Mechanism of Action of the Redox Affinity Reagent [(Trimethylammonio)methyl]catechol†

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ABSTRACT: The synthesis of 4- and 5-hydroxy-3-[(trimethylammonio)methyl]catechol (4- and 5-HTMC) was carried out to examine their proposed involvement as intermediates in the spontaneous redox-dependent half-of-sites inactivation of neurotoxin binding sites in the nicotinic acetylcholine receptor (nAcChR) mediated by the parent compound 3-[(trimethylammonio)methyl]catechol (TMC) [Nickoloff *et al.* (1985) *Biochemistry 24*, 999–1007]. Oxidation of 4- and 5-HTMC occurred with sodium periodate with facile conversion to the corresponding *p*-quinones which were intercepted with thiols and cyclopentadiene. Both 4- and 5-HTMC inactivated neurotoxin binding in the nAcChR in a time course and over a concentration range consistent with their involvement as intermediates in the TMC redox-dependent inactivation of neurotoxin ([^{125}I]-α-bungarotoxin) binding sites. Rapid concentration-dependent inactivation of neurotoxin sites occurred over a 10–1000 μM range and was resistant to further inactivation after 50% loss of available toxin binding sites on the nAcChR. Both 4- and 5-HTMC inactivated nAcChR neurotoxin sites much more rapidly and efficiently than was observed previously with TMC. The apparent binding constants for 4- and 5-HTMC with the nAcChR, calculated from their concentration-dependent inactivation behavior toward toxin binding sites, were K_d = 224 ± 98 and 39 ± 17 μM, respectively. The observed results and the redox potentials (vs Ag/AgCl reference electrode) measured by cyclic voltammetry at pH 1.8 for TMC (719 mV) and the 4- and 5-HTMC derivatives (519 and 443 mV, respectively) supported the previously proposed mechanism for inactivation of the nAcChR by TMC. Spectroelectrochemical studies of TMC and 4- and 5-HTMC under aerobic conditions confirmed the rapid and pH-dependent reversible formation of *o*- and *p*-quinones at appropriately low applied potentials. These mechanistic studies provide a basis for the design of additional redox-dependent affinity agents directed to other macromolecular sites.

Quaternary ammonium catechols represent a class of affinity reagents for cholinergic binding sites operating through a novel redox mechanism (Nickoloff *et al.*, 1982, 1985; Patel *et al.* 1991, 1993). These reagents may be directed to choline or acetylcholine binding macromolecules and may inhibit or selectively inactivate them through affinity-dependent reactions. Selective inactivation of the curarimimetic neurotoxin binding sites of the acetylcholine receptor occurs at one of the two neurotoxin sites using 3-[(trimethylammonio)methyl]-catechol (TMC).[1] These receptor sites competitively bind acetylcholine and *d*-tubocurarine [for a review of the nAcChR and binding of ligands, see Karlin (1991, 1993) and Changeux (1993)].

The reactivities of these catechol-based affinity reagents are much reduced and more selective when compared with those of the rapidly autocatalytically oxidized catecholamines. Reagents such as TMC and more reactive agents based on TMC may prove to be selective in their affinity-dependent reactions and therefore more useful in revealing chemical details in the macromolecular binding sites to which they are directed.

Earlier, we showed that the redox-reactive heterobifunctional affinity reagent 3-[(trimethylammonio)methyl]catechol (TMC, I) inactivated curarimimetic neurotoxin sites of the nicotinic acetylcholine receptor (nAcChR) with half-of-sites reactivity (Nickoloff *et al.*, 1985). The time course of this inactivation was consistent with the involvement of the progressively more reactive hydroxylated derivatives of TMC formed during its slow autooxidation (Figure 1). The specific involvement of one or both of the trihydroxy derivatives was proposed to mediate the redox-dependent covalent inactivation of the receptor. Mixtures of 4- and 5-hydroxy-3-[(trimethylammonio)methyl]catechol (4-HTMC, IV, and 5-HTMC, III, respectively] were formed *in situ* from TMC under carefully controlled oxidation conditions. The increased rate and efficiency of half-of-sites inactivation of toxin sites which occurred under these conditions were proposed to implicate these early TMC autooxidation intermediates in the inactivation of the nAcChR by TMC. Further, a redox-activated covalent mechanism was supported by the observation of half-of-sites radiolabeling of the receptor by [^{3}H]TMC. The nAcChR was known to possess a more easily reduced disulfide (Wolosin *et al.*, 1980) at one of the two toxin binding sites which could be affinity labeled after controlled reduction with DTT (Damle & Karlin, 1978). Figure 2 illustrates the scheme proposed for 5-HTMC. An entirely analogous inactivation could be mediated simultaneously by 4-HTMC, which was also thought to be formed in the early stages of TMC oxidation.

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[1] Abbreviations: TMC, 3-[(trimethylammonio)methyl]catechol; TMQ, 3-[(trimethylammonio)methyl]-*o*-benzoquinone; 4-HTMC, 4-hydroxy-3-[(trimethylammonio)methyl]catechol; 5-HTMC, 5-hydroxy-3-[(trimethylammonio)methyl]catechol; 5-HTMQ, 2-hydroxy-6-[(trimethylammonio)methyl]-*p*-benzoquinone; 4-HTMQ, 2-hydroxy-3-[(trimethylammonio)methyl]-*p*-benzoquinone; nAcChR, nicotinic acetylcholine receptor; DTT, dithiothreitol; [^{125}I]-α-Btx, [^{125}I]-α-bungarotoxin; DMF, *N,N*-dimethylformamide; NMR, nuclear magnetic resonance; ^{13}C-NMR, natural abundance ^{13}C-nuclear magnetic resonance spectrum; HOAc, acetic acid; ME, mercaptoethanol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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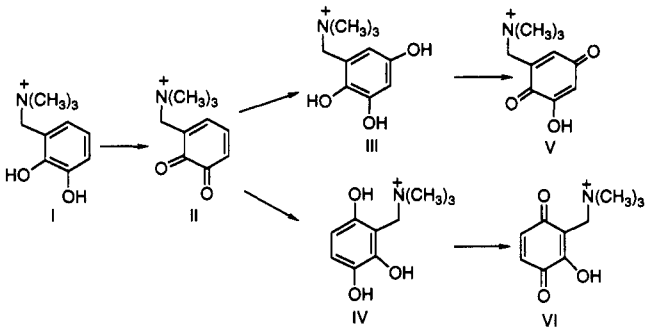

FIGURE 1: Reaction sequence occurring early in the oxidation of 3-[(trimethylammonio)methyl]catechol (TMC, I). The products shown are 3-[(trimethylammonio)methyl]-*o*-benzoquinone (TMQ, II), 5-hydroxy-3-[(trimethylammonio)methyl]catechol (5-HTMC, III), 4-hydroxy-3-[(trimethylammonio)methyl]catechol (4-HTMC, IV), 2-hydroxy-6-[(trimethylammonio)methyl]-*p*-benzoquinone (5-HTMQ, V), and 2-hydroxy-3-[(trimethylammonnio)methyl]-*p*-benzoquinone (4-HTMQ, VI).

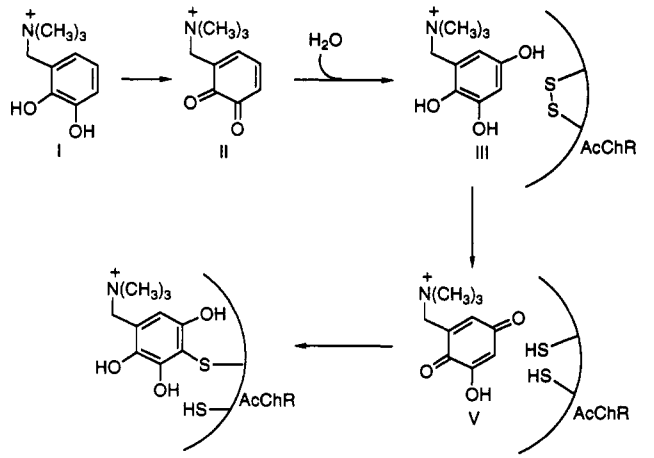

FIGURE 2: Proposed scheme for redox-dependent half-of-neurotoxin-site labeling of the nAcChR (Nickoloff *et al.*, 1985). Analogous scheme as shown for covalent modificationn of AcChR using 5-HTMC may be drawn for 4-HTMC.

While the complex mechanism of oxidation of TMC was characterized to an extent by our earlier spectroscopic and kinetic studies (Nickoloff *et al.*, 1985), neither the 4- and 5-HTMC intermediates nor their quinone oxidation products (V and VI) have been isolated or intercepted with trapping agents to give products which supported the proposed mechanism. Neither have the proposed intermediates been directly tested as redox-dependent inactivators of the acetylcholine receptor. Thus, we have prepared the proposed trihydroxy intermediates (III and IV) and demonstrated that their behavior was consistent with their direct involvement in the TMC-mediated half-of-sites inactivation at toxin binding sites in the nAcChR. Further, each of the synthetic trihydroxy compounds has been oxidized independently to a single quinone and, in each case, intercepted with cyclopentadiene to form characterizable adducts. An analysis of the reactions of synthetic 4- and 5-HTMC with the neurotoxin sites in the nAcChR reported here completely supported the role proposed for these intermediates in the toxin binding inactivating reaction mechanism proposed earlier. The explicit reactive nature of these interesting redox-dependent affinity reagents is now better defined. The experiments reported here provide the basis for the incorporation of the redox-dependent element into additional reagents which may be directed to other macromolecular targets.

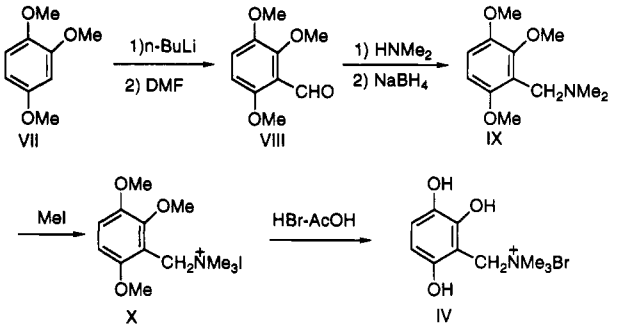

FIGURE 3: Chemical synthesis of 4-hydroxy-3-[(trimethylammonio)-methyl]catechol (4-HTMC, IV).

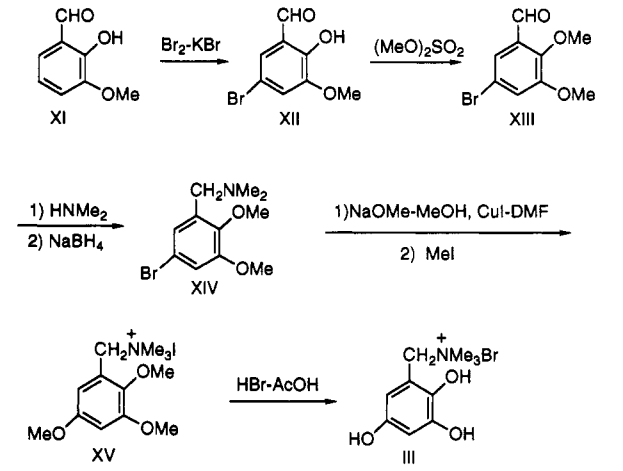

FIGURE 4: Chemical synthesis of 5-hydroxy-3-[(trimethylammonio)-methyl]catechol (5-HTMC, III).

RESULTS

4- and 5-HTMC (IV and III) were prepared according to the synthetic schemes shown in Figures 3 and 4. Assignment of the chemical structures of these hydroxycatechol derivatives was based on chemical analysis and nuclear magnetic resonance spectra. The redox behaviors of TMC (I), III, and IV were determined by spectroelectrochemical studies (Figure 5) and cyclic voltammetry (Figure 6). The redox potentials (vs Ag/AgCl reference electrode) measured by cyclic voltammetry at pH 1.8 were, for TMC and 4- and 5-HTMC (III and IV), 719, 519, and 443 mV, respectively. The comparative ease of oxidation of III and IV relative to that of TMC was consistent with a role for these hydroxycatechols in the reduction of an easily reduced protein disulfide (Allison & Schoup, 1983) in the proposed mechanism of TMC-directed inactivation of the nAcChR (Nickoloff *et al.*, 1985). When TMC was treated with 1 equiv of ceric ammonium nitrate, a compound (II) with λ_{max} 385 nm was formed. The spectrum rapidly decayed while a new peak appeared with λ_{max} 460 nm [*cf.* Nickoloff *et al.* (1985)]. In our earlier paper, we proposed that these maxima were due to the formation of the *o*-quinone II (λ_{max} 385 nm) and an unknown mixture of *p*-quinones V and VI (composite peak with λ_{max} 460 nm). The *o*-quinone was proposed to proceed through III and IV to form V and VI, respectively. These observations were consistent with both chemically and electrochemically induced transformations of the catechols I, III, and IV observed spectrophotometrically in Figure 5. The measured decay in the spectra of V and VI was pseudo first order with rate constants of 2.4×10^{-4} and 2.1×10^{-5} M/h, respectively. These rates of decay reflected stabilities in both *p*-quinones products sufficient for trapping with reactive dienes or nucleophiles (*cf.* Figure 5, top). When compared with our earlier observations, these measured decay rates allowed us to estimate by spectroscopic comparison that

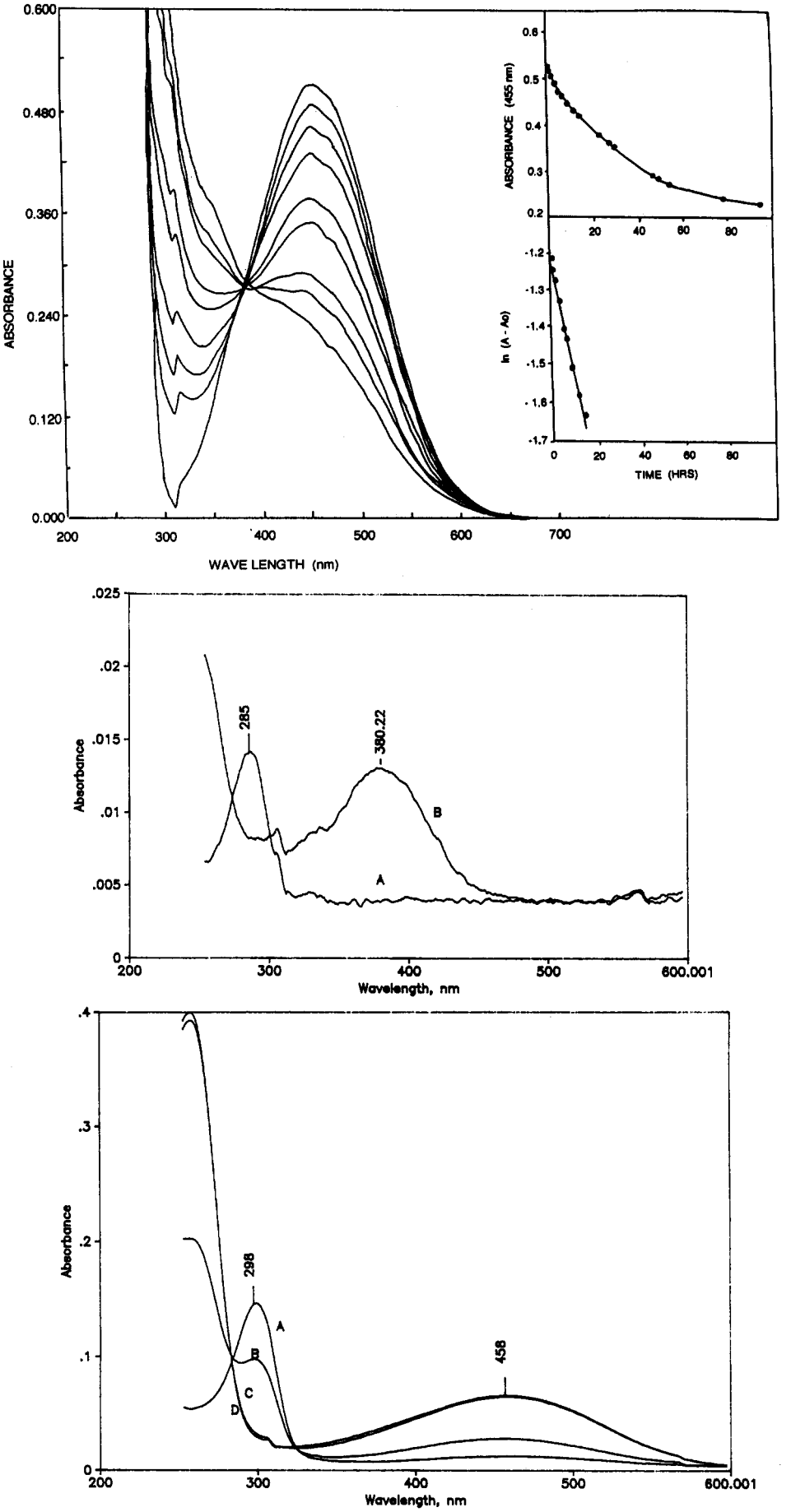

FIGURE 5: (top) Spectroscopic decay of 4-HTMQ (V). Spectroelectrochemical plots of TMC (middle) (A, without applied electrical potential; B, time = 1.5 min; E(V) applied vs Ag/AgCl = 0.9) and 4-HTMC (bottom) (A, time = 0, E(V) = –0.2; B, time = immediately, E(V) = 0.8; C, time = 1.33 min, E(V) = 0.8; D, time = 3 min, E(V) = 0.8).

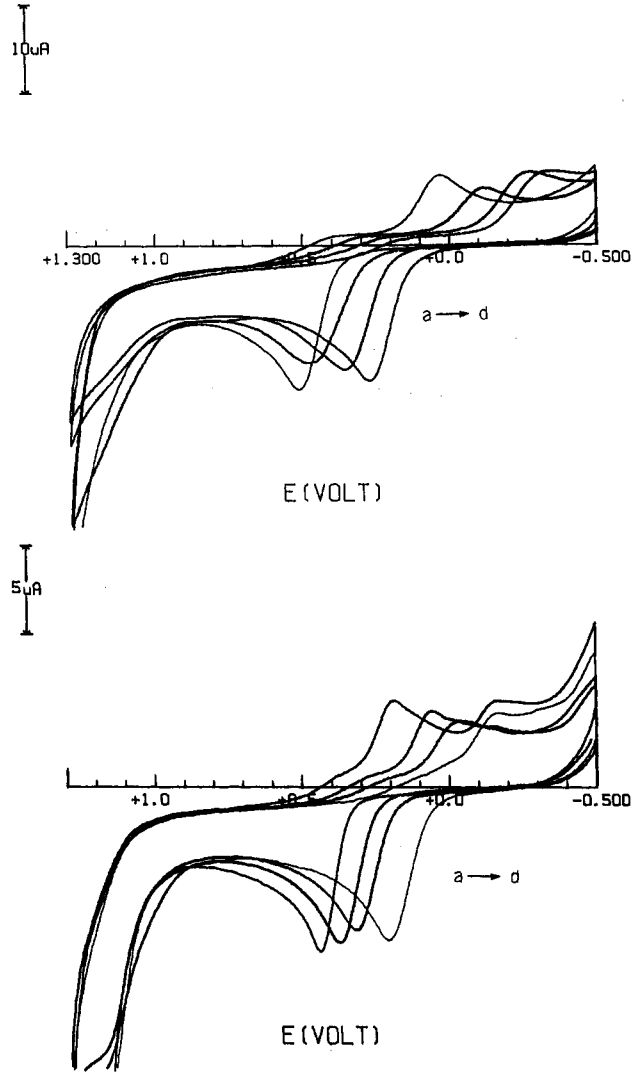

FIGURE 6: Cyclic voltammograms for 4-HTMC (top), pH 1.77, 3.43, 4.38, and 5.78 for a–d, respectively, and 5-HTMC (bottom), pH 1.77, 3.36, 4.36, and 5.16 for a–d, respectively.

TMC oxidation partitions about 80–85% along the IV and VI reaction pathway and about 15–20% along the III and V reaction pathway (Nickoloff *et al.*, 1985). The relatively small difference in rate underlying partition along these different reaction pathways may be due to a tendency for the OH^- or H_2O (which reacts by nucleophilic addition to the TMC-derived *o*-quinone II) to have more of a stabilizing effect in the transition state when reaction occurred at position 4, compared with reaction at position 5. In any event, these were not large effects. The estimated free energy differences in the transition states were small ($\Delta\Delta G^{\ddagger}$ = 0.82 kcal/mol, based on a 4-fold rate difference to account for the 4–5:1 product ratios). These results revealed an added complexity in the oxidation of TMC not fully appreciated earlier.

We intercepted both V and VI with cyclopentadiene after separately oxidizing 4- and 5-HTMC. The structures of the cyclopentadiene adducts shown in Figure 7 were determined by NMR analysis. Also, when TMC was oxidized in deuterated water to the *o*-quinone II and subsequently trapped with cyclopentadiene, two isomeric cycloadducts with a ratio of 2.5:1 were formed. These were the exo and endo isomers noted in Figure 7. For V and VI, only one isomer was formed. The quantitative retention of the tetraalkylammonium function in the trapped products derived from TMC and 4- and 5-HTMC precluded quinone methide-forming reactions prior

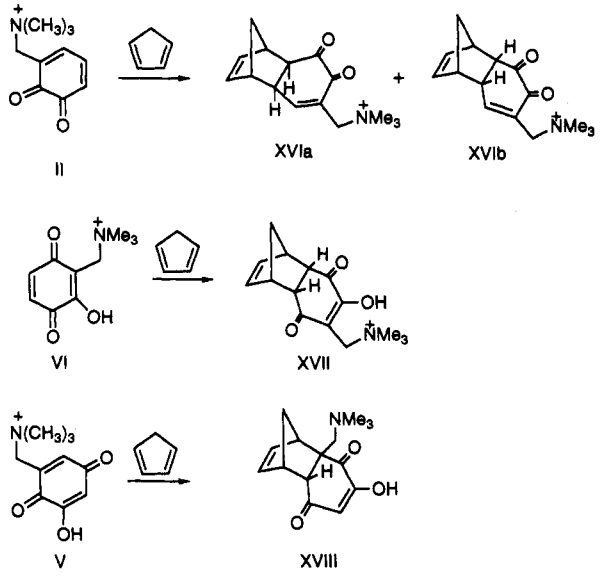

FIGURE 7: Chemical interception of transient quinone species with cyclopentadiene. Shown are the exo and endo adducts (XV1a and XV) of TMQ (II) and the endo adducts (XVII and XVIII) of 4-HTMQ (VI) and 5-HTMQ (V), respectively.

to trapping. In that case, the loss of trimethylamine would have been expected.

The reactions of 4- and 5-HTMC (IV and III) with the nAcChR were examined in order to compare their efficacy with that of TMC in the inactivation of neurotoxin binding sites. Figure 8 shows rapid inactivation of neurotoxin sites at all concentrations of reagent tested. Although residual sites were determined after dilution and rapid dialysis of reagents, these data may not be taken as an accurate reflection of the rate of toxin binding site inactivation. Measurable inactivation of neurotoxin sites occurred in the 10–1000 μM range for both 4- and 5-HTMC. The extent of inactivation was concentration-dependent, as is illustrated in Figure 8, middle and bottom. At concentrations in excess of 1000 μM, additional inactivation was not observed beyond 50% of all neurotoxin binding sites. This behavior parallels the earlier observation using what now appears to be approximately a 4:1 mixture of IV:III generated *in situ* from the controlled oxidation of TMC (Nickoloff *et al.*, 1985). This behavior shows that saturation of half-of-sites inactivation begins at about 300 μM and is effectively complete at about 1300 μM.

At all concentrations employing the independently synthesized 4- and 5-HTMC, a 10-fold molar excess of dithiothreitol (DTT) was added simultaneously to trap quinones formed in solution away from the receptor surface. However, the receptor was not preincubated with DTT prior to initiation of the reaction with 4- or 5-HTMC. In the absence of added DTT, a relatively rapid formation of quinone oligimers and polymers occurred, interfering, at the higher concentrations of 4- and 5-HTMC tested, with the determination of the concentration of toxin binding sites. In control experiments, in which receptors were first reduced with DTT, some inactivation of toxin sites occurred when 4- or 5-HTMC was later added even in the presence of DTT. Thus, we could not rule out that a portion of the observed inactivation of the toxin binding was due to reaction of the reduced form of the receptor with the oxidized form of the reagent, as shown in the mechanistic scheme (Figure 9). The effect of DTT alone appears to be mostly associated with its ability to trap polymerizing 4- or 5-HTMC-derived quinones and oligomers formed in the early stages of polymerization, a reaction which occurs away from the surface of the receptor as well as at the receptor surface. In the presence of DTT, polymerization of

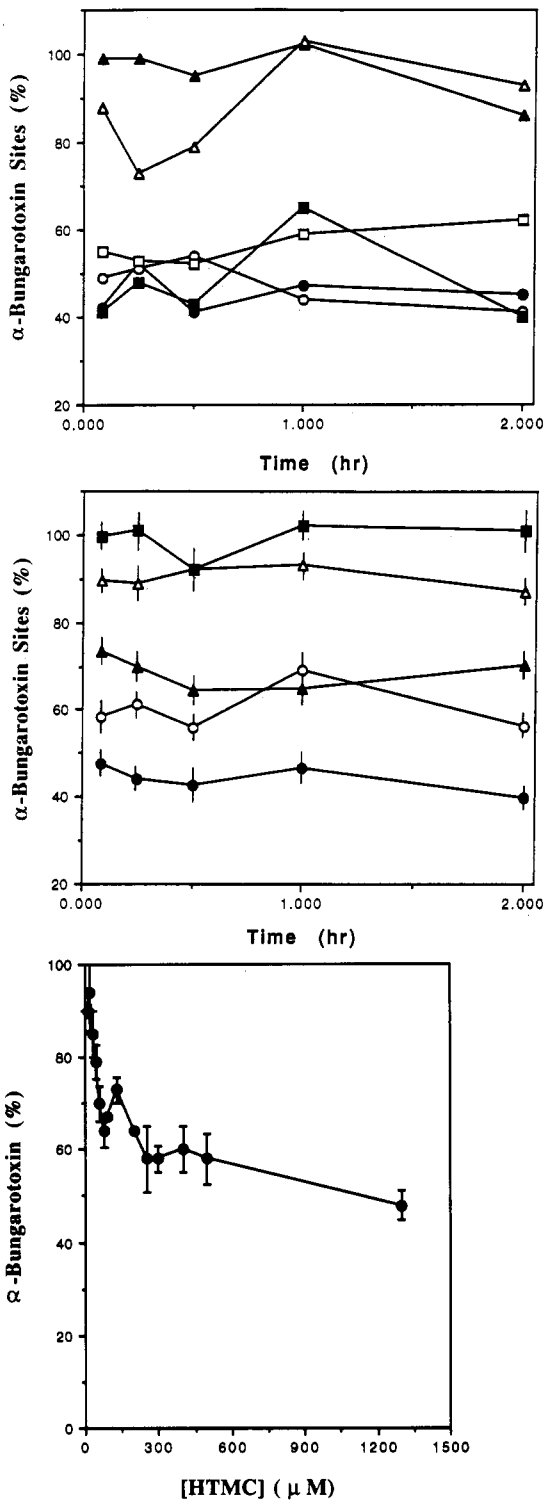

FIGURE 8: Inactivation of [^{125}I]-α-bungarotoxin-determined sites of the nAcChR plotted as a percentage of the residual sites/mg of protein against time of incubation with 4- or 5-HTMC. Top: 1.3 mM 4- or 5-HTMC in the presence of 10-fold excess of DIT or mercaptoethanol (ME) (●, 5-HTMC + DTT, ○, 5-HTMC + ME, ■, 4-HTMC + DIT, □, 4-HTMC + ME). Control plots for solutions not containing 4- or 5-HTMC show determination of [^{125}I]-α-bungarotoxin sites in the presence of 21.6 mM DTT (▲) or 21.5 mM ME (△). Middle: inactivation of sites observed as a function of time for 5-HTMC at 1.3 (■), 13 (△), 130 (▲), 500 (○), and 1300 μM (●). The data represented the average values of at least two trials for each data point. The [^{125}I]-α-Btx counts (cpm) were determined by subtracting experimental values from nonspecific binding. The calculation was based on the assumption that [^{125}I]-α-Btx binds to the nAcChR at 100% in the absence of competitor (4- or 5-HTMC) after nonspecific binding was subtracted. Bottom: inactivation of sites plotted as a function of concentration for 5-HTMC at a number of concentrations including those illustrated in the middle graph.

4- or 5-HTMC is thus less extensive than in the absence of DTT. Further, HTMC-derived polymers formed in the absence of DTT appear to produce a complex nondialyzable polymer which interacts with [^{125}I]-α-BTX and adds to apparent receptor binding. This interpretation is consistent with a higher level of binding of the radiolabeled toxin to disks, which occurs with time in solutions of polymerized HTMC with receptor (without added DTT). In the presence of DTT, polymerization of 4- or 5-HTMC proceeds but removal of the monomer and smaller DTT-trapped oligomers by dialysis is relatively complete. Thus, the extent of receptor inactivation was determined more accurately by carrying out the reaction in the presence of DTT. The affinity-dependent reactions occurring at the receptor surface were not altered by added DTT. The 4- or 5-HTMC concentration dependence on the extent of receptor inactivation was used in determining the apparent K_d's, which characterize the preliminary noncovalent interaction between 4- or 5-HTMC and the receptor.

Binding constants (K_d's) for 4- and 5-HTMC with the nAcChR were calculated for compounds III and IV and found to be 39 ± 17 and 224 ± 98 μM, respectively. These results represent the average calculated value ± standard deviation. The calculation of these equilibrium constants assumed rapid pre-equilibration of 4- or 5-HTMC with the nAcChR ($K_{-1} \gg K_{redox} + K_{inact}$) with rapid but rate-determining reduction of receptor and oxidation of catechol at the receptor surface leading to irreversible inactivation of toxin binding sites, as shown in Figure 9. In this scheme, the amount of the 4- or 5-HTMC derivative bound at equilibrium is essentially equivalent to the amount of receptor which eventually becomes inactivated. The calculated values were corrected by a factor of 2, assuming equal binding to the 50% of all toxin sites of the receptor where disulfide was not as easily reduced. These assumptions are consistent with the data observed in that a self-consistent equilibrium constant is calculated from each data point (the independently calculated K_d's cluster around an average value from the measurements undertaken over a several hundred fold range in reagent concentration). Catechol oxidation occurring away from the receptor surface before and after rapid establishment of the equilibrium with receptor was assumed to quantitatively partition the quinone products through formation of a covalent adduct with DTT. Thus, the determined ratio of the concentration of remaining toxin sites to inactivated toxin sites multiplied by the 4- or 5-HTMC concentrations employed gave an estimated equilibrium constant at every concentration measured. The possibility that some of the quinone product formed away from the receptor surface escapes trapping by DTT and that some DTT-induced reduction of receptor occurs allowing affinity-dependent reaction of quinone with receptor could not easily be taken into account quantitatively in the scheme used to calculate these apparent equilibrium constants. Thus, these calculated K_d's represent estimated composite values for the two complexes (illustrated in Figure 9), both of which were capable of carrying out the observed inactivation of the nAcChR. The narrow variation of values for the independently calculated K_d's determined over about a 50-fold concentration range taken together with the similarity of the K_d for III and IV to that directly measured for the less reactive TMC (K_d = 28 μM; Nickoloff *et al.*, 1985) suggested that the K_d values reported for III and IV with the nAcChR accurately reflected equilibrium behavior which could not be directly measured.

EXPERIMENTAL SECTION

General. NMR spectra were obtained in $CDCl_3$ on a Bruker AC-F 300 MHz instrument. Chemical shifts were expressed

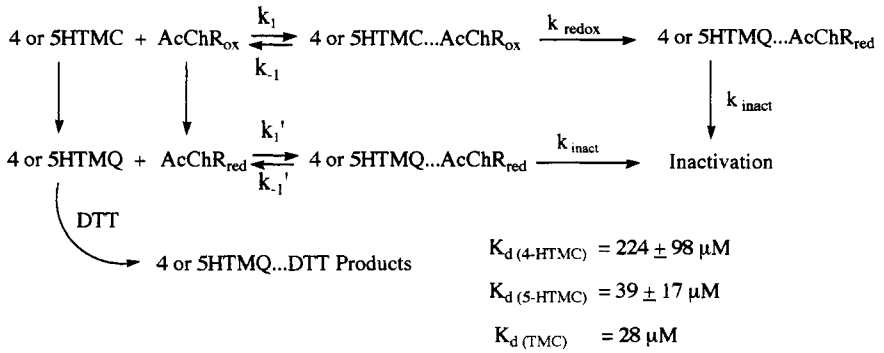

FIGURE 9: Proposed scheme for the concentration-dependent inactivation of neurotoxin sites of the n-AcChR by 4- or 5-HTMC.

in ppm downfield from internal tetramethylsilane. Coupling constants (J) were measured in hertz. For ^{13}C-NMR, attached proton tests (APT) were performed to distinguish different carbons. Ether was distilled from sodium benzophenone ketyl under nitrogen. Column chromatography was conducted using Florisil, 200 mesh. Melting points are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

2,3,6-Trimethoxybenzaldehyde (VIII). 1,2,4-Trimethoxybenzene (5 g, 0.03 mol) in 200 mL of dry tetrahydrofuran was cooled to 0 °C. *n*-Butyllithium (2 N in hexane, 15 mL) was added. The mixture was stirred at room temperature for 2 h and then cooled to −78 °C under dry ice–acetone. *N,N*-Dimethylformide (8.67 g, 0.12 mol) was added. The mixture was warmed to room temperature and stirred for 2 h. Hydrochloric acid (6 N, 50 mL) was slowly added and the solution stirred for 0.5 h. Tetrahydrofuran was removed by evaporation, and ether was added. The ether layer was separated and dried over magnesium sulfate. Evaporation gave a yellowish oil which was dissolved in hexane and passed over Florisil eluting with ethyl acetate:hexane (1:9). Evaporation of the product containing fractions *in vacuo* gave a colorless oil (4.15 g, 71% yield). ^{1}H-NMR ($CDCl_3$): δ 10.4 (s, 1H, CHO), 7.10 (d, 1H, J = 9.1 Hz), 6.66 (d, 1H, J = 9.1 Hz), 3.93 (s, 3H, OMe), 3.86 (s, 3H, OMe), 3.85 (s, 3H, OMe).

2,3,6-Trimethoxy-[(N,N-dimethylamino)methyl]benzene (IX). Compound VIII (1.96 g, 10 mmol) was mixed with 80 mL of an aqueous solution of dimethylamine (40%) and stirred overnight. Sodium borohydride (1.5 g, 31 mmol) was added and the solution stirred for 2 h. The solvent was evaporated *in vacuo* and cooled under ice bath while acidifying slowly with aqueous HCl (10%). After washing with ether twice, the acidic solution was neutralized with dilute NaOH and extracted subsequently with ether. The ether extract was dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give a yellowish oil: (1.52 g, 6.5 mmol, 67% yield). ^{1}H-NMR ($CDCl_3$): δ 6.81 (d, 1H, J = 8.9 Hz), 6.58 (d, 1H, J = 8.9 Hz), 3.84 (s, 3H, OMe), 3.82 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.48 (s, 2H), 2.59 (s, 6H).

2,3,6-Trimethoxy-[(N,N,N-trimethylammonio)methyl]benzene Iodide (X). Compound IX (1.12 g, 5 mmol) was dissolved in ether, and iodomethane (1 g, 6.9 mmol) was added. The solution became turbid, and a yellowish oil precipitated. The oil was taken up in ether and the ether solution extracted with water. Evaporation of the final water extract gave a glassy semisolid (1.7 g, 4.5 mmol, 90% yield). ^{1}H-NMR (D_2O): δ 7.15 (d, 1H, J = 9.2 Hz), 6.79 (d, 1H, J = 9.2 Hz), 4.38 (s, 2H), 3.74 (s, 3H, OMe), 3.70 (s, 6H, 2xOMe), 2.93 (s, 9H, 3xMe).

2,3,6–Trihydroxy-[(N,N,N–trimethylammonio)methyl]benzene Bromide (5-HTMC, IV). Compound X (1 g, 2.6 mmol) was refluxed for 3 h in a mixture of 35% HBr aqueous solution (15 mL) and HOAc (10 mL) under an argon atmosphere. The solvents were evaporated *in vacuo*, and the residue was crystallized from acetone (0.58 g, 2.0 mmol, 77% yield). ^{1}H-NMR (D_2O): δ 6.79 (d, 1H, J = 8.7 Hz), 6.28 (d, 1H, J = 8.7 Hz), 4.34 (s, 2H), 2.94 (s, 9H). ^{13}C-NMR (D_2O): δ 151.5 (C), 147.4 (C), 138.2 (C), 120.2 (CH), 107.4 (CH), 105.5 (C), 60.1 (CH_2), 53.8 (CH_3). Anal. Calcd for $C_{10}H_{16}O_3NBr \cdot {}^1/_4H_2O$: C, 42.48; H, 5.84; N, 4.95. Found: C, 42.58; H, 5.88; N, 4.67.

2-Hydroxy-3-methoxy-5-bromobenzaldehyde (XII). *o*-Vanillin (XI) (6.0 g, 40 mmol) was added in small portions to 200 mL of aqueous solution containing bromine (2.4 mL) and potassium bromide (16 g). The mixture was stirred at room temperature for 2 h. Methanol (50 mL) was added, and the solution was warmed to 50–60 °C. A pale solid (4.0 g) precipitated and was collected by filtration. From the mother liquor, an additional 1.8 g of yellowish solid was obtained (5.8 g, 25 mmol, 62% yield, mp 136–138 °C). ^{1}H-NMR ($CDCl_3$): δ 11.03 (s, 1H, CHO), 9.86 (s, 1H, OH), 7.32 (d, 1H, J = 2.1 Hz), 7.18 (d, 1H, J = 2.1 Hz, 3.92 (s, 3H, OMe).

2,3-Dimethoxy-5-bromobenzaldehyde (XIII). Compound XII (1 g, 4.3 mmol) was refluxed in 100 mL of dry THF containing dimethyl sulfate (0.3 g, 24 mmol) and anhydrous potassium carbonate (3 g) for 6 h. The reaction mixture was filtered and the filtrate evaporated to give a yellowish solid. Recrystallization from ether–hexane afforded white crystals (0.99 g, 4.0 mmol, 93% yield, mp 96–97 °C). ^{1}H-NMR ($CDCl_3$): δ 10.34 (s, 1H, CHO), 7.54 (d, 1H, J = 2.3 Hz), 7.23 (d, 1H, J = 2.3 Hz), 3.98 (s, 3H, OMe), 3.91 (s, 3H, OMe).

2,3-Dimethoxy-5-bromo-[(N,N-dimethylamino)methyl]benzene (XIV). This compound was prepared by the same procedure given for compound IX. From 0.5 g (2.0 mmol) of compound XIII, 0.35 g (1.3 mmol, 65% yield) of an oil was obtained. ^{1}H-NMR ($CDCl_3$): δ 7.15 (d, 1H, J = 2.3 Hz), 6.94 (d, 1H, J = 2.3 Hz), 3.85 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.42 (s, 2H), 2.25 (s, 6H, 2xMe).

2,3,5-Trimethoxy-[(N,N,N-trimethylammonio)methyl]benzene Iodide (XV). Sodium (0.3 g, 12 mmol) was dissolved in 30 mL of methanol. Compound XIV (0.3 g, 1 mmol) and CuI (0.38 g, 3 mmol) in DMF (10 mL) was added. The mixture was refluxed overnight under a nitrogen atmosphere. The greenish solution turned brown with some precipitation which was filtered. The filtate was evaporated, and water and ether were added. The ether solution was dried over magnesium sulfate, and methyl iodide (0.2 g, 1.3 mmol) was added. A white precipitate was formed and was filtered and washed with cold acetone (0.32 g, 0.81 mmol, 81% yield, mp 215–216 °C). ^{1}H-NMR (D_2O): δ 6.72 (d, 1H, J = 2.7 Hz), 6.48 (d, 1H, J = 2.7 Hz), 4.29 (s, 2H), 3.74 (s, 3H, OMe),

3.67 (s, 3H, OMe), 3.62 (s, 3H, OMe), 2.93 (s, 9H, 3xMe).

5-Hydroxy-3-[(trimethylammonio)methyl]catechol Bromide (4-HTMC, III). Compound XV (0.5 g, 1.3 mmol) was mixed with HBr (30 mL, 48% aqueous solution) and refluxed for 8 h under argon gas. The solvent was evaporated *in vacuo*. Recrystallization of the resulting solid from acetone and Et_2O gave white crystals (0.38 g, 1.1 mmol, 86% yield). ^{1}H-NMR (D_2O): δ 6.49 (d, 1H, J = 2.9 Hz), 6.31 (d, 1H, J = 2.9 Hz), 4.28 (s, 2H), 2.95 (s, 9H). ^{13}C-NMR (D_2O): δ 150.4 (C), 147.7 (C), 140.4 (C), 117.6 (C), 112.4 (CH), 107.7 (CH), 65.5 (CH_2), 54.2 (CH_3). Anal. Calcd for $C_{10}H_{16}O_3NBr$: C, 43.16; H, 5.41; N, 5.03. Found: C, 43.36; H, 5.76; N, 5.16.

General Procedure for Trapping Oxidation Products of TMC, 4-HTMC, and 5-HTMC with Cyclopentadiene. TMC, 4-HTMC, or 5-HTMC (0.5 mmol) was dissolved in deuterated water (1 mL). Excess of cyclopentadiene (0.1 mL) was added to the solution followed by the addition of sodium periodate (11 mg, 0.5 mmol). Cyclopentadiene was prepared by heating dicyclopentadiene at 170 °C and collected after distillation through a 1 m vigreaux column. The mixture was stirred well for 2–5 min. In the case of TMC, a yellowish solution was formed, while a dark red solution resulted in the case of 4-HTMC or 5-HTMC. Excess cyclopentadiene was removed from the reaction mixture extraction with methylene chloride twice (2 × 2 mL). The deuterated water phase of each reaction was examined by NMR. *Cycloadduct XVIa*: ^{1}H-NMR δ 7.8 (d, 1H, J = 4.4 Hz), 6.0 (q, 1H, J = 5.6, 2.9 Hz), 5.9 (q, 1H, J = 5.6, 2.9 Hz), 4.1 (d, 1H, J = 13.2 Hz), 4.0 (d, 1H, J = 13.2 Hz), 3.6 (m, 1H), 3.35–2.7 (m, 3H), 2.93 (s, 9H), 1.54–1.45 (m, 2H). *Cycloadduct XVIb*: ^{1}H-NMR δ 7.3 (d, 1H, J = 4.8 Hz), 5.8 (q, 1H, J = 2.9, 5.6 Hz), 5.7 (q, 1H, J = 2.9, 5.6 Hz), 4.2 (d, 1H, J = 13.1 Hz), 3.7 (d, 1H, J = 13.1 Hz), 3.4–2.7 (m, 4H), 2.91 (s, 9H), 1.4–1.3 (m, 2H). *Cycloadduct XVII*: ^{1}H-NMR δ 5.9 (m, 2H), 4.1 (m, 2H), 3.4–3.2 (m, 4H), 2.9 (s, 9H), 1.4 (m, 2H). *Cycloadduct XVIII*: ^{1}H-NMR δ 5.94 (q, 1H, J = 5.3, 2.8 Hz), 5.86 (q, 1H, J = 5.3, 2.8 Hz), 4.25 (d, 1H, J = 13.8 Hz), 3.55 (d, 1H, J = 13.8 Hz), 3.45 (d, 1H, J = 3.5 Hz), 3.31 (br s, 1H), 3.08 (br s, 1H), 2.88 (s, 9H), 1.60, (d, 1H, J = 9.6 Hz), 1.38 (d, 1H, J = 9.6 Hz).

Acetylcholine Receptor (AcChR): Purification and [^{125}I]-Bungarotoxin Assay. AcChR was purified from the electric organ of *Torpedo californica* (Pacific Biomarine, Venice, CA) by cobratoxin-affinity chromatography of the Triton X-100 solubilized electroplax using procedures given earlier (Nickoloff *et al.*, 1985; Wohlfeil & Hudson, 1991). The neurotoxin was purified by the method of Karlsson *et al.* (1971) from the lyophillized venom of the Thailand cobra, *Naja naja siamensis* (obtained from the Miami Serpentarium, Miami, FL). Purification of the nAcChR was followed by observed increases in [^{125}I]-α-bungarotoxin ([^{125}]-α-Btx) binding sites/mg of protein (Schmidt & Raftery, 1973), by SDS–PAGE on 7% acrylamide gels, and by separation from acetylcholine esterase, which was followed by the procedure of Ellman (1961). [^{125}I]-α-Btx was purchased from New England Nuclear. Protein was determined by the method of Lowry *et al.* (1978). The specific activity for most receptor preparations used in these experiments was 4.4 nM toxin sites/mg of protein. However, our preparations ranged from 4 to 7 nM toxin sites/mg of protein. Theory is usually considered to be about 8 nM toxin sites/mg of protein, although values of 8–12 have been reported. These higher values have been considered to represent partial proteolysis of receptor without loss of toxin binding capacity.

Spectroelectrochemistry and Cyclic Voltammetry. 2-Hydroxy-3-[(trimethylammonio)methyl]-*p*-benzoquinone and 2-Hydroxy-6-[(trimethylammonio)methyl]-*p*-benzoquinone were generated by oxidation of 4- and 5-HTMC in pH 3 buffer with 1 equiv of ceric ammonium nitrate in unbuffered nitric acid (pH 3) or in sodium iodate at neutral pH. The decay of the characteristic hydroxy-*p*-quinone spectrum was measured at 460 nm using a Varian DMS 200 spectrophotometer. The pH of solutions was measured with a Radiometer 26 pH meter. Stock solutions of ceric ammonium nitrate or sodium iodate were freshly prepared. Absorption spectra were obtained under a variety of conditions and compared with spectra of model hydroxy-*p*-quinones.

Voltammetric measurements were conducted with a Bioanalytical Systems Inc. (BAS) 100 B electrochemical analyzer, which was interfaced to a Gateway 386 PC and a Houston Instruments DMP-40 digital plotter. For the spectroelectrochemical measurements, potentials were applied with a BAS Model C-27 potentiostat and monitored with a Keithley 197A multimeter. The working electrode for voltammetry was a glassy carbon disk electrode (GCE, BAS), while the spectroelectrochemical measurements used an optically transparent thin-layer electrode (OTTLE). The OTTLE was constructed with 100 wires/in. of gold minigrid by the method of DeAngelis and Heineman (1976). An aqueous Ag/AgCl (3 M NaCl) electrode (BAS) and a platinum wire were used as reference and auxiliary electrodes, respectively. Absorption spectra were recorded on a Varian-Cary 5E UV–vis–NIR spectrophotometer. The cell compartment was modified to accommodate the electrical leads and an inert gas inlet for spectroelectrochemical experiments. All solutions were prepared from distilled and deionized water, which was purified to a resistivity of at least 18 MΩ cm by a Barnstead Organic pure water system. Reagent grade potassium nitrate was used as the supporting electrolyte without further purification. No significant electroactive impurities were observed in either the solvent or the supporting electrolyte.

Rate and Extent of Inactivation of α-Bungarotoxin Sites of the Acetylcholine Receptor by 4- and 5-HTMC. Determination of the Equilibrium Constant between 4- or 5-HTMC and the nAcChR. Dilute solutions of purified nAcChR previously titrated with known concentrations of [^{125}I]-α-Btx were adjusted to contain 1–2 pmol of toxin sites. Either 2-mercaptoethanol or dithiothreitol (DTT) prepared in receptor buffer (0.1% Triton X-100, 10 mM sodium phosphate, pH 7.4) was added in proportional amounts to the receptor solutions together with varying concentrations of 4- or 5-HTMC. Final concentrations of 4- or 4-HTMC varied from 1.3 mM to 1.3 μM, while DTT concentrations were adjusted to reflect a 10-fold excess over the corresponding 4- or 5-HTMC concentration. The 4- or 5-HTMC solutions were prepared fresh in concentrated form in pH 2.2 at room temperature and aliquoted to give the appropriate concentrations in receptor buffer. The nAcChR solutions were incubated alone or in the presence of added 4- or 5-HTMC–DTT in the receptor buffer. The receptor was also incubated alone as a control or with DTT or mercaptoethanol in the absence of 4- or 5-HTMC. The total reduction of toxin binding sites in these receptor solutions was determined by the [^{125}I]Btx procedure of Schmidt and Raftery (1973) after dialysis. Nonspecific binding of radiolabeled toxin in receptor buffer controls was subtracted from the measured counts observed in the presence of receptor and under various conditions with 4- or 5-HTMC present. To minimize the tendency of toxin to stick to plastic and glass, all toxin containers were coated with 1 mg/mL BSA (bovine serum albumin). In general, we carried out dialysis for 2 h. During this time, two changes of buffer were made. In controls, we showed that more

extensive dialysis did not change the results. The amount of radiolabeled toxin adhering to washed disks was the same as that in undialyzed and dialyzed buffer controls to which neither reagents nor receptor had been added. Also, when solutions of 4- or 5-HTMC and DTT were incubated alone in the absence of receptor and for various times in the presence of receptor, 2 h of dialysis with two exchanges of buffer was sufficient to remove all inhibiting substances; that is, after dialysis addition of receptor and [^{125}I]-α-BTX under the usual conditions, no inhibition of toxin binding to the receptor was observed.

The extents of toxin binding inactivation thus determined were used as a measure of the extent of 4- or 5-HTMC binding to receptor in the preequilibrium step. Thus, the ratio of the remaining active receptor to inactive receptor could be calculated and, when multiplied by the 4- or 5-HTMC concentration employed, gave an estimate of equilibrium constant after correction by a factor of 2. The latter assumed that the half-of-sites of receptor, not easily inactivated by the reagents, was capable of binding to reagent.

DISCUSSION

Covalent modification of proteins *via* quinones derived from catechols is well known (Sauer & Thoenen, 1971; Gupta & Vitayathil, 1980). These critical reactions underly melanogenesis [*cf.* review by Pawelek and Korner (1982)], the hardening of barnacle and other arthropod secretions (Lindler *et al.*, 1973), aspects of the selective catecholaminergic neuronal toxicity associated with 6-hydroxydopamine (Jonsson, 1981), and the initiating antigenic reactions in poison ivy-induced delayed-type hypersensitivity (Symes & Dawson, 1954). The importance of reactions of quinones in biological systems has been discussed recently by Peter (1989).

Examples of the covalent inactivation of enzymes and other protein active sites through catechol oxidation include tyrosinase (Duckworth & Coleman, 1970), DNA polymerase (Wick & Mui, 1980; Fitzgerald & Wick, 1983), catechol-*o*-methyltransferase (Borchardt & Bhatia, 1982), and the α-bungarotoxin binding site in the nicotinic acetylcholine receptor (Nickoloff *et al.*, 1985). The relatively clean half-of-sites inactivation of the nicotinic acetylcholine receptor using the reagent TMC was suggested to proceed through an affinity redox-initiated reaction pathway once early hydroxylated intermediates had formed. Although inactivation of half-of-sites of the receptor appears to be saturated in the 1–3 mM range for 4- or 5-HTMC, it was possible to go past half-of-sites inactivation using higher concentrations and/or longer incubation times. Indeed, this behavior was observed earlier using a mixture of the reagents employed here one at a time (Nickoloff *et al.*, 1985). Thus, it was clear that the first site was more sensitive to inactivation and was essentially cleanly titrated with 4- or 5-HTMC.

The present investigation has demonstrated the concentration-dependent inactivation of neurotoxin binding sites by 4- or 5-HTMC, the proposed intermediates in our earlier study demonstrating half-of-sites inactivation of neurotoxin sites in the nAcChR by TMC. These observations were consistent with a direct role for these agents as intermediates in the affinity-dependent redox-initiated inactivation of neurotoxin binding sites of the nAcChR by TMC (Nickoloff *et al.*, 1985) and thereby supported our earlier mechanistic proposal involving these hydroxylated derivatives. While the independently synthesized 4- and 5-HTMC possessed somewhat different concentration dependence in their inactivation of neurotoxin binding sites of the receptor, the quantitative differences were minor and certainly reflected their involvement as a mixture *in situ* in the toxin binding site inactivating reactions of TMC.

Redox-dependent inactivation of protein sites using catechol oxidation as a theme may be thus less ambiguously examined by directly utilizing trihydroxybenzenes analogous to 4- or 5-HTMC rather than dihydroxybenzenes analogous to TMC. Slow autooxidative formation of the more reactive hydroxylated intermediates capable of interacting with protein sites will clearly complicate the use of the dihydroxybenzene-based reagents analogous to TMC in affinity labeling reactions. Further, 4- and 5-HTMC and analogous agents have the capacity to redox inactivate functionality such as disulfides at proteins sites not normally labeled by affinity reagents. The greater ease of spontaneous oxidation of the trihydroxybenzene (*cf.* the dihydroxybenzene) makes the relatively more stable *p*-quinone (*cf. o*-quinone) form of the agent more directly accessible and also a more probable intermediate in these affinity-dependent reactions. Indeed, TMC appears to be unable to directly reduce the disulfide present at the neurotoxin site, whereas the 4- or 5-HTMC derivatives can. The oxidized forms of these affinity reagents will react easily with thiols and amines present at sites to which they are affinity directed.

In the present example, we believe the 4- or 5-HTMC reagent reduces the easily reduced disulfide (at half-of-all-sites) with a subsequent rapid reaction of the newly formed *p*-quinone at the site with the newly formed receptor thiol. It has been shown that the cysteinyl SH group is the strongest of nucleophiles reacting with quinones (Tse *et al.*, 1975). We could exclude the possibility that preliminary formation of the quinone from the 4- or 5-HTMC oxidation reaction may also occur with the reduced form of the receptor, formed through reaction with added thiols or with low concentrations of DTT (Damle & Karlin, 1978). Thus, a degree of versatility as well as complexity in these reagents may be achieved in the site-directed alkylation using these trihydroxybenzene-based reagents as a proreactive redox-activated element, or the corresponding *p*-quinone as a directly reactive moiety, in the affinity-dependent reactions.

The site-directing functionality employed here, a (trimethylammonio)methyl function, was important in two ways. First, it was the principal basis for the affinity of the reagents to the nAcChR, and it had to be stabily integrated into the trihydroxybenzene structure. Second, it had to be unreactive intramolecularly toward the *p*-quinone structure which was an obligatory intermediate for both the direct and indirect affinity labeling reactions considered above. While the trimethylammonium functional group was unreactive toward quinones, it remained possible that trimethylamine elimination could occur with the formation of quinone methides. This reaction did not occur and generally would not be expected to occur under the mild conditions employed in the use of these reagents as affinity ligands for macromolecular targets.

Reactive quinone methides may be formed when the alternative formation of *o*- or *p*-quinones is not as likely as it is in the present case (Peter, 1989). In substrates, appropriately substituted to afford the generation of quinone methides, their role in affinity-dependent reactions at biological sites of interest may be investigated (*cf.* Skibo, 1992). Here, it was important to demonstrate whether any portion of the oxidation of 4- or 5-HTMC or of the initial oxidation of TMC to the *o*-quinone was partitioned through the quinone methide. Two observations bear on this point. First, essentially quantitative retention of the trimethylammonium group was observed in the structure of the products formed through reaction with cyclopentadiene. Second, in our original observations of half-of-sites inactivation of toxin binding site to the nAcChR with TMC, receptor was labeled with [^{3}H]TMC to the extent expected given the level of toxin binding site inactivation (Nickoloff *et al.*, 1985). As

tritium was incorporated into one of the methyls of the (trimethylammonio)methyl substituent, retention of the intact (trimethylammonio)methyl function was thus demonstrated.

In summary, we have characterized in some detail the mechanistically complex reactions of TMC as they affect the course of the redox-dependent affinity-directed half-of-sites inactivation of curarimimetic toxin sites of the nAcChR. While these results provide no further insight into the nature of these sites in the receptor, the course of the redox-dependent affinity reactions of this interesting class of reagents is now better understood and the groundwork for the application of the chemistry in affinity-dependent redox reactions with other macromolecular targets has been established.

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