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CHEMICAL MODIFICATION OF α_2-ADRENOCEPTORS

POSSIBLE ROLE FOR TYROSINE IN THE LIGAND BINDING SITE

HIROYASU NAKATA,* JOHN W. REGAN† and ROBERT J. LEFKOWITZ

Howard Hughes Medical Institute, Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, NC 27710, U.S.A.

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Abstract—Tetranitromethane (TNM) is a reagent which reacts with the tyrosine and cysteine residues of proteins. Chemical modification of partially purified human platelet α_2-adrenoceptors with TNM resulted in an irreversible loss of binding activity. Typically, an 80–90% decrease in binding activity occurred with a 60-min exposure to 320 μM TNM. The loss of α_2-adrenoceptor activity caused by TNM could be prevented if α_2-adrenergic ligands were present during exposure of the receptor to TNM. The protection afforded by α_2-adrenergic ligands was dose-dependent and showed a positive correlation with the affinity of the ligand for the α_2-adrenoceptor. Prazosin, an α_1-specific antagonist, and propranolol, a β-adrenergic antagonist, did not protect α_2-adrenoceptors against the inactivation caused by TNM. Saturation curve analysis revealed that the decrease in α_2-adrenoceptor activity caused by TNM was due to a decrease in B_{max} with no change in K_d. α_2-Adrenoceptors were also inactivated with the sulfhydryl-specific reagent phenylmercuric chloride (PMC). The receptor inactivation caused by PMC could be reversed completely by subsequent treatment with dithiothreitol. Treatment of α_2-adrenoceptors with combinations of TNM and PMC showed that the receptor inactivation caused by TNM was most likely due to an interaction with tyrosine residues. These results indicate that tyrosine residues have a function in the conformational stability of α_2-adrenoceptors and may be directly involved with ligand binding to the receptor.

Human platelets contain α_2-adrenoceptors which are coupled to the inhibition of adenylate cyclase (reviewed in Ref. 1). α_2-Adrenoceptors have traditionally been distinguished from α_1-adrenoceptors on functional and pharmacological grounds [2]. The most useful α-adrenergic agents for the pharmacologic classification have been the antagonists yohimbine, which is α_2-specific, and prazosin, which is α_1-specific. Additionally, the agonists clonidine and phenylephrine have been useful for the classification of α-adrenoceptors. Thus, the order of potency at α_1-adrenoceptors is phenylephrine > clonidine, whereas at α_2-adrenoceptors the order is clonidine > phenylephrine. Ultimately, a biochemical understanding will provide a foundation for the pharmacologic characteristics of these adrenergic receptors.

Relatively little is known about the biochemistry of the α_2-adrenoceptor as a whole and even less about the ligand binding site itself. Recently, human platelet α_2-adrenoceptors have been purified to homogeneity and have been covalently labeled with [^{3}H]phenoxybenzamine, a chemically reactive β-chloro-alkylamine [3]. From the latter it would seem probable that the ligand binding site of the α_2-adrenoceptor contains a nucleophilic residue which can react with the aziridium ion generated by [^{3}H]phenoxybenzamine. Such a nucleophile could be the sulfhydryl group of cysteine. In this regard purified α_2-adrenoceptors [3] as well as those present in membranes [4, 5] have been shown to contain essential sulfhydryl residues. The characteristics of α_2-adrenoceptor inactivation caused by exposure to mercuric compounds suggest that a sulfhydryl residue may be in or around the ligand binding site.

Tetranitromethane (TNM) is another reagent which has been used for the chemical modification of proteins [6]. Under neutral or slightly basic conditions, TNM is capable of nitrating the phenolic side chain of tyrosine; however, it can also oxidize the sulfhydryl group of cysteine. The present report describes the chemical modification of partially purified α_2-adrenoceptors with TNM. The characteristics of this chemical modification imply a significant role for tyrosine residues in the biochemistry of α_2-adrenoceptors.

EXPERIMENTAL PROCEDURES

Materials. Most of the drugs and chemicals were from the same sources as reported previously [3, 7]. TNM was from Sigma. Phenylmercuric chloride was from Aldrich, sodium metabisulfite was from MCB, sodium iodide was from Fisher, chloramine T was from Eastman, and *p*-nitrobenzenesulfonyl fluoride was from Pierce. SKF 101605 (3-allyl-6-chloro-2,3,4,5-tetrahydro-1*H*-3-benzazepine) was provided

* Present address: Histopharmacology Section, Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, MD 20892.

† Address all correspondence to: Dr. John W. Regan, Box 3821, Department of Medicine, Duke University Medical Center, Durham, NC 27710.

by Dr. Robert M. DeMarinis (Department of Medicinal Chemistry, Smith Kline & French Laboratories, Philadelphia, PA 19101). [^{3}H]Yohimbine (85 Ci/mmole) was from New England Nuclear. Human platelet membranes were prepared and solubilized with digitonin, and α_2-adrenoceptors were partially purified by a combination of affinity and heparin chromatography [3]. α_2-Adrenoceptor activity was eluted from the heparin-agarose column and was stored in a buffer containing 300 mM KCl, 100 μM phentolamine, 100 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES), 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.1% digitonin (pH 7.2). The concentration of α_2-adrenoceptors in the eluate was typically in the range of 50–100 pmoles/ml; the specific activity was in the range of 150–200 pmoles/mg protein which represents a 700- to 1000-fold purification of the α_2-adrenoceptor from human platelet membranes.

Chemical modification with TNM. Typically, an aliquot (50 μl) of α_2-adrenoceptors from the heparin-agarose chromatography was desalted by Sephadex G-50 chromatography into a buffer consisting of 50 mM Tris–HCl, 1 mM EDTA, 0.1% digitonin (pH 7.2). Depending upon the experiment, the α_2-adrenoceptors were diluted further (2- to 10-fold) with the same buffer. A working solution of TNM was made by diluting the stock TNM with absolute ethanol. Diluted α_2-adrenoceptors (~100 fmoles) were mixed with the working solution of TNM to give the desired concentration of TNM in a final volume of 500 μl. Absolute ethanol was added to all control samples to equal the concentration of ethanol present in the TNM-treated samples. The samples were incubated on ice for the amount of time listed in each experiment and were desalted by Sephadex G-50 chromatography into 50 mM Tris–HCl, 1 mM EDTA, 0.1% digitonin (pH 7.2). The α_2-adrenoceptor activity was then determined by the binding of [^{3}H]yohimbine.

Binding assays. The binding of [^{3}H]yohimbine was routinely measured at a final concentration of ~6 nM and in a final volume of 500 μl. Non-specific binding was determined in the presence of 10 μM phentolamine. For saturation analysis, the binding of [^{3}H]yohimbine was measured at seven concentrations between 0.25 and 7.5 nM. Incubations were done overnight (14–16 hr) at 4–6°. Bound and free [^{3}H]yohimbine were separated by gel filtration over Sephadex G-50. Radioactivity was determined by liquid scintillation spectrometry (efficiency 45%). Saturation data were analyzed by computer using a non-linear least squares curve fitting procedure [8].

Sephadex G-50 chromatography. Desalting, prior to and following TNM treatment, and the separation of bound and free [^{3}H]yohimbine were done by Sephadex G-50 chromatography. The samples in a final volume of 500 μl were applied to a 3.4 ml column (0.6 × 12 cm) of Sephadex G-50 (fine) pre-equilibrated with 50 mM Tris–HCl, 1 mM EDTA, 0.1% digitonin (pH 7.2). Then, 600 μl of the latter buffer was applied and the flow-through was discarded. Another 1 ml of buffer was applied and the eluate (void volume) was collected.

Other. Protein was determined by the method of Schaffner and Weissmann [9]. Time was kept with a Seiko watch. Temperatures were measured with a mercury-filled thermometer (American Scientific Products, No. T2002-4).

RESULTS

Figure 1 shows a time course for the exposure of α_2-adrenoceptors to TNM. It is clearly evident that chemical modification of α_2-adrenoceptors by TNM results in a significant loss of binding activity. As one would expect of a bimolecular reaction, the loss of binding activity was both time and concentration dependent. Thus, at the earliest time point (10 min) a 35% loss of binding activity occurred in the presence of 80 μM TNM and a 70% loss in the presence of 320 μM TNM. After 90 min of exposure, a nearly complete loss of α_2-adrenoceptor activity was obtained with 320 μM TNM. Exposure to 2% ethanol alone (control) resulted in a slight loss of binding activity over the same time course.

The concentration dependence of α_2-adrenoceptor inactivation by TNM was investigated in the presence and absence of 1 μM phentolamine. The results of these experiments, done for 1 hr on ice, are shown in Fig. 2. Again a marked decrease in binding activity occurred with exposure of the α_2-adrenoceptor to increasing concentrations of TNM. Interestingly, however, the loss of binding activity could be prevented when the exposure of α_2-adrenoceptors to TNM was done in the presence of phentolamine, an α-adrenergic ligand. The pharmacologic properties of this protection against receptor inactivation were examined, and the results are shown in Fig. 3. These results clearly show an α_2-adrenergic pattern of protection. Thus, at 200 nM, both yohimbine and phen-

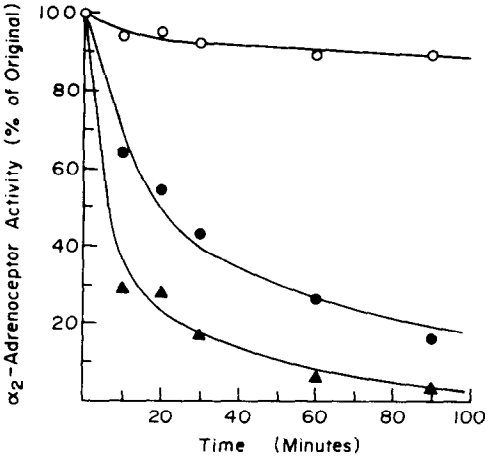

Fig. 1. Time course for the inactivation of partially purified α_2-adrenoceptors using two concentrations of TNM. Partially purified α_2-adrenoceptors were mixed with TNM to give final concentrations of either 80 (●) or 320 (▲) μM TNM. The control (○) contained a final concentration of 2% ethanol. The samples were incubated on ice for the indicated amount of time and were then desalted by Sephadex G-50 chromatography to remove free TNM. The binding of [^{3}H]yohimbine was then measured at a final concentration of 6 nM. One hundred percent equals 34 fmoles of [^{3}H]yohimbine binding activity.

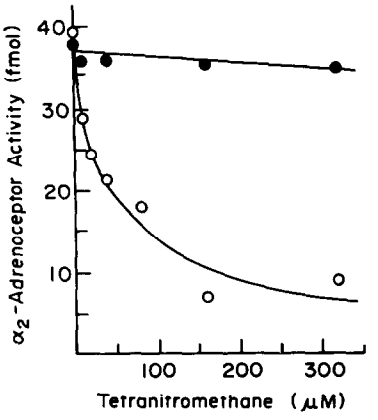

Fig. 2. Concentration dependence for the inactivation of α_2-adrenoceptors by TNM in the presence (●) or absence (○) of 1 μM phentolamine. A solution of TNM in ethanol was added to a partially purified receptor preparation to give the final concentrations of TNM listed in the figure. After a 60-min incubation on ice, the samples were subjected to gel filtration over Sephadex G-50, and the binding of [^{3}H]yohimbine was then determined.

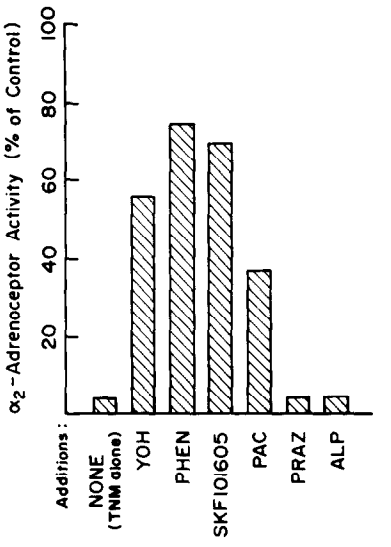

Fig. 3. Protection against TNM-induced α_2-adrenoceptor inactivation by various adrenergic ligands. Adrenergic ligands were added to samples of partially purified α_2-adrenoceptors to give final concentrations of 200 nM. The samples were incubated on ice for 10 min and TNM was added to give a final concentration of 320 μM. The samples, in a final volume of 500 μl, were left on ice for another 60 min and were then desalted by Sephadex G-50 chromatography. The binding of [^{3}H]yohimbine was determined and the results were expressed as a percentage of a control sample containing α_2-adrenoceptors and a final concentration of 2% ethanol. One hundred percent equals 35 fmoles of [^{3}H]yohimbine binding activity. Abbreviations: YOH, yohimbine; PHEN, phentolamine; PAC, *p*-aminoclonidine; PRAZ, prazosin; and ALP, (−)alprenolol.

tolamine were effective at preventing α_2-adrenoceptor inactivation while prazosin (α_1-specific) and (−)alprenolol (a β-adrenergic antagonist) were completely inactive. SKF 101605, an α_2-selective antagonist with an affinity comparable to that of phentolamine [10], was also quite effective in preventing α_2-adrenoceptor inactivation. In the presence of 200 nM *p*-aminoclonidine, an α_2-selective agonist, ~40% of the α_2-adrenoceptors were protected against inactivation by TNM. As compared with phentolamine, this lower level of protection by *p*-aminoclonidine probably reflects its lower affinity for solubilized α_2-adrenoceptors ($K_I \sim 80$ nM, unpublished results). At 2 μM *p*-aminoclonidine, ~75% of the original α_2-adrenoceptor activity was present following exposure to TNM (data not shown). The use of other adrenergic agonists for protection experiments was difficult to evaluate since most of these ligands are catecholamines which are capable of reacting directly with TNM.

Saturation curve analysis was used to determine if the decrease in α_2-adrenoceptor activity was due to allosteric effects or to blockade of the ligand binding site. The data from these studies were plotted according to Scatchard [11], and the results are shown in Fig. 4. Exposure of α_2-adrenoceptors to either 20 or 100 μM TNM caused respective 29 or 46% decreases in the B_{max} of [^{3}H]yohimbine binding. In marked contrast, chemical modification with TNM had no effect upon the K_d of [^{3}H]yohimbine binding to the α_2-adrenoceptor. These results are entirely consistent with the supposition that the receptor inactivation caused by TNM is due to a direct effect upon the ligand binding site of α_2-adrenergic receptors.

The most likely amino acids to be chemically modified by TNM are tyrosine and cysteine. Previous studies have shown that membrane bound α_2-adrenoceptors can be inactivated by reagents which react specifically with the sulfhydryl group of cysteine [4, 5]. We have also found that solubilized, partially purified, and purified α_2-adrenoceptors can be inactivated with phenylmercuric chloride (PMC). In addition, we have found that the PMC-induced inactivation of α_2-adrenoceptors is reversible by subsequent treatment with the low molecular weight thiol, DTT [3]. The reaction of PMC is specific for cysteine residues only, and we used the reversible nature of the PMC reaction to explore the specificity of the TNM reaction. Thus, partially purified α_2-adrenoceptors were first reacted with PMC. Then the receptors were subjected to a reaction with TNM; at this point since the cysteines are already blocked, TNM can only react with the tyrosine residues. Finally, the binding of [^{3}H]yohimbine was measured in the presence and absence of DTT. The results of these experiments are shown in Fig. 5. The control shows that the presence of DTT itself had no effect upon the binding of [^{3}H]yohimbine to α_2-adrenoceptors. When the partially purified α_2-adrenoceptors were exposed to PMC, a 90% loss in binding activity resulted; however, in the presence of 5 mM DTT, the binding activity was restored almost completely. On the other hand, exposure of α_2-adrenoceptors to TNM alone resulted in a 95% loss of binding activity which remained unchanged when binding was measured in the presence of DTT. When receptors were first reacted with PMC and then with TNM, there was a 98% loss of binding activity which was decreased to 75% in the presence of DTT. This

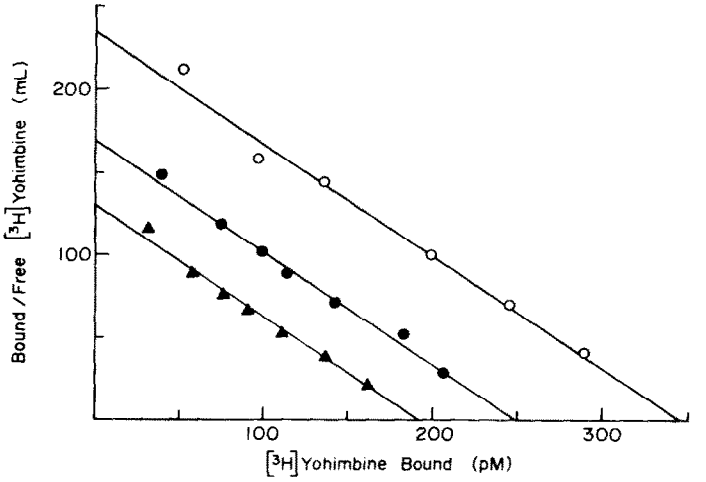

Fig. 4. Scatchard plots of the inactivation of α_2-adrenoceptors by TNM. Partially purified α_2-adrenoceptors were incubated for 60 min on ice with a final concentration of either 20 μM TNM (●), 100 TNM μM (▲) or 2% ethanol (○, control). The samples were then chromatographed over Sephadex G-50, and the binding of [^{3}H]yohimbine was determined at seven concentrations between 0.3 and 7.3 nM. Computerized nonlinear regression analysis yielded the following parameter estimates: control, K_d = 1.5 nM, B_{max} = 350 pm; 20 μM TNM, K_d = 1.5 nM, B_{max} = 250 pM; and 100 μM TNM, K_d = 1.5 nM, B_{max} = 190 pM.

partial reversal by DTT suggests that some of the residues modified by TNM are cysteines; however, the inability of DTT to completely restore binding activity indicates that the inactivation of α_2-adrenoceptors is probably due to a predominant reaction with tyrosine residues. Recently the experiments depicted in Fig. 5 have been repeated using α_2-adrenoceptors purified to apparent homogeneity. Virtually identical results were obtained, demonstrating that the findings obtained with the partially purified receptors were not due to nonspecific reactions with other proteins.

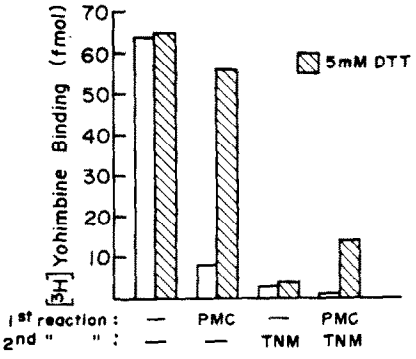

Fig. 5. Effect of DTT following α_2-adrenoceptor inactivation by PMC, TNM, and a successive treatment with PMC and TNM. Partially purified α_2-adrenoceptors were preincubated for 30 min on ice either alone or with 10 μM PMC present. The samples were desalted by Sephadex G-50 chromatography, and aliquots were taken and incubated either alone or with 320 μM TNM present. After 60 min on ice, the samples were chromatographed over Sephadex G-50, and the binding of [^{3}H]yohimbine was measured in the presence (shaded bars) and absence (open bars) of 5 mM DTT.

Other reagents that may also be used to chemically modify tyrosine include iodine [12] and *p*-nitrobenzenesulfonyl fluoride [13]. The results of chemical modification of α_2-adrenoceptors with these reagents are shown in Fig. 6. In part A, α_2-adrenoceptors were inactivated in the presence of sodium iodide and chloramine T. The inactivation was rapid and it was prevented when the antagonist ligand, yohimbine, was present during the reaction. The presence of both sodium iodide and chloramine T was necessary for receptor inactivation which is consistent with a mechanism involving an initial oxidation of iodide to iodine, followed by iodination and inactivation of the α_2-adrenoceptor [12]. In Fig. 6B, the results of the reaction of *p*-nitrobenzenesulfonyl fluoride (NBSF) with partially purified α_2-adrenoceptors are shown. After a 2-hr reaction at 4–6°, there was a 75% loss of binding activity at 500 μM NBSF which increased to 95% at 3 mM NBSF. As was the case with TNM, the presence of the α_1-antagonist prazosin did not prevent this loss of binding activity, whereas the presence of phentolamine prevented any significant α_2-adrenoceptor inactivation by NBSF. The loss of binding activity due to exposure of the α_2-adrenoceptor to NBSF is most likely the result of a specific sulfonylation of the aromatic hydroxyl group of tyrosine by NBSF [13].

DISCUSSION

The results of this study show that treatment of unliganded α_2-adrenoceptors with reagents that can covalently modify tyrosine residues caused an irreversible loss of binding activity. In the case of exposure of the α_2-adrenoceptor to TNM, inactivation is probably the result of electronic or steric changes arising from nitration of tyrosine side

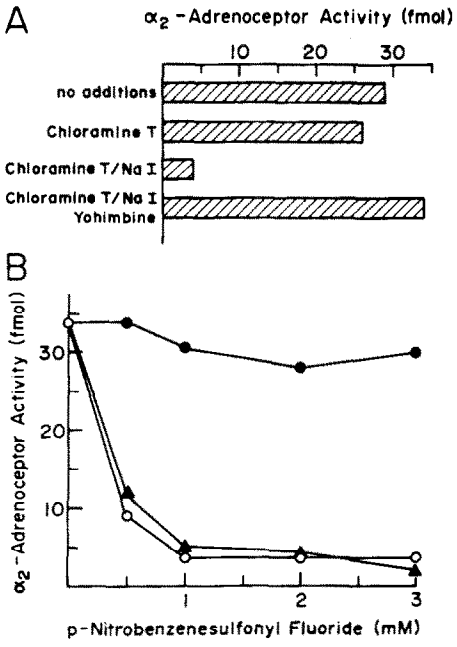

Fig. 6. Iodination (A) and sulfonylation (B) of α_2-adrenoceptors. (A) Partially purified α_2-adrenoceptors were incubated at room temperature (~24°) for 60 sec with the compounds listed in the figure. The final concentrations of the compounds were as follows: chloramine T, 570 μM; NaI, 1 μM; and yohimbine, 2 μM. Following the incubation, sodium metabisulfite was added to give a final concentration of 5.9 mM, and the samples were cooled on ice and then desalted by Sephadex G-50 chromatography. The binding of [^{3}H]yohimbine was then determined. (B) A solution of *p*-nitrobenzenesulfonyl fluoride (NBSF) in ethanol was added to partially purified α_2-adrenoceptors to give the final concentrations listed in the figure. In addition to the samples containing NBSF alone (○), samples were also prepared which contained NBSF and 1 μM phentolamine (●), and NBSF and 1 μM prazosin (▲). Following a 2-hr incubation at 4–6°, the samples were desalted over Sephadex G-50, and the binding of [^{3}H]yohimbine was assessed.

chain(s). The loss of binding activity could be prevented or reduced if exposure of the receptor to TNM were done in the presence of ligands which occupy the ligand binding site. There did not appear to be any significant qualitative differences between agonists and antagonists in their abilities to protect the α_2-adrenoceptor against inactivation. The results of saturation studies indicate that the loss of binding activity caused by TNM is due to direct, as opposed to allosteric, effects upon the ligand binding site of the α_2-adrenoceptor.

Recently, a selective effect on agonist binding was obtained following TNM treatment of muscarinic acetylcholine receptors [14]. Thus, treatment of membrane bound muscarinic receptors with TNM resulted in an increase in the affinity of some agonists but had no effect on the binding of antagonists. A similar effect has also been shown following the treatment of muscarinic receptors with sulfhydryl oxidizing reagents [15, 16] and with sulfhydryl alkylating reagents [17]. It appeared that the effects of TNM were due to a reaction with tyrosine and they were consistent with the modification of a residue involved with ligand binding [14]; however, an allosteric effect involving the formation of the agonist high affinity state also seems quite possible. In any event, the effects of TNM and of sulfhydryl specific reagents upon the α_2-adrenoceptor seem to be fundamentally different from their effects upon muscarinic acetylcholine receptors. These differences may reflect different susceptibilities of the tyrosine residues present in the ligand binding sites, as compared with the guanine nucleotide protein-coupling sites, of these adenylate cyclase inhibitory receptors.

As with many of the reagents that can react with tyrosine, TNM is also capable of oxidizing the sulfhydryl group of cysteine. In fact, in some cases the reaction of TNM with cysteine exceeds its reaction with tyrosine; however, inactivation of enzymes caused by the oxidation of cysteine residues is usually partially or completely reversible following treatment of the modified enzyme with DTT [18]. In the present experiments, exposure of the TNM-treated α_2-adrenoceptor to DTT did not restore any binding activity which suggests that the loss of binding activity was not due to reaction with cysteines. On the other hand, the experiments done with phenylmercuric chloride suggest that part of the receptor inactivation may be due to the reaction of TNM with cysteine. In all probability, the effects of TNM on the α_2-adrenoceptor are due to reactions with both tyrosine and cysteine residues; however, the predominant reaction is probably with tyrosine. If TNM does react with cysteine, it appears to involve oxidation of the sulfhydryl beyond the formation of simple disulfides.

It appears likely from the present results and from other studies [4, 5] that both tyrosine and cysteine are located in close proximity to the ligand binding site of α_2-adrenoceptors. The precise function of these amino acids is presently unknown but one possibility is that their side chains are directly involved in ligand binding. Analogous effects have been documented in studies of the chemical modification of enzymes by TNM. For example, a tyrosine residue participates in the binding of the inhibitory substrate, deoxythymidine 3′,5′-diphosphate, to staphylococcal nuclease [19]. Treatment of staphylococcal nuclease with TNM in the absence of the inhibitory substrate results in nitration of this tyrosine and a loss of enzymatic activity; treatment in its presence prevents nitration and the loss of enzymatic activity [20]. An interesting effect of TNM on carboxypeptidase is to enhance its esterase activity while reducing its peptidase activity [21]. This effect is prevented when the nitration is performed in the presence of the inhibitory substrate β-phenylpropionate. With regard to the α_2-adrenoceptor, an obvious question is how TNM treatment affects other functions, such as its ability to inhibit adenylate cyclase via its interaction with the guanine nucleotide binding protein, N_i. Future studies with TNM and other reagents should provide answers to these and other questions regarding structural aspects of the α_2-adrenoceptor and other adrenergic receptors.

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