Biochemical Pharmacology, Vol. 58, pp. 623–632, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00135-5

Mechanisms of Inhibitory Effects of Zinc and Cadmium Ions on Agonist Binding to Adenosine A1 Receptors in Rat Brain

*Anna Maria Rosati and Ugo Traversa**

BASIC RESEARCH AND INTEGRATIVE NEUROSCIENCE CENTRE–DEPARTMENT OF BIOMEDICAL SCIENCES, UNIVERSITY OF TRIESTE, 34127 TRIESTE, ITALY

ABSTRACT. The dose-dependent inhibition of zinc and cadmium ions of agonist binding to A1 adenosine receptors in rat brain is prevented by histidine and cysteine, respectively. In the present study, the possible different mechanisms of Zn^{2+} and Cd^{2+} inhibitions were examined. The effects of Zn^{2+} and Cd^{2+} on equilibrium binding parameters of the agonists N^6-cyclohexyl-[2,8-^{3}H]-adenosine ([^{3}H]CHA) or chloro-N^6-cyclopentyl-adenosine ([^{3}H]CCPA) and the antagonist cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) were compared with those effects of reagents or binding conditions which altered histidyl or cysteinyl residues of the A1 receptor. Zn^{2+} pretreatment did not change A1 agonist or antagonist affinity, but did reduce the B_{max}. The inhibitory effects of Zn^{2+} pretreatments were also maintained after several membrane washings. Diethylpyrocarbonate, a histidine-specific alkylating reagent, behaved like zinc ions: pretreatment with A1 agonist protected the histidyl residues of the [^{3}H]CHA binding site against modification by Zn^{2+}, while the modification of the protonation state of the nitrogen of the imidazole group of histidines by changing pH indicated that the interactions of Zn^{2+} with the histidyl residues were feasible with their unprotonated form. These findings suggest the formation of coordination bonds between Zn^{2+} and histidines critical for [^{3}H]CHA or [^{3}H]DPCPX binding, which may prevent the ligand interaction with the specific sites without modifying the binding kinetics of radioligand to the non-chelated recognition sites. Cd^{2+} pretreatment reduced the [^{3}H]CCPA affinity, but did not modify the affinity of the antagonist [^{3}H]DPCPX, the B_{max} remaining unaffected. As with cadmium effects, the oxidation of the thiol group of cysteine by dithionitrobenzoic acid (DTNB) reduced [^{3}H]CCPA affinity without changing the number of binding sites. The reducing reagent dithiothreitol, which alone was unable to modify [^{3}H]CCPA binding, overcame the inhibiting effects of both Cd^{2+} and DTNB. These findings suggest that cadmium ions may oxidize SH groups of cysteines localized on the A1 receptor molecule or a cysteine localized in the region of $G_i\alpha$ subunit involved in the coupling with receptors. This mechanism can justify potential conformational modifications of the receptor molecule producing the decrease in affinity. BIOCHEM PHARMACOL **58**;4:623–632, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. A1 receptor binding; zinc and cadmium; histidine; cysteine; alkylating agent; redox agents

A number of reports have shown modulations of adenosine receptors by divalent ions. Divalent cations can modulate agonist binding to A1 adenosine receptors in opposite ways. Among divalent cations, Mg^{2+} Ca^{2+} and Mn^{2+} increase [1], but Cu^{2+} Zn^{2+} and Cd^{2+} decrease agonist binding to receptors [2]. Whereas the increasing effect of Mg^{2+} on binding is related to a stimulation of GTP hydrolysis at the α_i subunit of G-protein, the stimulatory effect of Ca^{2+} and Mn^{2+} does not appear to be exerted at the level of G-protein, but rather at the receptor itself [2–5]. The reduction in A1 agonist binding by Zn^{2+} Cu^{2+} and Cd^{2+} paralleled that observed with opioid receptors, where their inhibiting effects were explained by an oxidation of sulfhydryl groups essential for opioid agonist binding [6, 7]. Moreover, zinc ions showed a non-competitive inhibition of $GABA_A$ (γ-aminobutyric acid) receptors, probably binding to some putative histidine groups present in the $\alpha_1\beta_1$ subunits of the receptor molecule [8, 9]. Therefore, we hypothesized that the effects of divalent cations on A1 adenosine receptors might depend on an action on the agonist-binding component of the receptor molecule which could be independent from and superimposed on the coupling of the receptor to G-protein [2, 10].

Adenosine receptor subtypes show the characteristic secondary structure of all G-protein-coupled receptors, i.e. seven predominantly hydrophobic stretches of 20–25 amino acids that span the cell membrane as α helices. A number of reports have indicated that histidyl residues are essential in the A1 ligand recognition binding site [11, 12], and evidence has recently emerged that the two histidine residues (251 and 278) of transmembrane domains VI and

* Corresponding author: Dr. Ugo Traversa, Department of Biomedical Sciences, University of Trieste, via L. Giorgieri 7, 34127 Trieste, Italy. Tel. +39-040-6763533; FAX +39-040-577435; E-mail: traversa@univ.trieste.it

Received 27 October 1998; accepted 19 February 1999.

VII are critical for agonist binding to A1 receptors [13]. Moreover, several cysteine residues are localized in the extracellular domain, in III-IV-VI transmembranal α helices, and in the intracellular loop between I and II α helices. The cysteine thiol groups can be important for the receptor structure.

The aim of the present study was to investigate whether the mechanism of the inhibitory action of zinc and cadmium ions might depend on their effects at the level of the agonist recognition site of A1 adenosine receptors. Zinc and cadmium ions form complexes with amino acids and peptides. The stability constants of zinc complexes are greater than those of cadmium when nitrogen serves as a ligand, whereas cadmium binds more firmly to a free sulfur group [14]. Therefore, both the imidazole group of histidine and the thiol group of cysteine could be a target for the inhibitory actions of the zinc or cadmium ions on the binding of agonists to A1 receptors.

MATERIALS AND METHODS

[^{3}H]CHA* (34.4 Ci/mmol), [^{3}H]CCPA (30.0 Ci/mmol), and [^{3}H]DPCPX (98.1 Ci/mmol) were supplied by NEN-Life Science Products. R-PIA, S-PIA, and DPCPX were supplied by Research Biochemical Inc. Adenosine deaminase from bovine intestine (200 IU/mg) was from Boeringher. Cadmium and zinc, as chloride salts, were from Merck. DEP, DTNB, and DTT were supplied by Sigma-Aldrich. Filter-Count scintillation cocktail was supplied by Camberra Packard. All other chemicals were of analytical grade or the best commercially available.

Preparation of Membranes from Cerebral Cortex

Male Wistar rats, weighing 150–250 g, were killed by decapitation. The brains were quickly removed and the cerebral cortices were dissected on ice and washed with ice-cold 50 mM Tris–HCl buffer, pH 7.4. The tissue was homogenized in 10 volumes of ice-cold buffer with a Ultra-Turrax (Ika-Werk) and centrifuged at 40,000 *g* for 20 min at 4°; the pelleted membrane fraction was washed with 10 volumes of buffer and centrifuged at 40,000 *g* for 20 min at 4°. The final pellet was suspended in 10 volumes of 50 mM Tris–HCl buffer and the membrane suspension, divided into 1 mL aliquots, was stored at −80° for at most 3 months or used immediately in the receptor assays. No difference in binding characteristics were found between stored and fresh tissue. Before use, the membrane suspension was pretreated with adenosine deaminase (2 IU/mL) at 25° for 30 min. Protein content was measured by the method of Lowry *et al.* [15], using BSA as a standard.

Pretreatment of Membranes with Ions

Aliquots of 1 mL of membrane suspension (about 5 mg protein) were treated for 20 min at room temperature with different concentrations of zinc or cadmium ions. The tubes were rapidly spun at maximal speed in an Eppendorf 5412 centrifuge and pellets were washed twice with 1.5 mL cold Tris–HCl buffer to remove the ions. The final pellets were resuspended in 1 mL buffer and used in the binding assays. Protection of [^{3}H]CHA binding against Zn^{2+} modifications was investigated as follows: the membranes were incubated at room temperature with 1 nM [^{3}H]CHA for 120 min to reach binding equilibrium, and thereafter increasing concentrations of $ZnCl_2$ were added and the reaction stopped after a further 20-min incubation.

Modification of Histidyl Groups

Histidyl groups were modified by treatment with the histidine-specific reagent DEP [16]. According to Garritsen *et al.* [17], DEP was freshly dissolved in ethanol to 200 times the required concentration. The reaction was initiated by addition of this solution (5 μL/mL) to the membrane suspension in 50 mM Tris–HCl, pH 7.4. After treatment for 15 min at room temperature, the membrane suspension was used in a binding assay. As the half-life of DEP is very short in aqueous solutions, the hydrolysis of DEP terminated the reaction.

Redox Modifications of Thiol Groups

The redox state of thiol groups of cysteines was modified by treatment with the oxidizing agent DTNB or the reducing agent DTT. After treatment for 15 min at room temperature with various concentrations of the oxidizing or reducing compound, 1 mL of membrane suspension aliquots was rapidly spun at maximal speed in an Eppendorf 5412 centrifuge. The pellet was washed twice with 1.5 mL cold Tris–HCl buffer and centrifuged as before. The pellet was resuspended up to 1 mL with buffer and used in a binding assay. These procedures were also used in experiments combining the effects of reduction, oxidation, and cadmium ions: i) membrane aliquots were pretreated with 50 μM DTNB, after washing procedures were treated with 1 mM DTT or 10 μM $CdCl_2$, ii) membrane aliquots were pretreated with 1 mM DTT, after washing procedures were treated with 50 μM DTNB or 10 μM $CdCl_2$, and iii) membrane aliquots were pretreated with 10 μM $CdCl_2$, after washing procedures were treated with 50 μM DTNB or 1 mM DTT.

* *Abbreviations:* [^{3}H]CHA, N^6-cyclohexyl-[2,8-^{3}H]-adenosine; [^{3}H]CCPA, chloro-N^6-cyclopentyl-adenosine, 2-[cyclopentyl-2,3,4,5-^{3}H]; [^{3}H]DPCPX, cyclopentyl-1,3-dipropylxanthine, 8-[dipropyl-2,3-^{3}H]; R-PIA, N^6-(*R*-phenylisopropyl)adenosine; S-PIA, N^6-(*S*-phenylisopropyl)adenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DEP, diethylpyrocarbonate; DTNB, dithionitrobenzoic acid; and DTT, dithiothreitol.

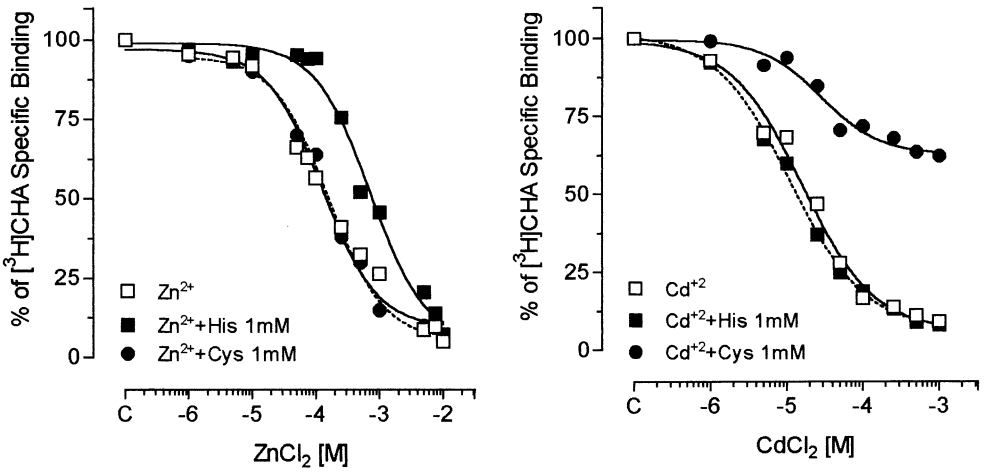

FIG. 1. Concentration-dependent inhibition of 1 nM [^{3}H]CHA binding in rat cerebral cortex membranes by zinc and cadmium ions in the absence or presence of 1 mM histidine (His) or 1 mM cysteine (Cys). Data points are from a representative experiment that was repeated at least three times, each dose in triplicate, with similar results. SEM was less than 5% in all cases.

Binding Assay for A1 Receptors

Standard assays were performed in 50 mM Tris–HCl, pH 7.4, at 25°, 1 mL total volume containing 200–300 μg of protein; 10 μM R-PIA was used to define non-specific binding. The incubation was started by adding cortical membranes, carried out for 120 min ([^{3}H]CHA and [^{3}H]D-PCPX) or 180 min ([^{3}H]CCPA) and stopped by adding 2.5 mL aliquots of ice-cold Tris–HCl buffer and by vacuum filtration through Whatman GF/B glass fibre filters previously soaked in Tris–HCl buffer. The filters were washed 3 times with 2.5 mL aliquots of buffer, dried and placed in miniature polyethylene vials containing 4 mL Filter Count cocktail, and counted by a liquid scintillation analyzer. The dose–effect curves were performed using 1 nM [^{3}H]CHA or 0.5 nM [^{3}H]CCPA and membrane preparations pretreated with increasing concentrations of $ZnCl_2$, $CdCl_2$, DEP, DTNB, and DTT. Saturation experiments with the agonists [^{3}H]CHA and [^{3}H]CCPA or the antagonist [^{3}H]DPCPX were performed with concentrations ranging from 0.1 to 20 nM, 0.01 to 3 nM, and 0.075 to 1.5 nM, respectively. Competition experiments were carried out using 1 nM [^{3}H]CHA or 0.5 nM [^{3}H]CCPA and increasing concentrations of R-PIA or S-PIA. Membranes pretreated with 100 μM $ZnCl_2$ or 100 μM $CdCl_2$ were used to detect the variations of binding characteristics in saturation or competition curves. Furthermore, saturation experiments were performed after the pretreatment of rat cortical membranes with DEP or DTNB.

Statistical Analysis

The analyses of saturation and competition curves were performed by the RADLIG program (version 4.0, Biosoft) or by PRISM (version 2.0, GraphPad). The dose–effect curves were evaluated by the PRISM program. The significant difference for IC_{50} or K_d or B_{max} values was assessed by Student's *t*-test. The significant difference of the percentage variations was calculated by Anova followed by Tukey's multiple comparison test applied to arcosin transformed percentages.

RESULTS

Inhibition of A1 Agonist Binding by Zinc

It was observed that $ZnCl_2$ inhibited the specific binding of [^{3}H]CHA to A1 adenosine receptors in the cortex of the rat in a dose-dependent manner. The calculated IC_{50} was 123.0 ± 3.7 μM. The presence of 1 mM histidine, a strong metal coordinator, in the incubation medium counteracted the inhibition of zinc ions by significantly ($P < 0.0001$) increasing the IC_{50} value (719.3 ± 15.6 μM), whereas the addition of 1 mM cysteine did not affect zinc effects (IC_{50} = 158.5 ± 47 μM) (Fig. 1). This suggests that zinc ions could chelate the histidyl residues present on the receptor molecule.

Effect of Zinc Pretreatment on Saturation Experiments

The effects of the zinc on the binding parameters of the A1 agonist [^{3}H]CHA in rat cortex membranes are shown in Table 1 and Fig. 2A. Pretreatment with 100 μM $ZnCl_2$ significantly reduced the number of binding sites to about 25% of the control without a change in affinity. When saturation assays were performed in the presence of 100 μM Zn^{2+}, the addition of 1 mM histidine in the incubation buffer completely reversed the effects of the zinc ions (K_d = 0.81 ± 0.09 nM; B_{max} = 0.40 ± 0.02 pmol mg^{-1} protein), while 1 mM histidine alone did not affect the binding parameters of [^{3}H]CHA (data not shown). The effects of 100 μM $ZnCl_2$ on the binding of the A1 antagonist, [^{3}H]DPCPX, in rat cortex membranes are reported in Table 1 and Fig. 2B. Here as well, Zn^{2+} significantly reduced the number of binding sites to about 20% of the control without a change in affinity.

TABLE 1. Effects of pretreatments with $ZnCl_2$ and $CdCl_2$ on saturation isotherm binding of [^{3}H]CHA or [^{3}H]CCPA and [^{3}H]DPCPX in membrane preparations of rat cerebral cortex

	Controls		100 μM $ZnCl_2$	
	K_d	B_{max}	K_d	B_{max}
[^{3}H]CHA	0.98 ± 0.11	0.418 ± 0.013	1.02 ± 0.14	0.318 ± 0.010*
[^{3}H]DPCPX	0.42 ± 0.03	0.695 ± 0.028	0.39 ± 0.02	0.573 ± 0.013†
	Controls		100 μM $CdCl_2$	
	K_d	B_{max}	K_d	B_{max}
[^{3}H]CCPA	0.36 ± 0.01	0.259 ± 0.014	1.24 ± 0.1‡	0.220 ± 0.010
[^{3}H]DPCPX	0.70 ± 0.01	0.249 ± 0.008	0.63 ± 0.03	0.238 ± 0.001

The K_d (nM) and B_{max} (pmol mg^{-1} protein) values are means ± SEM of 3–5 experiments, each dose in triplicate. The significant difference in the treatments versus respective controls was calculated by Student's *t*-test.

*$P < 0.005$.

†$P < 0.02$.

‡$P < 0.0001$.

Effect of Zinc Pretreatment on the Competition Experiments

In Table 2 are reported the apparent K_i values of the agonist stereoisomers R-PIA and S-PIA on displacement of 1 nM [^{3}H]CHA binding. Consistent with the results obtained in the saturation experiments, Zn^{2+} did not change the apparent affinity of PIA stereoisomers or their potency ratio. The ratio between K_i values of *S*- and *R*-stereoisomers of PIA (S/R) for controls and Zn^{2+} pretreatment was 21.4 and 23.9, respectively. Since the inhibitory effects of the ions were maintained after several washings of Zn^{2+}-pretreated cortical membranes (data not shown), the observed reduction in A1 receptor number could be due to a chelating effect of zinc ions on the two histidyl residues of the A1 receptor essential for agonist binding [11, 12]. To verify this possible mechanism of action of zinc ions, experiments were performed under various conditions modifying the histidyl residues.

Effect of pH Changes in the Incubation Medium on the Inhibition of [^{3}H]CHA Binding by Zinc

The change in pH in the incubation medium influenced the protonated/unprotonated form of the histidines residues essential for agonist binding. In Fig. 3, the effects of pH on the inhibitory potency of 100 μM $ZnCl_2$ are shown. At pH 5, Zn^{2+} had a weak effect, with an inhibition of approx. 10%; at pH 6 and 7, the inhibition was 30% and 56% respectively, with the greatest decrease (78%) at pH 8.

Protection Experiments

In order to assess whether zinc ions bind to the same histidines as CHA, a protection experiment was performed in which the membranes were incubated with 1 nM [^{3}H]CHA for 120 min; thereafter, increasing concentrations of $ZnCl_2$ were added and the reaction stopped after a further 20-min incubation. Alternatively, the membranes were preincubated with increasing $ZnCl_2$ concentrations for 20 min, washed, the tracer then added, and the reaction stopped after a further 120-min incubation. The preincubation with [^{3}H]CHA protected the A1 receptors from the inhibitory effects of Zn^{2+}, as shown in Fig. 4.

Effects of Chemical Modifications with DEP on [^{3}H]CCPA Binding

The effects of chemical modifications with DEP, a specific histidyl-alkylating reagent, are presented in Figs. 5 and 6. Treatment of membranes for 15 min at room temperature with DEP decreased specific [^{3}H]CCPA binding in a concentration-dependent manner up to 95% at a concentration of 4 mM (Fig. 5) The calculated IC_{50} value was 359.5 ± 0.72 μM. The reduction in specific [^{3}H]CCPA binding by DEP treatment was mainly due to a significant decrease ($P < 0.0001$) in maximal binding capacity, whereas the reagent did not alter the affinity (Fig. 6). The saturation experiments were performed with 2 mM DEP, a condition which caused a great reduction in the binding of the A1 agonist. In fact, the apparent number of receptors was 0.424 ± 0.011 pmol mg^{-1} protein in controls and 0.038 ± 0.007 pmol mg^{-1} protein after the pretreatment with DEP. The K_d values did not change, being 0.50 ± 0.015 nM for controls and 0.52 ± 0.04 nM for DEP.

Inhibition of A1 Agonist Binding by Cadmium

It was observed that $CdCl_2$ inhibited the specific binding of 0.5 nM [^{3}H]CCPA to A1 adenosine receptors in the cortex of the rat in a dose-dependent manner. The calculated IC_{50} was 25.0 ± 5.0 μM. The presence of 1 mM histidine in the incubation medium was unable to modify the inhibition of cadmium (IC_{50} = 24.9 ± 7.4 μM), whereas the addition of 1 mM cysteine abolished the effect of $CdCl_2$ (Fig. 1). This may suggest a possible interaction of cadmium ions with the thiol group of cysteines present on the receptor molecule whose redox state has an important structural function.

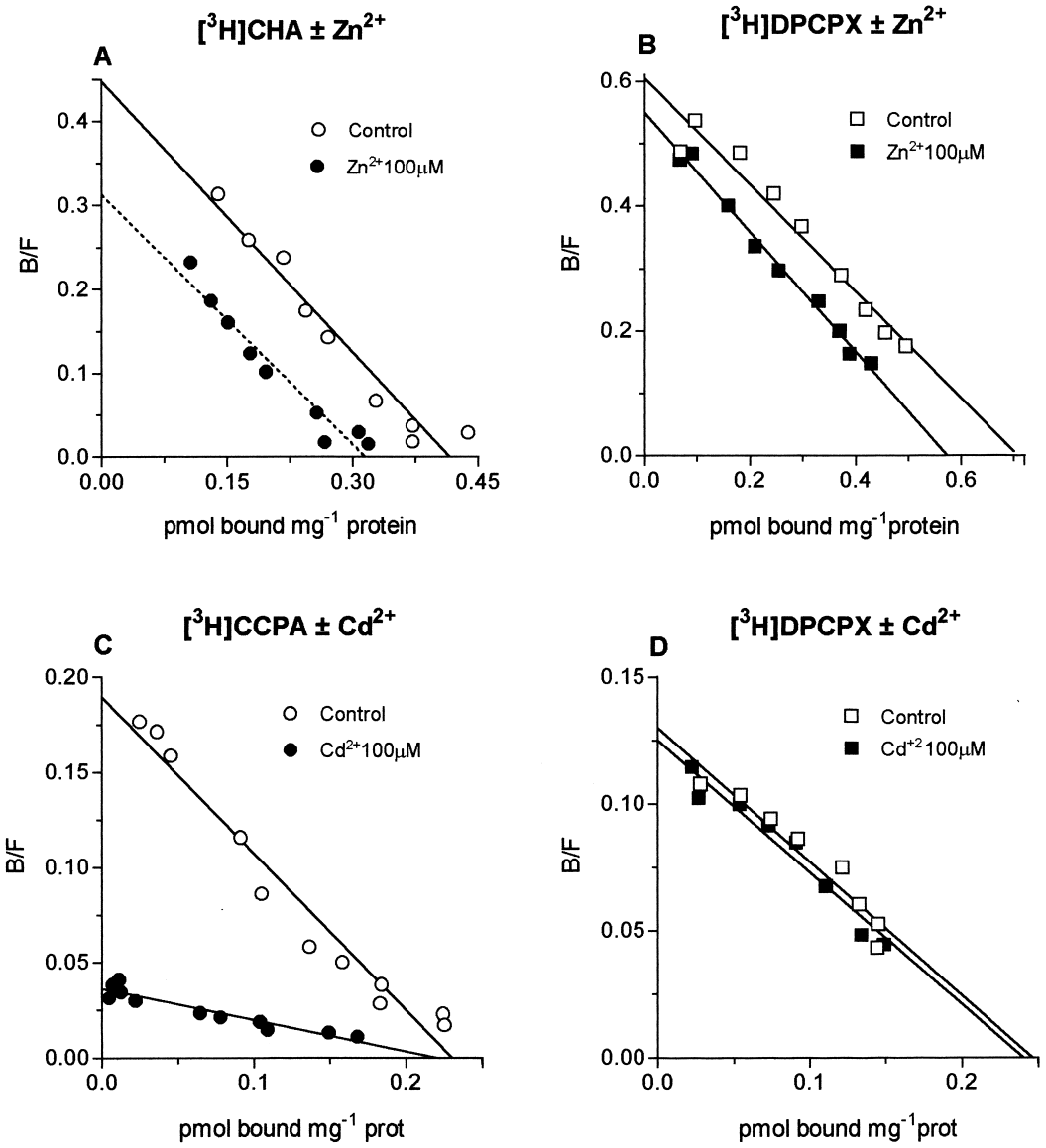

FIG. 2. Effects of zinc and cadmium ions on saturation of [^{3}H]CHA or [^{3}H]CCPA and [^{3}H]DPCPX in membrane preparations from cerebral cortex of the rat. The pretreatment of membranes with 100 μM each of $ZnCl_2$ and $CdCl_2$ was performed as described in Materials and Methods. The saturation data were fitted by a computerized non-linear regression analysis and shown as Scatchard plots. B/F is the ratio between specific binding and free radioligand concentration. Data points are from a representative experiment that was repeated at least three times, each dose in triplicate. SEM was less than 10% in all cases.

Effect of Cadmium Pretreatment on Saturation Experiments

As shown in Fig. 2C, the reduction in specific [^{3}H]CCPA binding by pretreatment with 100 μM $CdCl_2$ was mainly due to a significant decrease ($P < 0.0001$) in affinity, with a marginal decrease in the maximal binding capacity. In contrast to the effects of zinc ions, Cd^{2+} did not alter either the affinity or the number of binding sites of the A1

TABLE 2. Effects of pretreatments with $ZnCl_2$ and $CdCl_2$ on competition experiments using 1 nM [^{3}H]CHA and 0.5 nM [^{3}H]CCPA, respectively, in membrane preparations of rat cerebral cortex

	Controls		100 μM $ZnCl_2$		Controls		100 μM $CdCl_2$	
	K_i [nM]	S/R	K_i [nM]	S/R	K_i [nM]	S/R	K_i [nM]	S/R
R-PIA	1.17 ± 0.10	21	1.08 ± 0.12	23.9	1.29 ± 0.29	19	5.94 ± 0.87*	9.7
S-PIA	25.0 ± 1.30		25.8 ± 1.60		24.4 ± 1.90		57.9 ± 15.2*	

Values are means ± SEM of three experiments, each dose in triplicate. S/R: ratio between K_i values of S-PIA and R-PIA. The significant difference in the treatments versus controls was calculated by Student's *t*-test.

*$P < 0.02$.

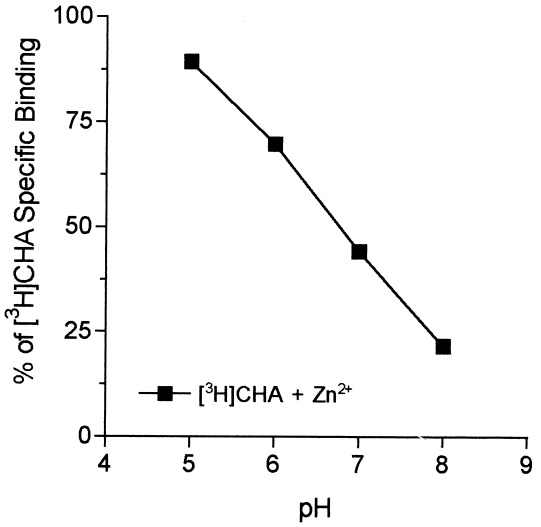

FIG. 3. Effects of pH on the inhibitory potency of 100 μM $ZnCl_2$ on 1 nM [^{3}H]CHA binding to cerebral cortex membranes of the rat. The percentages are calculated versus respective controls from two separate experiments, each value in triplicate. SEM is less than 5%.

antagonist [^{3}H]DPCPX (Fig. 2D). The results are summarized in Table 1.

Effect of Cadmium Pretreatment on the Competition Experiments

In Table 2 are reported the apparent K_i values of the agonist stereoisomers R-PIA and S-PIA on displacement of 0.5 nM

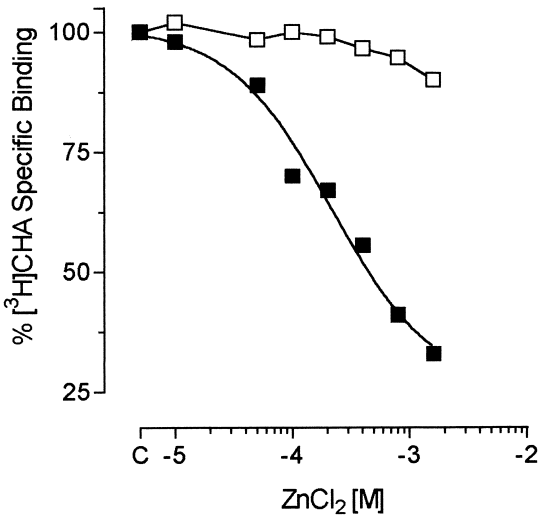

FIG. 4. Protection of [^{3}H]CHA binding from the inhibitory effects of zinc ions. (■) Preincubation with different doses of $ZnCl_2$ for 20 min. The membranes were washed twice, the tracer (1 nM) added, and the reaction stopped after a 120-min incubation. (□) Preincubation with [^{3}H]CHA for 120 min, after which 100 μM $ZnCl_2$ was added and the reaction stopped after a further 20-min incubation. The percentages are calculated versus respective controls from two separate experiments, each value in triplicate. SEM is less than 5% and is not shown.

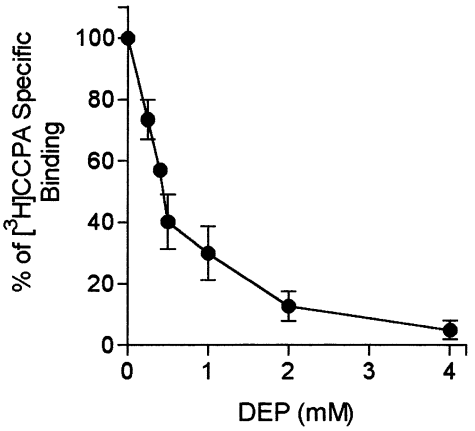

FIG. 5. Concentration dependency of the modifications of 0.5 nM [^{3}H]CCPA binding by DEP. Cerebral cortex membranes of the rat were treated with various DEP concentrations for 15 min at room temperature. Data points are means ± SEM of four experiments, each dose in triplicate.

[^{3}H]CCPA binding. Consistent with the results obtained in the saturation experiments, Cd^{2+} changed the apparent affinity of PIA stereoisomers and as a consequence their potency ratio. The S/R ratio for controls and Cd^{2+} pretreatment was 19 and 9.7, respectively.

Effects of Sulfhydryl Reagents on Inhibition of [^{3}H]CCPA Binding by Cadmium

The reducing agent DTT in a concentration range from 10 μM to 10 mM was unable to modify [^{3}H]CCPA specific binding (data not shown). The oxidizing reagent DTNB inhibited this binding, in dose-dependent manner, up to 65% at a concentration of 1 mM (Fig. 7A). The reduction in specific [^{3}H]CCPA binding by DTNB pretreatment was mainly due to a significant decrease in the affinity, whereas the reagent did not alter maximal binding capacity (Fig. 7B). The [^{3}H]CCPA saturation experiments were performed after membrane pretreatment with 100 μM DTNB per 15 min. The K_d values changed, being 0.34 ± 0.01 nM for controls and 0.71 ± 0.03 nM for DTNB ($P < 0.0001$). The apparent number of receptors did not change, being 0.293 ± 0.019 and 0.276 ± 0.01 pmol mg^{-1} protein in controls and DTNB pretreatment, respectively.

Combined Effects of Cd^{2+}, DTT, or DTNB on 0.5nM [^{3}H]CCPA Binding

Experiments were performed under different conditions that affect the redox state of A1 receptors. The treatments with the different reagents were performed as described in Materials and Methods. As shown in Fig. 8, the significant percentage decrease in [^{3}H]CCPA binding upon oxidation with 50 μM DTNB (40%) was potentiated by 10 μM Cd^{2+} (63%), but upon reduction with 1 mM DTT the effect of DTNB was completely reversed, the percentage being not

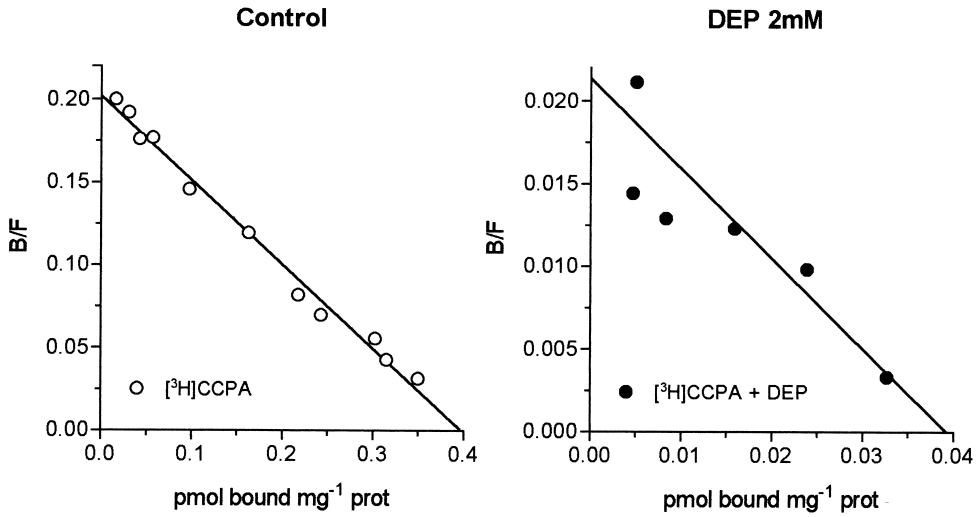

FIG. 6. Effects of DEP on saturation of [^{3}H]CCPA in membrane preparations of rat cerebral cortex. The pretreatment of membranes with 2 mM DEP was performed as described in Materials and Methods. The saturation data were fitted by a computerized non-linear regression analysis and shown as Scatchard plots. B/F is the ratio between specific binding and free radioligand concentration. Data points are from a representative experiment that was repeated three times with similar results, each dose in triplicate. SEM was less than 10% in all cases.

significant different from control value. DTT preincubation did not change the binding, and the subsequent treatments with DTNB or Cd^{2+} significantly reduced agonist binding, reaching percentage levels similar to those obtained with DTNB or Cd^{2+} alone. The significant percentage decrease in [^{3}H]CCPA binding induced by Cd^{2+} pretreatment (20%) was potentiated by DTNB (60%), but after DTT treatment the effect of Cd^{2+} was completely blocked.

DISCUSSION

In the present study, results concerning the different mechanisms of the inhibitory regulation of zinc and cadmium ions on agonist binding to the A1 adenosine receptor are shown. It has been reported that the cerebral cortex, the hippocampus, and the basal ganglia contain 0.8–1.0 μmol zinc ions per gram tissue [18], corresponding to approximately 1 mM. However, the functional significance of these

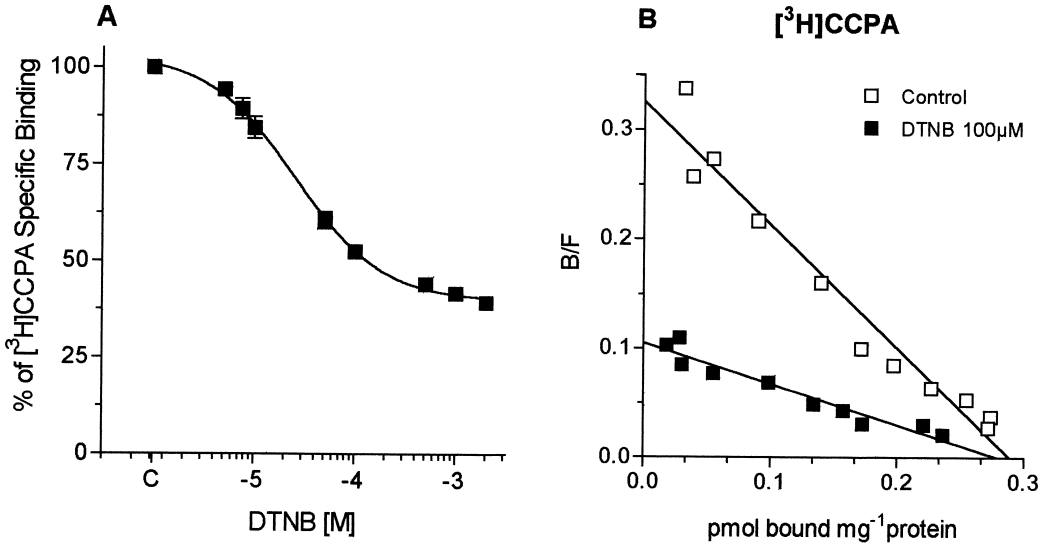

FIG. 7. Effects of DTNB on [^{3}H]CCPA binding to membrane preparations of rat cerebral cortex. (A) the concentration-dependent inhibition of 0.5 nM [^{3}H]CCPA binding is reported. Rat brain cortex membranes were treated with DTNB as described in Materials and Methods. Data points are means ± SEM of four experiments, each dose in triplicate. (B) the effects of 100 μM DTNB on the saturation of [^{3}H]CCPA are reported. The saturation data were fitted by a computerized non-linear regression analysis and shown as Scatchard plots. B/F is the ratio between specific binding and free radioligand concentration. Data points are from a representative experiment that was repeated twice with similar results, each dose in triplicate. SEM was less than 10% in all cases.

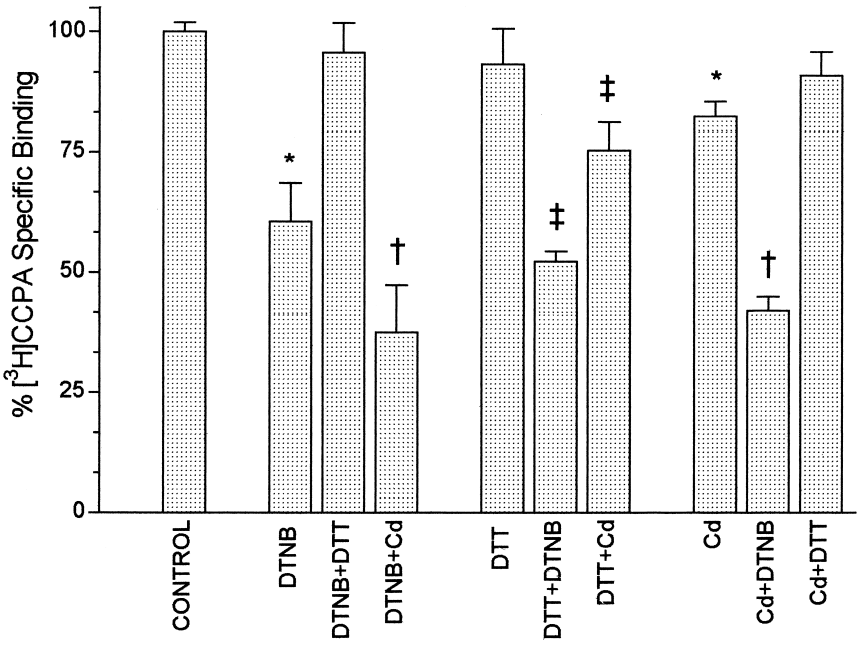

FIG. 8. Effects of combined treatments with 50 μM DTNB, 1 mM DTT, and 10 μM $CdCl_2$ on 0.5 nM [^{3}H]CCPA binding to membrane preparations of rat cerebral cortex. For details of the treatments see Materials and Methods. The significant difference in the percentage variations was calculated by Anova ($F = 69.46$; $P < 0.0001$) followed by Tukey's multiple comparison test applied to arcosin-transformed percentages that were obtained from three experiments, each dose in triplicate. *$P < 0.001$ for DTNB and Cd^{2+} versus control; †$P < 0.001$ for DTNB + Cd^{2+} versus DTNB, and for Cd^{2+} + DTNB versus Cd^{2+}; and ‡$P < 0.001$ for DTT + DTNB versus DTT, and DTT + Cd^{2+} versus DTT.

findings is unclear and the involvement of zinc in neural function remains to be clarified. Zinc ions form complexes with amino acids and the stability constants indicate that preferred complexes are with nitrogen (as of the imidazole group of histidine) [14]. It is known that histidine is a strong metal coordinator and that the histidine residues present in the A1 adenosine receptor are essential for agonist binding [11, 19, 20]. The decrease in A1 agonist binding induced by Zn^{2+} was due to a decrease in the number of binding sites without changes in affinity. The effect of zinc was abolished by histidine, which alone did not affect binding parameters. This suggests that histidine was exerting most of its effects by complexing with free zinc ions and acting as a chelator. The histidine increased the IC_{50} of Zn^{2+} 5.8-fold. Similar results were obtained with the opioid receptor system, where histidine increased the IC_{50} of Zn^{2+} for the binding of [^{3}H]naloxone in the rat cortex 5.3-fold [6]. Two histidines (His^{251} and His^{278}) essential for agonist binding are present on the A1 receptor molecule. The decrease in the number of binding sites could depend on zinc chelation of the nitrogen of the imidazole group of the histidines, thereby possibly preventing ligand interaction with the specific agonist recognition sites without modifying the binding kinetics of radioligand to the free (not chelated) recognition sites. This chelation does not change the stereostructure of A1 receptors, as confirmed by competition studies with the *R*- and *S*-isomers of PIA.

Interestingly, Zn^{2+} treatment decreased the receptor number, but did not affect the receptor affinity for the antagonist DPCPX. This result is consistent with a modification of receptors at the ligand-binding domain. In fact, the cyclopentyl moiety of DPCPX may bind to the same region on the adenosine A1 receptor as the N^6 exocyclic substituent of agonists [21]. One of the histidines present on the agonist binding domain may also be involved in the binding of the antagonist [11, 17]. In particular, according to the N^6/C8 model explaining the competitive interaction between agonist and antagonist on adenosine A1 receptors, the His^{251} (helix VI) seems important for antagonist binding, whereas His^{278} does not [22]. On the other hand, the absence of a histidine in helix VI, as in mutated adenosine A1 receptors, prevents antagonist binding and reduces the number of receptors without perturbing agonist affinity [13].

The finding that the zinc inhibitory effects were also maintained after several washings of the cortical membranes pretreated with the ion supports the hypothesis of the formation of stable bonds between zinc and the receptor molecule. The formation of coordination bonds with histidines seems to be confirmed by some experimental conditions that can modify the histidyl residues. First, the proposed interactions of Zn^{2+} with the histidine residues are only feasible with the unprotonated form of these amino acids. Variations in the pH of the incubation medium induced changes in the inhibitory potency of Zn^{2+}, according to the Henderson–Hesselbach equation. Assuming that the pK_a of the imidazole group of histidine is 6, at pH 5 the concentration of the protonated form is 10-fold higher than

the unprotonated form and Zn^{2+} induced a marginal reduction in [^{3}H]CHA binding; at pH 8 the unprotonated form is 100-fold higher than the protonated form and Zn^{2+} induced the highest reduction; at pH 6 and 7.4 the inhibitory potency of Zn^{2+} was intermediate. Allende *et al.* [11] concluded that the pK_a values of the histidines involved in the bonds with agonists could be between 6.0 and 7.4. This suggests that at physiologic pH the majority of the histidine residues would be unprotonated upon binding. Second, the prior incubation of membranes with [^{3}H]CHA completely protected the binding sites against modifications by Zn^{2+}. This protection from inactivation implies that Zn^{2+} and agonists occupy the same domains of the ligand recognition site: agonist binding to the histidines could *de facto* prevent the chelation of histidine residues by Zn^{2+}. Third, it has been shown that the alkylation by DEP of histidine residues in the domain of the ligand recognition site resulted in a decrease in [^{3}H]PIA binding [11, 17, 19]. Under our experimental conditions, DEP treatment reduced the receptor number, whereas it did not affect receptor affinity. This result is analogous to that obtained with zinc pretreatment and, again, is consistent with the hypothesis that histidines in the domain of the ligand recognition site can be a target for Zn^{2+} actions. It has been described that the G-protein contains histidine at the nucleotide binding site [23]. Under our experimental conditions, neither Zn^{2+} nor DEP changed the affinity of the agonist. This led us to the conclusion, taking into account the results of Allende *et al.* [11], that the possible histidine G-protein modification did not have any influence on the agonist binding to the A1 receptor.

Cadmium is a body trace element as well as an important environmental contaminant. Cadmium ions form complexes with amino acids, and the stability constants are greater when free sulfur groups serve as ligands (as thiol groups of cysteines) than constants when nitrogen or oxygen serve as ligands. The interaction of cadmium with polythiols is particularly strong and likely of considerable biological significance. For example, preferential binding of cadmium versus zinc has been suggested as a diagnostic index for a dithiol configuration of an enzyme [14]. The pretreatment of cortical membranes with increasing concentrations of $CdCl_2$ potently decreased [^{3}H]CCPA binding. Cysteine, but not histidine, was capable of preventing the effect of Cd^{2+} on the A1 adenosine receptor. The reduction in specific agonist binding by Cd^{2+} treatment was mainly due to a decrease in affinity. This pattern, opposite to that of Zn^{2+}, is confirmed by competition studies with R- and S-PIA. These findings could suggest that cadmium modifies the stereostructure of the A1 receptors and that cysteines localized in several regions of intracellular and extracellular domains may be involved in the action of cadmium. The oxidation-reduction state of the SH group of cysteines has an important structural function related to receptor activity, as shown for opiate and *N*-methyl-D-asparate receptors [7, 24]. The SH-oxidizing reagent, DTNB, behaved in a similar fashion as cadmium: the dose-dependent inhibition of [^{3}H]CCPA binding was mainly due to a decrease in affinity without a change in the B_{max} value. The reducing reagent DTT, which alone was unable to modify [^{3}H]CCPA binding, reversed the inhibiting effects of both Cd^{2+} and DTNB. These results could support the hypothesis that Cd^{2+} modifies the SH group of cysteines on A1 receptors. An analogous hypothesis was put forth to explain the inhibitory effects of copper on opioid receptors [25].

In contrast to the results obtained with the agonist, the saturation experiments performed with the antagonist [^{3}H]DPCPX showed that the Cd^{2+} pretreatment did not modify either the affinity or the maximal binding capacity of the antagonist. This could suggest that the action of cadmium ions may be targeted toward the cysteine residue known to be involved in receptor/G-protein coupling [19, 26, 27]. Although specific experiments have not been performed, the decrease in agonist affinity could depend on modifications of the thiol group of this cysteine. In fact, *N*-ethylmaleimide, an alkylating agent of thiol groups, produces effects analogous to those observed with Cd^{2+}. *N*-Ethylmaleimide treatment abolishes the coupling between G_i-protein and A1 receptor, and this reduced high-affinity agonist binding but did not affect the interaction with antagonists as shown by Garritsen *et al.* [17] and Klotz *et al.* [19]. Under our experimental conditions, pretreatment of membranes with Cd^{2+} reduced the affinity of the agonist [^{3}H]CCPA, but the affinity of the antagonist [^{3}H]DPCPX was unaltered. The reversal of the Cd^{2+} effects on agonist binding with DTT lends further support to the idea that modification of the SH group of cysteines may be the mechanism for the cadmium action. On the other hand, the carboxyl-terminal region from Val^{336} to Leu^{354} in the $G_i\alpha$ subunit has been proposed as the site of interaction with receptors. The cysteine residue located in position 352 is the site for ADP ribosylation by pertussis toxin and the covalent modification uncouples receptor and $G_i\alpha$ subunit, preventing this protein from responding to stimulation by its respective receptors [27].

In conclusion, the results suggest that the mechanisms of zinc and cadmium on the inhibition of the binding of agonists to A1 receptors are different. Zinc ions may chelate the histidines critical for agonist binding, preventing hydrogen bonds between the non-protonated nitrogen atom of His^{251} and the exocyclic N^6-H in CHA or CCPA, and between His^{278} and 2′- and 3′-OH of the ribose ring [28]. This mechanism can explain the reduction in the number of binding sites without a change in affinity. Cadmium ions may oxidize SH groups of cysteines localized in several domains of the A1 receptor molecule or of a cysteine localized in the region of the $G_i\alpha$ subunit specifically involved in the coupling with receptors. This mechanism can justify the conformational modifications of the receptor molecule that result in a decrease in affinity. The high potency of cadmium in complexing free sulfur groups [14] and the strong blockade of the Cd^{2+} effects by cysteine could suggest that the redox reaction between Cd^{2+} and

cysteine's thiol group may result in binding of the metal into thiol–cadmium complexes rather than in the formation of disulfide and liberation of the reduced metal. However, our experiments did not distinguish between these possibilities.

This work was supported by a grant from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST).

References

1. Goodman RR, Cooper MJ, Gavish M and Snyder SH, Guanine nucleotide and cation regulation of the binding of [^{3}H]cyclohexyladenosine and [^{3}H]diethylphenylxanthine to adenosine A1 receptors in rat membranes. *Mol Pharmacol* **21:** 329–335, 1982.
2. Traversa U, Rosati AM, Florio C and Vertua R, Effects of divalent cations on adenosine agonist binding to A1 receptors and non-A1/non-A2 sites in rat cerebral cortex. *Pharmacol Toxicol* **75:** 28–35, 1994.
3. Klotz KN, Lohse MJ and Schwabe U, Characterization of the solubilized A1 adenosine receptor from rat brain membranes. *J Neurochem* **46:** 1528–1534, 1986.
4. Garritsen A and Cooper DMF, Evaluation of the role of GTP hydrolysis in the interaction between Mg^{+2} and GTP in regulating agonist binding to the adenosine A1 receptor. *J Receptor Res* **11:** 849–864, 1991.
5. Johansson B, Parkinson FE and Fredholm B, Effects of mono- and divalent ions on the binding of the adenosine analogue CGS 21680 to adenosine A2 receptors in rat striatum. *Biochem Pharmacol* **44:** 2365–2370, 1992.
6. Hanissian SH and Tejwani GA, Histidine abolishes the inhibition by zinc of naloxone binding to opioid receptors in rat brain. *Neuropharmacology* **27:** 1145–1149, 1988.
7. Tejwani GA and Hanissian SH, Modulation of mu, delta and kappa opioid receptors in rat brain by metal ions and histidine. *Neuropharmacology* **29:** 445–452, 1990.
8. Smart TG and Constanti A, Differential effect of zinc on the vertebrate $GABA_A$–receptor complex. *Br J Pharmacol* **99:** 643–654, 1990.
9. Smart TG, Moss SJ, Xie X and Huganir RL, $GABA_A$ receptors are differentially sensitive to zinc: Dependence on subunit composition. *Br J Pharmacol* **103:** 1837–1839, 1991.
10. Reddington M, Alexander SP, Hoppe E and Kreutzberg GW, Sensitivity to Mg^{2+} of ligand binding to A1 adenosine receptors in rat brain and adipocytes. In: *Adenosine Receptors in the Nervous System, Proceedings of the Satellite Symposium of 12th International Society for Neurochemistry Biennial Meeting*, Albufeira, Portugal, 20–22 April 1989 (Ed. Ribeiro JA), pp. 95–102. Taylor & Francis, London-New York-Philadelphia, 1989.
11. Allende G, Casadó V, Mallol J, Franco R, Lluis C and Canala EI, Role of histidine residues in agonist and antagonist binding sites of A1 adenosine receptor. *J Neurochem* **60:** 1525–1533, 1993.
12. IJzerman AP, van Galen PJM and Jacobson KA, Molecular modeling of adenosine receptors. I. The ligand binding site on the A1 receptor. *Drug Des Discov* **9:** 49–67, 1992.
13. Olah ME, Ren H, Ostrowski J, Jacobson KA and Stiles GL, Cloning, expression, and characterization of the unique bovine A1 adenosine receptor. *J Biol Chem* **267:** 10764–10770, 1992.
14. Vallee BL and Ulmer DD, Biochemical effects of mercury, cadmium and lead. *Annu Rev Biochem* **41:** 91–128, 1972.
15. Lowry OH, Rosebrough NJ, Farr AR and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **93:** 265–275, 1951.
16. Miles EW, Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods Enzymol* **47:** 431–442, 1977.
17. Garritsen A, IJzerman AP, Beukers MW and Soudijn W, Chemical modification of adenosine A1 receptors. Implications for the interaction with R-PIA, DPCPX, and amiloride. *Biochem Pharmacol* **40:** 835–842, 1990.
18. Donaldson J, St. Pierre T, Minnich JL and Barbeau A, Determination of Na^+, Mg^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} in rat brain regions. *Can J Biochem* **51:** 87–92, 1973.
19. Klotz K-N, Lohse MJ and Schwabe U, Chemical modification of A1 adenosine receptors in rat brain. Evidence for histidine in different domains of the ligand binding site. *J Biol Chem* **263:** 17522–17526, 1988.
20. Jacobson MA, Molecular biology of adenosine receptors. In: *Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology* (Eds. Belardinelli L and Pelleg A) pp. 5–14. Kluwer Academic Publishers, Boston-Dordrecht-London, 1994.
21. van der Wenden EM, van Galen PJM, IJzerman AP and Soudijn W, Mapping the xanthine C8-region of the adenosine A1 receptor with computer graphics. *Eur J Pharmacol* **206:** 315–323, 1991.
22. van der Wenden EM, IJzerman AP and Soudijn W, A steric and electrostatic comparison of three models for the agonist/antagonist binding site on the adenosine A1 receptor. *J Med Chem* **35:** 629–635, 1992.
23. Kaziro Y, Itoh H, Kozasa T, Masato N and Satoh T, Structure and function of signal-transducing GTP-binding proteins. *Annu Rev Biochem* **60:** 349–400, 1991.
24. Gozlan H and Ben-Ari Y, NMDA receptor redox sites: Are they targets for selective neuronal protection? *TIPS* **16:** 368–374, 1995.
25. Marzullo G and Hine B, Opiate receptor function may be modulated through an oxidation-reduction mechanism. *Science* **208:** 1171–1173, 1980.
26. Watson S and Arkinstall S, The G-protein-linked receptor. Academic Press, London, 1994.
27. Olate J and Allende JE, Structure and function of G protein. In: *Intracellular Messangers* (Ed. Taylor CW), pp. 25–46 Pergamon Press, Oxford, 1993.
28. IJzerman P, van der Wenden NM, van Galen PJM and Jacobson KA, Molecular modeling of adenosine A1 and A2a receptors. In: *Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology* (Eds. Belardinelli L and Pelleg A) pp. 27–37. Kluwer Academic Publishers, Boston-Dordrecht-London, 1994.