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Chemical modifications of striatal A_{2A} adenosine receptors: a possible role for tyrosine at the ligand binding sites

Claudia Martini *, Letizia Trincavelli, Antonio Lucacchini

Istituto Policattedra di Discipline Biologiche, Università di Pisa, Via Bonanno 6, 56126 Pisa, Italy

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Abstract

A_{2A} adenosine receptors were examined in bovine striatal membranes following exposure to tetranitromethane (TNM) which modifies tyrosine and cysteine residues. TNM (0.05–0.5 mM) treatment caused an irreversible, concentration-dependent decrease in the binding activity of the selective A_{2A} agonist [^{3}H]CGS 21680. Protection studies showed that TNM inactivation could be prevented by the adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) and by the antagonist xanthine amine congener (XAC), suggesting that TNM modified residues at the ligand-binding sites. Scatchard analysis of the binding data showed that 0.15 mM TNM decreased the [^{3}H]CGS 21680 B_{max} value from 447 ± 39 to 273 ± 21 fmol/mg of proteins without any significant change in the K_d values (13.5 ± 1.4 and 14.7 ± 1.5 for control and treated membranes, respectively). We carried out a series of successive chemical modifications with the reducing agent dithiothreitol (DTT), which indicated that the residues modified by TNM, under our experimental conditions, are tyrosine residues and not cysteine residues.

Keywords: A_{2A} adenosine receptor; Tyrosine residue; Chemical modification

1. Introduction

Adenosine acts as a neuromodulator in the central and peripheral nervous system and as a homeostatic regulator in a variety of other tissues, including heart, kidney and immune system [1]. These effects involve a receptor-mediated mechanism including direct interaction with second messenger systems (via G proteins) [2,3], transmembrane ion fluxes [4] and neurotrasmitter release [5,6]. Four subtypes of adenosine receptors (ARs), referred to as A_1, A_{2A}, A_{2B} and A_3, have been cloned and expressed [7] from several species including rats [8–11], dogs [12,13], mice and humans [14].

Pharmacologically, the A_{2A} receptor is associated with hypotensive [15], immnunosuppressive [16], platelet antiaggregatory [17] and locomotor depressant effects [18].

Relatively little is known about the biochemistry of A_{2A} ARs as a whole, and even less about the ligand-binding site itself. A model [19] featuring seven transmembrane helices characteristic of G protein-linked receptors, based on structural analysis of canine A_{2A} ARs, has been proposed. Briefly, this model predicts that the receptor consists of seven alpha-helical membrane-spanning domains connected by three extracellular and three intracellular loops; the N- and C-termini of the protein are situated on

* Corresponding author. Fax. +39-50-40517; E-mail: cmartini@farm.unipi.it

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the extracellular and intracellular sides of the plasma membrane, respectively. Furthermore, A_{2A} ARs contain several features common to all G-protein-coupled receptors which display this structure: these include the presence of cysteine residues on the extracellular loops which, by analogy with other receptors, may be involved in disulfide bond formation. A sensitivity of [^{3}H]CGS 21680 binding in rabbit striatum to the reducing agents dithiothreitol and sodium dithionite suggests that such bonds are present in A_{2A} ARs [20]. A role for histidine residues in mediating ligand-binding at A_{2A} ARs has been suggested by the ability of diethylpyrocarbonate (DEP), a His-reactive compound, to diminish both the agonist [^{3}H]CGS 21680 and the antagonist [^{3}H]XAC binding in treated rabbit striatal membranes [20].

The aim of this study was to further characterize the site of interaction of the agonist [^{3}H]CGS 21680 with A_{2A} ARs, in bovine striatum, by treatment with tetranitromethane (TNM).

TNM has been shown to be a convenient reagent for the nitration of tyrosine residues in proteins [21], although specificity studies have shown that other residues, in particular sulfhydryl groups, are potentially reactive towards TNM.

2. Materials and methods

[^{3}H]CGS 21680 (47.2 Ci/mmol) was obtained from Dupont–New England Nuclear (Boston, MA, USA); Adenosine deaminase, NECA and XAC were purchased from Boehringer-Mannheim (Mannheim, Germany); TNM and other chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA).

2.1. Membrane preparation

Bovine striatal membrane preparation was performed as previously described [22]. Briefly, the tissue was homogenised in 20 volumes of ice-cold 50 mM Tris HCl buffer at pH 7.5 containing 10 mM $MgCl_2$ (T_1) in the presence of protease inhibitors (160 μg/ml benzamidine, 20 μg/ml soybean trypsin inhibitor and 200 μg/ml bacitracine) and centrifuged at $48\,000 \times g$ for 10 min at 4°C. The resulting pellet was resuspended in T_1 buffer containing protease inhibitors and 2 IU/ml of adenosine deaminase and incubated at 37°C for 30 min to remove endogenous adenosine.

This preparation was recentrifuged and the final pellet was frozen at −70°C until the time of assay.

2.2. TNM treatment

Nitration of striatal membranes (2–3 mg/ml proteins) was performed with TNM at pH 8.1 as previously described [23]. Briefly, aliquots of TNM in ethanol were added to the receptor preparations to yield a final TNM concentration of 0.05–0.5 mM in 2% ethanol. Control membranes were subjected to the same procedure, but only ethanol was added.

Incubation with gentle mixing was carried out at constant pH, at room temperature for 30 min. Following incubation with TNM, the membrane suspensions were pelleted by centrifugation and washed three times with reaction buffer, 50 mM Tris HCl at pH 8.1 (1:20) (T_2) to remove the excess of the reagent.

In order to evaluate the site specificity of the inactivation reaction, the protection of [^{3}H]CGS 21680 binding against TNM modification was investigated as follows: membranes were preincubated separately with NECA (1 μM) or XAC (5 μM), and then 0.15 mM TNM was added to the samples. At the end of the modification reaction, membranes were centrifuged and the ligands were removed by washing. Control samples were prepared by incubating aliquots of membranes in the same experimental conditions without the reagent.

TNM modification and protection experiments were carried out with the same procedure at pH 6.5 too.

In some experiments, treatment with DTT, a sulfhydryl reagent (10 mM), preceded or followed the nitration step. This treatment was carried out at 25°C, pH 8.1, for 30 min and was separated from the nitration step by washing of the membranes in buffer T_2.

2.3. Radioligand binding

The [^{3}H]CGS 21680 binding assay was carried out in triplicate as previously described [24]. Briefly, aliquots of striatal membranes (0.3 mg of proteins) were incubated in 500 μl T_1 buffer with [^{3}H]CGS

21680 (5 nM), both in the presence and in the absence of unlabeled NECA (100 μM) for non-specific binding determination. After 90 min at 25°C, the binding reaction was stopped by filtration through Whatman GF/C fiber glass filters, and then five washes with 5 ml ice-cold buffer were performed.

Modification by TNM was evaluated by assaying the [^{3}H]CGS 21680 binding to control and treated membranes.

Saturation analysis of [^{3}H]CGS 21680 binding sites was performed on control and treated membranes using 1.6–46 nM radioligand concentrations.

For competition studies, 6–8 different concentrations of the agonist NECA or the antagonist XAC were incubated with the T_1 buffer containing 5 nM [^{3}H]CGS 21680, both in control and in TNM-treated membranes.

Protein concentration was determined by the method of Lowry et al. [25] using bovine serum albumin as a standard.

2.4. Data analysis

Statistical analysis and curve-fitting were carried out on an IBM-compatible personal computer using the non-linear multipurpose curve-fitting program KINETICS, EBDA and LIGAND [26], from which the values of K_{ob}, the dissociation constant (K_d) and the maximum number of receptor sites (B_{max}) were generated. Accordingly, a partial F test ($P < 0.01$) was used to determine whether the binding data were best fitted by a one- or two-site model. IC_{50} values were derived from semilog plots of data from agonist/antagonist displacement experiments. The Cheng-Prusoff equation was used to calculate K_i values from IC_{50} values [27]. Values represent the means ± SE derived from (n) experiments conducted in triplicate. The difference among binding parameters obtained in untreated and TNM-treated membranes was evaluated by one-way analysis of variance (ANOVA).

3. Results

3.1. Effects of TNM treatment on radioligand binding

The chemical modification of A_{2A} ARs by TNM, carried out as described in the Material and Methods section, resulted in a significant loss of [^{3}H]CGS 21680 binding activity, which was both concentration- and time-dependent.

After 15 min, a nearly complete loss of A_{2A} AR activity was obtained with 0.5 mM TNM (Fig. 1). The IC_{50} value of TNM for inhibition of [^{3}H]CGS 21680 binding was found to be 0.18 ± 0.013 mM.

When B/B_o (B is the binding after pretreatment of membranes with TNM for the periods indicated, and B_o is control binding) was plotted on a logarithmic scale, the decrease appeared to be linearly related to the preincubation time (Fig. 2). Therefore, the inactivation proceeded according to apparent first-order kinetics. The apparent first-order constant (K_{ob}) is defined by the equation $\ln B/B_o = -K_{ob}t$, as shown in Fig. 2.

Exposure to 2% ethanol alone (control) resulted in a slight drop of binding activity over the same time course.

The reduction in agonist-binding induced by TNM could be due to maximum binding or affinity decrement, or both factors together. To establish which of these factors were affected after TNM treatment, equilibrium parameters of agonist-binding were assayed by saturation analysis. The data were analyzed in accord with the Scatchard method [28], and are shown in Fig. 3. The one-site model provided a

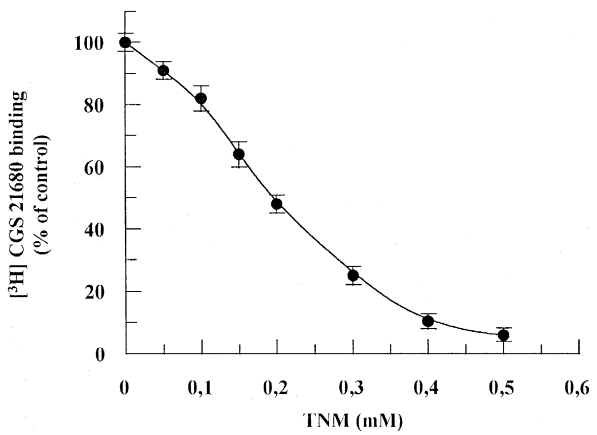

Fig. 1. Concentration dependence of the inactivation of A_{2A} ARs by TNM. Membranes were preincubated with increasing concentrations of TNM (0.05–0.5 mM) for 15 min at 25°C, washed three times with reaction buffer and incubated with 5 nM [^{3}H]CGS 21680. The subsequent binding assay involved 90 min incubation followed by rapid filtration. Values represent means ± standard errors of three separate experiments.

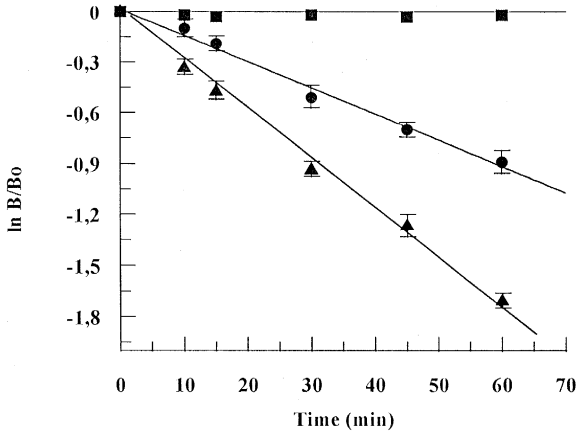

Fig. 2. Time-dependent inactivation of [^{3}H]CGS 21680 binding sites by TNM. Membranes were pretreated with: (■) only 2% ethanol in buffer, control; (●) 0.1 mM TNM and (▲) 0.2 mM TNM for the periods of time indicated at 25°C, washed and assayed with [^{3}H]CGS 21680 as described in Section 2. Control binding referred to binding of [^{3}H]CGS 21680 to membranes treated as described in Section 2. The pseudo-first order rate constants calculated from the slopes of the lines shown are $1.3\times10^{-2}\pm7.8\times10^{-4}$ and $2.8\times10^{-2}\pm1.9\times10^{-3}$ min^{-1}. Values represent means ± standard errors of at least six determinations carried out in triplicate.

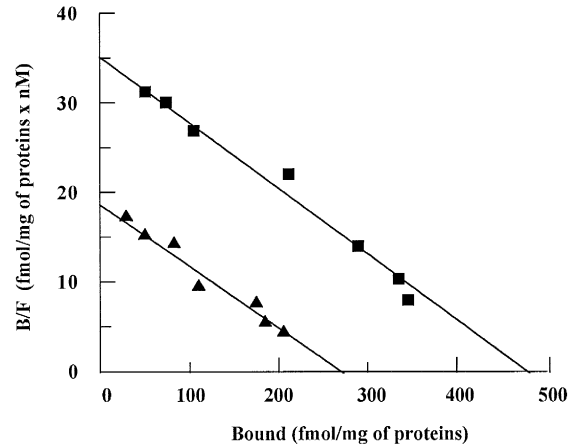

Fig. 3. Representative Scatchard plots of specifically bound [^{3}H]CGS 21680 to control (■) and 0.15 mM TNM-treated (30 min) (▲) membrane preparations. The membranes (0.3 mg of proteins) were incubated in triplicate with increasing concentrations of [^{3}H]CGS 21680 (1.6–46 nM) in the absence and in the presence of unlabeled NECA (100 μM) for 90 min at 25°C as described in Section 2. The data of specifically bound [^{3}H]CGS 21680 were plotted according to Scatchard [28]. Data are from a single experiment carried out in triplicate. Three such experiments yielded similar results.

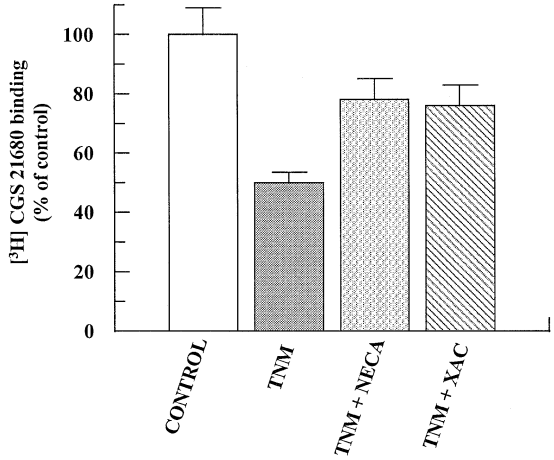

Fig. 4. Protection from inactivation by TNM. Membranes were preincubated with buffer alone as control, with 1 μM NECA or 5 μM XAC for 10 min. Then 0.15 mM TNM-treatment for 30 min was performed as described in Section 2. After extensive washing, the binding of [^{3}H]CGS 21680 was determined. Data points are means ± standard errors of three experiments carried out in triplicate and are presented as the percentage of specific [^{3}H]CGS 21680 binding versus control.

significantly ($P < 0.05$) better fit and allowed detection of high affinity binding sites both in control and in TNM-treated membranes. Exposure of bovine A_{2A} ARs to 0.15 mM TNM for 30 min caused a significant decrease in the maximum density of [^{3}H]CGS 21680 binding sites against the control value (273 ± 21 vs. 477 ± 39 fmol/mg of proteins respectively) ($P = 0.025$). By contrast, the chemical modification had no effect upon the K_d of the [^{3}H]CGS 21680 binding to A_{2A} ARs (13.5 ± 1.4 nM for control and 14.7 ± 1.5 nM for treated membranes).

The addition of specific adenosine receptor ligands, 1 μM NECA or 5 μM XAC, to the preincubation medium, was significantly suitable to prevent the inactivation of [^{3}H]CGS 21680 radioligand binding (Fig. 4) ($P = 0.0057$ for TNM + NECA and $P = 0.0036$ for TNM + XAC). As shown, both the agonist and the antagonist had the same ability to protect [^{3}H]CGS 21680 binding sites by inactivation.

Fig. 5 (panels A and B) shows the displacement of [^{3}H]CGS 21680 binding to control and 0.15 mM TNM treated membranes by increasing concentrations of NECA and XAC. The shape of the displacement curve was not significantly altered for either ligand by chemical modification; the K_i values for NECA were 14 ± 1.1 nM and 12.3 ± 1.0 nM in

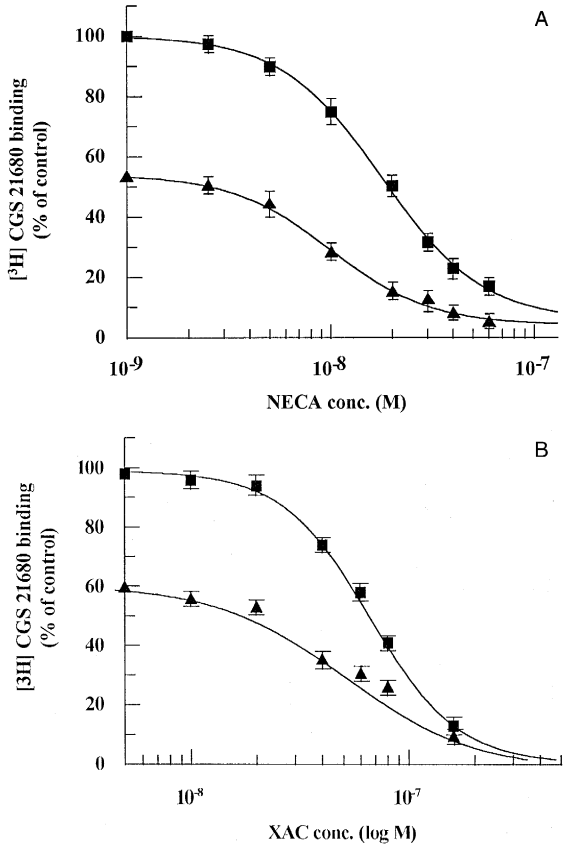

Fig. 5. Inhibition curves of [^{3}H]CGS 21680 displaced by NECA (panel A) and XAC (panel B) to bovine striatal A_{2A} ARs before and after TNM treatment. Binding was carried out after 30 min preincubation at 25°C in the presence of 0.15 mM TNM (▲) and in control membranes (■). Membranes were washed three times with reaction buffer before radioligand binding. Membranes were incubated with the radioligand at 25°C for 90 min. Data points are means ± standard errors of three experiments conducted in triplicate.

control and treated membranes, respectively, and for XAC they were 50 ± 4.2 nM and 58 ± 4.9 nM, respectively.

To demonstrate that TNM inactivation of A_{2A} ARs was due to the nitration of tyrosine residues at the ligand binding site and/or oxidation of SH groups, we carried out a series of successive chemical modifications of the membranes.

If TNM treatment at pH 8.1 induced oxidation of SH to S–S at the binding site, the reducing agent DTT should restore their original properties. As shown in Table 1, the sulfhydryl-reducing agent DTT (10 mM) did not restore the properties of TNM-modified receptors. Furthermore, pretreatment with DTT (protecting the sulfhydryl groups) did not prevent TNM inactivation. These results would appear to exclude the possibility that TNM modified cysteine residues in our experimental conditions (at pH 8.1).

Table 1
Combined effects of TNM and DTT on A_{2A} adenosine receptors from bovine striatal membranes

Membrane preincubation		[^{3}H]CGS 21680 binding (% of control)
First preincubation	Second preincubation	
Buffer	Buffer	100 ± 7.0
TNM	Buffer	37 ± 2.1
TNM	DTT	35 ± 1.9
DTT	Buffer	61 ± 4.8
DTT	TNM	17 ± 1.1

Membranes were preincubated with buffer or TNM (0.2 mM) or DTT (10 mM) for 30 min at 25°C and washed as described in the text. Then, a second incubation with buffer or with indicated compounds were performed followed by washings as indicated. Aliquots of control and treated membranes were monitored by [^{3}H]CGS 21680 binding. Data are means ± standard error of three experiments.

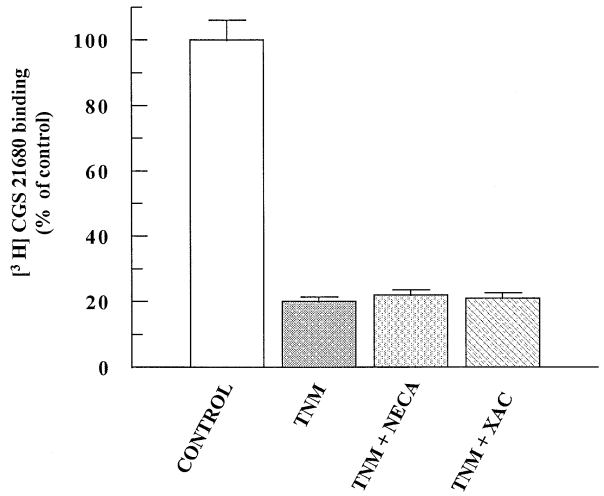

Fig. 6. Inactivation of [^{3}H]CGS 21680 binding sites by TNM at pH 6.5 and protection by NECA and XAC. Membranes were preincubated with buffer alone, with 1 μM NECA or 5 μM XAC for 10 min. Then 0.15 mM TNM treatment for 15 min was performed as described in Section 2. After extensive washing, the binding of [^{3}H]CGS 21680 was determined. Data points are means ± standard errors of three experiments carried out in triplicate and are presented as the percentage of specific [^{3}H]CGS 21680 binding versus control.

The nitration of tyrosine residues is expected to proceed much more slowly at pH 6.5 than at pH 8.1, whereas the oxidation of cysteine is efficient at the lower pH [21]. Unexpectedly, when we subjected an aliquot of the membrane receptor preparation to treatment with 0.15 mM TNM at pH 6.5 the loss of [^{3}H]CGS 21680 binding activity was more marked than at pH 8.1: 30% of [^{3}H]CGS 21680 binding capacity was lost after 15 min at pH 8.1 while the corresponding value at pH 6.5 was 80% (Fig. 6).

The inhibition of radioligand binding by TNM at pH 6.5 was not significantly prevented by the addition of A_{2A} AR ligands, 1 μM NECA or 5 μM XAC, to the preincubation medium (Fig. 6). This suggests that the residues that can be modified at this pH, probably cysteines, are not located at the [^{3}H]CGS 21680 adenosine-binding sites.

4. Discussion

Over the past few years, the synthesis of adenosine derivatives as agonists, and xanthine and non-xanthine derivatives as antagonists has made it possible to carry out a rigorous examination of the molecular structure and regulation of adenosine receptors. The subsequent use of gene cloning and molecular biology technology has advanced insight into the molecular characteristics of these receptors, such as their primary sequence, structural homology between species, distribution in tissues and cellular expression. A pivotal point in the field of purinergic receptors was the cloning and expression of RDC7 and RDC8 from a dog thyroid cDNA library, using a screening strategy based on sequence homology with other G-protein-coupled receptors. RDC7 and RDC8 were found to encode the A_1 and A_2 adenosine receptor subtypes, respectively, through expression in COS and CHO cells in which functional coupling to adenylyl cyclase and radioligand binding was demonstrated [29,30].

A considerable body of work studying the binding at cloned biogenic amine receptors (particularly adrenergic and muscarinic receptor subtypes) has demonstrated the importance of the amino acids present in the transmembrane-spanning regions in mediating agonist and antagonist recognition [31].

Owing to the proteic nature of these receptor sites, their chemical modification by protein-group selective reagents may disclose the chemical topography of the recognition sites and their regulatory interrelationship as well. In the present study, we used TNM as a modifying reagent in a receptor–ligand assay to delineate specific tyrosine residues involved in high-affinity [^{3}H]CGS 21680 binding and A_{2A} ARs. Under mild conditions, in a pH-dependent fashion, at pH 8.1 and above, TNM is an efficient, specific reagent for the nitration of tyrosine residues.

The treatment of A_{2A} ARs with TNM at pH 8.1 caused an irreversible loss of [^{3}H]CGS 21680 binding activity of more than 90%. The loss of the binding activity could be reduced if exposure of receptors to chemical modification was performed after preincubation with ligands which occupied the ligand-binding site. Both the agonist NECA and the antagonist XAC were able to protect the receptor against TNM inactivation. These results indicated a direct, rather than allosteric, effect of the TNM agent upon the ligand site of A_{2A} ARs, suggesting the existence at the binding sites of TNM-modified residues with an important role, for both agonist and antagonist binding.

The results of saturation studies for A_{2A} ARs showed that TNM treatment reduced the number of [^{3}H]CGS 21680 binding sites, with no change in the affinity for the agonist. Furthermore, the binding site characteristics before and after TNM treatment were investigated by competition experiments with the agonist NECA and the antagonist XAC. The affinity constant (K_i) obtained for both ligands in native and TNM-modified receptors was unchanged.

These results provided evidence that TNM modification was specific and occurred in or near the binding sites of A_{2A} ARs, probably without any conformational change.

Also A_1 adenosine receptors were modified by TNM treatment, as reported by Klotz et al. [32]. As the inactivation of agonist and antagonist binding could not be prevented by adenosine receptor ligands, the modification probably does not occur at the A_1 binding sites.

Recently, Ijzerman and co-workers have described a three-dimensional model for rat A_{2A} ARs, using the A_{2A} amino-acid sequence and the atomic coordinates of bacteriorhodopsin [33]. This model predicted the

presence in the A_2a receptor of a hydrophobic domain, consisting of Ile^{139}, Phe^{177}, Phe^{252}, Cys^{249} and Tyr^{174}, which is responsible for A_{2A} selectivity. These data could justify the different effects obtained by the chemical modification of tyrosine residues for A_1 and A_2 receptors.

TNM is well known for its ability to nitrate tyrosine residues as well as to oxidize free sulfhydryl groups. A number of successive chemical modifications were performed in order to determine whether cysteine residues are involved in the nitration procedures.

As summarized in Table 1, the protection of the S–H groups obtained with DTT pretreatment did not account for the functional consequences of TNM treatment. Preincubation with the sulfhydryl-reducing agent induced a decrease in [^{3}H]CGS 21680 binding (probably due to the reduction of the S–S bonds present in A_{2A} ARS) without any effects on TNM inactivation. In fact, TNM induced the same decrease in [^{3}H]CGS 21680 binding in native and DTT-treated membranes.

Furthermore, if TNM induced oxidation of cysteine residues at binding sites, the reducing agent DTT, applied to modified receptors, should restore their original properties. Treatment with DTT did not restore the properties of the TNM-modified receptors, thus excluding the possibility that TNM modified cysteine residues under these experimental conditions.

Experiments carried out at pH 6.5 did not exclude the presence of other modifiable residues, probably cysteines: when TNM modification was performed at pH 6.5, we observed a higher level of [^{3}H]CGS 21680 binding inactivation than at pH 8.1. However, the agonist and antagonist ligands were unable to protect the sites from reagent modifications, indicating that no residues are located on the adenosine A_{2A} ARs sites. Studies are required to better clarify the involvement of cysteine residues in [^{3}H]CGS 21680 binding.

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