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Increased Agonist Affinity Is Induced in Tetranitromethane-Modified Muscarinic Receptors

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ABSTRACT: Tetranitromethane (TNM) modifies the muscarinic receptors from rat cerebral cortex. The modified receptor possesses an increased binding affinity (6–9-fold) toward several agonists such as acetylcholine, carbamoylcholine, arecoline, etc. The binding of antagonists (B_{max} and K_d) is only slightly altered. The effects of TNM treatment can be prevented by atropine, thus indicating that TNM modifies residue(s) at the binding site. We carried out a series of successive chemical modifications which indicated that the modified residue(s) is (are) most probably a tyrosyl and not a cysteinyl residue. This conclusion gains support from the pH profile of agonist binding, which suggests the involvement of a residue with an apparent pK comparable to that of the phenolic hydroxyl of a nitrotyrosyl residue. The binding properties of the modified receptor, when compared to those of the native one, clearly indicate that the response to TNM modification with respect to the binding of agonists such as acetylcholine and carbamoylcholine is different from that when oxotremorine and its analogue are employed. This is interpreted as being the result of different binding modes exhibited by the various agonists. Nitration of the receptors can be prevented by the presence of an antagonist but not by an agonist. We propose that this differential response is due to the formation of ligand–receptor complexes that differ with respect to the microenvironment of the modified tyrosyl residue.

Previous studies on ligand binding to muscarinic receptors have demonstrated that the binding of antagonists from the benzilate or tropate classes yields binding isotherms that fit a homogeneous population of binding sites [reviewed in Sokolovsky (1984)]. Studies on the binding of agonists, on the other hand, point to the existence of a heterogeneous population of binding sites that differ in their affinities toward agonists [for review, see Ehlert et al. (1982), Birdsall & Hulme (1983), and Sokolovsky (1984)]. It thus appears that agonist binding is the more sensitive to the state of the receptor.

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Chemical modification of functional groups in receptors for neurotransmitters and hormones can shed light on the structure of the receptor binding site(s). The binding properties of muscarinic receptors can be altered either by blocking a sulfhydryl residue(s) or by sulfhydryl–disulfide transformations (Aronstam et al., 1978; Hedlund & Bartfai, 1979; Wei & Sulakhe, 1980; Ehlert et al., 1980; Ikeda et al., 1980; Abd-Elfattah & Shamoo, 1981; Aronstram & Carrier, 1982; Vanquelin et al., 1982; Harden et al., 1982; Uchida et al., 1982; Birdsall et al., 1983; Gurwitz et al., 1984a). Tetranitromethane (TNM)[1] has been shown to be a convenient reagent for the nitration of tyrosyl residues in proteins (Riordan et al., 1966; Sokolovsky et al., 1966; Riordan & Sokolovsky, 1971a). We therefore employed TNM in an effort to investigate whether tyrosyl residues play a role in the binding of agonists and/or antagonists to brain muscarinic receptors. We report here that low TNM concentrations modify rat cortex membranes, resulting in an increased affinity for the binding of certain agonists while affecting antagonist binding only very slightly. TNM is not totally selective for tyrosine. Previous specificity studies have shown that other residues, in particular, sulfhydryl groups, are potentially reactive toward TNM (Riordan et al., 1966; Sokolovsky & Riordan, 1970). We therefore carried out a series of successive chemical modifications of the membranal receptor, which enabled us to report here that the functional changes observed upon treatment of cortical membranes with TNM are unlikely to be a result of oxidation of a cysteinyl residue(s) but are most probably related to nitration of a tyrosyl residue(s). Our results strongly suggest that tyrosine is involved in the binding of agonists to the high-affinity binding site of the muscarinic receptors in the brain.

Materials and Methods

Materials. [^{3}H]AcCh and [^{3}H]oxotremorine M of high specific radioactivities (86 and 80 Ci/mmol, respectively, 98% purity) were purchased from Amersham. Small aliquots of the radiochemicals in ethanol/water (1:1 v/v) were kept at −70 °C and subjected to drying by a gentle stream of nitrogen prior to use. [^{3}H]-*N*-Methyl-4-piperidyl benzilate (4-NMPB, 70 Ci/mmol, 97% purity) was the same preparation described and used previously (Gurwitz et al., 1984a). Tetranitromethane was purchased from Fluka. Most of the reagents and drugs tested were purchased from Sigma, except for (−)-quinuclidinyl benzilate [(−)-QNB] and (+)-quinuclidinyl benzilate [(+)-QNB] which were prepared as described elsewhere (Rehavi et al., 1977).

Tissue Preparation. Adult male rats of the CD strain were obtained from Levinstein's Farm (Yokneam, Israel) and maintained in an air conditioned room at 24 ± 2 °C for 14 h (0500–1900) under fluorescent illumination and in darkness for 10 h. Food from Assia Maabarot Ltd. (Tel Aviv, Israel) and water were supplied ad libitum. Rats aged 3–4 months and weighing 190–250 g were decapitated (between 0900 and 1000 h), and their cerebral cortices were immediately dissected out in a cold room and immersed in cold 50 mM Tris-HCl buffer, pH 7.4 (buffer A). Tissue (pooled from two or three rats) was homogenized in 50 volumes of buffer A by using a glass–Teflon homogenizer (10 strokes). The homogenates were incubated for 30 min at 25 °C in buffer A with gentle shaking and then centrifuged at 30000*g* for 15 min. This procedure was repeated twice.

Nitration of Membranes. Freshly prepared membranes were resuspended in 50 mM Tris-HCl buffer, pH 8.1, to yield a concentration of 0.5 mg of protein/mL. TNM (in ethanol) was added to yield the final concentrations indicated in Figures 1 and 4, and the preparations were incubated at 25 °C for 20 min. The reaction was stopped by washing the membranes twice in buffer A (30000*g* for 15 min). Control membranes were subjected to the same procedure, but ethanol alone was added (1% during preincubation); ethanol pretreatment had no effect on any of the parameters measured. In some experiments treatment with sulfhydryl agents (2 mM diamide or 10 mM DTT) preceded or followed the nitration step. These treatments were also carried out at 25 °C, pH 8.1, for 20 min and were separated from the nitration step by washing of the membranes in buffer A.

Nitration of Tissue Slices. In some experiments freshly prepared rat cerebral cortex slices [prepared according to Gonzales & Crews (1984)] were subjected to TNM treatment. Cerebral cortices (pooled from two rats) were sliced to a thickness of 200 μM in two perpendicular directions by using a Sorvall tissue chopper. The minced tissue was rapidly transferred to a flask containing Krebs–bicarbonate buffer at 37 °C and dispersed. The tissue was subjected to continuous gentle agitation while O_2/CO_2 (19:1) was bubbled through the flask. Following dispersion the slices were washed twice with fresh buffer and then treated with TNM (300 μM) for 20 min at 37 °C. The reaction was stopped by changing the buffer 3 times (separated by 10-min incubations with gentle agitation). Following the washing step, the slices were homogenized in buffer A as described under Tissue Preparation, and the membranes thus prepared were subjected to binding assays.

After completion of the above procedures, the final pellets were resuspended in modified Krebs buffer containing 25 mM Tris-HCl, 118 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM $MgCl_2$, and 1.9 mM $CaCl_2$ (pH 7.4, 25 °C). A fresh solution of diisopropyl fluorophosphate (DFP; Sigma lot 93F-0101) in water was added to the homogenate to achieve a concentration of 200 μM. The homogenate was incubated for a further 20 min at 25 °C prior to binding assay.

Equilibrium [^{3}H]AcCh Binding Assays (Gurwitz et al., 1984b). Aliquots (20 μL) of freshly prepared membranes were added to tubes containing 20 μL of modified Krebs buffer, 200 μM DFP, and various concentrations of [^{3}H]AcCh. After 1 h of incubation with gentle shaking at 25 °C, 3 mL of ice-cold modified Krebs buffer was added, and the contents of the tubes were filtered under high pressure through GF/C filters (Whatman; 25-mm diameter). The filters were immediately washed with an additional 3 mL of buffer. Filters were counted for tritium by using a scintillation cocktail (Hydroluma) and a scintillation spectrometer (LKB-1218) at 48% efficiency. An incubation period of 1 h was chosen, since after this period even the lowest concentration of [^{3}H]AcCh employed has reached equilibrium. Ice-cold buffer was used for washes to prevent dissociation of [^{3}H]AcCh from the receptors, since at 0 °C the dissociation half-time was relatively slow (90 s) (Gurwitz et al., 1984b).

All determinations were carried out in quadruplicate and varied by less than 15%.

pH Profile Studies of [^{3}H]AcCh Binding. Pellets were resuspended in the same medium as that described under the preceding subheading, except that Tris was omitted. Aliquots

[1] Abbreviations: TNM, tetranitromethane; AcCh, acetylcholine; 4-NMPB, *N*-methyl-4-piperidyl benzilate; QNB, 3-quinuclidinyl benzilate; OXO-M, oxotremorine M; DFP, diisopropyl fluorophosphate; Gpp(NH)p, guanylyl imidodiphosphate; diamide, 1,1′-azobis(*N*,*N*-dimethylformamide); DTT, 1,4-dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

(20 μL) were added to tubes containing 20 μL of modified Krebs buffer including 50 mM Tris, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 200 μM DFP, and 48 nM [^{3}H]AcCh, and the mixtures were titrated with NaOH to yield the desired pH. Incubation and filtration were as described. Nonspecific binding was determined separately for each pH value and subtracted from the total binding to yield the specific binding of [^{3}H]AcCh.

Kinetic Studies of [^{3}H]AcCh Binding. The association kinetics of specific [^{3}H]AcCh binding were studied by employing the same incubation conditions described under Equilibrium [^{3}H]AcCh Binding Assay except that the reaction was stopped by filtration following the various incubation periods at 25 °C. Nonspecific binding was determined separately for each incubation period and subtracted from the corresponding total binding to yield the specific binding of [^{3}H]AcCh at time *t*. Dissociation kinetics of specific [^{3}H]-AcCh binding were studied by preincubating the membranes with 12 nM [^{3}H]AcCh for 1 h at 25 °C, then adding 10 μM atropine (10 μL of 50 μM atropine), and incubating the membranes at 25 °C for a further period indicated in Figure 6 prior to filtration.

Equilibrium [^{3}H]Oxotremorine M Binding Assay. For this ligand, the procedure outlined for [^{3}H]AcCh binding was followed, except that DFP was omitted.

Equilibrium [^{3}H]-4-NMPB Binding. Aliquots (20 μL) of the membrane preparation were incubated for 60 min at 25 °C with various concentrations of [^{3}H]-4-NMPB in 1 mL of modified Krebs buffer. Assays were terminated by filtration through GF/C filters and washing 3 times with 4 mL of ice-cold buffer. Nonspecific binding was determined with 10 μM atropine. These assays were routinely carried out in parallel with [^{3}H]AcCh binding assays in the same preparations.

Protein Determination. Protein concentration was determined according to Lowry et al. (1951) with bovine serum albumin as a standard.

Data Analysis. Results of binding experiments are presented as mean values or means ± one standard deviation. Values for maximal binding capacity (B_{max}) and dissociation constants (K_d) were obtained by linear regression analysis of binding isotherms. The inhibition constants (K_i) for unlabeled ligands were calculated from the competition assays with [^{3}H]AcCh according to the equation $K_i = I_{50}/(1 + L/K_d)$, in which I_{50} is the drug concentration inhibiting half the specific binding of [^{3}H]AcCh (at concentration L) to the muscarinic receptor and K_d is the dissociation constant for [^{3}H]AcCh determined as described.

Results

Modification of Muscarinic Receptors by TNM. The effects of various concentrations of TNM on the binding properties of antagonist [^{3}H]-4-NMPB and of agonist [^{3}H]AcCh to muscarinic receptors in the rat cortex homogenates were examined. As shown in Figure 1, low concentrations of TNM bring about a selective change in the binding of the agonist and only a small effect on the binding of the antagonist. As the concentration of TNM is increased, the binding of [^{3}H]-AcCh is increased by 2.5-fold. At a concentration of TNM higher than 100 μM, binding of agonist is greatly reduced and is abolished at 10 mM. On the other hand, binding of the tritiated antagonist is reduced by only 15% of that of the control value at 100 μM TNM and is drastically reduced at higher concentrations. We therefore selected TNM concentration of 100 μM, the concentration that induces the maximal changes in agonist binding (20 min, pH 8.1, 50 mM Tris buffer) for all subsequent modifications of the cortical membranes. The effects were not reversed by extensive washing.

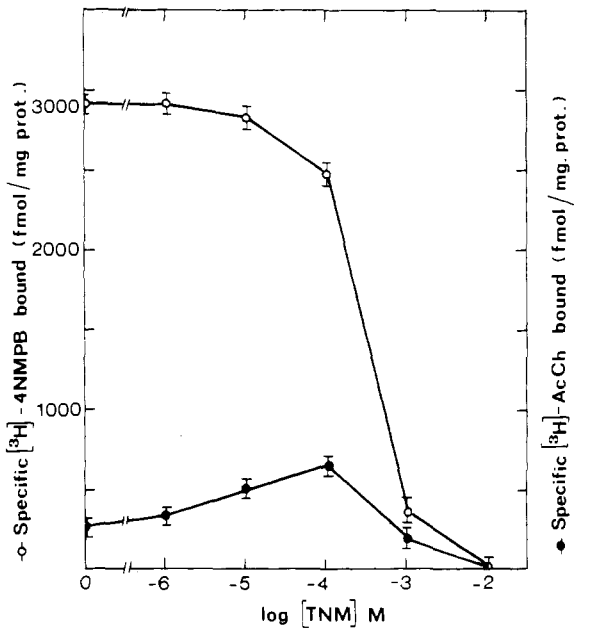

FIGURE 1: Effect of TNM pretreatment on muscarinic receptor binding to rat cerebral cortex membranes. Hypotonically washed membranes (0.5 mg of protein/mL) were pretreated for 20 min at 25 °C with the indicated concentrations of TNM at 50 mM Tris-HCl, pH 8.1. Pretreated membranes were subsequently washed twice (see Materials and Methods) and assayed for the specific binding of 25 mM [^{3}H]-4-NMPB (○) or 24 nM [^{3}H]AcCh (●). Values represent mean ± standard deviation of a typical experiment carried out in parallel for the two ligands.

It should be noted that the B_{max} values obtained from direct binding of [^{3}H]AcCh are significantly lower than those observed for antagonist binding in the same tissue preparations (Gurwitz et al., 1984b, 1985). This is due to the fact that [^{3}H]AcCh sites measured in the direct binding experiments represent the population of high-affinity agonist binding sites. Thus, in tissues enriched with low-affinity sites, i.e., cortex, hippocampus, and striatum, the density of [^{3}H]AcCh site is 20–25% of that of ^{3}H-labeled antagonist sites, whereas in tissues enriched with high-affinity sites, i.e., brain stem, cerebellum, and atrium, the density of [^{3}H]AcCh sites is 45–71% (Gurwitz et al., 1985). Moreover, under our experimental conditions [^{3}H]AcCh binds to a homogeneous population of sites, which are the high-affinity muscarinic agonist binding sites (Gurwitz et al., 1984a,b, 1985). Unlike the labeled neurotransmitter, the synthetic agonist oxotremorine M exhibits binding to super-high- and high-affinity sites (Birdsall et al., 1980; see Discussion).

The binding characteristics of the resulting modified receptor are shown in Figures 2 and 3. Scatchard plots of the specific binding of [^{3}H]-4-NMPB are linear and indicate a decrease of about 15% in the binding capacity of the ligand, accompanied by an increase in the K_d value from 0.4 (control) to 0.82 nM (modified receptor). As shown in Figure 3 (left), Scatchard plots of the binding data of [^{3}H]AcCh are linear for both the control and the modified receptor, while Hill coefficients of the saturation isotherms are close to unity, indicating that, over the concentration range investigated (2–160 nM), the labeled acetylcholine binds to a single class of sites. Binding was characterized by a dramatic increase of about 8-fold in the affinity, i.e., K_d = 40 nM for the control as compared to K_d = 5 nM for the modified receptors (Table I). This effect was accompanied by only a slight increase in its binding capacity (Figure 3, left, and Table I). It is im-

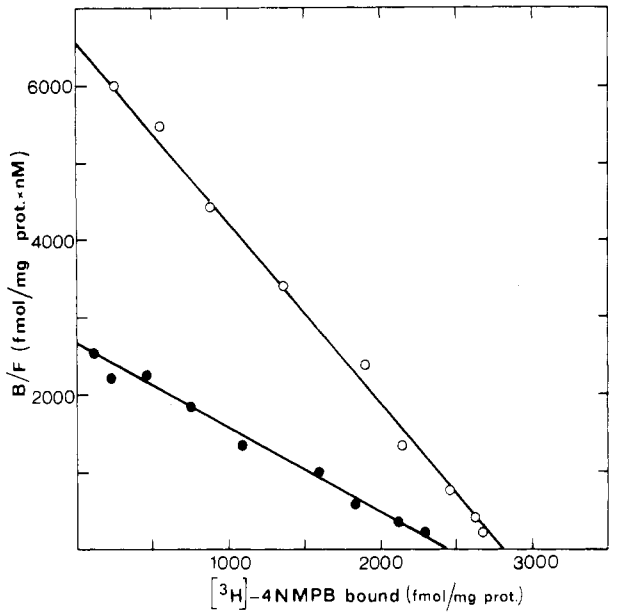

FIGURE 2: Scatchard plot of binding isotherms for [^{3}H]-4-NMPB to control (○) and 0.1 mM TNM pretreated (●) rat cerebral cortex membranes. See Materials and Methods for details. Data represent a typical experiment, carried out in triplicate, agreeing within 10% and employing 64 μg of protein per tube.

portant to note that 1 μM atropine prevented the changes induced by TNM modification (Table I), indicating that TNM indeed modifies residues at the binding site. Moreover, as shown in Figure 4, the dependence of the dissociation constant of binding of ^{3}[H]AcCh to membranes modified with various concentrations of TNM indicates a saturable reaction, again pointing to specific chemical modifications.

The effect of TNM on the muscarinic receptors was also investigated in intact cells by using brain slices. Therefore, cortical slices were incubated for 20 min with 300 μM TNM followed by extensive washing to remove the reagent and homogenization as described under Materials and Methods. A comparison between the binding properties of the modified and the unmodified receptor indicated that there was no change in [^{3}H]-4-NMPB binding while [^{3}H]AcCh binding showed a 6-fold increase in affinity, i.e., from K_d = 42 ± 4

Table I: Equilibrium Specific Binding of [^{3}H]AcCh and [^{3}H]-4-NMPB to Rat Cerebral Cortex Membranes[a,b]

	[^{3}H]AcCH		[^{3}H]-4-NMPB	
	B_{max} (fmol/mg of protein)	K_d (nM)	B_{max} (fmol/mg of protein)	K_d (nM)
control[c]	678 ± 82	40 ± 6	2844 ± 320	0.40 ± 0.08
0.1 mM TNM	805 ± 88	5 ± 1.6	2446 ± 276	0.82 ± 0.12
1 μM atropine[d] + 0.1 mM TNM	736 ± 96	36 ± 7	2730 ± 304	0.46 ± 0.10

[a] Values are means ± one standard deviation of three to four experiments, each carried out employing triplicate samples of 10 concentrations of [^{3}H]AcCh or 9 concentrations for [^{3}H]-4-NMPB as described in legends to Figures 2 and 3. Binding assays for the two radiolabeled ligands were routinely carried out by employing the same preparations. [b] Pretreatments were carried out by employing 0.5 mg of protein/mL washed cerebral cortex membranes, resuspended in 50 mM Tris-HCl, pH 8.1, for 20 min at 25 °C, and were followed by washing the membranes twice by centrifugation (30000g for 15 min) prior to binding assay. [c] One percent ethanol was added during the pretreatment period to correct for ethanol present in the TNM pretreatment. [d] Atropine (1 μM) was added 10 min prior to TNM.

Table II: Combined Effects of TNM and Sulfhydryl Reagents on Muscarinic Receptors from Rat Cerebral Cortex[a]

pretreatments[b]			specific binding of 24 nM [^{3}H]AcCh	
1st	2nd	3rd	fmol/mg of protein	% of control
control	control	control	252 ± 20	100
control	control	DTT	282 ± 36	112
diamide	control	control	386 ± 47	153
diamide	control	DTT	278 ± 32	110
control	TNM	control	643 ± 58	255
control	TNM	DTT	578 ± 56	229
diamide	TNM	control	610 ± 78	242
diamide	TNM	DTT	552 ± 63	219

[a] Results are means ± one standard deviation of two experiments. [b] All pretreatments were carried out on hypotonically washed cerebral cortex membranes, as described under Materials and Methods.

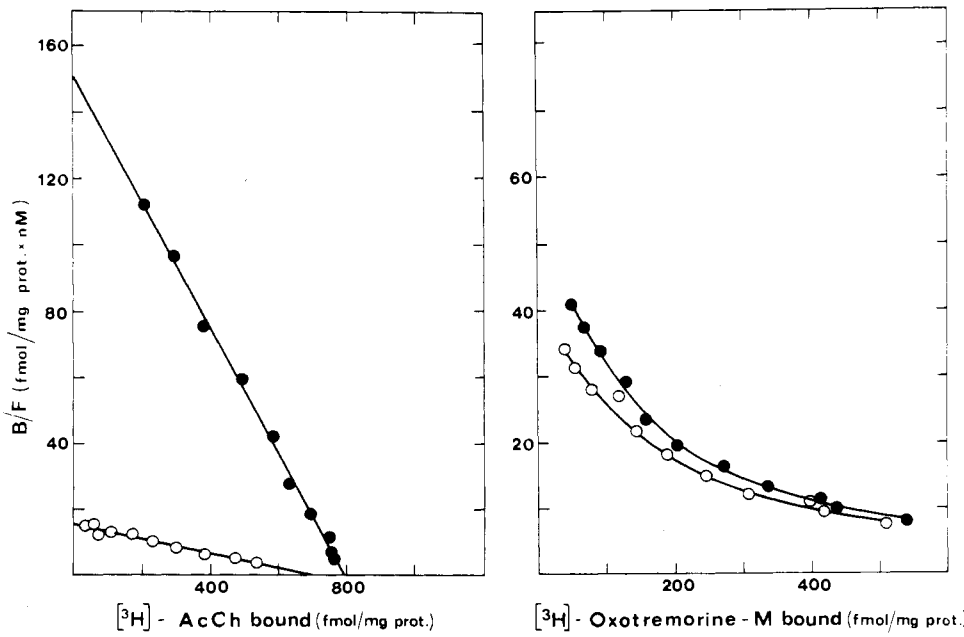

FIGURE 3: Scatchard plot of binding isotherms for [^{3}H]AcCh (left) and [^{3}H]oxotremorine M (right) to control (○) and 0.1 mM TNM pretreated (●) rat cerebral cortex membranes. Data represent a typical experiment carried out in parallel for the two ligands and employing 72 μg of protein per tube.

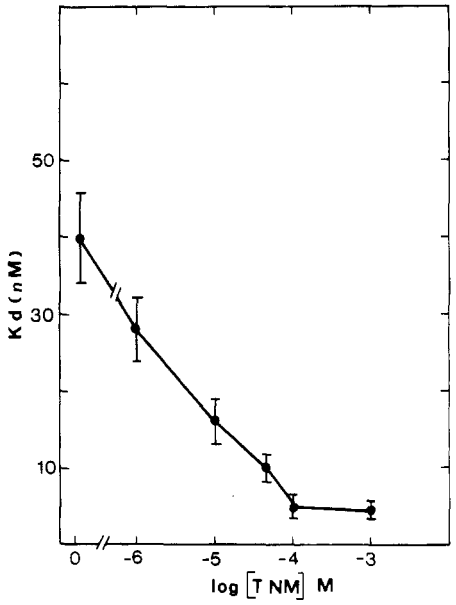

FIGURE 4: Relationship between TNM concentration and affinity of [^{3}H]AcCh for muscarinic receptors. Data are means ± standard deviations derived from three to four binding isotherms for each point.

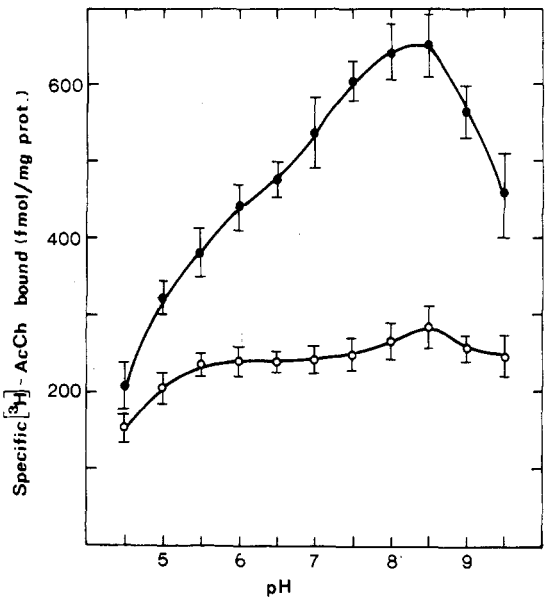

FIGURE 5: pH profile of specific [^{3}H]AcCh binding to control (○) and 0.1 mM TNM pretreated (●) rat cerebral cortex membranes. See Materials and Methods for details. Mean results ± standard deviations of three separate experiments are shown.

nM to K_d = 7 ± 2 nM, similar to the results obtained with membranes.

Nature of the Residue Modified by TNM. We cannot yet directly demonstrate the product formed as a result of nitration of the muscarinic receptors by TNM, i.e., the formation of a NO_2–Tyr residue and/or oxidation of SH. Hence, successive chemical modifications were employed in order to circumvent this problem and identify the residue modified. If TNM induces oxidation of SH to S–S at the binding site(s), then a reducing agent applied to the modified receptors should restore their originial properties.[2] As shown in Table II, a sulfhydryl-reducing agent (10 mM DTT) previously shown to be capable of inducing reduction of S–S bonds in muscarinic receptors (Gurwitz et al., 1984a) did not restore the properties of the TNM-modified receptors (compare lines 5 and 6). In another set of experiments we first treated the muscarinic receptors with diamide, an oxidizing agent that is a structural analogue of the agonist acetylcholine and that oxidizes SH to S–S at the agonist binding sites (Gurwitz et al., 1984a). As demonstrated by us previously, treatment of cortical membranes with diamide results in an increase of about 50% in the specific [^{3}H]AcCh binding. As shown in Table II, oxidation induced by diamide (third line) is indeed accompanied by an increase in [^{3}H]AcCh binding which is reversed by the use of DTT (fourth line). Furthermore, pretreatment with diamide [thereby oxidizing the sulfhydryl groups to disulfide(s)] did not prevent the effect induced by TNM (second line from the bottom), and again treatment of the modified receptors with DTT (last line) did not restore their original properties.

These results would appear to exclude the possibility that TNM modifies a cysteine residue. It thus seems reasonable to conclude that the most likely candidate for the site of modification is a tyrosyl residue(s), through the formation of 3-nitrotyrosine. The latter can be reduced by sodium dithionite ($Na_2S_2O_4$) to 3-aminotyrosine (Sokolovsky et al., 1967; Riordan & Sokolovsky, 1971b), which increases the pK of the phenolic hydroxyl group to that of unmodified Tyr. Thus, if the effect on [^{3}H]AcCh binding by TNM is related to the pK of the phenolic hydroxyl group of a Tyr residue, the reduction by dithionite might restore the changes to the control level. Treatment of TNM-modified receptors with 10 mM $Na_2S_2O_4$ (pH 8.1) did not change the binding properties of either agonist or antagonist of the modified receptor. However, in view of the fact that sodium dithionite is a lipid-insoluble ionic reducing agent, and until a lipid-soluble reagent that is capable of reducing NO_2-Tyr is obtained, these results should be treated with reservation.

As noted above, the presence of 1 μM antagonist (atropine and/or scopolamine) protected the receptor from the changes induced by TNM. Surprisingly, the agonist carbamoylcholine (at the concentration range of 1 μM–1 mM) did not afford such protection; other agonists, such as oxotremorine and arecoline, were not tested in view of their chemical susceptibility to TNM [a reaction with their unsaturated bonds is expected (Sokolovsky et al., 1966; Riordan et al., 1966)].

The presence of 1 mM GTP or Gpp(NH)p during the nitration procedure afforded no protection against modification, and the resulting modified receptor was identical in its binding properties to the receptor modified in the absence of these drugs. Unlike the situation with the above-mentioned agonists, no reaction between TNM and guanyl nucleotides was observed under the experimental condition employed here.

Characterization of the Nitrated Muscarinic Receptor. The pH profile of the specific binding of [^{3}H]AcCh to muscarinic receptors is virtually constant in the pH range 5–9.5 (Figure 5). For agonist binding to the modified receptor, however, the pH profile shows a dramatic change: the specific binding increases sharply from pH 5.5 to reach an apparent maximum at pH 7.5–8. The sharp increase in binding of [^{3}H]AcCh to the modified receptor might reflect ionization of a group(s) of a protein side chain(s) with an apparent pK of ~6.5 (Figure 5).

The 8-fold increase in affinity of [^{3}H]AcCh observed on nitration might result from changes in the on- and off-rates of the modified receptor. Kinetic experiments were carried out to further characterize the modified receptor. As shown in Figure 6A, the rate of association of the ligand to the TNM-treated receptor is twice as fast as that with the control

[2] Assuming comparable accessibility of TNM, diamide, and DTT to the modified residues in the receptor.

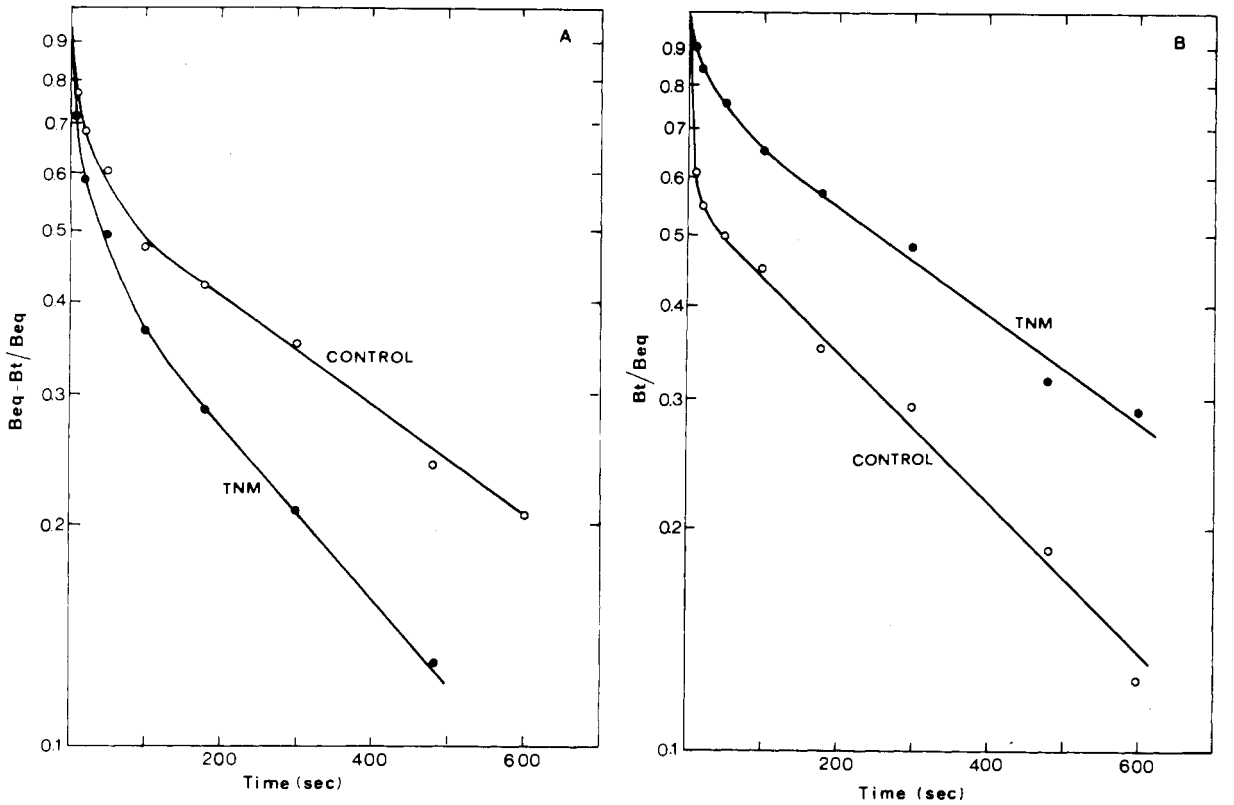

FIGURE 6: Association (left) and dissociation (right) kinetics of [^{3}H]AcCh binding to muscarinic receptors in control (○) and 0.1 mM TNM pretreated (●) rat cerebral cortex membranes. See Materials and Methods for details. Results represent a typical experiment in which both association and dissociation kinetics were studied by employing 12 nM [^{3}H]AcCh and 74 μg of protein per tube. B_{eq} was taken as the specific binding observed following a 60-min incubation period and was 163 and 594 fmol/mg of protein for control and TNM-pretreated membranes, respectively.

($t_{1/2}$ = 40 and 90 s for the modified and control receptors, respectively). The dissociation rate (Figure 6B) is about 6-fold slower in the modified receptor ($t_{1/2}$ = 250 and 40 s for the modified and control receptors, respectively). These changes in the rates of association and dissociation are in excellent agreement with the observed value of the affinity at equilibrium, i.e., an increase of 8-fold (Figure 3 and Table I). As shown in Figure 6, the plots of the association and dissociation rates show deviations of the first-order curves from linearity as observed previously (Gurwitz et al., 1984b), indicating that the binding of the triatiated agonist to both the native and the modified receptor does not follow a simple bimolecular mechanism. We did not attempt to perform a quantitative analysis of these kinetic data, since such an analysis would vary according to the specific model chosen. Selection of an appropriate model requires more data than are presently available and is currently under study.

Specific binding of [^{3}H]AcCh to control membranes is sensitive to guanyl nucleotides (Gurwitz et al., 1984b); i.e., they induce interconversion of high-affinity to low-affinity sites with no concomitant effect on the antagonist binding sites. Binding of [^{3}H]AcCh to the modified receptors was insensitive to 200 μM of either GTP or Gpp(NH)p, and there was no change either in the B_{max} value or in the affinity.

The characteristics of the binding sites were determined by measuring the inhibitory effects of various drugs on the binding of [^{3}H]AcCh. As summarized in Table III, muscarinic antagonists were more potent inhibitors than agonists in both the modified and the native receptor. Any changes in the calculated K_i values of the unlabeled antagonist are only minor (Table III). A high degree of stereospecifity is preserved in the modified receptor: (−)-QNB is more potent than (+)-QNB (Table III). Binding site characteristics were also determined by competition experiments with various agonists. Figure 7 depicts representative competition curves for two agonists, carbamoylcholine and oxotremorine, competing for the [^{3}H]AcCh sites. The K_i values calculated from these data are given in Table III. Two interesting phenomena can be clearly observed. First, the increased affinity seen in the modified receptors occurs not only with acetylcholine but also with agonists such as carbamoylcholine, carbamoylmethylcholine, arecoline, and pilocarpine. Second, when oxotremorine or

Table III: Inhibition Constants of Muscarinic Ligands to the High-Affinity State of Rat Cerebral Cortex Muscarinic Receptors[a]

competing ligand	control K_i (nM)[b]	TNM-treated K_i (nM)	K_i(control)/K_i(TNM)
antagonists			
(−)-QNB	3.2 ± 0.8	3.8 ± 1.2[c]	0.84
(+)-QNB	36 ± 9	72 ± 14[d]	0.50
(−)-*N*-methylscopolamine	2.6 ± 0.7	2.9 ± 0.8[c]	0.90
atropine	3.5 ± 0.9	4.3 ± 1.1[c]	0.81
agonists			
carbamoylcholine	132 ± 25	21 ± 5[d]	6.3
carbamoylmethylcholine	1370 ± 260	238 ± 62[d]	5.8
arecoline	155 ± 32	24 ± 6[d]	6.5
pilocarpine	590 ± 70	66 ± 14[d]	8.9
oxotremorine	15 ± 6	10 ± 4[c]	1.5
oxotremorine M	28 ± 7	25 ± 6[c]	1.1

[a] Values are means ± one standard deviation of two to three experiments for each ligand, each employing 12 concentrations (in triplicate) of the indicated unlabeled ligand and 10 nM [^{3}H]AcCh. Membranes were prepared and assayed for [^{3}H]AcCh binding as described under Materials and Methods. [b] See Data Analysis under Materials and Methods: K_d's for [^{3}H]AcCh were taken as 40 nM and 5 nM for control and TNM-pretreated membranes, respectively. [c] Not significantly different from control K_i. [d] Significantly different from control K_i ($p < 0.01$).

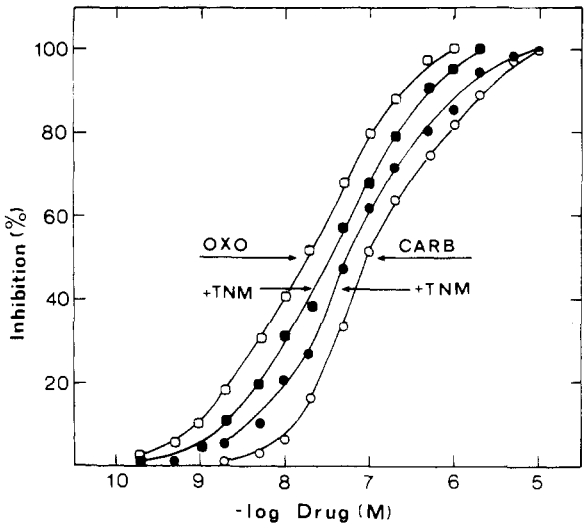

FIGURE 7: Inhibition by carbamoylcholine (○, ●) and oxotremorine (□, ■) of specific [^{3}H]AcCh binding to control (open symbols) and 0.1 mM TNM pretreated (closed symbols) rat cerebral cortex membranes. Membranes (69 μg of protein) were incubated for 60 min at 25 °C with 12 nM [^{3}H]AcCh and the indicated concentrations of the drugs. Results are shown as means of three separate experiments, each carried out in triplicate. Standard deviations (which were within 2–4% of inhibition values shown) are omitted for the sake of clarity.

oxotremorine M are used, the affinity of the modified receptor toward them is very similar to that of the native receptor. This observation is confirmed by direct binding studies using [^{3}H]-oxotremorine M (Figure 3, right, and Table III).

Discussion

The functional importance of tyrosyl residue(s) in muscarinic receptors emerges from a study of chemical modification using TNM, which is known to modify tyrosyl residues. The resulting affinity of agonists binding is 6–9 times higher than that of the unmodified control, while the binding of antagonists is only slightly altered. The effects of TNM treatment can be prevented by atropine and/or scopolamine, thus providing evidence that TNM modifies a residue(s) at the binding sites. Moreover, the fact that binding of agonists is altered without any concomitant effect on antagonist binding, together with the preservation of stereoselectivity in binding, is a strong indication that the structural integrity of the membrane preparation is preserved upon treatment with TNM (100 μM, pH 8.1, 20 min). Further support for this conclusion arises from the observation that TNM modifies the binding properties of muscarinic receptors in rat cortical slices in a manner identical with the modification of the receptors in homogenates. Thus, the modification induced by TNM seems to be specific and to occur at the binding site of the muscarinic receptors.

A number of successive chemical modifications were performed in order to determine whether cysteinyl residues are involved in the nitration procedures. As summarized in Table II, oxidation of the sulfhydryl groups to disulfide would not account for the functional consequences of TNM treatment, even though such a reaction might conceivably occur with TNM (Riordan et al., 1966; Sokolovsky et al., 1969, 1970). However, a reducing agent would only reverse the effects of TNM if the reaction resulted in formation of a disulfide which is the more likely product. If a sulfinic acid derivative had formed, reduction would of course have no effect (Sokolovsky et al., 1969). The failure of diamide to prevent modification by TNM strongly supports the conclusion that the effect on agonist binding is not due to oxidation of a cysteinyl residue. Thus, a tyrosyl residue(s) is (are) the most likely candidate as the residue responsible for the increased affinity in the binding of agonists to cortical muscarinic receptors. This is supported by the pH profile of agonist binding to the modified receptor (Figure 5), which suggests an ionization in the vicinity of about pH 6.5 and which is not detected in the unmodified receptor. This could be consistent with a phenolic hydroxyl of a nitrotyrosyl residue(s).

The binding of [^{3}H]AcCh displays the typical characteristics of specific interaction with high affinity between the ligand and muscarinic receptors (Gurwitz et al., 1984a,b). This property is retained in the modified receptor, but the affinity of most agonists tested is increased by 6–9-fold (Table III), with one exception, i.e., oxotremorine and its analogue oxotremorine M, for which the affinity is not altered after TNM treatment. As shown in Figure 3 (right), the Scatchard plots obtained for [^{3}H]oxotremorine M binding in both native and modified receptors are curvilinear [in agreement with previous studies for the native recetors (Birdsall et al., 1980; Harden et al., 1983)], while those obtained for [^{3}H]AcCh are linear (Figure 3, left). These results could stem from the fact that the interaction of oxotremorine M with the receptor, unlike that of AcCh, is characterized by binding to two subtypes of sites (super high and high affinity) (Birdsall et al., 1980). The exceptional findings for oxotremorine and its analogues (i.e., that their affinity for TNM-modified receptor is unchanged) might thus be explained by the fact that this agonist possesses a different mode of binding to the muscarinic site from that of other agonists such as acetylcholine or carbamoylcholine. This difference is also reflected in the lack of ability of oxotremorine and its analogues to sense the changes induced in the receptor as a result of the introduction of an NO_2–Tyr residue, i.e., hydrophobic/hydrophilic changes in the microenvironment of the binding sites. This hypothesis is in agreement with recent kinetic studies in our laboratory (Schreiber et al., 1985) that indicate that the interaction of oxotremorine with the high-affinity sites is different from that of other agonists, e.g., AcCh and carbamoylcholine. It is also in line with the observation that binding of the latter two agonists is modulated by neurotoxin batrachotoxin while the binding of oxotremorine is not affected (Cohen-Armon et al., 1985). It is worth noting that Brown & Brown (1984) observed major differences in the effects of carbamoylcholine and oxotremorine on both cAMP formation and phosphatidylinositol hydrolysis in embryonic chicken heart cells. They suggested that the receptor state associated with the inhibition of adenylate cyclase is the state common to the two agonists, while only carbamoylcholine is associated with the phosphatidylinositol response.

It is generally accepted that the process of receptor coupling with other components influences agonist affinity. Thus, binding of agonists to muscarinic receptors was previously shown to be modulated by guanine nucleotides (Berrie et al., 1979; Wei & Sulakhe, 1979; Sokolovsky et al., 1980; Ehlert et al., 1982; Waelbroeck et al., 1982; Harden et al., 1982; Burgisser et al., 1982). The fact that TNM treatment of the receptor is followed by an increase in agonist affinity might therefore lead one to postulate that the sensitive groups may be located on the receptor molecule as well as on a guanine nucleotide binding protein. However, since neither GTP nor Gpp(NH)p protected against nitration, this possibility seems unlikely.

Also of interest here is the observation that while antagonists protect the muscarinic receptors from the chemical modification by TNM, agonists do not. One likely explanation is

that this results stem from differences in the conformation of the receptor–ligand complex when agonists or antagonists are employed. Thus, in the antagonist–receptor complex the tyrosyl residue is not exposed to the reagent, while in the free receptor and in the agonist–receptor complex the tyrosyl residue is present in a position in which its microenvironment facilitates the chemical modification. Previous studies on nitration of copolymers (Riordan & Sokolovsky, 1971a) have shown, for example, that if tyrosyl is surrounded by positively charged lysyl groups, the rate of nitration is much faster and the pK of the resulting nitrotyrosyl group is lower than when it is surrounded by negatively charged aspartate or glutamate residues. Thus, the tyrosyl residue might be in close proximity to a negatively charged microenvironment in the antagonist–receptor complex and to a positively charged group(s) in the free and in the agonist–receptor complex. In this context it is worth citing the recent report of Vanderheyden et al. (1984), who suggested that agonists mediate a conformational change of the low-affinity state, resulting in their increased susceptibility toward N-ethylmaleimide alkylation. Further experimentation will obviously be needed in order to examine this suggestion.

Registry No. AcCh, 51-84-3; 4-NMPB, 3608-67-1; carbamoylcholine, 462-58-8; arecoline, 63-75-2; oxotremorine, 70-22-4.

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