MOLECULAR PHARMACOLOGY, 8, 786–789

Interactions of a Cobra Neurotoxin and Affinity Labels of the Acetylcholine Receptor in the Electroplax

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(Received September 15, 1972)

SUMMARY

The principal neurotoxin of *Naja naja siamensis* alters interactions with the reduced acetylcholine receptor in the electroplax of one inactivating and two activating affinity reagents. Prior application of this cobra toxin to the dithiothreitol-treated electroplax prevents the depolarization otherwise resulting from the reaction with the activating affinity reagents and blocks the reaction with the receptor of the inactivating affinity reagent. Subsequent application of the toxin reverses the depolarization following the reaction of one of the activating affinity reagents only. It has been previously found that reversibly acting competitive inhibitors such as *d*-tubocurarine reverse the depolarization following the reaction of either of these activating reagents; the depolarization recurs when the competitive inhibitor is removed by washing. It is inferred that the neurotoxin and all three affinity reagents have overlapping although not identical sites or modes of attachment to the receptor. All bind to the negative subsite of the acetylcholine-binding site but also have other subsites of attachment. *d*-Tubocurarine binds either to a site with a common subsite or to a wholly distinct but strongly interacting site.

Certain similar polypeptide neurotoxins present in the venom of elapid snakes have been shown to block completely and irreversibly the acetylcholine-induced depolarization of the postsynaptic membrane of vertebrate neuromuscular junctions and of homologous synapses in electric tissue of *Torpedo*, *Raja*, and *Electrophorus* (1–4). Protection of the response is afforded by cholinergic activators and inhibitors such as carbamylcholine and *d*-tubocurarine (1–3), suggesting that the toxins act at the receptor for acetylcholine. Such considerations have led to the use of these toxins as specific labels in attempts to localize (1, 5–7) and isolate this receptor (2, 4, 5, 7–10).

This work was supported by grants from the United States Public Health Service, the National Science Foundation, and The New York Heart Association, Inc.

In the electroplax of *Electrophorus* certain quaternary ammonium alkylating and acylating agents appear to react at the acetylcholine-binding site of the receptor following reduction of a disulfide group near the site with dithiothreitol (11, 12). Apparently, for each of these affinity reagents, a moiety is covalently bound by a reactive carbon atom to a sulfhydryl group at the periphery of the site while its quaternary ammonium group is noncovalently bound to the negative subsite within the site. In the case of two agents, bromoacetylcholine bromide and the *p*-nitrophenyl ester of (*p*-carboxyphenyl trimethylammonium iodide, the covalent attachment at the site of $(CH_3)_3N^+CH_2CH_2OCOCH_2$— (moiety I) and of $(CH_3)_3N^+C_6H_4CO$— (moiety II), respectively, results in strong activation of the receptor as evidenced by depolarization

of the electroplax (13). In the case of 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide attachment of the succinimido-α-benzyltrimethylammonium group does not cause depolarization but does prevent depolarization by subsequently added reversible activators such as carbamylcholine (11). Among the affinity agents tried, the extent of depolarization increases as the length of the covalently attached quaternary ammonium moiety decreases (11). Tritiated MBTA[1] has been used to label the reduced receptor in the electroplax (14), and solubilized labeled receptor has been separated from nonspecifically labeled components by gel electrophoresis (15). We report on the interaction in the electroplax of the binding of purified toxin 3 from *Naja naja siamensis* (16) (a gift of Drs. D. Cooper and E. Reich) with the reactions of these affinity agents.

The application of 1 μg/ml of toxin (0.125 μM) for 10 min to the innervated membrane of an isolated cell (electroplax) from the organ of Sachs of *E. electricus* results in approximately 90 % inhibition of the depolarizing response to 40 μM carbamylcholine (Fig. 1). The response is taken as the depolarization across the innervated membrane (ΔV_I). The inhibition is not diminished by washing for more than 1 hr. A similar effect of α-bungarotoxin on the electroplax has been reported (2). Prior or subsequent application of dithiothreitol has little effect on the inhibition by toxin 3, in agreement with work on frog muscle (3).

The application of 0.1 mM NPTMB subsequent to dithiothreitol results, as shown before (13), in depolarization of the electroplax which increases after excess, unreacted NPTMB is removed by washing (Fig. 2). Unreacted NPTMB is a reversible competitive inhibitor of the unreduced receptor. It appears also to compete with the covalently attached moiety II for binding to the negative subsite. Subsequent addition of toxin 3 has no effect on this depolarization. V_I does not change during 6 min of application of 1 μg/ml of toxin 3. A second application of 2 μg/ml of toxin 3 for 10 min is also without appreciable effect on V_I. This is in contrast to the effect of unreacted NPTMB, which is able to inhibit the response to the covalently attached activator both before and after toxin 3. Conversely, the addition of toxin 3 to the dithiothreitol-treated electroplax prior to NPTMB prevents activation by and presumably reaction with NPTMB (Fig. 3).

BAC, like acetylcholine, is a potent, reversible activator of the receptor (13). Added subsequently to dithiothreitol, BAC causes a large depolarization, most of which is not reversed by washing and undoubtedly is due to the covalent attachment of moiety I (13) (Fig. 4). In this case 1 μg/ml of toxin 3 added

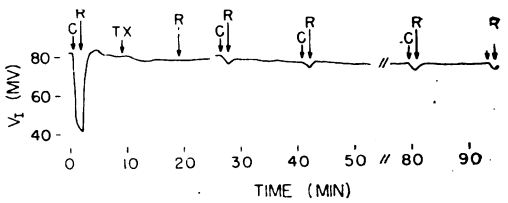

FIG. 1. *Responses of single cells (electroplax) dissected from the organ of Sachs of Electrophorus electricus to covalent and noncovalent cholinergic agents*

The potential difference across the cell from the solution bathing the noninnervated membrane to the solution bathing the innervated membrane (V_{N-I}) and the potential difference between the solution bathing the noninnervated membrane and an intra-cellular microelectrode (V_N) were recorded simultaneously (11). The potential difference across the innervated membrane (V_I) is the algebraic difference, $V_N - V_{N-I}$. The response of the electroplax to a depolarizing agent is taken to be ΔV_I. All solutions were continuously perfused past a portion of the innervated membrane exposed through a window in a supporting sheet of plastic (11). Solution changes were complete within seconds, and the concentration of agents in the continuously flowing solutions remained constant during application. All agents were dissolved in Ringer's solution (R) containing 165 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2.3 mM KCl, 1.2 mM K_2HPO_4, and 0.3 mM KH_2PO_4 (pH 7.1). In addition, Ringer's solution alone contained 10 mM glucose, which was omitted from the solutions containing reagents. C, 40 μM carbamylcholine chloride; TX, 1 μg/ml of toxin 3 (*Naja naja siamensis*).

[1] The abbreviations used are: MBTA, 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide; NPTMB, the *p*-nitrophenyl ester of (*p*-carboxyphenyl)trimethylammonium iodide; BAC, bromoacetylcholine bromide.

subsequently does cause a slow reversal of the depolarization. ΔV_I decreases approximately 50% in 20 min. *d*-Tubocurarine (0.1 mM) causes a more rapid reversal of the depolarization, which, however, returns after the *d*-tubocurarine is washed out (13). [Toxin 3 at 1 μg/ml added to 40 μM carbamylcholine causes a 50% decrease in the depolarization in 5 min (not shown).] In the reverse order, there is little irreversible ac-

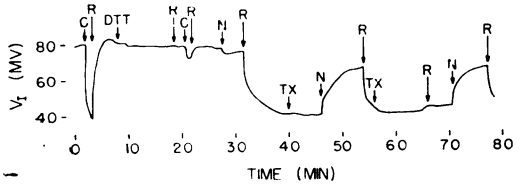

FIG. 2. *Responses of electroplax to covalent and noncovalent cholinergic agents*

The procedure was the same as in Fig. 1, except that dithiothreitol (DTT) was dissolved at 1 mM in Ringer's solution containing 5 mM KCl and with Tris-HCl (pH 8.0) replacing the phosphate buffer, and the concentration of toxin 3 was 2 μg/ml for the second application. N, 100 μM NPTMB.

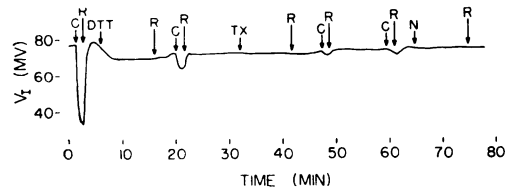

FIG. 3. *Responses of electroplax to covalent and noncovalent cholinergic agents*

Conditions were the same as described in Figs. 1 and 2. The concentration of toxin 3 was 1 μg/ml.

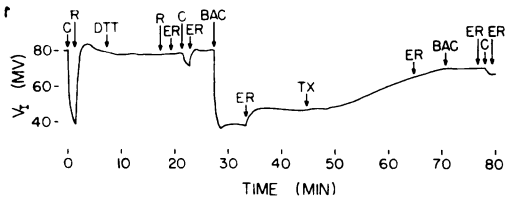

FIG. 4. *Responses of electroplax to covalent and noncovalent cholinergic agents*

Conditions were the same as described in Figs. 1 and 2. The concentration of toxin 3 was 1 μg/ml. ER, 50 μM eserine (to prevent hydrolysis of BAC by acetylcholinesterase); BAC, 20 μM BAC in eserine.

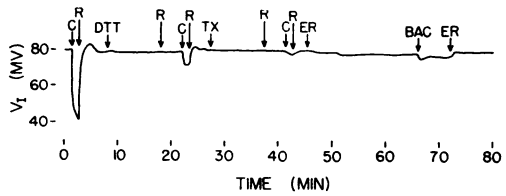

FIG. 5. *Responses of electroplax to covalent and noncovalent cholinergic agents*

Conditions were the same as described in Figs. 1, 2, and 4. The concentration of toxin 3 was 1 μg/ml.

tivation of the dithiothreitol-treated electroplax by BAC added subsequently to toxin 3 (Fig. 5).

Since both toxin 3 and MBTA inhibit the response to carbamylcholine irreversibly, their interaction cannot be monitored physiologically; however, a direct approach is possible. A fraction of the labeling of the dithiothreitol-treated electroplax by [^{3}H]-MBTA is specific for the receptor: 16% at 10 nM [^{3}H]MBTA added for 10 min (14). Furthermore, all of the specifically labeled component is found in one peak on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (15). Application of 1 μg/ml of toxin 3 for 10 min prior to the application of 10 nM [^{3}H]MBTA blocks on the average 8 ± 3% (n = 7) of the labeling of the electroplax. This effect of toxin 3 on the *total* labeling is variable; however, the electrophoretic peak containing labeled receptor is in all cases substantially diminished in extracts of toxin-treated electroplax (15). Toxin 3 also appears to cause an *increase* in the nonspecific labeling of some high molecular weight component(s) of the electroplax, which accounts for the observed variable decrease in total labeling (15).

The prior binding of toxin 3 appears to block the reaction with the reduced receptor of three affinity agents. The reaction of two of these agents (NPTMB and BAC) results in activation of the receptor, and conceivably toxin 3 could block this manifestation of their reactions, and of that of reversible cholinergic activators, by binding to some nonreceptor component involved in the mechanism of the permeability change. The block by toxin 3 of the reaction with the nonactivating [^{3}H]MBTA, measured directly,

suggests that the toxin in fact binds to the receptor at a site strongly coupled to or overlapping the site of reaction of these affinity reagents. A similar conclusion has been reached with regard to the relationship between the toxin-binding site and the site(s) binding reversible cholinergic activators and inhibitors (2).

Toxin 3 reverses the response due to the covalently attached moiety I but not II. Both responses are reversed, however, by 0.1 mM *d*-tubocurarine, so that the affinities of moieties I and II for the negative subsite are probably not markedly different. If toxin 3 were binding at a distinct but strongly coupled site, it would be expected to displace moiety II as well as I. The alternative explanation, that the toxin-binding site includes the acetylcholine-binding site, appears more likely. The guanidinium group of arginine at position 33 of the toxin is a probable candidate for the group binding to the postulated negative subsite of the receptor (17). Given the extremely low dissociation constant of the toxin-receptor complex, it seems likely that there would be a number of other specific side chain interactions, some of them outside the acetylcholine-binding site. It seems plausible that the toxin could accommodate itself to the presence of the flexible moiety I, rotated away from the negative subsite, but not to the presence of the more rigid moiety II, with its phenyl ring, even though moiety II apparently can also rotate away from the negative subsite.

It appears that reversible cholinergic activators such as acetylcholine and carbamylcholine, covalent affinity labels such as BAC, NPTMB, and MBTA (11, 12), and curariform neurotoxins such as toxin 3 all bind at sites on the receptor with a common intersection. Whether *d*-tubocurarine binds to an overlapping or to a distinct but strongly coupled site remains an open question. *d*-Tubocurarine, being considerably smaller than toxin 3 (molecular weights 625 and 8000, respectively), might reasonably be expected to accommodate itself more readily to the presence at the site (but rotated away from the negative subsite) of moieties I and II, so that an overlapping site is not excluded by these results. An allosteric mechanism of inhibition by *d*-tubocurarine, in which *d*-tubocurarine binding at a distinct site suppresses all binding at the acetylcholine site, including that of toxin 3, is also consistent with the data. More than two conformational states would have to be involved in the allosteric mechanism, however, since *d*-tubocurarine and the toxin would have to be bound to two different *inactive* states of the receptor. In the absence of evidence to the contrary, it is simplest to assume for the present that all cholinergic agents—activators and inhibitors, reversible and irreversible—have overlapping sites of action.

ACKNOWLEDGMENTS

We thank Drs. D. Cooper and E. Reich for their gift of purified toxin, and Mrs. C. Silaghy for her expert technical assistance.

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