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BINDING OF DEPOLARIZING DRUGS TO THE IONIC CHANNEL SITES OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

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The electric organs of fish, derived embryonically from myoblasts, are innervated by cholinergic motor neurons, and have the highest concentrations of nicotinic acetylcholine (ACh) receptors with similar pharmacology to those of motor endplates (1,2). Thus, they offer the biochemist a unique opportunity to study the molecular and pharmacological properties of these receptors (3). Membrane preparations from the electric organs of the electric ray Torpedo sp. and the electric eel Electrophorus electricus have been used to study the kinetics of drug-induced receptor changes such as measurements of fluxes of radioactive ions (4), changes in fluorescence of drug-receptor complexes (5), and changes in the rate of binding of α-bungarotoxin (α-BGT) (6). The finding that this receptor/channel molecule had two different kinds of sites--"receptor sites" that bound ACh and α-BGT in a voltage- and almost temperature-independent manner and "channel sites" that bound perhydrohistrionicotoxin (H_{12}-HTX) and phencyclidine in a voltage- and temperature-dependent manner (7,8)--led to the study of the allosteric interactions between these sites (9). Receptor agonists were found to increase dramatically the initial rate of binding of [^{3}H]H_{12}-HTX to the channel sites, due partially to increased affinity, but mostly to increased accessibility of the sites for the drug (9). It was also possible to block irreversibly the receptor sites with α-BGT for the duration of the experiment and study directly the interaction of an antagonist such as d-tubocurarine with the channel sites without interference from the receptor sites (10). The study of kinetics of binding of the channel drug in the absence and presence of receptor agonists and antagonists allowed the detection of four receptor conformations: resting, active, desensitized and antagonist induced.

Both succinylcholine and decamethonium are presented in pharmacology texts as depolarizing blockers and though not stated clearly, it is implied that the mechanism of postsynaptic blockade by the two drugs is the same. Recent investigation of the effects of decamethonium using miniature endplate currents, membrane noise, and voltage jump techniques suggested that decamethonium inhibited the initial depolarization by blocking the receptor-regulated ionic channel (11). It was also found to open and block the endplate ACh-receptor channels, acting like an agonist at low concentrations by stimulating binding of [^{3}H]H_{12}-HTX to the channel sites and at higher concentrations like a channel blocker by inhibiting the stimulation of [^{3}H]H_{12}-HTX binding (12). Therefore, the present study was initiated to probe further into, and compare, the molecular actions of different agonists of the nicotinic ACh-receptor using the same biochemical approaches.

MATERIALS AND METHODS

Tissue preparation. Membranes were prepared from Torpedo ocellata electric organ stored at -90° as described (12). The tissue was homogenized (1 g/ml) in 10 mM Tris-HCl, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid, 0.1 mM diisopropylfluorophosphate, 0.1 mM phenylmethylsulfonyl fluoride, and 0.02% NaN_3. The homogenate was centrifuged at 3000 g for 10 min, and the pellets were rehomogenized and centrifuged as before. The supernatant fractions of these two centrifugations were combined and centrifuged at 30,000 g for 60 min, and the pellets were reconstituted in 10 mM Tris-HCl, pH 7.4, 0.1 mM diisopropylfluorophosphate, and 0.02% NaN_3 (1 ml/g original tissue). Final protein concentration averaged about 4 mg protein/ml.

Binding assay. Specific binding of [^{3}H]H_{12}-HTX to the channel sites was determined by a filter assay as described (12). Torpedo membranes (50 μl) were added to a test tube containing 2 nM [^{3}H]H_{12}-HTX (sp. act. 21 Ci/mmole (8)) in 50 mM Tris-HCl, pH 7.4, in the absence or presence of drugs under study, in a final volume of 1 ml. After incubation at 23° for 30 sec, the mixture was filtered over Whatman GF/B glass fiber filters, which had been immersed in 1% Prosil-28 (PCR Research Chemicals, Gainesville, Fla.) to reduce nonspecific binding. Each filter was washed with 7-8 ml of ice-cold 50 mM Tris-HCl, pH 7.4, and placed in 5 ml of toluene-based scintillation solution, and its radioactivity was counted after 8 hr. The binding of [^{3}H]H_{12}-HTX not inhibited by 5 mM amantadine, a competitive inhibitor of the specific binding (13), was considered as nonspecific. To eliminate any contributions from receptor

sites in certain experiments, Torpedo membranes were preincubated with α-BGT (10 μM) for 1 hr prior to their use for [^{3}H]H_{12}-HTX binding to the channel sites.

RESULTS

As shown earlier (12), the actions of the depolarizing blockers, decamethonium and succinylcholine, at 10^{-7} - 10^{-5} M, were similar to that of the full agonist carbamylcholine, i.e. stimulating the binding of [^{3}H]H_{12}-HTX in a dose-dependent manner. Although the stimulation by decamethonium was shown to be reduced above 10^{-5} M (12), this continued with increasing decamethonium concentrations such that at 10^{-2} M, binding of [^{3}H]H_{12}-HTX was reduced to below control level (i.e. binding in absence of drugs) (Fig. 1). This suggests that at high concentrations decamethonium may be inhibiting [^{3}H]H_{12}-HTX binding directly. By contrast, only a small reduction in the stimulation of [^{3}H]H_{12}-HTX binding was observed with 10^{-2} M succinylcholine (Fig. 1). We had found that preincubation of Torpedo membranes with carbamylcholine reduced its stimulation of [^{3}H]H_{12}-HTX binding in a concentration- and time-dependent manner, and suggested that it was most likely due to receptor desensitization (12). Larger reductions were induced by preincubation with succinylcholine and decamethonium (open symbols, Fig. 1). At 1 μM, succinylcholine was the most potent in producing the

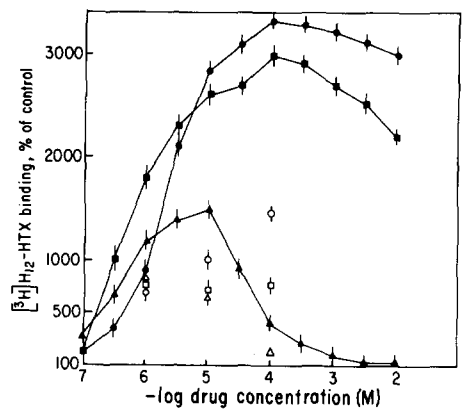

Fig. 1. Effect of carbamylcholine (●,○), succinylcholine (■,□) and decamethonium (▲,△) on the binding of [^{3}H]H_{12}-HTX to the channel sites of the ACh-receptor in Torpedo membranes. Solid symbols represent the simultaneous addition of agonist and tissue to 2 nM [^{3}H]H_{12}-HTX in 50 mM Tris buffer and then incubation for 30 sec; open symbols represent tissue preincubated for 1 min with the indicated concentration of the agonist, then 2 nM [^{3}H]H_{12}-HTX was added, and the mixture was filtered after 30 sec. Symbols and bars are means and standard deviations of triplicate experiments.

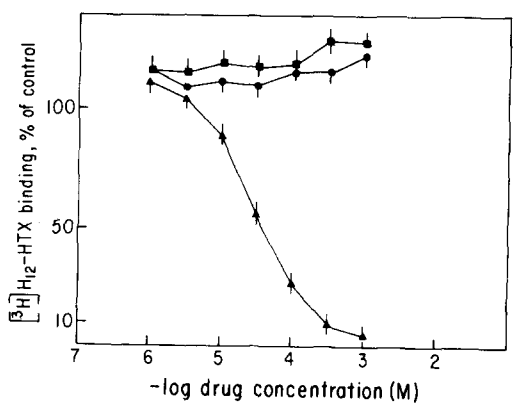

Fig. 2. Effect of carbamylcholine (●), succinylcholine (■) and decamethonium (▲) on the binding of [^{3}H]H_{12}-HTX to the channel sites in Torpedo membranes that were preincubated for 60 min with 10 μM α-BGT. The membranes, drugs and [^{3}H]H_{12}-HTX (2 nM) in 50 mM Tris buffer were incubated for 2 hr before filtration. Symbols and bars are means and standard deviations of triplicate experiments.

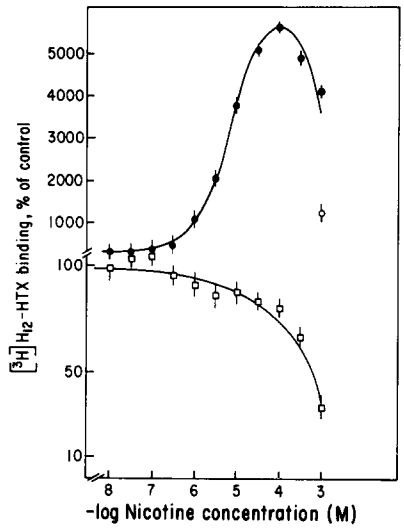

Fig. 3. Effect of nicotine on the binding of [^{3}H]H_{12}-HTX (2 nM) to Torpedo membranes, untreated (●) or preincubated for 60 min with 10 μM α-BGT (□). Incubation time with [^{3}H]-H_{12}-HTX and nicotine was 30 sec for the untreated and 2 hr for the α-BGT-treated membranes. Binding to untreated Torpedo membranes that had been preincubated with 1 mM nicotine for 1 min prior to addition of [^{3}H]H_{12}-HTX (o) and measurement of binding after 30 sec. Symbols and bars are means and standard deviations of triplicate experiments.

"desensitizing" effect, reducing maximum [^{3}H]H_{12}-HTX binding by 58% followed by decamethonium (30%) and carbamylcholine (23%) with 1-min preincubations. Stronger reductions were obtained by 100 μM of these drugs, 76, 65 and 56% respectively.

We had found that the kinetics of binding of [^{3}H]H_{12}-HTX were affected little by the presence of α-BGT at saturating concentrations which blocked all receptor sites and their allosteric effects on [^{3}H]H_{12}-HTX binding (12). Preincubation of Torpedo membranes with 10 μM α-BGT for 60 min eliminated the dramatic stimulation of [^{3}H]H_{12}-HTX binding induced by the three agonists (Fig. 2). Since binding of [^{3}H]H_{12}-HTX to the channel sites in presence of agonist could reach equilibrium in seconds yet in absence of agonist, or when receptor sites were occupied with α-BGT, reached equilibrium in about 2 hr (12), binding of [^{3}H]H_{12}-HTX to the α-BGT-treated membranes was measured after a 2-hr incubation, compared to 30 sec for the untreated membranes. Long incubation of the α-BGT-treated membranes with agonist did not cause desensitization because the receptor sites were occupied by α-BGT. Carbamylcholine and succinylcholine at concentrations of 1-1000 μM did not inhibit [^{3}H]H_{12}-HTX binding to the α-BGT-treated membranes, but decamethonium inhibited it in a dose-dependent manner with a K_i of 50 μM.

Nicotine produced effects on [^{3}H]H_{12}-HTX binding that were intermediate between those of succinylcholine and decamethonium (Fig. 3). At lower concentrations (<100 μM), nicotine behaved like an agonist, stimulating the binding of [^{3}H]H_{12}-HTX to untreated Torpedo membranes. At higher concentrations, however, there was a sharp reversal in effect that suggested that nicotine might be inhibiting [^{3}H]H_{12}-HTX binding directly. To distinguish this from the time-dependent desensitization-like effect, we studied the effect of nicotine, at the same range of concentrations, on the binding of [^{3}H]H_{12}-HTX to α-BGT-treated membranes and found that nicotine up to 1 μM had no significant effect, but at higher concentrations displaced [^{3}H]-H_{12}-HTX binding with a K_i of 650 μM, suggesting a direct interaction with the ionic channel sites. Like the other agonists, preincubation of the membranes for 1 min with 1, 10 and 100 μM nicotine caused a reduction in stimulated [^{3}H]H_{12}-HTX binding by 22, 59 and 72% respectively.

DISCUSSION

The results of this study suggest that the interactions of decamethonium and succinylcholine with the nicotinic ACh-receptor are quite different. Decamethonium interacts with the receptor sites as shown by its activation of [^{3}H]H_{12}-HTX binding (Fig. 1), and with the ionic channel sites as evidenced by its inhibition of [^{3}H]H_{12}-HTX binding to the α-BGT-treated Torpedo receptors (Fig. 2). However, the other depolarizing blocker, succinylcholine, binds only to the receptor sites, causing stimulation of the binding of [^{3}H]H_{12}-HTX to the channel sites (Fig. 1), and fails to inhibit [^{3}H]H_{12}-HTX binding to the α-BGT-treated Torpedo receptors (Fig. 2). This suggests that the mechanism of blockade of postsynaptic depolarization by succinylcholine is not through interaction with the channel sites. It is possible that the blockade by succinylcholine of neuromuscular transmission may be due to its promotion of receptor desensitization, since preincubation of Torpedo receptor with it results in

reduced stimulation of [^{3}H]H_{12}-HTX binding even more so than preincubation with carbamylcholine or decamethonium (Fig. 1).

The stimulation of [^{3}H]H_{12}-HTX binding to the ionic channel sites by nicotine (Fig. 3) supports its agonistic activity on motor endplates. Its inhibition of neuromuscular transmission at higher concentrations may result from desensitization as well as its blockade of the ionic channel, which is suggested from the data on its inhibition of [^{3}H]H_{12}-HTX binding to the α-BGT-treated Torpedo membranes (Fig. 3). Thus, nicotine interacts with the ACh-receptor/channel molecule like decamethonium, through binding to the receptor as well as the channel sites, though its affinity for the latter sites is 13-fold lower than that of decamethonium.

Agonist-induced receptor desensitization and drug-induced channel blockade have some similarities in their effects on neuromuscular transmission (14) and result in reduced [^{3}H]-H_{12}-HTX binding to the channel sites (7,12). However, the two phenomena may be distinguishable biochemically. Desensitization by an agonist is caused by binding to the receptor sites and is detectable by the time-dependent reduction in [^{3}H]H_{12}-HTX binding to the channel sites, while the receptor sites are uninhibited. Channel blockade by a drug is detectable by the reduction in [^{3}H]H_{12}-HTX binding, while the receptor sites are inhibited by α-BGT. If this is true, then receptor desensitization and channel blockade result from drug occupation of different sites in the receptor/channel molecule, though the two induced conformations may be similar. It is not known if the metaphilic effect produced by decamethonium derivatives on muscle endplates (15) is due, in part, to channel blockade.

In summary, it is suggested that nicotine and decamethonium interact with both the receptor and channel sites of the nicotinic ACh-receptor, though with lower affinity for the latter sites, while succinylcholine, like ACh and carbamylcholine (12), interacts only with the former sites. Also, it is suggested that the depolarizing blockade of muscle endplates produced by decamethonium produces depolarizing blockade by a mechanism that is different from that of succinylcholine.

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