to all of these diterpenes might not be the sole determinants of irreversible blockade, since these groups are present on inactive analogs II and III. Rather, reactivity for the analogs may reside in the terminal isopropenyl region. For this region of active analog IV, one suspects an epoxide nucleophilic addition mechanism. Furthermore, this region of active analogs I and V possesses an α-β-unsaturated carbonyl which would be subject to attack by a receptor-associated nucleophile by way of Michael addition mechanism. This reaction would result in covalent bonding to the receptor:



A similar configuration exists in the furan ring of lophotoxin:



Our observation that ethanol extraction of *Lophogorgia* yields a mixture of lophotoxin and the ethanol adduct depicted below provides supporting evidence for the proposed reactivity of the furanoaldehyde moiety:



In evaluating the reactivity of lophotoxin and analog I, consideration should also be given to the possibility of receptor modification by a Schiff base reaction between the aldehyde moiety of these compounds and a receptor-associated primary amine. However, this mechanism would not explain the partial activity exhibited by analog IV or V.

It is puzzling that analog II was found to be inactive in all of the nicotinic receptor preparations utilized in this study, even though it possesses an isopropenyl aldehyde moiety identical to that of the active analog I. The inactivity of analog II might be related to the absence of an acetoxy moiety between the isopropenyl and furan groups. Apparently, an acetoxy group at this position may be critical for activity among the analogs, although this is not the case for lophotoxin.

We have demonstrated that lophotoxin blockade of [^{125}I]-α-toxin sites on nicotinic receptors is restricted to intact cells. One explanation for this finding would be that the lophotoxin-binding site is altered when the receptor is present in an environment other than its native state on an intact cell. This would imply that the critical point(s) of contact between lophotoxin and receptor is (are) not identical with that for the active analog I, since the latter compound was equally effective in intact and disrupted cells. We cannot completely exclude the possibility that the requirement of an intact cell for lophotoxin reactivity might reflect a necessity for metabolic activation of lophotoxin by the cell. Since quaternary agonists and antagonists protect against lophotoxin inactivation, the site of lophotoxin association should be on the extracellular surface of the receptor. Others have demonstrated oxidative opening of the furan ring for a variety of toxic methylfuran compounds (25). We have examined the possibility of cellular activation of lophotoxin by incubating BC3H-1 monolayers with the toxin, then transferring the incubation solutions to Triton X-100-solubilized receptor preparations. Such preconditioning treatments did not potentiate subsequent lophotoxin inhibition of detergent-extracted receptor (data not

shown). However, it is possible that the activated lophotoxin species, if it exists, might not attain sufficient concentration in the extracellular medium to allow its detection by our experimental format.

In addition to its occupation of surface nicotinic receptors of BC3H-1 cells, externally applied lophotoxin also occupies a population of Triton-extractable receptors that are not accessible to externally applied α-toxin. These "hidden" entities very likely represent a population of newly synthsized nicotinic receptors which have not yet been incorporated into the plasma membrane (23). Lophotoxin's capacity to permeate intact cells and thereby gain access to nonsurface receptors probably derives from its uncharged, lipophilic nature. Lophotoxin might therefore provide a uniquely suited ligand for the labeling of internal receptor pools in intact cells. By protecting surface receptors with saturating concentrations of reversible nicotinic agonists or antagonists during incubations with lophotoxin, selective labeling of intracellular receptor pools could be achieved, thus providing a novel approach to the study of nicotinic receptor biosynthesis and degradation. Our preliminary experiments have affirmed the feasibility of this approach.[3]

We plan to characterize the receptor binding site(s) for diterpene lactones in greater detail through the use of radiolabeled derivatives of these compounds. From the studies presented here, it will be necessary to utilize intact BC3H-1 cells for determinations of radiolabeled lophotoxin binding. For more detailed analyses which necessitate the greater receptor quantities provided by membrane fragment preparations from *Torpedo* electric organs, analog I could be developed as a radiolabeled probe of the binding site.

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[3] P. Culver, unpublished results.