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Modulation of Phencyclidine-Sensitive Ethidium Binding to the *Torpedo* Acetylcholine Receptor: Interaction of Noncompetitive Inhibitors with Carbamylcholine and Cobra α-Toxin

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SUMMARY

Ethidium is one of two fluorescent ligands known to bind to the noncompetitive inhibitor (NCI) site in the central ion channel of the *Torpedo* acetylcholine receptor with a micromolar dissociation constant. To further characterize heterotropic allosteric regulation of ligand binding in general, and of ethidium binding in particular, to the *Torpedo* receptor, we measured the effects of three liquid anesthetics (diethyl ether, halothane, and butanol), two barbiturates (secobarbital and thiamylal), and urethane. The phencyclidine-sensitive chromatic shift and the quantum yield increase associated with ethidium binding to the channel NCI site were used as indicators of ethidium binding. In the absence of other ligands, halothane, diethyl ether, and butanol increased the affinity of ethidium toward the channel NCI site to the same extent as carbamylcholine (400–600-fold), whereas the barbiturates and urethane were without effect. Cobra α-toxin blocked anesthetic-induced ethidium binding, confirming that cobra α-toxin stabilizes the AcChR in the resting-like state. In the presence of carbamylcholine, when ethidium was bound to the channel NCI site, several ligand-dependent effects were observed. 1) Without affecting further the affinity of ethidium for the NCI binding site, diethyl ether and halothane increased and butanol had no effect on the fluorescence emission of channel-bound ethidium. This indicated that there is little relation between the affinity and the quantum yield of the channel-bound ethidium. 2) Addition of secobarbital and thiamylal had no effect, beyond the effect of carbamylcholine, on ethidium binding to the channel NCI site, indicating that the barbiturates did not bind to the channel NCI site. 3) Urethane inhibited carbamylcholine-induced ethidium binding to the channel NCI binding site, suggesting direct interaction of urethane with the channel NCI binding site, at least when the receptor is in a desensitized state. The results confirm the conformational sensitivity of ethidium binding to the channel NCI binding site and demonstrate at least three different modes of action of anesthetics to inhibit the *Torpedo* receptor noncompetitively.

The muscle-type nicotinic AcChR is a pentameric ligand-gated cation channel that is composed of four types of subunits, designated α, β, γ, and δ, in the stoichiometric ratio of 2:1:1:1. Each subunit is thought to snake back and forth through the membrane at least four times. Together, the subunits are arranged like barrel staves around the central ion channel (1). Ligands interact with at least three classes of binding sites on the AcChR. Agonists, like carbamylcholine, and competitive antagonists, like snake venom α-toxins, bind to two sites that are located, in part, on the α subunits (2). A second class of binding sites are located in the transmembrane domain of the AcChR, in or near the central ion channel, and are variously designated the allosteric, local anesthetic, high affinity, or channel NCI binding sites (2). PCP and histrionicotoxin bind to this channel NCI binding site (3, 4). A diverse group of hydrophobic agents, including many general anesthetics, interact with a third class of binding sites, designated the low affinity NCI binding sites, at 10–20 undetermined sites on the AcChR (2, 3).

The AcChR exists in at least four interconvertible states, resting, open-channel, fast-desensitized, and slow-desensitized (5, 6). Ligands transiently induce and/or stabilize one or more of these states. Understanding how the various classes of ligands interact with each other to alter the state of the AcChR is a major focus of current research. Much is known about the effects of ligand occupation of one class of binding sites on ligand binding to another class of binding site(s). For example, the channel NCIs, which display greater affinity for the slow-desensitized state, enhance the rate of conversion of the AcChR from a resting to slow-desensitized state (3, 4, 7). Reciprocally, agonists increase the affinity of this same group of channel NCIs toward the AcChR (4, 8). Low affinity NCIs increase the binding affinity of both agonists and channel NCIs, presumably

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ABBREVIATIONS: AcChR, acetylcholine receptor; PCP, phencyclidine; cobra α-toxin, cobra α-toxin (II); NCI, noncompetitive inhibitor; dansyltrimethylamine, dimethylaminonaphthalene-5-sulfonamidoethyltrimethylammonium.

by inducing the slow-desensitized state, which is associated with higher affinity for the agonists and channel NCIs (4, 7, 9–12). Much less is known about the effects of concurrent occupation of two classes of binding sites on ligand binding to the third class of sites.

Because of the diversity and availability of fluorescent probes for the agonist/competitive antagonist binding sites, fluorescence spectrometric techniques have elucidated structural and functional features of the receptor, as well as offering a less expensive alternative to radionuclides for receptor quantification (8, 13–15). Currently, only two relatively high affinity fluorescent probes of the channel NCI binding site have been identified, ethidium (8) and quinacrine (16). Pharmacological and spectrofluorometric characterization of these channel NCI probes is incomplete. For example, although agonist occupation of the acetylcholine binding sites produces a dramatic increase in the affinity of ethidium toward the channel NCI binding site, the effects of ligand occupation of the low affinity NCI binding sites on ethidium binding are unknown. To further characterize ligand regulation of ethidium binding to the AcChR and to gain a greater understanding of the heterotropic allosteric regulation of ligand binding to the AcChR in general, we examined the effect of carbamylcholine, cobra α-toxin, and several classes of NCIs on ethidium binding to the channel NCI binding site. The PCP-sensitive chromatic shift and the quantum yield increase associated with ethidium binding to the channel NCI site were used as indicators of ethidium binding. Viewed from the NCI binding site, we found that anesthetics can inhibit the receptor by at least three mechanisms, 1) induction of a desensitized state by ligand binding to the low affinity NCI sites, 2) stabilization of the resting state by ligand binding to the low affinity NCI sites or unique barbiturate site(s), and 3) direct anesthetic binding to the channel NCI site.

Experimental Procedures

Materials. Ethidium bromide was purchased from Calbiochem (La Jolla, CA). Carbamylcholine, PCP, suberyldicholine, urethane, and secobarbital sodium were obtained from Sigma (St. Louis, MO) and halothane from Halocarbon Laboratories (Hackensack, NJ). Diethyl ether and butanol were acquired from Aldrich (St. Louis, MO) and dansyl-trimethylamine perchlorate from Pierce Chemical Co. (Rockford, IL). [^{125}I]Iodo-α-bungarotoxin was purchased from Amersham (Arlington Heights, IL) and thiamylal sodium from Boehringer Ingelheim Animal Health Inc. (St. Joseph, MO). Cobra α-toxin was isolated, following the method of Karlsson *et al.* (17), from *Naja naja siamensis* venom purchased from Miami Serpentarium (Salt Lake City, UT). Frozen *Torpedo californica* electric organ was purchased from Marinus Inc. (Long Beach, CA).

Receptor isolation. Receptor-enriched membranes were isolated from *T. californica* electric organ by established procedures (18, 19). The specific binding activities of the receptor preparations were measured by adsorption of [^{125}I]iodo-α-bungarotoxin receptor complexes onto DEAE-cellulose filters (20) or by measurement of the decrease of dansyl-trimethylamine fluorescence (6.6 μM) produced by the titration of suberyldicholine into suspensions of AcChR (0.3 mg/ml) in the presence of PCP (100 μM), following the method of Neubig and Cohen (21). The specific activities ranged between 0.9 and 1.3 nmol of [^{125}I] iodo-α-bungarotoxin or suberyldicholine binding sites/mg of protein. Each membrane preparation was also evaluated for the ability of carbamylcholine to increase the affinity of ethidium toward the PCP-sensitive binding site on the receptor. Only membrane preparations that displayed carbamylcholine-induced affinity increases of ethidium toward the AcChR were utilized.

Steady state spectroscopy. Steady state fluorescence and absorption measurements were made with a Perkin-Elmer MPF 66 spectrofluorometer and a Perkin-Elmer Lambda 3B spectrophotometer, respectively. Emission correction factors were generated with the aid of a calibrated 150-W reference lamp, from Optronics Laboratories, Inc. (Orlando, FL).

Fluorescence titrations. Titrations were carried out in 0.5- or 1-cm stoppered cuvettes. Except where noted, all fluorescence values were corrected for dilution resulting from added titrant and for the intrinsic fluorescence of the sample. Also, except where noted, excitation and emission wavelengths were 520 and 595 nm, respectively, and a Corning 2-63 cut-off filter was placed in the path of the emission beam to reduce stray-light effects. In the case of direct titrations with ethidium, fluorescence contributions of the free ligand were subtracted from total fluorescence, and the specific fluorescence enhancements versus ligand concentration were plotted directly. Specific (PCP-sensitive) fluorescence associated with the binding of ethidium to the channel NCI site was defined in all cases by the change in ethidium fluorescence associated with the addition of PCP (100–500 μM). Estimates of the equilibrium dissociation constants, EC_{50}, or IC_{50} were made by fitting plots of the specific (PCP-sensitive) changes in ethidium fluorescence versus the logarithm of ligand concentration to the equation for a sigmoid, by using a Marquardt algorithm (22) in the GraphPAD InPlot computer program (23). All samples were suspended in 100 mM NaCl, 10 mM $NaPO_4$, pH 7.4 (buffer I), except for the experiments with barbiturates, where 100 mM $NaPO_4$, pH 7.4 (buffer II), was used to control the alkaline barbiturate stock solutions. Stock solutions of diethyl ether, butanol, and halothane were dissolved in distilled H_2O. The stock solutions of volatile anesthetics were handled so as to minimize evaporative loss, by preparation just before each titration and storage on ice in light-protected sealed vials during the titrations. Aliquots were withdrawn through Teflon-lined diaphragms in the vial caps with a Hamilton syringe. Stock solutions of thiamylal sodium and secobarbital sodium were prepared with distilled water and had pH values of 10.0 and 8.8, respectively.

Results

Effects of NCIs on ethidium binding. The liquid anesthetics (halothane, diethyl ether, and butanol) induced a concentration-dependent increase in PCP-sensitive ethidium fluorescence, suggesting increased binding of ethidium to the channel NCI binding site (Fig. 1). These effects cannot be explained by a direct interaction of ethidium with the anesthetics, because in the absence of AcChR the anesthetics had no effect on ethidium fluorescence (Fig. 1). To provide a semiquantitative description of the results, the apparent EC_{50} values and Hill coefficients (n_H) for halothane, diethyl ether, and butanol to increase ethidium fluorescence were estimated to be 2.9 ± 2.5 mM (n_H = 2.8 ± 1.2; three experiments), 14 ± 6.0 mM (n_H = 1.9 ± 0.5; two experiments), and 47.2 ± 23.8 mM (n_H = 2.8 ± 1.3; three experiments), respectively. In all cases, the apparent Hill coefficients were greater than unity, consistent with previous reports on the ability of low affinity NCIs to induce agonist binding (3, 24). The calculated EC_{50} values and Hill coefficients for diethyl ether and butanol are approximate, because of the uncertainty in determining the plateau values for the effects of these agents. In the case of diethyl ether, miscibility constraints did not allow the addition of sufficient diethyl ether to achieve a plateau response (Fig. 1B). Butanol displayed more than one effect. At lower concentrations butanol increased PCP-sensitive ethidium fluorescence, and at high concentrations ethidium fluorescence decreased (Fig. 1C).

To confirm that the liquid anesthetics induced ethidium binding, the effects of the anesthetics on the K_D of ethidium

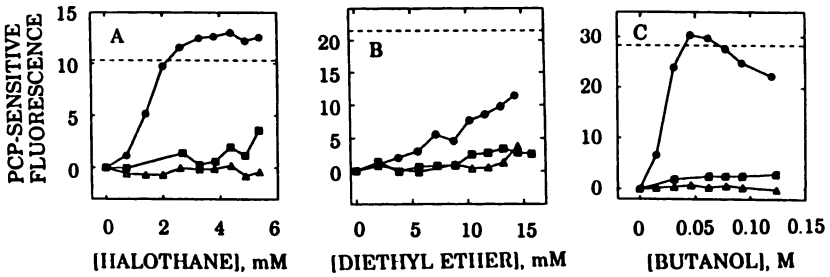

Fig. 1. Interaction of liquid anesthetics and α-toxin in the modulation of PCP-sensitive ethidium binding to *Torpedo* membranes. All samples contained ethidium (2.5 μM) suspended in buffer I, plus specified anesthetic concentrations. ●, Samples that also contained the AcChR (0.3 μM in α-toxin binding sites); ■, samples that contained AcChR plus α-toxin (3.3 μM). For samples that contained AcChR, the results are expressed as the difference in ethidium fluorescence in the absence minus presence of PCP (100 μM). For the sample that contained only buffer and ethidium (Δ), the results are also expressed as the difference in ethidium fluorescence in the presence minus absence of PCP. Fluorescence was measured with excitation at 520 nm and emission at 595 nm, and corrections were made for dilution effects. All values represent the average intensities from duplicate samples. *Dashed line near the top of each panel*, maximum PCP-sensitive change in ethidium fluorescence produced by carbamylcholine for the particular preparation of *Torpedo* membranes used for the results presented below it. A, Halothane; B, diethyl ether; C, butanol.

binding to the AcChR were measured. Direct titrations of ethidium into suspensions of AcChR-enriched membranes, in the presence of a fixed concentration of the liquid anesthetics, were performed. The fluorescence of ethidium in the absence and presence of PCP (500 μM) was monitored and plotted versus the added concentration of ethidium, and the apparent K_D was calculated (Table 1). Halothane, diethyl ether, and butanol increased the affinity of ethidium for the channel NCI binding site to about the same extent as carbamylcholine (K_D = 1.9 ± 1.3 μM; 18 experiments).

The magnitude of the anesthetic-induced increase in PCP-sensitive fluorescence was ligand dependent. Compared with the maximum change in PCP-sensitive ethidium fluorescence produced by carbamylcholine, the effect of halothane was ~20% higher, that of diethyl ether was ~50% lower, and that of butanol was not different. Combining the aforementioned observations with the finding that there is no difference in the ability of halothane, diethyl ether, butanol, or carbamylcholine to increase the affinity of ethidium toward the channel NCI binding site, these results indicate little relation between the binding affinity and the quantum yield of channel-bound ethidium.

TABLE 1

Effect of various ligands on the apparent equilibrium dissociation constant (K_D) of ethidium toward the channel NCI binding site on the AcChR

Values are mean ± standard deviation of the number of determinations given in parentheses, determined by direct fluorescence titration of ethidium into suspensions of AcChR (0.3 μM in α-toxin binding sites), in the absence and presence of PCP (500 μM). The concentration of carbamylcholine was 0.5 mM.

Ligands added	K_D	
	Without carbamylcholine	With carbamylcholine
	μM	
Control	~1000[a]	1.9 ± 1.3 (18)
Butanol (0.1 M)	1.5 ± 1.1 (3)	2.0 ± 1.3 (3)
Halothane (7.5 mM)	1.6 ± 0.5 (3)	1.2 ± 0.4 (3)
Diethyl ether (20 mM)	2.5 ± 1.1 (3)	2.5 ± 0.6 (3)
Thiamylal (200 μM)	ND[b]	2.5 ± 1.4 (4)
Secobarbital (286 μM)	ND[b]	1.3 ± 0.9 (3)
Urethane (300 mM)	ND[b]	4.9 ± 2.1 (3)[c]

[a] Value from Herz *et al.* (8).
[b] ND, value could not be determined because inner filter effects limited the maximum concentration of ethidium that could be added to each cuvette.
[c] $p < 0.02$ for paired comparison with carbamylcholine control.

The barbiturate thiamylal and the carbamate ester urethane did not significantly affect PCP-sensitive ethidium fluorescence (Fig. 2, A and C). The barbiturate secobarbital produced a small nonsignificant decrease in PCP-sensitive ethidium fluorescence (Fig. 2B).

For comparison, the effect of carbamylcholine to induce the maximum PCP-sensitive increase in ethidium fluorescence is represented as a dashed line near the top of each graph (Figs. 1 and 2). Because of differences in the viability and specific activity of each preparation, the maximum change in ethidium fluorescence induced by carbamylcholine differs from one membrane preparation to another. Carbamylcholine displayed an EC_{50} of 0.09 ± 0.06 μM (n_H = 1.2; six experiments) to induce ethidium binding (data not shown).

Modulation of NCI effects by cobra α-toxin. To assess an interaction of snake α-toxins with anesthetics, sufficient cobra α-toxin (3.3 μM) to occupy all α-toxin binding sites was added to the suspensions of AcChR-enriched membranes and ethidium, and the effects of the various anesthetics on PCP-sensitive ethidium fluorescence were measured. Interestingly, cobra α-toxin binding to the agonist/competitive antagonist sites blocked anesthetic-induced ethidium binding, consistent with previous observations that cobra α-toxin stabilizes the receptor in a low affinity or resting-like state (25) (Figs. 1 and 2). Also, secobarbital, thiamylal, and urethane produced no effects on AcChR-enriched membranes pretreated with cobra α-toxin (Fig. 2).

Carbamylcholine and NCI interaction. To assess interactions between the conformational states induced by general anesthetics and by carbamylcholine, the anesthetics were titrated into ethidium-AcChR suspensions, in the presence or absence of carbamylcholine (1 mM). The results of these experiments were complex (Figs. 3 and 4). Both halothane and diethyl ether enhanced PCP-sensitive ethidium fluorescence beyond the maximum effect of carbamylcholine alone (Fig. 3, A and B). A direct titration of ethidium into suspensions of AcChR, carbamylcholine, and halothane or diethyl ether showed that the addition of carbamylcholine did not significantly alter the K_D of ethidium toward the channel NCI binding site (Table 1). This suggests that, in the presence of carbamylcholine, halothane and diethyl ether induce a conformational state that is associated with a slightly greater quantum yield for AcChR-bound ethidium. It also shows that there is little

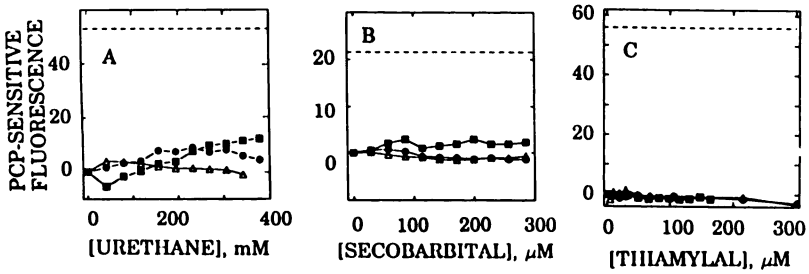

Fig. 2. Interaction of urethane, secobarbital, and thiamylal with α-toxin in the modulation of PCP-sensitive ethidium binding to *Torpedo* membranes. All samples contained ethidium (2.5 μM) suspended in buffer I (urethane) or buffer II (barbiturates), plus specified anesthetic concentrations. ●, Samples that also contained the AcChR (0.3 μM in α-toxin binding sites); ■, samples that contained AcChR plus α-toxin (3.3 μM). For samples that contained AcChR, the results are expressed as the difference in ethidium fluorescence in the absence minus presence of PCP (100 μM). For the sample that contained only buffer and ethidium (△), the results are also expressed as the difference in ethidium fluorescence in the presence minus absence of PCP. See legend to Fig. 1 for experimental details. A, Urethane; B, secobarbital; C, thiamylal.

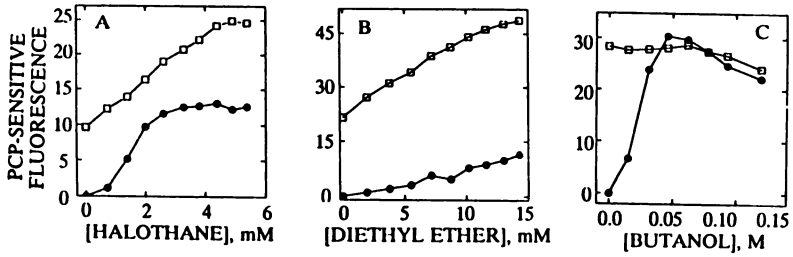

Fig. 3. Interaction of liquid anesthetics and carbamylcholine in the modulation of PCP-sensitive ethidium binding to *Torpedo* membranes. All samples contained ethidium (2.5 μM) suspended in buffer I, plus specified anesthetic concentrations. ●, Samples that contained the AcChR (0.3 μM in α-toxin binding sites); □, samples that contained both AcChR and carbamylcholine (1 mM). The results are expressed as the difference in ethidium fluorescence in the absence minus presence of PCP (100 μM). Fluorescence was measured with excitation at 520 nm and emission at 595 nm, and corrections were made for dilution effects. The data points of the carbamylcholine-containing samples are initially higher, because the effect of the addition of carbamylcholine was measured before the anesthetic titration, in order to compare the carbamylcholine with the anesthetic effects. All values represent the average intensities from duplicate samples. A, Halothane; B, diethyl ether; C, butanol.

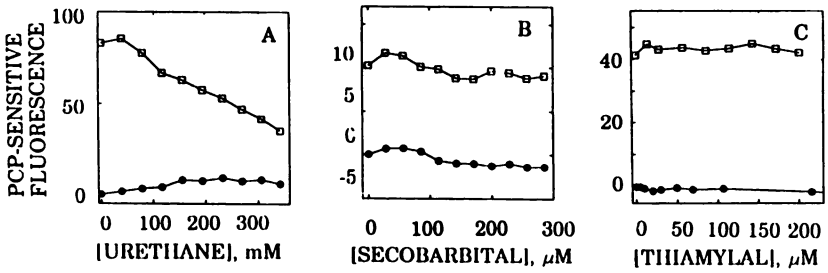

Fig. 4. Interaction of urethane, secobarbital, and thiamylal with carbamylcholine in the modulation of PCP-sensitive ethidium binding to *Torpedo* membranes. All samples contained ethidium (2.5 μM) suspended in buffer I (urethane) or buffer II (barbiturates), plus specified anesthetic concentrations. ●, Samples that also contained the AcChR (0.3 μM in α-toxin binding sites); □, samples that contained both AcChR and carbamylcholine (1 mM). See legend to Fig. 3 for experimental details. A, Urethane; B, secobarbital; C, thiamylal.

relation between the binding affinity and the quantum yield of channel-bound ethidium (see above).

Addition of butanol to samples containing carbamylcholine produced no further effect (Fig. 3C). Neither PCP-sensitive ethidium fluorescence (Fig. 3C) nor the K_D of ethidium toward the channel NCI binding site (Table 1) changed, relative to the effect of carbamylcholine alone. Secobarbital and thiamylal did not affect either PCP-sensitive ethidium fluorescence (Fig. 4, B and C) or K_D in the presence of carbamylcholine (Table 1). Urethane, on the other hand, decreased carbamylcholine-induced ethidium fluorescence (Fig. 4A) and the apparent affinity of ethidium for the channel NCI binding site (Table 1), suggesting a direct interaction of urethane with the channel or low affinity NCI binding sites.

Effects of anesthetics on fluorescence spectrum of AcChR-bound ethidium. Halothane and diethyl ether appeared to affect the quantum yield of channel-bound ethidium. To assess further ligand-dependent differences in the spectral properties of ethidium-AcChR complexes, the excitation and emission spectra of ethidium and AcChR were measured in the absence and presence of PCP (500 μM). Difference spectra were computed, and excitation and emission difference maxima were determined (Table 2). As previously reported (8), carbamylcholine induces a ~40-nm red shift in the excitation difference spectrum of ethidium bound to channel NCI sites and a smaller blue shift (~20 nm) in the emission difference spectrum, compared with ethidium in buffer. Butanol, halothane, and diethyl ether induced less of a red shift (35, 32, and 25 nm, respectively) in the excitation difference spectrum of channel-bound ethidium. In the emission difference spectrum, butanol induced a slightly larger blue shift (10 nm), diethyl ether produced a smaller blue shift (5 nm), and halothane did not induce a

TABLE 2

Effect of NCIs on the difference spectral maxima of AcChR-bound ethidium

Corrected excitation and emission spectra were taken in the absence and presence of PCP (500 μM). Difference spectra were then computed and the maxima were determined. AcChR (1.0 μM in toxin binding sites) and ethidium (1 μM) were suspended in buffer I. For reference, using a Perkin-Elmer MPF-66 spectrofluorometer, the corrected excitation and emission maxima of ethidium in buffer I are 482 and 628 nm, respectively. For ethidium and AcChR, the corrected excitation and emission maxima are 500 and 612 nm, respectively.

Condition	Excitation maxima	Emission maxima
	nm	
Ethidium and AcChR and carbamylcholine (0.6 mM)	525	608
Ethidium and AcChR and butanol (0.22 M)	517	610
Ethidium and AcChR and diethyl ether (20 mM)	507	611
Ethidium and AcChR and halothane (7.5 mM)	514	614

significant effect. No clear NCI effect, distinct from the effect of carbamylcholine, was observed on either the excitation or the emission spectra of ethidium.

Discussion

Because the affinity of ethidium is remarkably dependent on the receptor conformation, ethidium is a unique probe of the channel NCI binding site on the *Torpedo* AcChR. When agonists like carbamylcholine bind to the AcChR and induce a desensitized state, the affinity of ethidium toward the channel NCI site is >500-fold higher than when the agonist/competitive antagonist binding sites are unoccupied or when competitive antagonists are bound. This compares with only a ~100-fold difference in affinity of quinacrine toward the desensitized versus resting states.[1]

In this manuscript, we describe the use of ethidium to evaluate the effect of low affinity NCIs on ligand binding to the channel NCI site. Ethidium binding to the channel NCI site was assayed under conditions that either favor ethidium binding or are not normally associated with significant ethidium binding. Occupation of the agonist/competitive antagonist binding site with an agonist, like carbamylcholine, induces a high affinity (desensitized) state that normally favors ethidium binding to the channel NCI site. When the agonist/competitive antagonist sites are unoccupied or occupied by cobra α-toxin, the AcChR is usually in a low affinity conformation for ethidium binding to the NCI site.

The effects of NCI on ethidium binding to the channel NCI binding site were ligand dependent. When the agonist/competitive antagonist binding sites are unoccupied, the liquid anesthetics (halothane, diethyl ether, and butanol) increased the affinity of ethidium toward the PCP-sensitive channel NCI binding site 400–600-fold. This affinity increase is essentially identical to the effect of carbamylcholine. The concentrations of the liquid anesthetics needed to induce ethidium binding to the channel NCI site are generally significantly higher than the ED_{50} concentrations for anesthesia for tadpole preparations (24), clearly indicating that these anesthetics do not affect the AcChR channel NCI binding site at pharmacologically relevant concentrations. The two barbiturates and urethane had no detectable effect on PCP-sensitive ethidium binding, indicating a mechanism of action different from that of the liquid anesthetics.

The interrelation between ligand occupation of the 1) agonist/competitive antagonist, 2) channel NCI, and 3) low affinity NCI binding sites was examined by determining the effect of ligand occupation of the agonist/competitive antagonist binding sites on the ability of the general anesthetics to modulate ethidium binding to the channel NCI site. When cobra α-toxin was bound to the agonist/competitive antagonist binding sites, the liquid anesthetics did not induce ethidium binding, indicating that the liquid anesthetics cannot surmount the effects of cobra α-toxin on the conformation of the channel NCI binding site. This confirms that snake α-toxins stabilize the AcChR in a resting-like state (25). These results should also expand our notions on the mechanism of action of snake α-toxins to include both the competitive inhibition of acetylcholine binding and stabilization of the receptor in a closed channel conformation, i.e., resting-like state.

When carbamylcholine occupied the agonist/competitive antagonist binding sites, several ligand-dependent responses were observed. 1) Halothane and diethyl ether increased the quantum yield of AcChR-bound ethidium, beyond the effect of carbamylcholine, without altering the affinity of ethidium. 2) Butanol, secobarbital, and thiamylal had no effect beyond the effect of carbamylcholine on the fluorescence of ethidium bound to the channel NCI site. 3) Urethane decreased ethidium binding to the channel NCI site.

The effects of halothane and diethyl ether in the presence of carbamylcholine suggest that the conformation of the receptor induced by halothane or diethyl ether is somewhat different from the conformation induced by carbamylcholine. It is unclear whether this difference in conformation extends beyond the immediate area or microenvironment of the ethidium binding site. Not all the liquid anesthetics affect the quantum yield of AcChR-bound ethidium. Butanol, for example, did not influence ethidium binding or quantum yield beyond the effects of carbamylcholine.

Barbiturates generally decrease the affinity of [^{3}H]acetylcholine toward the agonist/competitive antagonist binding sites and inhibit [^{3}H]histrionicotoxin binding to the channel NCI site (4, 10, 26, 27). These effects have been explained as an induction of the resting state through direct barbiturate binding either to the channel NCI or to unique barbiturate site(s) (4, 10, 26). Most barbiturates bind preferentially to the resting conformation of the AcChR, with their effects on [^{3}H]acetylcholine binding being explained by a simple two-state model (10, 26, 27). Secobarbital is relatively unique, because it binds with about equal affinity to the resting and desensitized states of the receptor and, consequently, has a minimal effect on [^{3}H]acetylcholine binding (26). Although secobarbital does not decrease [^{3}H]acetylcholine binding, it inhibits the binding of radiolabeled barbiturates that do, strongly suggesting that secobarbital binds to the same site(s) as other barbiturates (26). We evaluated the effects of secobarbital and its derivative thiamylal, to determine whether barbiturates bind to the channel NCI site. If the barbiturates had interacted with the channel NCI site, then secobarbital and possibly thiamylal would have necessarily displaced channel-bound ethidium, but they did not, indicating that these barbiturates do not bind to the

[1] Unpublished observations, Valenzuela, C. F., Kerr, J. A., and Johnson, D. A.

channel NCI site. Possible sites for barbiturate binding would include the low affinity NCI sites or unique barbiturate site(s).

Urethane has been shown to increase [^{3}H]acetylcholine binding with an EC_{50} of 96 mM, suggesting that it acts allosterically to induce desensitization by binding to either the channel or the low affinity NCI sites (24). However, the inability of urethane alone to increase ethidium binding suggests that urethane does not simply bind to the low affinity NCI binding sites to induce desensitization. A urethane-induced desensitization would have increased ethidium binding, which we did not observe (Fig. 2A). When ethidium was bound to the channel NCI site, i.e., in the presence of carbamylcholine, urethane inhibited ethidium binding, suggesting that it acts by binding to the channel NCI site and not to the low affinity NCI sites.

Viewed from the perspective of PCP-sensitive ethidium binding to the channel NCI site, the results provide additional evidence for at least three different modes of action of general anesthetics on the *Torpedo* AcChR. One mode of action involves the induction of a desensitized state that is associated with the enhancement of ligand binding to the channel NCI site. The liquid anesthetics examined here acted in this manner. A second mode is associated with barbiturates and entails stabilization of the resting state of the receptor by ligand binding at the low affinity NCI sites or unique barbiturate site(s). A third mode is produced by drugs like urethane and involves direct anesthetic binding to the channel NCI site. The net effect of each of these modes of action is to inhibit ligand-induced channel opening by induction of a closed-channel state (resting or desensitized) and/or by direct interaction and blockade of the cation channel of the receptor.

Note Added in Proof

Improvements in our sample mixing technique have allowed us to add sufficient diethyl ether (200 mM) to achieve a plateau in the change of the PCP-sensitive ethidium fluorescence. The effect of ether on ethidium binding in the absence of other ligands reached a plateau at 100 mM (EC_{50} = 49 mM; n_H = 2.3). The K_D of ethidium toward the receptor in the presence of diethyl ether (200 mM) or diethyl ether (200 mM) and carbamylcholine (0.5 mM) were 1.5 and 1.8 μM, respectively.

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