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OF NICOTINIC ACETYLCHOLINE RECEPTOR FROM TORPEDO CALIFORNICA ELECTROPLAX

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EFFECTS OF THIOL MODIFICATION AND CA++ ON AGONIST-SPECIFIC STATE TRANSITIONS OF NICOTINIC ACETYLCHOLINE RECEPTOR FROM TORPEDO CALIFORNICA ELECTROPLAX

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Summary

The agonist binding affinity of nicotinic acetylcholine receptor (nAChR) from Torpedo californica electroplax, as inferred from ability of agonist to inhibit specific curaremimetic neurotoxin binding to nAChR, is sensitive to the duration of exposure to agonist. The concentration of carbachol necessary to prevent one-half of toxin binding over a 30 min incubation with nAChR (K30) is 10 μM when toxin and carbachol are simultaneously added to membrane-bound nAChR, and 3 μM when nAChR are pretreated with carbachol for 30 min prior to the addition of toxin. These alterations in agonist affinity may be mimicked by modification of nAChR thiol groups. Affinity of nAChR for carbachol is decreased following treatment with dithiothreitol (DTT). Dithio-bis-nitrobenzoic acid treatment of DTT-reduced membranes yields K30 values of 5 μM for carbachol, while N-ethylmaleimide treatment of DTT-reduced nAChR produces nAChR with reduced affinity for carbachol, reflected in K30 values of about 400 μM. In the absence of Ca++, K30 values for carbachol binding to native and DTT-reduced nAChR are diminished 3-6 fold. These affinity alterations are not observed with d-tubocurarine (antagonist) binding to nAChR. Thus, Ca++ and the oxidation state of nAChR thiols appear to affect the affinity of nAChR for agonists (but not antagonists), and may therefore be related to agonist-mediated events in receptor activation and/or desensitization.

The study of acetylcholine receptor-ligand interactions has been facilitated by the use of neurotoxins that bind to those receptors with high affinity. Recent reports have appeared describing agonist-specific changes in affinity of nicotinic acetylcholine receptor (nAChR) for cholinergic agonists, as detected by inhibition of curaremimetic neurotoxin binding to peripheral (1-6) and central (7,8) nAChR. These reports suggest that nAChR selectively and actively responds to agonists, and that the observed affinity changes correlate with the different physiologically relevant states of the receptor. In all of these studies, the low-affinity state of the receptor was found to exist transiently; application of cholinergic agonists to nAChR causes a time-dependent transition to a high affinity state toward agonist binding.

Chemical modification of nAChR and its consequences on the physical properties of the receptor have contributed toward the elucidation of the molecular mechanisms underlying receptor function. The involvement of thiol groups was established when it was found that reduction of Electrophorus electricus electroplax with dithiothreitol (DTT) inactivated the physiological re-
response to acetylcholine (ACh) (9). Furthermore, application of N-ethylmaleimide (NEM) to DTT-reduced electroplax rendered this inactivation irreversible, but 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) treatment following DTT fully restored the response to ACh. There is also evidence that Ca++ may participate in the natural deactivation and desensitization of nAChR (10) and that Ca++ interacts with nAChR (11). Recent studies in this laboratory have revealed that chemical modification of disulfide groups and the presence of Ca++ have a significant effect on competition potency of various cholinergic drugs toward α-bungarotoxin binding to membrane-bound nAChR from rat brain subcellular fractions (12).

In this report we describe the effects of thiol modification and Ca++ on cholinergic drug competition effectiveness toward [125-I]-dendrotoxin binding to membranes prepared from Torpedo californica electroplax. The utility of dendrotoxin 4.7.3, an α-toxin from the venom of Dendroaspis viridis (13), as a cholinergic probe has been demonstrated (14). The toxin apparently binds to the same number of sites on Torpedo nAChR as α-bungarotoxin, and the two toxins exhibit virtually identical pharmacological properties. The data presented here show that agonist-specific transitions of Torpedo nAChR are affected by Ca++ and thiol group oxidation-reduction state, and that appropriate chemical modification of sulfhydryl groups can fix nAChR in non-transitory, high or low affinity states.

Materials

Dendrotoxin 4.7.3 was a generous gift of Dr. R. A. Shipolini. Bungarus multicinctus crude venom was obtained from Miami Serpentarium and α-bungarotoxin was purified as described previously (15). Liquid nitrogen-frozen Torpedo california electric tissue was obtained from Pacific Bio-Marine and stored at -70°C until use.

All homogenizations and binding assays were performed in Ringer's medium containing 115 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1.3 mM MgSO₄, 33 mM Tris (pH 7.4), 0.02% NaN₃, and 0.1% phenylmethylsulfonyl fluoride (full Ringer's medium), or with 1.8 mM MgCl₂ and 4 mM ethylene glycol-bis-(β-aminoethyl ether) N,N-tetraacetic acid substituted for CaCl₂ (Ca++-free medium).

Methods

Dendrotoxin was radioactively labeled with 125-I as described previously (14,16). Torpedo californica nAChR-rich membranes were prepared by the technique of Hazelbauer and Changeux (17), and resuspended in the appropriate buffer.

Thiol-group modification. Membrane preparations at ~2 mg/ml were treated with 0.3 mM DTT for 20 min. DTT-treated membranes were then treated with either 0.3 mM DTNB for 20 min, or with 0.3 mM NEM for 5 min followed by DTNB. In all cases, the thiol reagent treatment was terminated by dilution in the appropriate Ringer's medium followed by centrifugation at 36,000 x G for 20 min. Thus, excess reagent was removed prior to subsequent chemical modification or preparation of samples for binding assays.

Binding assays. The binding of radiolabeled toxin to Torpedo membranes was measured by a microfuge assay. Fifty μl of membrane suspension (10 μg membrane protein/ml) were added to polypropylene microfuge tubes containing Ringer's solution and cholinergic drug for preincubation samples and incubated for 30 min at 21°C prior to addition of radiolabeled toxin. For coincubation samples, membrane suspension was added to tubes containing Ringer solution,
cholinergic drug, and radiolabeled toxin. All samples were then incubated for 30 min at 21°C in a total volume of 400 μl. The reaction was terminated by centrifugation at 4°C for 6 min in a Beckman 152 microfuge, followed by aspiration of the supernatant and resuspension of the pellet in 200 μl of Ringer, recentrifugation, and aspiration to a dry pellet. The microfuge tubes were placed directly in γ-well tubes and counted in a Nuclear Chicago γ-ray counter (41% efficiency). Specifically bound toxin was measured as the difference between the total amount of radiolabeled toxin bound, and blank samples treated with 0.2 μM α-bungarotoxin for 30 min prior to the addition of [125-I]-dendrotoxin and/or cholinergic ligand. (This specific binding is typically 80-95% of total binding). A detailed theoretical treatment of the effects of ligand on toxin binding to nAChR, which demonstrates the validity and usefulness of this experimental paradigm to yield information on relative affinities of nAChR for ligand,is to appear elsewhere (12).

Results and Discussion

Levels of [125-I]-dendrotoxin specifically bound to nAChR after 30 min incubation decrease as the concentration of cholinergic ligand in the assay medium increases. The concentration of ligand that blocks 50% of specific toxin binding (K30) is an indication of the relative affinity of nAChR for ligand. K30 values for carbachol vary with duration of exposure of carbachol to nAChR, thiol modification of nAChR, and the concentration of Ca++ in the assay medium.

In Ca++-containing full Ringer's medium, the K30 value for carbachol is 10 μM when carbachol and toxin are simultaneously exposed to nAChR (coincubation), and 3 μM when membranes are pretreated with carbachol for 30 min (preincubation) prior to the addition of toxin (Fig. 1 and Table I). Thus, there is a time-dependent increase in apparent affinity of nAChR for carbachol on exposure to carbachol. K30 on preincubation represents an estimate of the dissociation constant for carbachol binding to nAChR in a high-affinity state. K30 on coincubation provides a lower limit for the true, instantaneous coincubation dissociation constant for carbachol binding to the low-affinity state, as it reflects a constantly changing ligand affinity (or rate of toxin binding) over the course of the assay. These time-dependent changes in nAChR state are observed only for cholinergic agonists, as K30 for d-tubocurarine, a nicotinic cholinergic antagonist, is 10 μM independent of duration of exposure to nAChR (Table I). These results are consistent with those reported in previous investigations based on time-dependent increase in agonist-mediated inhibition of the initial rate of curaremimetic neurotoxin binding to nAChR on preexposure to agonist (1-6).

Thiol modification of Torpedo membrane proteins in the presence of Ca++ produces a significant effect on the characteristics of carbachol interaction with nAChR (Table I). Exposure of membranes to DTT, which cleaves disulfide bonds and reduces sulfhydryls, followed by treatment with DTNB, which favors oxidation of sulfhydryls and presumably promotes reformation of disulfide bonds, yields nAChR with K30 value for carbachol of 5 μM for both coincubation and preincubation assays. In contrast, NEM treatment of DTT-reduced membranes, where sulfhydryl groups are irreversibly alkylated, greatly reduces the ability of carbachol to compete for toxin binding; K30 values are about 400 μM for both pre- and coincubated samples. DTT treatment alone appears to have little effect on the affinity for carbachol when reduced nAChR is preincubated with carbachol, but K30 values are about 30 μM on coincubation with DTT-treated nAChR. That is, K30 values are about 3-fold higher than for similar coincubation assays with native nAChR. None of these thiol group-mediated state changes affect toxin binding competition by d-tubocurarine. The K30 value for d-tubo-
Cholinergic ligand competition toward specific toxin binding. Percent of specific \(^{125}\text{I}\)-dendrotoxin sites occupied by toxin is plotted against the molar concentration (logarithmic scale) of drug. Carbachol competition (upper panel): Preincubation assay in full Ringer's medium with native membranes (○); preincubation assay in Ca\(^{++}\)-free medium with native membranes (●); preincubation assay in Ca\(^{++}\)-free medium with DTT-NEM-treated membranes (▲); coincubation assay in Ca\(^{++}\)-free medium with DTT-DTNB-treated membrane (●). \(\alpha\)-Tubocurarine competition (lower panel): Coincubation assay in Ca\(^{++}\)-free medium with native membrane (○); preincubation assay in Ca\(^{++}\)-free medium with DTT-NEM-treated membranes (▲); coincubation assay in Ca\(^{++}\)-free medium using DTT-DTNB-treated membranes (●); preincubation assay in full Ringer's medium using DTT-treated membranes (○).

curarine is 10 μM, and remains essentially the same for preincubation or coincubation assay, whether native nAChR, or DTT-, DTT-NEM-, or DTT-DTNB-treated membranes are used (Table I and Fig. 1). These results indicate that
TABLE I

K₃₀ Values (µM) for Competition vs. 8nM [¹²⁵-I]-Dendrotoxin

<table>
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<th>Drug</th>
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<td>CF</td>
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K₃₀ values are determined from inspection of data as exemplified in Fig. 1. Data are for experiments conducted in Ca²⁺-containing, full Ringer's medium (FR), or in Ca²⁺-free medium (CF). Preincubation samples are exposed to cholinergic ligand for 30 min prior to addition of [¹²⁵-I]-dendrotoxin. Coincubation samples are exposed to ligand and toxin simultaneously. nAChR are untreated (native), treated with OTT only (OTT), or with OTT-DTNB (DTNB), or OTT-NEM (NEM).

A transient, low-affinity state (toward agonist) is preserved by DTT-reduction of nAChR. The rate of transformation to the high-affinity state is apparently retarded, relative to native nAChR, for DTT-treated receptor. As is the case for coincubation K₃₀ values for carbachol binding to native nAChR, K₃₀ values for coincubation in the presence of DTT-reduced membranes reflect a constantly changing affinity of receptor for ligand. Thus, these K₃₀ values are a lower limit for the true, instantaneous dissociation constant for carbachol binding to nAChR in a low-affinity state. The true dissociation constant is more closely approximated by K₃₀ values for carbachol binding to DTT-NEM-treated nAChR. NEM alkylation of DTT-reduced nAChR produces an irreversibly stable low-affinity state. Effects of DTT on receptor affinity for carbachol are reversed by oxidation with DTNB, leaving nAChR in a high-affinity state toward agonist. Thiol group modification by DTT-DTNB or DTT-NEM abolishes preincubation responsiveness to agonist.

Similar effects of thiol group-directed reagents on affinity of nAChR for agonist have been measured directly (18,19). The present results illustrate agonist specificity of these receptor state changes, and the usefulness of toxin binding inhibition assays toward their characterization (see also 12). Modification of receptor thiol groups has been shown to affect in vivo (9,20-23) and in vitro (19,24) responses of chemically excitable nAChR to cholinergic agonist. Time-dependent changes in receptor state on exposure to agonist, as measured by toxin binding assays, have been related to physiologically important changes in receptor state (1-6). The parallel nature of thiol reagent effects on biochemically measurable receptor affinity for agonist and on agonist-specific physiological responses of receptor further suggests possible participation of specific receptor sulfhydryls/disulfides in agonist-mediated activation and desensitization of nAChR.

Physiological concentrations of Ca²⁺ in assay media are found to specifically affect affinity of receptor for carbachol (Table I and Fig. 1). K₃₀ values for carbachol inhibition of toxin binding to native nAChR are increased about 3-fold in Ca²⁺-free medium, relative to control experiments in full Ringer's medium, for both co- and preincubation assay paradigms. For both
pre- and coincubation conditions, K30 values for carbachol binding to DTT-reduced nAChR are also higher in Ca++-free medium. Nevertheless, even in Ca++ free medium, both native and reduced nAChR show sensitivity to pretreatment with agonist. Ca++ concentration has little effect on K30 values for carbachol binding to DTT-DTNB or DTT-NEM treated nAChR, and, in each case, response of nAChR to preincubation with agonist is not observed (Table I). Moreover, d-tubocurarine binding remains largely unaffected by the presence or absence of Ca++ in the assay medium (Table I and Fig. 1). Thus, effects of Ca++ are specific for agonist binding to native or reduced nAChR.

The effect of Ca++ on agonist binding to nAChR has been recognized in peripheral (25,26) and central (12) nervous systems. In each case, the affinity of nAChR for agonist is increased with increasing concentration of Ca++. Again, there is parallel physiological evidence that Ca++ may accelerate, if not induce, nAChR desensitization in vivo (27-30) and in vitro, (31), which has led to specific hypotheses (32) regarding its possible mechanism of action. The data presented here support the contention that Ca++ increases the affinity of agonist-responsive nAChR for agonist (but not for antagonist) and are consistent with postulated roles of Ca++ in receptor desensitization.

The exact relationship between the observed affinity alterations, as detected in this study, and the physiologically identifiable states of resting, activated and desensitized receptor remain unclear. Nevertheless, this study provides insight into possible molecular mechanisms of agonist-mediated nAChR activity, and offers promising experimental approaches toward ultimate elucidation of those mechanisms.

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References
