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8-(3-Isothiocyanatostyryl)caffeine Is a Selective, Irreversible Inhibitor of Striatal A₂-Adenosine Receptors

Xiao-duo Ji, Carola Gallo-Rodriguez, and Kenneth A. Jacobson

Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland

Abstract

8-(3-Isothiocyanatostyryl)caffeine (ISC) was synthesized and shown to inhibit selectively the binding of [³H]CGS 21680 (an A_{2a}-selective agonist) at adenosine receptors in striatal membranes. The K_i value at A_{2a}-receptors was found to be 110 nM (rat), with selectivity ratios for A_{2a} versus A₁-receptors in rat, guinea pig, bovine, and rabbit striatum of >100-fold. Preincubation of membranes with ISC caused a dose-dependent, irreversible antagonism of the binding of [³H]CGS 21680, with an IC₅₀ value of 3 μM. The irreversibility is likely due to the presence of the chemically reactive isothiocyanate group, since the binding of the corresponding analogue in which the isothiocyanate was replaced with a chloro group was completely reversible. The potency of ISC to irreversibly inhibit the binding of [³H]CGS 21680 in several species varied in the order rat ≈ guinea pig > bovine ≈ rabbit. In all four species, binding of the A₁-selective agonist [³H]R-N⁶-phenylisopropyladenosine was not diminished by pre-treatment with 2 μM ISC. The kinetics of irreversible inhibition of rat A_{2a}-receptors by 2 μM ISC gave a t_{1/2} of approximately 3 min. Following partial inactivation, the remaining rat A_{2a}-binding sites retained the same K_d value as in control membranes for saturation by [³H]CGS 21680. Thus, ISC appears to be a selective affinity label for A_{2a}- versus A₁-receptors in the brain.

Keywords

xanthines; affinity label; adenosine receptors; radioligand binding

INTRODUCTION

Adenosine acts as a neuromodulator in the central and peripheral nervous systems [reviewed in Ferré et al., 1992, Olsson and Pearson, 1990]. As a local hormone in a variety of organs, it decreases the oxygen consumption (as in its negative chronotropic action in the heart, through activation of A₁ receptors) and increases oxygen supply (for example, through activation of vascular A_{2a} receptors) in response to stress [Bruns, 1991]. In the CNS, A_{2a} receptors are present in high density in the striatum, where they have been studied using radioligand binding techniques [Bruns et al., 1986; Barrington et al., 1989]. Other tissues in which radioligand binding to A_{2a} receptors has been characterized include platelets [Lohse et al., 1988], pheochromocytoma (PC12) cells [Hide et al., 1992], and smooth muscle (DDT₁-MF2) cells [Ramkumar et al., 1991]. A_{2a} receptors are linked via G_s guanine nucleotide binding proteins to the stimulation of adenylate cyclase. A_{2b} receptors [Bruns et

al., 1986], through which adenosine agonists also stimulate adenylate cyclase, but at higher concentrations, are found in the brain, fibroblasts, and intestines [Stehle et al., 1992].

We have developed novel pharmacological probes, including radioligands and photoaffinity labels [Barrington et al., 1990], fluorescent labels [Mc-Cabe et al., 1993], and chemically reactive affinity labels [Jacobson et al., 1989, 1992], as tools for the characterization of adenosine receptors. Binding site-directed affinity labels for A₁-receptors (agonists and antagonists) and A_{2a}-receptors (agonists) have been synthesized based on a functionalized congener approach. Recently, 8-styrylxanthine derivatives that act as A_{2a}-selective adenosine antagonists have been reported [Shimada et al., 1992]. We now introduce an 8-styrylxanthine isothiocyanate derivative that acts as a selective irreversible inhibitor of binding to A_{2a}-receptors. The design of this agent was based on a detailed structure activity study [Jacobson et al., 1993] of this class of xanthines.

EXPERIMENTAL PROCEDURES

Materials

2-Chloroadenosine was obtained from Research Biochemicals, Inc. (Natick, MA). 1,3,7-Trimethyl-8-(3-aminostyryl)xanthine, **1**, and the 3-chloro analogue, **3**, were prepared as described [Jacobson et al., 1993]. [³H]N⁶-phenylisopropyladenosine, and [³H]CGS 21680 were obtained from Dupont NEN (Boston, MA).

Synthesis of 8-(3-Isothiocyanatostyryl)caffeine, **2**

1,3,7-Trimethyl-8-(3-aminostyryl)xanthine (50 mg, 0.16 mmol) was dissolved in 2 ml chloroform, and saturated sodium bicarbonate solution (1 ml) was added. After cooling the mixture in an ice bath, thio-phosgene (0.1 ml, 1.3 mmol) was added at once with vigorous stirring. After 5 min, the reaction was complete and additional solvent was added to break the emulsion. The phases were separated, and the organic phase was washed several times with water and dried (MgSO₄). The solvent was evaporated, and the solid yellow residue was recrystallized from chloroform/acetonitrile to provide 32 mg (57% yield) of the homogeneous product, 8-(3-isothiocyanatostyryl)caffeine or 1,3,7-trimethyl-8-(3-isothiocyanatostyryl)xanthine hemi-hydrate (TLC system chloroform: methanol: acetic acid, 95:4:1, R_f = 0.41) Mp 268–271°C. ¹H NMR CDCl₃ δ 3.43 (s, 3H N-CH₃); 3.63 (s, 3H N-CH₃); 4.07 (s, 3H N7-CH₃); 6.93 (d, 1H J = 16Hz, olefin); 7.21 (d, 1H J = 8Hz); 7.39 (t, 1H J = 8Hz, C5 arom); 7.44 (s, 1H, C2 arom); 7.47 (d, 1H J = 8Hz); 7.75 (d, 1H J = 16Hz, olefin). MS (EI) M⁺ 353. IR (NaBr) 2124 cm⁻¹. Elemental analysis (C₁₇H₁₅N₅O₂S·0.5H₂O): calculated, 56.34% C, 4.45% H, 19.33% N; found 56.43% C, 4.16% H, 19.07% N.

Biochemical Assays

Preparation of striatal membranes—Striatal tissue was isolated by dissection of rabbit, bovine, and rat brain, obtained frozen from Pel-Freeze Biologicals Co. (Rogers, AS), and guinea pig brain, obtained frozen from Keystone Biologicals (Cleveland, OK). Membranes were homogenized in 20 volumes of ice cold 50 mM Tris HCl (pH 7.4) using a Polytron (Kinematica GmbH, Luzern, Switzerland) at a setting of 6 for 10 sec. For each species except rat, the homogenization was carried out in the presence of protease inhibitors (5 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 0.01 mg/ml soybean trypsin inhibitor, 5 µg/ml leupeptin, 1 µg/ml pepstatin A). The membrane suspension was then centrifuged at 37,000g for 10 min at 4°C. The pellet was resuspended (20 mg tissue/ml) in the above buffer solution, preincubated at 30°C for 30 min with 3 IU/ml of adenosine deaminase, and the membranes were again homogenized and centrifuged. Finally the pellet was suspended in buffer (100 mg wet weight per ml) and stored frozen for no longer than 2

weeks at -70°C . Protein was determined using the BCA protein assay reagents (Pierce Chemical Co., Rock-ford, IL).

Treatment of striatal membranes with inhibitor—Membranes were incubated with ISC in pH 7.4 Tris buffer containing adenosine deaminase, for 1 h at 25°C , subjected to three washing cycles, consisting of centrifugation at $37,000g$ and resuspended of the pellet in Tris buffer, prior to radioligand binding. For kinetic experiments with the affinity label, aliquots were removed periodically and quenched with a large volume of buffer solution ($30\times$) prior to radioligand binding. For protection experiments, membranes were preincubated with theophylline at 25°C for 20 min, and then ISC was added immediately for an additional incubation at 25°C for 30 min. At the end of this sequence, the membranes were washed by repeated centrifugation and resuspension and subjected to 2-(carboxyethylphenylethylamino)adenosine-5'-carboxamide ($[^3\text{H}]\text{CGS 21680}$) binding.

Washing cycles for inhibition experiments required resuspending the membrane pellet by gentle vortex mixing. At the final step, prior to radioligand binding, the membranes were homogenized manually using a glass tissue grinder.

Radioligand binding— $[^3\text{H}]\text{CGS 21680}$ binding to striatal $\text{A}_{2\text{a}}$ -receptors from rat, guinea pig, rabbit and bovine brains was carried out as described for rat brain [Jarvis et al., 1989] using $10\text{ }\mu\text{M}$ 2-chloroadenosine to determine non-specific binding. Adenosine deaminase was present (3 IU/ml) during the incubation with radioligand.

The binding of $[^3\text{H}]\text{R-PIA}$ to striatal A_1 -receptors in four species was measured by a modification of the method described for rat cortex [Jacobson et al., 1993]. In competition studies, to avoid precipitation of the xanthine in the $100\text{ }\mu\text{M}$ concentration range, the tubes in that range containing all components were warmed to $\sim 50^{\circ}\text{C}$, prior to the incubation carried out for 90 min at 37°C .

For saturation and competition studies, B_{max} , K_{d} , and IC_{50} values were determined using the Ligand [Munson and Rodbard, 1980] and Inplot (Graphpad, San Diego, CA) computer programs. IC_{50} values were converted to apparent K_{i} values using K_{D} values in rat striatum of 1.0 and 15 nM for $[^3\text{H}]\text{PIA}$ and $[^3\text{H}]\text{CGS 21680}$ binding, respectively, and the Cheng-Prusoff equation [Cheng and Prusoff, 1973].

RESULTS

An isothiocyanate derivative (1,3,7-trimethyl-8-(3-isothiocyanato-styryl)xanthine, ISC, **2**) in the A_2 -selective, 8-styrylxanthine series of adenosine antagonists [Shimada et al., 1992; Jacobson et al., 1993] was prepared as a potential receptor affinity label. It was synthesized by reaction of the corresponding 3-amino derivative [Jacobson et al., 1993], **1**, with thiophosgene (Fig. 1). The previously noted difficulty of treating 1,3-disubstituted xanthines with thiophosgene [Jacobson et al., 1989] was not encountered with this 1,3,7-trisubstituted xanthine. The decomposition noted previously was likely due to involvement of the imidazole group, which is N-alkylated in this case. The meta-position was selected as the site of styryl substitution with an isothiocyanate group, based on structure activity relationships [Jacobson et al., 1993], suggesting that the mono-, meta-substitution favored selectivity and potency at rat striatal $\text{A}_{2\text{a}}$ -receptors.

Competition by ISC of binding of $[^3\text{H}]\text{CGS 21680}$ (an $\text{A}_{2\text{a}}$ -selective agonist) and $[^3\text{H}]\text{R-PIA}$ (an A_1 -selective agonist) in striatal membranes from four species was measured (Table 1) under “reversible” conditions. Major species differences have been noted previously for xanthines binding at $\text{A}_{2\text{a}}$ -adenosine receptors [Stone et al., 1988]. In rat striatum, the IC_{50} at

A_{2a} -receptors was found to be 146 nM (corresponding to an apparent K_i value of 111 nM, assuming reversibility). At A_1 -receptors the IC_{50} was found to be 43 μ M (corresponding to a K_i value of 20 μ M). Thus, the selectivity ratio of ISC for A_{2a} - versus A_1 -receptors in the rat based on IC_{50} values was 290-fold (180-fold, based on K_i values). The selectivity ratio in guinea pig striatum was nearly identical. In other species A_{2a} -selectivity was maintained (bovine, 120-fold, and rabbit, 180-fold), although the affinity was diminished. At rabbit A_{2a} receptors, the apparent K_i value of ISC was 290 nM based on the reported K_d value of 28.6 nM for binding of [3 H]CGS 21680 [Jacobson et al., 1992]. The Hill coefficients for displacement of binding of [3 H]CGS 21680 in the four species were approximately equal to 1. The A_2 -selectivity of ISC was consistent with the previously determined A_2 -selectivity [Jacobson et al., 1993] of the amino precursor, **1**, and the 3-chloro derivative, **3** (30-fold and 520-fold selectivity, respectively, based on K_i values).

ISC was examined for the ability to irreversibly inhibit A_{2a} -receptors. Preincubation of rat striatal membranes with ISC caused a dose-dependent, irreversible antagonism of the binding of 5 nM [3 H]CGS 21680 (an A_{2a} -selective agonist), with an IC_{50} value of 2.7 μ M (Fig. 2A). This IC_{50} value was 18-times greater than the IC_{50} value in competitive displacement of [3 H]CGS 21680 in the same tissue (Table 1). Preincubation with 20 μ M ISC resulted in the loss of approximately 80% of the specific binding of [3 H]CGS 21680. The irreversible nature of inhibition by the isothiocyanate derivative was demonstrated by the failure of repeated washing to regenerate the A_{2a} -receptor binding site. Nearly all of the binding of [3 H]N⁶-phenylisopropyladenosine (PIA) to striatal A_1 receptors was recovered following washout by repeated cycles (4x) of centrifugation and resuspension of the membranes in fresh buffer. Thus, at A_1 -adenosine receptors in rat striatal membranes, ISC at a high concentration of 20 μ M was barely effective as an irreversible inhibitor. At this concentration only $12 \pm 3\%$ of [3 H]PIA binding was lost compared to $81 \pm 2\%$ of [3 H]CGS 21680 binding.

Exposure of the ISC-treated striatal membranes to the weak adenosine antagonist 3-isobutyl-1-methylxanthine (IBMX, 100 (μ M) overnight also did not regenerate any A_{2a} -receptor binding (data not shown). Treatment with IBMX was used to removed non-chemically bound ligand from the membranes in a previous study of chemically reactive xanthines as irreversible inhibitors of A_1 receptors [Jacobson et al., 1989]. Such treatment was found to be unnecessary in this study, since no difference in binding was observed. Increasing the temperature of preincubation with ISC to 37°C also did not affect significantly the fraction of binding irreversibly inhibited (data not shown).

The irreversibility was examined in three other species (Figs. 2B–D). In the guinea pig striatum, the inhibition occurred at concentrations similar to those used with rat striatum (EC_{50} value 2.8 (μ M). In rabbit and bovine striatum, ISC caused an irreversible inhibition of A_{2a} receptors, but at considerably higher concentrations than in rat striatum. The EC_{50} values for ISC irreversibly inhibiting bovine and rabbit A_{2a} receptors were 8 and 10 μ M, respectively.

The irreversibility is likely due to the presence of the chemically reactive isothiocyanate group, since the binding of the corresponding analogue in which the isothiocyanate was replaced by a chloro group, **3**, was completely reversible (data not shown).

To study the time course for inactivation of rat A_{2a} receptors a concentration of 2 (μ M ISC was used. This concentration corresponds to 14-fold greater than the IC_{50} value for ISC in this species in the “competitive” binding assay vs. [3 H]CGS 21680 (Table 1). The inactivation was rapid (Fig. 3), although the degree of irreversible inhibition was not complete even after 2 h. Approximately 3 min was required for inhibition of 50% of its final

value at 2 h (at 2 h approximately 55% of the specific [^3H]CGS 21680 binding relative to control membranes was lost). The fraction of receptors inactivated by this isothiocyanate derivative increased as the concentration of ISC was raised (Fig. 2).

Saturation of binding of [^3H]CGS 21680 to rat striatal receptors following treatment with ISC (Fig. 4) and washing was measured. Following a preincubation with 5 μM ISC resulting in partial inhibition, at the remaining A_{2a} sites the B_{max} value relative to control membranes was reduced without a significant effect on the K_d value. The K_d value for [^3H]CGS 21680 binding was 15.7 nM, and the B_{max} value was 450 fmol/mg protein after pretreatment with ISC, versus 14.3 nM and 900 fmol/mg protein for control.

Inhibition of binding of [^3H]CGS 21680 at A_{2a} -receptors by ISC could be prevented by the adenosine receptor antagonist theophylline. The receptor was protected in the presence of 1 mM theophylline, with degrees of protection of 45% and 37% at 0.5 μM and 2 μM ISC, respectively (Fig. 5).

DISCUSSION

The isothiocyanate ($-\text{N}=\text{C}=\text{S}$) group is frequently used as a chemically reactive group for incorporation into receptor affinity labels, including irreversible inhibitors of adenosine receptors [Jacobson et al., 1989]. An aryl isothiocyanate has a favorable balance of stability in aqueous medium and reactivity towards nucleophiles present on proteins, such as amino and thiol groups.

ISC appears to be a selective affinity label of moderate affinity for A_{2a} - versus A_1 -receptors in four species. The affinity of ISC at low affinity A_{2b} -receptors [Bruns et al., 1986; Hide et al., 1992] has yet to be determined.

The chemical mechanism for the irreversibility is presumably acylation by the reactive isothiocyanate group of a nucleophilic group located on or in the vicinity of the antagonist binding site of the receptor protein. Following partial inactivation, the remaining rat A_{2a} -binding sites retained the same K_d value for saturation by [^3H]CGS 21680. Thus, the inhibition is all-or-none, consistent with covalent anchoring of the ligand in its usual binding site.

ISC is of potential utility in characterization of the binding of xanthines to adenosine receptors at the molecular level. The amino acid sequence of A_{2a} receptors in several species is known [Maenhaut et al., 1990; reviewed in van Galen et al., 1992; Linden et al., 1993]. The site of presumed reaction with the receptor protein may be characterized in future studies through radiolabeling the isothiocyanate derivative for purposes of peptide mapping and by site-directed mutagenesis. Unlike previous irreversible inhibitors of adenosine receptors [Jacobson et al., 1989, 1992], in which an isothiocyanate group was positioned on a functionalized chain at a large distance from the pharmacophore, this NCS group is located close to the purine moiety. Thus, ISC may be especially helpful in identifying amino acid residues that are in proximity to the xanthine binding site.

In order to confirm that ISC is an irreversible antagonist of adenosine receptor activation, it will be necessary to study its functional effects on adenylate cyclase activity. Such selective inhibitors are also potentially of interest in physiological studies, in defining the roles of adenosine receptor subtypes. By analogy, an A_{2a} agonist-derived affinity label, 2-[2-[4-[2-[[(4 - isothiocyanatophenyl)aminothiocarbonylamino-ethyl]-amino]carbonyl]ethyl]phenyl]ethylamino]-5'-N-ethylcarboxamidoadenosine (*p*-DITC-APEC) has been studied in the isolated, perfused guinea pig heart [Niiya et al., 1993] and found to cause a prolonged coronary vasodilatation. An A_1 antagonist-derived affinity label,

(8-[4-[[[2-(3-isothiocyanatophenyl) aminothiocabonylaminoethyl]-amino] carbonyl] methyl]oxy]phenyl]-1,3-dipropylxanthine) (*m*-DITC-XAC) was used to demonstrate the presence of spare adenosine receptors in the guinea pig atrioventricular node [Dennis et al., 1992]. It is possible that ISC will also be useful in vivo. Other aryl isothiocyanate-bearing affinity labels have been administered in vivo [de Costa et al., 1989] and found to be sufficiently stable to label their receptor sites in the brain and elsewhere.

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Figure 1. Structures and synthesis of xanthine derivatives used in this study. The 3-amino derivative, **1**, was converted to the isothiocyanate, ISC, **2**, using thiophosgene. The 3-chloro derivative, **3**, is also selective for A_{2a}-receptors, but is not chemically reactive.

Figure 2.

Dose-dependent irreversible inhibition by ISC of radioligand binding at A₁- (open symbols) and A_{2a}- (solid symbols) adenosine receptors in A) rat, B) guinea pig, C) bovine, and D) rabbit striatal membranes (n = 4 or more). The preincubation with ISC or control was carried out for 1 h at 25°C, and the subsequent binding assay involved a 90 min incubation followed by rapid filtration. The radioligand binding step consisted of incubation in triplicate with 5 nM [³H]CGS 21680 for A_{2a}-receptors or 1 nM [³H]PIA for A₁-receptors. Each data point represents the mean ± s.d. specific binding for 3–4 separate experiments. In A, error bars at 10 and 20 μM ISC are obscured by the data point.

Figure 3.

Time course for inhibition of specific binding to rabbit striatal A_{2a} -adenosine receptors at 25°C by 2 μ M ISC. The membranes were washed by centrifugation (3x) prior to radioligand binding. [3 H]CGS 21680 was used at a concentration of 5 nM. This curve is representative of data from three separate experiments.

Figure 4.

Saturation curve (A) and Scatchard transformation (B) for the specific binding of [³H]CGS 21680 to A_{2a}-adenosine receptors in rat striatal membranes, in control membranes (open circles) and following preincubation at 25°C for 1 h with 2 μM ISC (closed circles). The volume of incubation for radioligand binding (approximately 150 μg protein/tube) was 1 ml. Membranes were incubated with radioligand at 25°C for 90 min. Specific binding in control (○) and treated (●) membranes is shown. Non-specific binding in control and treated membranes was nearly identical and amounted to 8–10% of total binding at 5 nM [³H]CGS 21680.

Figure 5.

Protection by theophylline of rat striatal A_{2a} receptors from inhibition by ISC. Percent irreversible inhibition relative to the level of specific binding of 5 nM [3 H]CGS 21680 in control membranes is shown ($n = 3$). Hatched bars are for ISC alone, at the indicated concentration. Solid bars are for the combination of ISC and theophylline (1 mM).

TABLE 1

Potencies of ISC in Inhibiting Radioligand Binding at Central A₁ and A_{2a} Receptors in Four Mammalian Species. *

Species	IC ₅₀ (A ₁)	IC ₅₀ (A ₂)	A ₁ /A ₂ ratio
Rat	42,600 ± 3600 ^a	146 ± 3 ^b	291
Guinea pig	51,400 ± 17,700	160 ± 2	320
Bovine	63,400 ± 5,900	516 ± 64	122
Rabbit	89,500 ± 2,000	413 ± 135 ^c	217

* Expressed in nM (mean ± s.d. for 3 or more determinations) vs. [³H]PIA (1 nM) at striatal A₁-receptors and vs. [³H]CGS 21680 (5 nM) at striatal A_{2a}-receptors. Non-specific binding was determined in the presence of 10 μM 2-chloroadenosine.

^a Corresponds to a K_i value of 20,300 ± 1700 nM.

^b Corresponds to a K_i value of 111 ± 0.5 nM and a selectivity ratio of 182.

^c Corresponds to a K_i value of 347 ± 112 nM.