Tetranitromethane Modification of the Muscarinic Receptors in Bovine Adrenal Medulla

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Abstract—Muscarinic receptor binding was examined in bovine adrenal medullary microsomes following exposure to tetranitromethane (TNM) that modifies tyrosine and cysteine residues in proteins. The TNM (10–100 μM) treatment of adrenal medullary microsomes caused a concentration-dependent and irreversible reduction in the maximum number of binding sites \( (B_{\text{max}}) \) for \( \text{(-)} \left( ^{3} \text{H} \right) \text{quinuclidinyl benzilate} \) (QNB), with a slight increase in the equilibrium dissociation constant \( (K_D) \). Typically, about a 36% decrease and a 1.3-fold increase in the corresponding values were obtained at 50 μM of TNM. The alteration in the \( B_{\text{max}} \) was partially prevented by atropine but not carbamylcholine, and it was not reversed by subsequent treatment with dithiothreitol, a disulfide reducing agent. The change in the \( K_D \) was unaffected by these agents. The TNM (50 μM) treatment also caused a slight decrease in the affinity of atropine and pirenzepine (for both the high and low affinity sites), and it caused a slight decrease in the affinity of carbamylcholine at the high affinity site, with a large loss of the low affinity site. Thus, the results indicate that TNM causes a loss of muscarinic binding sites and a decrease in the binding affinity of muscarinic receptors in bovine adrenal medulla, probably through modifications of functional groups such as tyrosine residues.

The muscarinic receptors in various tissues are mainly subclassified into \( M_1 \)- and \( M_2 \)-subtypes according to the respective high and low affinity for a unique antagonist pirenzepine (1–3), while each subtype receptor has two or three distinct affinity sites (or states) for agonist binding (4–7). The two subtypes of receptors were demonstrated to be derived from distinct muscarinic receptor genes (8, 9); and in membranes, agonist binding to these receptors are modulated differentially by divalent cations and guanine nucleotides (6, 7, 10), indicating their variable degree of coupling with guanine nucleotide binding proteins (G-proteins), which mediate diverse signal transduction of muscarinic responses (11, 12).

Interestingly, chemical modification of sulfhydryl and tyrosine residues in proteins has been shown to alter the binding properties of muscarinic receptors, suggesting some roles of these functional groups within receptors and/or G-proteins. Thus, alkylation of sulfhydryl groups with lower doses of N-ethylmaleimide induces a marked decrease in the agonist affinity, especially toward muscarinic receptors in the brain stem and cerebellum (rich in \( M_2 \)-subtype) (7, 13, 14), whereas nitration of tyrosine residues with tetranitromethane (TNM) causes an increase in the agonist affinity, especially toward the receptors in the cerebral cortex (rich in \( M_1 \)-subtype) (15, 16).

Recently, we found that muscarinic receptors in bovine adrenal medulla are rich in the \( M_1 \)-subtype (17), based on the affinity of pirenzepine and the effects of modulators. In the present study, we examined the effect of TNM on the binding properties of adrenal medullary muscarinic receptors, in order to have an insight into the role of functional groups sensitive to this reagent.

Materials and Methods

Tissue preparation and TNM treatment: Bovine adrenal medulla was dissected on ice
from fresh adrenal glands (3–5 glands for each experiment) obtained at a local slaughterhouse, within 1 hr postmortem. The tissue was homogenized in ice-cold 0.25 M sucrose containing 5 mM Tris-HCl (pH 7.5 at 25°C) as described previously (17, 18), and the homogenate was centrifuged at 22,600×g for 20 min and at 160,500×g for 30 min, consecutively. The final pellet (which contained purified microsomes) was suspended in 2.5 vol. (as tissue wet weight) of 50 mM Tris-HCl buffer (pH 8.0 at 25°C, buffer A) and divided into two or three equal fractions. Following centrifugation of these fractions at 160,500×g for 30 min, the pellets were resuspended in 12.5 vol. (as tissue wet weight) of buffer A containing TNM, and incubated for 20 min at 25°C, with rapid swinging (112 times/min). The control suspension was incubated with ethanol (final concentration of less than 1%), the solvent for dissolving TNM. The TNM treatment was terminated by centrifugation at 160,500×g for 30 min, the pellets were washed twice by resuspending them in 5 vol. of 50 mM Tris-HCl buffer (pH 7.7, buffer B) at 25°C and subsequent centrifugation at 160,500×g for 30 min. The final pellets (microsomes) were suspended in 2–2.5 vol. (as tissue wet weight) of the ice-cold Tris buffer B and used for the binding assays. Each process of suspending the pellets was done by homogenization using a Kinematica Polytron PT-10 (setting at 6.0 for 10 sec), and each centrifugation was performed at 4°C.

In the protection experiments against the TNM treatment, the microsomal fractions were incubated with a muscarinic agent in 12.5 vol. of buffer A for 20 min at 25°C, prior to the incubation with TNM. In some experiments, the pellets obtained after centrifugation following the TNM treatment were again suspended in 12.5 vol. of buffer A containing dithiothreitol and incubated for 20 min at 25°C. These incubation mixtures were then centrifuged at 160,500×g for 30 min, and the resultant pellets were washed twice as described above.

**Binding assays:** The binding assays of muscarinic receptors were performed as previously described (17, 18). Briefly, the microsomes (120–220 μg of protein) were incubated with 7–9 different concentrations of (3H)QNB (20–650 pM) in the saturation experiments or incubated with a fixed concentration of the radioligand and 16–18 different concentrations of a displacer in the drug competition experiments. The incubations were carried out for 60 min at 37°C, in a total 2-ml volume of 50 mM Tris-HCl buffer (pH 7.4). After the 60-min incubation, bound and free radioligands were separated by rapid filtration over Whatman GF/B filters, followed by 4×4 ml rinses of the filters with ice cold buffer. (3H)QNB retained on the filters after drying overnight was extracted with scintillation fluid, and the radioactivity was counted in a liquid scintillation spectrometer at efficiencies of 32–35%. Nonspecific binding of (3H)QNB was taken as the binding in the presence of 10 μM atropine, and it was less than 10% (0.02–1.17 fmol/tube) at concentrations up to 500 pM and 10.6–19.0% (1.31–3.35 fmol/tube) at the higher concentrations examined. The treatment of microsomes with 50 μM TNM produced no significant changes in the absolute values of the nonspecific (3H)QNB binding. All assays were done in duplicate. The determination of protein was performed according to the method of Lowry et al. (19), using bovine serum albumin as a standard.

**Data analysis:** Saturation isotherms were analyzed by Scatchard transformation and Hill plots, as described previously (20). This analyses gave the maximum number of binding sites (Bmax), the apparent dissociation constant (Kd) and the Hill coefficient (nH). The drug competition curves were subjected to log-probit analysis (21) in order to estimate the IC50 (the drug concentration for half-maximal inhibition of specific ligand binding) and nH values. The competition curves were further tested by a nonlinear least-squares regression analysis, LIGAND (22), to determine whether the curves would fit a one-site or two-site model. In this analysis, the data for carbamylcholine were analyzed individually, using the affinity (equilibrium association constant) of (3H)QNB binding determined in the same preparation. Also, the data for muscarinic antagonists were analyzed individually, but the
affinity of the radioligand was set at the mean value obtained from six or four separate experiments. These analyses gave equilibrium association constants for drug binding and populations of different affinity sites. The results, therefore, were expressed as transformed equilibrium dissociation constants \((K_1, K_2)\) for each agent and percentage of the high affinity site \((R_1)\). Statistical evaluation of the fit between one-site and two-site models was performed by a partial \(F\)-test, as described previously (17, 22, 23). Statistical examination of the data was done by the paired or unpaired Student’s \(t\)-test and one way of analysis of variance with the Tukey test.

**Drugs:** \((^3\text{H})\text{QNB} (\beta\text{-isomer, 33.2 Ci/mmol})\) was purchased from New England Nuclear (Boston, MA, U.S.A.). The following agents were also obtained from commercial sources: atropine sulfate (Nakarai Chemicals, Ltd., Kyoto, Japan); carbamylcholine chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); and TNM (Maruwaka Kagaku Co., Ltd., Osaka, Japan). TNM was dissolved in 99.5% ethanol as a 10 mM stock solution. Pirenzepine dihydrochloride was donated by the Japan Boehringer Ingelheim Co., Ltd. (Hyogo, Japan).

**Results**

\((^3\text{H})\text{QNB} binding in TNM-treated microsomes:** The specific binding of \((^3\text{H})\text{QNB}\) was saturable and of high affinity in adrenal medullary microsomes, in agreement with our previous reports (17, 18). Figure 1A shows typical saturation isotherms of specific \((^3\text{H})\text{QNB}\) binding in the control and TNM-treated microsomes prepared from the same adrenal medulla. The specific ligand binding was significantly lower in the TNM-treated microsomes than in the control, at all concentrations of \((^3\text{H})\text{QNB}\) examined. This indicates an irreversible action of TNM because the TNM-treated samples were washed twice prior to the binding assays. Thus, Scatchard plots of these data shown in Fig. 1B revealed marked decreases in the \(B_{\text{max}}\) value for \((^3\text{H})\text{QNB}\) binding, by 21.5% and 70.5% in the microsomes treated with 50 and 100 \(\mu\text{M}\) TNM, respectively. These changes were accompanied by slight increases in the corresponding \(K_D\) values to about 1.3- and 1.2-fold, respectively. Hill analysis of these data indicated the existence of a single population of binding sites for \((^3\text{H})\text{QNB}\) in the control and 50 \(\mu\text{M}\) TNM treated samples (\(n_H\) values of close to unity), whereas a possibility of

![Fig. 1. Typical saturation isotherms (A) and Scatchard plots (B) of specific \((^3\text{H})\text{QNB}\) binding in control and TNM-treated microsomes of bovine adrenal medulla. Saturation experiments were performed in duplicate with 20–650 pM of \((^3\text{H})\text{QNB}.\) Scatchard and Hill analyses gave \(B_{\text{max}}\) (fmol/mg protein), \(K_D\) (pM) and \(n_H\) values of 110.2, 87.7 and 1.08 in the control; 86.6, 115.3 and 1.02 in the 50 \(\mu\text{M}\) TNM sample; and 32.5, 103.3 and 1.28 in the 100 \(\mu\text{M}\) TNM sample, respectively.](image-url)
positive cooperative interaction in the \((^3\text{H})\)-QNB binding was suggested in the 100 \(\mu\)M TNM treated sample (\(n_H=1.28\), \(P<0.05\) as compared with unity). In several paired experiments, the effect of TNM on the \(B_{\text{max}}\) value was concentration-dependent (Table 1): the \(B_{\text{max}}\) value decreased by 10.4%, 35.7% and 65.2% at concentrations of 10, 50 and 100 \(\mu\)M TNM, respectively. The change in the \(K_D\) value was constantly observed only at a concentration of 50 \(\mu\)M TNM (which caused about 1.3-fold increase), while it was variable at 100 \(\mu\)M TNM. Increase in the \(n_H\) value was significant (\(P<0.05\)) only at 100 \(\mu\)M TNM (Table 1).

### Table 1. Parameters for saturable specific binding of \((^3\text{H})\)QNB in control and TNM-treated microsomes of bovine adrenal medulla

| TNM-treatment | \(B_{\text{max}}\) : fmol/mg protein (% decrease) | \(K_D\) : pM (-fold increase) | \(n_H\) |
|---------------|-----------------|-----------------|-----|
| None (n=6)    | 105.6±13.6      | 92.8±7.5        | 1.04±0.02 |
| 10 \(\mu\)M (n=3) | 99.4±6.1       | 117.6±24.5      | 1.03±0.03 |
|               | (10.4±4.4)      | (1.09±0.25)     |     |
| 50 \(\mu\)M (n=4) | 67.4±10.1       | 137.9±21.4*     | 1.04±0.03 |
|               | (35.7±8.4)      | (1.34±0.19)     |     |
| 100 \(\mu\)M (n=3) | 41.1±8.7        | 195.9±92.7      | 1.22±0.06* |
|               | (65.2±2.7)      | (2.52±1.16)     |     |

The values given are the mean±S.E. from separate experiments shown by n. The values in the parenthesis are the percent decrease and -fold increase in the \(B_{\text{max}}\) and \(K_D\) values as compared with the corresponding paired control values. *: \(P<0.05\), as compared with the control value or with unity by the paired Student's \(t\)-test.

### Effects of atropine, carbamylcholine and dithiothreitol on the loss of \((^3\text{H})\)QNB sites induced by TNM: The following saturation studies were performed to determine where TNM modifies the microsomes to cause a loss of the \((^3\text{H})\)QNB binding sites. Namely, adrenal medullary microsomes were preincubated with either atropine or carbamylcholine for 20 min prior to and during the TNM treatment. As shown in Fig. 2, 2 \(\mu\)M of atropine partially protected \((^3\text{H})\)QNB sites against the loss induced by TNM (50 \(\mu\)M). Similar results were obtained in an additional 2 experiments, and \(B_{\text{max}}\) values showed decreases of 25.6% and 58.8% in the paired TNM-treated microsomes with and without exposure to atropine, respectively (Table 2).

One way analysis of variance showed that TNM treatment had significant effects (\(P<0.05\)) by \(F(2, 6) = 7.68\) in the case of the control, TNM alone and TNM with atropine) on the \(B_{\text{max}}\) values. The subsequent Tukey test indicated a marked difference (\(P<0.05\)) in...
the data between the control and TNM groups, but not between the groups of the control and TNM with atropine. Also, the \( B_{\text{max}} \) value of TNM with atropine significantly \((P<0.05)\) differed from the values of the control and TNM alone by the unpaired Student’s \( t \)-test. Thus, atropine was active in the protection experiments. On the other hand, 2 mM of carbamylcholine was almost inactive (Table 2) when similar analyses were applied to the data from paired experiments. The \( K_d \) value of \((^3\text{H})\text{QNB} \) binding increased to about 1.8-fold following the TNM treatment \((P<0.05)\), and this change was unaffected by these muscarinic agents (Table 2).

Further experiments were done to determine if the loss of \((^3\text{H})\text{QNB} \) sites might be due to oxidation of sulfhydryl groups by TNM. In this case, TNM \((50 \mu\text{M})\)-treated microsomes were incubated with the disulfide reducing agent dithiothreitol \((10 \text{ mM})\) for 20 min and then washed. However, no restoration of the \((^3\text{H})\text{QNB} \) binding was observed: The \( B_{\text{max}} \) (fmol/mg protein) and \( K_d \) (pM) values from 3 paired experiments were 83.4±14.9 and 126.7±17.2 in the control samples, 87.2±13.6 and 140.9±13.1 in the samples with dithiothreitol alone, 54.8±11.2 and 168.5±26.9 in the samples with TNM alone, and 49.6±11.4 and 151.3±14.3 in the samples with both TNM and dithiothreitol, respectively.

**Carbamylcholine, pirenzepine and atropine binding in TNM-treated microsomes:** Previous studies have shown that TNM significantly changes agonist binding to muscarinic receptors in the brain at lower concentrations without affecting antagonist binding \((15, 16)\). Therefore, we examined carbamylcholine binding in competition experiments.

Figure 3A shows typical carbamylcholine/\((^3\text{H})\text{QNB} \) competition curves in the control and TNM-treated microsomes prepared from the same adrenal medulla. In this example, the treatment with TNM at concentrations of 10 and 50 \( \mu\text{M} \) decreased the specific binding of \((^3\text{H})\text{QNB} \) (59.3 pM) by 12.1% and 54.3%, respectively. Nevertheless, carbamylcholine displaced all of the specific \((^3\text{H})\text{QNB} \) binding with increasing concentrations in every preparation. Similar observations were obtained in additional experiments, which were shown in the transformed competition curves of Fig. 3B. From these data, 10 \( \mu\text{M} \) of TNM tended to increase the steepness of the curve and therefore the \( n_H \) value from 0.59 to 0.64. No change was observed in the IC50 value. A computerized nonlinear curve fitting analysis (LIGAND) revealed the presence of two affinity sites for carbamylcholine in the control and 10 \( \mu\text{M} \) TNM groups \((P<0.01)\) by a partial \( F \)-test as compared to a one-site model.
Table 3), with a high (K₁) and a low affinity (K₂). As the result, there was a trend of an increase in the K₁ value from 0.95 μM to 2.59 μM after the TNM treatment (Table 3), indicating about a 2.7-fold (4.27±1.98-fold as compared with paired control values, n=3) decrease in the affinity. No alterations were estimated in the K₂ value (about 80 μM) and percentage (R₁=about 48%) of the high affinity site.

Increasing concentration of TNM to 50 μM caused a leftward shift of the lower portion of the curve and decreased the IC₅₀ value from 19.3 μM to 8.6 μM (Fig. 3B, Table 3). This curve gave a significantly (P<0.05) increased nᵢₙ value of 0.76. In this case, the two curves fit a one-site model and exhibited only a high affinity site. An additional two curves fit a two-site model at the level of P<0.05. Even though, there was a marked increase in the R₁ value besides an increased K₁ value. Thus, 50 μM of TNM caused increases in the K₁ and R₁ values to 3.92 μM (3.84±0.89-fold decrease in the affinity as compared with the paired control values, n=4) and 87.7%, respectively (Table 3). Similar findings were obtained in two experiments with higher doses of TNM (100 μM) and (³H)ONB (138.8 pM), which gave a decreased IC₅₀ value (3.9 μM) and an increased nᵢₙ value (0.82) as compared with the control (13.8 μM and 0.58). Accordingly, 10 μM of TNM tended to decrease the agonist affinity at the high affinity site, and 50 μM of TNM exerted an additional effect to increase the percentage of the high affinity site. It should be noted that the latter change was due to a large loss of the low affinity site, but not to a conversion of the low to the high affinity site, because a marked reduction of (³H)ONB sites occurred in the preparations treated with higher doses of TNM, and no indication of such conversion was observed at 10 μM TNM (Fig. 3A, Table 3).

In separate experiments with pirenzepine, no significant differences were observed in the competition curves between the control and TNM (50 μM)-treated preparations, as shown by the similar IC₅₀ and nᵢₙ values (Table 3). Computer-aided analysis of these curves revealed the presence of two affinity sites for pirenzepine (P<0.01 by a partial F-test, except one of the three curves of each group gave the fit to a two-site model at the level of P<0.05) in both control and TNM groups. K₁ and K₂ values were 7.4 nM and 169 nM in the control, but 22.1 nM and 515 nM in the TNM group, respectively, because
Table 3. Parameters for carbamylcholine, pirenzepine and atropine binding estimated from the competition curves of specific (\(^3\)H)QNB binding in control and TNM-treated microsomes of bovine adrenal medulla

| Drug          | TNM-treatment | IC50 : \(\mu\)M | \(K_1 : \mu\)M | \(K_2 : \mu\)M | \(R_1 : \%\) | \(n\) |
|---------------|---------------|-----------------|----------------|----------------|--------------|------|
| Carbamylcholine | None (\(n=4\)) | 19.3±5.5 | 0.95±0.36 | 82.3±16.3 | 47.9±6.1 | 0.59±0.04 |
|                | 10 \(\mu\)M (\(n=3\)) | 23.0±5.3 | 2.59±0.88 | 80.4±31.4 | 47.1±3.2 | 0.64±0.02 |
|                | 50 \(\mu\)M (\(n=4\)) | 8.6±2.6 | 3.92±1.92 | 78.7±46.3 | 87.7±7.7* | 0.76±0.03* |
| Pirenzepine    | None (\(n=3\)) | 0.271±0.062 | 0.0074±0.0021 | 0.169±0.038 | 31.1±1.7 | 0.75±0.03 |
|                | 50 \(\mu\)M (\(n=3\)) | 0.264±0.098 | 0.0221±0.0028* | 0.515±0.011* | 61.0±4.2* | 0.70±0.05* |
| Atropine       | None (\(n=3\)) | 0.0014±0.0003 | 0.00041±0.00003 |          | 100       | 1.01±0.02 |
|                | 50 \(\mu\)M (\(n=3\)) | 0.0020±0.0002 | 0.00085±0.00018 |          | 100       | 0.90±0.06 |

The values given are the mean±S.E. from three or four separate experiments shown by \(n\). \(K_1\), \(K_2\) and \(R_1\) values are the equilibrium dissociation constants at high and low affinity sites and the percentage of the high affinity site, respectively. *: \(P<0.05\), as compared with the control value.
the affinity of (3H)QNB binding in the TNM-treated samples was lower than that in the control (see Table 1). This result indicated about 3-fold decreases in the affinity of pirenzepine after the TNM treatment for both the high and low affinity sites. Interestingly, such computer-aided analysis gave an increased $R_1$ value from about 30% in the control to 60% in the TNM group. The competition curves of atropine fit a one-site model and showed no change in the TNM samples, while about a 2-fold decrease in the affinity was estimated; the $K_I$ value was 0.41 nM in the control and 0.85 nM in the TNM (50 μM) group (Table 3).

Discussion

The TNM treatment of bovine adrenal medullary microsomes induced a marked decrease in the specific (3H)QNB binding. This effect was concentration-dependent, irreversible and resulted in a decrease in $B_{max}$. In addition, there was a slight decrease in the affinity of (3H)QNB binding in the TNM-treated preparations. Such a decrease in the affinity was also estimated for atropine and pirenzepine (for both the high and low affinity sites). These results are consistent with the loss of (3H)antagonist binding sites and a slight decrease in the affinity shown by Gurwitz and Sokolovsky (15) in the rat cerebral cortex membranes treated with TNM. However, these authors demonstrated more significant changes of agonist binding; that is, marked increases in the affinity and the number of high agonist affinity sites in the TNM (low dose) treated samples, with no change of antagonist binding (15). These observations were extended to other brain regions (striatum and hippocampus) (16) rich in $M_1$-subtype receptors. In our study, a lower dose (10 μM) of TNM caused a slight decrease in the agonist affinity at the high affinity site, and increasing doses (50-100 μM) of TNM further induced a decrease in the population of the low affinity site with a marked loss of the antagonist binding sites. Thus, the alteration of agonist binding induced by TNM in the adrenal medulla appeared to be quite different from the case in the cerebral cortex, although both tissues are rich in $M_1$-subtype receptors.

The reason for the difference remains unclear. It may be due to different experimental conditions such as the preparation and assay medium. Alternatively, the high affinity site for the agonist binding may reflect distinct forms of muscarinic receptors between the two tissues, as suggested by the difference in the affinity; that is, less than 100 nM in the cerebral cortex (15) and about 1 μM in the adrenal medulla (17, 18, present study).

Interestingly, it was shown that the monomeric state of glucose dehydrogenase has two tyrosine residues susceptible to TNM, while the tetrameric state has only one tyrosine residue (24). If muscarinic receptors in the adrenal medulla may exist in oligomeric states such as dimeric (low agonist affinity) and tetrameric (high agonist affinity) states as proposed by Sokolovsky (25), the low affinity site is likely to be more susceptible to the modification by TNM as compared with the high affinity site. This may be consistent with a large loss of the low affinity site observed in this study. However, further studies are necessary to clarify such a possibility.

The loss of (3H)QNB binding sites was partially prevented by the pretreatment of preparations with atropine but not carbamylcholine, whereas the change in the affinity was unaffected by these muscarinic agents. It is still unclear whether TNM directly acts on the antagonist binding sites (except agonist recognition sites) or not. Nevertheless, these results suggest that the complex of muscarinic receptors with an antagonist may be less sensitive to TNM as compared with free receptors or the receptors complexed with an agonist.

TNM has been shown to cause nitration of tyrosine residues (26) and oxidation of the sulfhydryl residue of cysteine (27). In this study, neither the loss of (3H)QNB sites nor the decrease in the affinity was reversed by subsequent treatment of TNM samples with dithiothreitol, a disulfide reducing agent. Therefore, the functional groups modified by TNM appeared to be tyrosine residues, although we did not directly demonstrate this in the present study. Nitration of tyrosine residues with TNM in bacteriorhodopsin has been shown to affect the absorption spec-
trum and photoreaction kinetics of the chromophore (28). Muscarinic receptors have been shown to have a primary amino acid sequence and transmembrane structure similar to the photoreceptors (opsin) (8, 9). At present, however, it remains unclear how the loss of $^{3}H$QNB sites induced by TNM modifies the function mediated by adrenal medullary muscarinic receptors.

Conclusively, we have demonstrated that TNM causes a loss of muscarinic binding sites and a slight decrease in the binding affinity of muscarinic receptors in bovine adrenal medulla, and therefore, the functional groups (probably tyrosine residues) sensitive to TNM may have a role in the conformational stability of muscarinic receptors in the interactions with muscarinic agents.

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