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Modulation of A_1 adenosine receptor signaling by peroxynitrite

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Abstract

Nitric oxide (NO) is a gaseous free radical involved in many pathophysiological processes. During oxidative stress, NO, its derivatives and adenosine are released. Considering adenosine neuroprotective role in the central nervous system (CNS) and toxicity of NO, we investigated the effect of a NO/peroxynitrite ($ONOO^-$) donor, 3-morpholinosydnonimine (SIN-1), on A_1 adenosine receptor (A_1AR) signaling pathway in rat cortical membranes. Membrane treatment with 0.5 mM SIN-1 for various periods of time (0–240 min) decreased specific binding of the radiolabeled A_1AR agonist, [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA), in a time-dependent manner, reaching the steady state after 120 min. The inhibitory effect of SIN-1 was concentration-dependent, with an EC_{50} value of 0.60 ± 0.30 mM ($N = 3$). Membrane pre-incubation with the superoxide anion ($O_2^{\bullet -}$) scavenger superoxide dismutase (SOD) followed by SIN-1 addition, abolished SIN-1 inhibition of [^{3}H]CHA binding. Membrane treatment with 0.5 mM SIN-1 for 120 min caused a significant 2-fold increase of the K_D value for [^{3}H]CHA without changing the B_{max} value. Moreover, pre-incubation of membranes with A_1AR agonists, CHA or N^6-(2-phenylisopropyl)-adenosine (R-PIA) before SIN-1 addition increased the inhibitory effect while the selective A_1AR antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) had no activity. Membrane treatment with SIN-1 decreased receptor-stimulated guanosine 5′-*O*-(γ[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding in a concentration-dependent manner. This treatment influenced [^{35}S]GTPγS binding affinity for A_1AR activated G_i proteins in cortical membranes. These findings suggest that $ONOO^-$ modulates A_1AR signaling pathways by affecting receptor G_i protein coupling.

Keywords: A_1 adenosine receptors; G_i proteins; Nitric oxide; Peroxynitrite; 3-Morpholinosydnonimine

1. Introduction

NO is a major signaling molecule in neurons and immune system either acting within cells in which it is produced or penetrating adjacent cells to affect their activity. It is generated from L-arginine by the action of three different forms of the enzyme NO synthase (NOS) referred to neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). NO is involved in several physiological functions such as vasodilatation, neurotransmission and immune defense [1]. In these processes, the main signal transduction pathway for NO is the high-affinity binding to the ferrous heme prosthetic group of soluble guanylate cyclase (sGC) [2,3] enhancing the catalytic activity of this enzyme and increasing cGMP production [4]. In the extracellular milieu, NO reacts with oxygen and water to produce nitrates and nitrites. Indeed, NO has been implicated in many pathophysiological processes and some of its cytotoxic effects are mediated via a rapid reaction with $O_2^{\bullet -}$ to form the highly reactive oxidant species $ONOO^-$ [5]. $ONOO^-$ can cause lipid peroxydation [6], inhibition of mitochondrial respiration [7,8] and membrane pumps [9], depletion of glutathione [10] and damage of DNA [11].

Apart from its effect on sGC, NO also leads to nitrosylation of thiol groups on cysteine residues [12,13]. It has been suggested that nitrosylation causes protein conformational changes [12,13], facilitation of ADP-ribosylation [14], inhibition of palmitoylation [15] and thereby modulation of their functions. A number of proteins involved in intracellular signaling are palmitoylated including many

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Abbreviations: NO, nitric oxide; $ONOO^-$, peroxynitrite; SIN-1, 3-morpholinosydnonimine; A_1AR, A_1 adenosine receptor; [^{3}H]CHA, [^{3}H]N^6-cyclohexyladenosine; $O_2^{\bullet -}$, superoxide anion; $^{\bullet}OH$, hydroxyl radical; SOD, superoxide dismutase; R-PIA, N^6-(2-phenylisopropyl)-adenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxantine; [^{35}S]GTPγS, guanosine 5′-*O*-(γ[^{35}S]thio)triphosphate; ROS, reactive oxygen species; GPCRs, G-protein-coupled receptors; β_2AR, β_2 adrenergic receptor; PMSF, phenylmethanesulphonylfluoride; ADA, adenosine deaminase; GDP, guanosine 5′-*O*-diphosphate; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNOG, *S*-nitroso-glutathione.

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GPCRs and the α-subunit of most heterotrimeric G proteins. This post-translational lipid modification plays a role in membrane anchorage but also in other protein functions such as receptor desensitisation and coupling. In the case of the β_2-adrenergic receptor (β_2AR), receptor activation by an agonist results in a greater incorporation of [^{3}H]palmitate into both the receptor [16] and the cognate G_s protein [17]. This observation is consistent with agonist-induced turnover of palmitate [16,17]. In fact, protein palmitoylation is mostly reversible [18] and therefore a potential site for regulation. In HEK293 and COS7 cells transfected with the wild-type β_2AR, NO appears to decrease receptor signaling via inhibition of its palmitoylation, thus promoting receptor G_s uncoupling [19]. A similar uncoupling between another GPCR, the bradykinin receptor and its cognate G proteins, G_q and G_i, has also been observed following treatment with NO donors [20].

The production of free radicals (e.g. NO and reactive oxygen species, ROS) during ischemia-reperfusion injury triggers the release of adenosine into the extracellular milieu [21]. Adenosine, activating A_1ARs which are abundant in the CNS, is known to inhibit the release of many neurotransmitters and thereby acts as a neuroprotective substance. In the G_i/G_q-coupled A_1AR, a cysteine residue in position 309 on the carboxyl tail can be palmitoylated [22]. In the case of the recombinant human A_1AR [23] palmitoylation has little effect on receptor–effector coupling and down-regulation. However, a possible modulation by NO cannot be excluded.

The aim of the present study was to analyze whether NO or its derivative $ONOO^-$ was able to modulate A_1AR signaling in rat cortical membranes. We found that membrane pre-treatment with the NO donor SIN-1 has a negative influence on agonist binding to A_1ARs and affects G_i protein activation. These SIN-1 effects appear to be mediated by $ONOO^-$ production.

2. Materials and methods

2.1. Preparation of rat cortical membranes

Brain cortices from young male Wistar rats (150–200 g) that had been subjected to cervical dislocation were rapidly removed and homogenised in 10 volumes of ice-cold 10 mM Tris–HCl buffer, pH 7.5, 5 mM EDTA (buffer A), containing 0.25 M sucrose, 0.1 mM PMSF, 1 mM benzamidine, 100 μg/mL bacitracin using a polytron homogenizer. The membrane homogenate was centrifuged at 1000 *g* for 10 min at 4°. The supernatant was collected and centrifuged at 48,000 *g* for 20 min at 4°. The resulting pellet was resuspended in 10 volumes of 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$ (buffer B), containing protease inhibitors and centrifuged again. The pellet was resuspended in 5 volumes of buffer B containing protease inhibitors and 2 IU/mL of ADA and incubated at 37° for 30 min to remove endogenous adenosine. The membrane homogenate was centrifuged at 48,000 *g* for 20 min at 4° and the final pellet was stored as aliquots at −80° until needed. Protein concentrations were determined by the method of Lowry *et al*. [24], using bovine serum albumin as a standard.

2.2. Membrane treatment with NO donors

Cortical membranes were resuspended (1.5–1.8 mg/mL of membrane proteins) in buffer B containing ADA (1 IU/mL) and incubated in the presence and absence of increasing concentrations of each one of the following NO donors, SNP, SNAP, SNOG and SIN-1 at 25° for 60 min under constant shaking. The reaction mixtures were then transferred to 1.5 mL microfuge tubes and centrifuged at 15,000 *g* at 4° for 5 min. The resulting pellets were resuspended in buffer B containing PMSF (0.1 mM) and benzamidine (160 μg/mL) and centrifuged again. This washing step was repeated twice. Pellets were resuspended in buffer B containing ADA (1 IU/mL) at a protein concentration of 1.5 mg/mL and used in binding assays.

To evaluate the time course of agonist ([^{3}H]CHA) binding inhibition by SIN-1, membrane suspensions (1.5–1.8 mg/mL of membrane proteins) were incubated in the presence and absence of 0.5 mM of this NO donor at 25° under continuous shaking for various periods of time (0–240 min). For saturation and inhibition experiments membrane suspensions were incubated with and without 0.5 mM SIN-1 at 25° for 120 min under continuous shaking. In order to assess the influence of agonists and antagonists on [^{3}H]CHA binding inhibition, membrane suspensions were incubated with and without these agents for 10 min at room temperature before the addition of SIN-1 (0.5 mM final concentration). The effect of SOD on [^{3}H]CHA binding inhibition was evaluated by co-incubating membrane suspensions (1.5–1.8 mg/mL of membrane proteins) with this enzyme (1000 IU/mL) and 0.5 mM SIN-1 at 25° for 2 hr under continuous shaking.

2.3. Radioligand binding assays

Routine binding assays were performed by incubating rat cortical membranes (100–150 μg of protein) with either 1.2 nM [^{3}H]CHA or 0.8 nM [^{3}H]DPCPX and ADA (1 IU/mL) in 0.5 mL of buffer B for 60 or 120 min at 25°. Binding reactions were terminated by filtration through Whatman GF/C glass fiber filters under reduced pressure. Filters were washed either three ([^{3}H]CHA binding) or five ([^{3}H]DPCPX binding) times with 5 mL of buffer B. Unspecific binding was defined in the presence of 15 μM R-PIA. Specific binding was 85–90% of total binding in all experiments.

For saturation studies, control membranes and membranes treated with SIN-1 (100 μg of protein) were incubated in buffer B containing ADA (1 IU/mL) and 7

different concentrations of [^{3}H]CHA ranging from 0.1 to 50 nM. Competition studies were carried out by incubating control membranes and membranes treated with SIN-1 (80–100 μg of protein) in buffer B containing ADA (1 IU/mL), 0.8 nM [^{3}H]DPCPX and 14 different concentrations of CHA ranging from 0.01 nM to 1 μM.

2.4. Rat cortical membrane preparation for [^{35}S]GTPγS binding

Brain cortices from young male Wistar rats were homogenised in 20 volumes of ice-cold 10 mM Hepes–NaOH, pH 7.4 (buffer C) containing 0.32 M sucrose, 160 μg/mL benzamidine, 200 μg/mL bacitracin, 20 μg/mL trypsin inhibitor, 0.1 mM PMSF using a polytron homogenizer. The homogenate was centrifuged at 1000 *g* for 5 min at 4°. The supernatant was collected and centrifuged at 48,000 *g* at 4° for 15 min. The pellet was resuspended in buffer C containing 160 μg/mL benzamidine, 200 μg/mL bacitracin, 20 μg/mL trypsin inhibitor, 0.1 mM PMSF and centrifuged again. The resulting pellet was resuspended in 20 volumes of 50 mM Hepes–Tris, pH 7.4 (buffer D) containing 100 μM GDP and incubated at room temperature for 60 min under constant shaking. Afterwards, the membrane homogenate was centrifuged at 48,000 *g* for 30 min at 4°. The pellet was collected and washed three times with buffer D. The final pellet was stored as aliquots at −80° until the time of assay. Protein concentrations were determined by the Coomassie blue binding method [25] using bovine γ-globulin as a standard.

2.5. [^{35}S]GTPγS binding

Rat cortical membranes (approximately 250 μg/mL of membrane proteins) were suspended in 25 mM Hepes–NaOH, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl (buffer E) containing 1 mM dithiothreitol (DTT). An aliquot of membrane homogenate (20 μL) was incubated in 0.1 mL of buffer E with ADA (1 IU/mL), 30 μM GDP and 0.3 nM [^{35}S]GTPγS in the presence (stimulated) and absence (basal) of an agonist. Incubation was carried out at 25° for 120 min. Unspecific binding was defined in the presence of 100 μM GTPγS. Specific binding was 97–98% of total binding in all experiments. Binding reactions were terminated by filtration through GF/C glass fiber filters using a multiscreen separation system (Millipore Corporation). Filters were washed three times with 200 μL of 20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 25 mM $MgCl_2$ (buffer F).

In order to investigate the time course of [^{35}S]GTPγS binding to rat cortical membranes, membrane suspensions were incubated with 0.3 nM [^{35}S]GTPγS for various periods of time (0–240 min) in the presence and absence of 1 μM CHA. Dose-dependence experiments were carried out by incubating membrane suspensions with concentrations of CHA and R-PIA ranging from 0.1 nM to 50 μM. For dilution studies, membrane suspensions were incubated as described above with 0.3 nM [^{35}S]GTPγS and 10 different concentrations (0.25 nM to 1 μM) of GTPγS, in the presence and absence of 1 μM CHA.

For studying the effect of SIN-1 on basal and stimulated [^{35}S]GTPγS binding, membranes were resuspended (1.5–1.8 mg/mL of membrane proteins) in buffer E containing ADA (1 IU/mL) and incubated with various concentrations of SIN-1 at 25° for 2 hr as described. SIN-1 influence on [^{35}S]GTPγS binding parameters was assessed by incubating membranes with 1 mM SIN-1 followed by dilution experiments of [^{35}S]GTPγS with unlabeled GTPγS as above.

2.6. Data analysis

A non-linear multipurpose curve-fitting computer program (EBDA/LIGAND, Elsevier-Biosoft) [26] was used for analysis of saturation data. Saturation curves were also fitted by the non-linear regression analysis of the GraphPad Prism Version 3.00 computer program (GraphPad Software). Single- and multiple-site models were statistically compared to determine the best fit and differences between models were tested by comparing the residual variance using a partial *F*-test and a significance level of $P < 0.05$ (GraphPad Prism Version 3.00). Data from concentration–response or displacement curves were analyzed by a non-linear least squares curve-fitting computer program (GraphPad Prism Version 3.00), and the EC_{50} values were derived. The K_i values for competition binding assays were calculated from the EC_{50} values by the Cheng and Prusoff equation [27]. Data are reported as means ± SEM of three experiments, except when otherwise stated. Statistical analysis (Student's *t*-test) was performed using the GraphPad Prism Version 3.00 computer program (GraphPad Software).

2.7. Materials

[^{3}H]CHA (32.2 Ci/mmol), [^{3}H]DPCPX (109 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were obtained from NEN Life Science Products. SNAP, SNOG and SIN-1 were purchased from Tocris. CHA and R-PIA were obtained from RBI. ADA was purchased from Boeringher-Mannheim. Benzamidine, SNP, GDP, GTPγS, DPCPX and SOD were obtained from Sigma Chemical Co. Bacitracin and PMSF were purchased from Fluka Chemie AG and DTT was obtained from Calbiochem. Other agents and reagents were from standard commercial sources.

3. Results

3.1. Inhibitory effect of NO donors on [^{3}H]CHA binding to rat cortical membranes

In order to evaluate the effect of NO on specific binding of [^{3}H]CHA to A_1 receptors, rat cortical membranes were

treated with NO donors e.g. SNP, SNAP, SNOG and SIN-1. SNP decreased agonist specific binding in a concentration-dependent manner reaching the maximal effect of 20–30% inhibition at 200 mM (Fig. 1, panel A). SNAP caused variable increases of agonist specific binding at all tested concentrations with a maximal stimulation of 48% at 5 mM (Fig. 1, panel B). On the other hand, *N*-acetylpenicillamine, which is the other substance released during SNAP decomposition, led to a decrease of agonist binding especially at the concentration of 10 mM (Fig. 1, panel B).

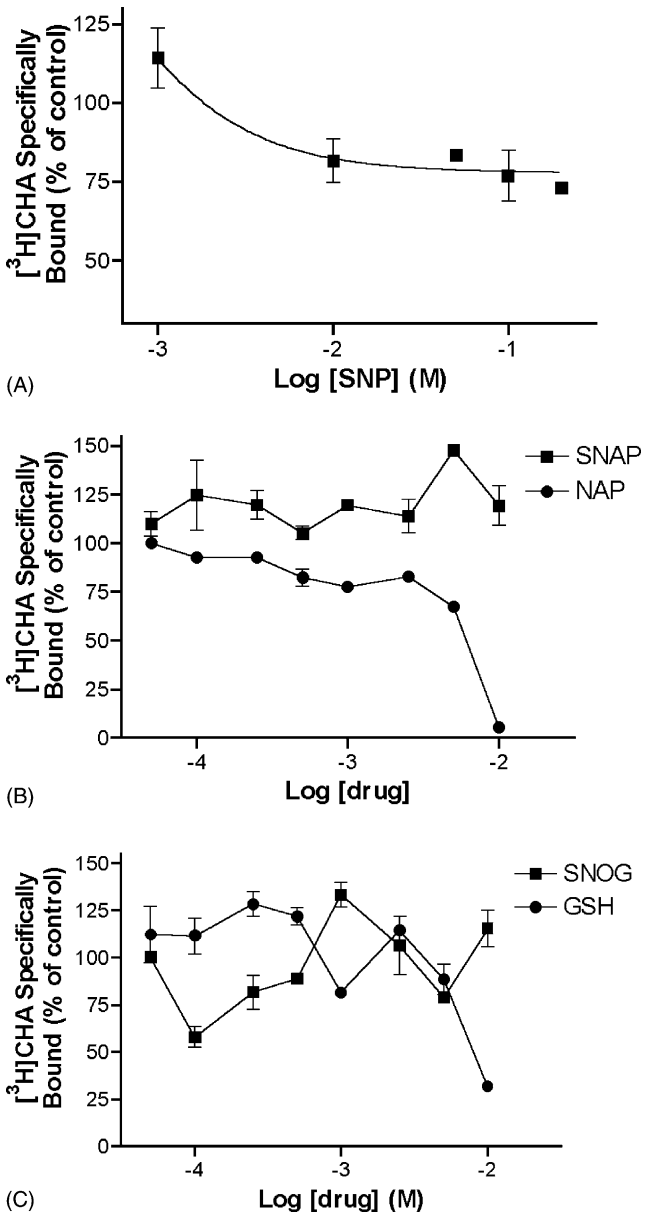

Fig. 1. Effects of NO donors on [^{3}H]CHA binding to rat cortical membranes. Membranes were treated with increasing concentrations of SNP (1–200 mM) (panel A), SNAP, *N*-acetylpenicillamine (NAP) (50 μM to 10 mM) (panel B), SNOG or glutathione (GSH) (50 μM to 10 mM) (panel C) for 1 hr at 25° (see Section 2). After treatment, specific binding of 1.2 nM [^{3}H]CHA to membranes was measured and compared to the appropriate control. Data points are means ± SEM of three independent experiments performed in duplicate.

SNOG produced variable effects decreasing or increasing agonist specific binding in a concentration-independent manner while glutathione, which is also released during SNOG decomposition, caused a marked inhibition (~70%) at a concentration of 10 mM (Fig. 1, panel C). These data suggest that NO by itself cannot significantly alter agonist specific binding to A_1 receptors.

3.2. Inhibitory effect of SIN-1 on [^{3}H]CHA binding to rat cortical membranes

The only NO donor that produced a marked decrease of agonist binding in a time and concentration-dependent manner was SIN-1. This compound, the metabolite of the vasodilator molsidomine, reduced agonist binding in a time-dependent fashion. At 0.5 mM a steady-state of 50% inhibition was reached after 2 hr (Fig. 2, panel A). Membrane incubation with various concentrations of SIN-1 for 2 hr caused a rapid linear decrease of agonist binding up to 0.5 mM. The concentration–response curve showed a maximal effect of 70% inhibition at 10 mM SIN-1 while

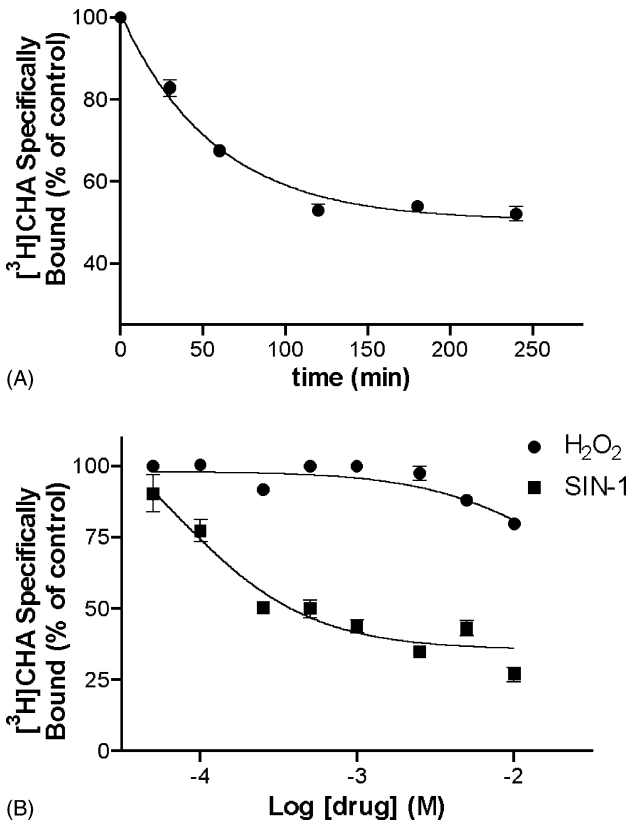

Fig. 2. SIN-1 affects [^{3}H]CHA specific binding to rat cortical membranes in a time- and concentration-dependent fashion. Panel A, membranes were treated with SIN-1 for various periods of time (0–240 min) as described under Section 2. Panel B, membranes were treated with increasing concentrations (0.05–10 mM) of SIN-1 or H_2O_2 for 2 hr at room temperature (see Section 2). After treatment, specific binding of 1.2 nM [^{3}H]CHA to membranes was measured and compared to corresponding controls. Data points represent means ± SEM of at least three independent experiments performed in duplicate.

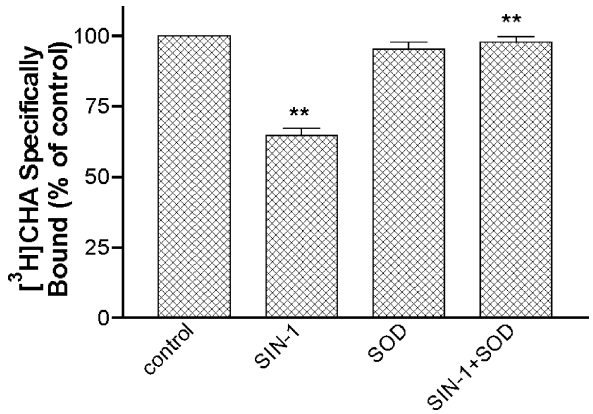

Fig. 3. Influence of SOD on SIN-1 induced inhibition of [^{3}H]CHA-specific binding to rat cortical membranes. Treatment with 0.5 mM SIN-1 for 120 min in the presence and absence of SOD (1000 IU/mL) was carried out as described under Section 2. After treatment, specific binding of 1.2 nM [^{3}H]CHA to membranes was measured and compared to the appropriate control. Data points are means ± SEM of three independent experiments performed in duplicate. SIN-1 treated membranes: $^{**}P < 0.05$ vs. untreated membranes (control). SIN-1 treated membranes in the presence of SOD: $^{**}P < 0.05$ vs. SIN-1 treated membranes.

the derived EC_{50} was 0.60 ± 0.30 mM (N = 3) (Fig. 2, panel B).

SIN-1 releases both NO and $O_2^{\bullet -}$. The last one reacts with H_2O to form hydrogen peroxide (H_2O_2) which did not considerably inhibit [^{3}H]CHA binding (Fig. 2, panel B). Since SIN-1 is the only NO donor releasing both NO and $O_2^{\bullet -}$, which rapidly react to form $ONOO^-$, the inhibition of agonist binding may be caused by the derivative, $ONOO^-$. In order to confirm our hypothesis for an involvement of $ONOO^-$ in SIN-1 effects, we studied the influence of 1000 IU/mL SOD on SIN-1 induced inhibition of agonist binding. The presence of this enzyme, which converts $O_2^{\bullet -}$ to H_2O_2 and thereby prevents $ONOO^-$ formation, completely abolished SIN-1 effects, strongly accounting for an involvement of $ONOO^-$ (Fig. 3). As a consequence of these observations, our studies focused on investigating SIN-1 on A_1AR activation and signal transduction.

Saturation studies with increasing concentrations of [^{3}H]CHA (0.1–50 nM) revealed the presence of a single class of binding sites (Fig. 4, panels A and B). Membrane treatment with SIN-1 modified the dissociation constant (K_D) value without significantly affecting the total number of binding sites (B_{max}) (Fig. 4, panels A and B). In fact, treatment with 0.5 mM SIN-1 for 2 hr at 25° caused a significant increase of the mean K_D value from 1.09 ± 0.36 nM to 2.59 ± 0.14 nM (N = 4; $P < 0.01$). For untreated and SIN-1 treated membranes, mean B_{max} values were 271 ± 80 fmol/mg and 204 ± 56 fmol/mg protein (N = 4), respectively.

Competition studies of [^{3}H]DPCPX (0.8 nM) binding to membranes by CHA (0.01 nM–1 μM) showed two affinity states of receptor binding sites. In fact, the best fit for competition curves was achieved using the two site model

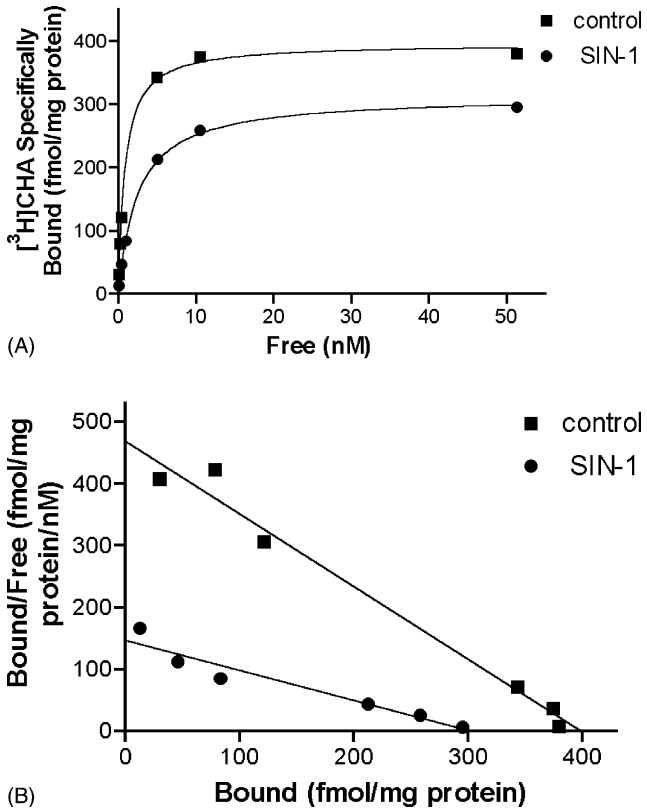

Fig. 4. Equilibrium binding of [^{3}H]CHA to rat cortical membranes untreated (control) or treated with 0.5 mM SIN-1 for 120 min. Membranes (100 μg of protein) were incubated with increasing concentrations of [^{3}H]CHA, ranging from 0.1 to 50 nM as described under Section 2. Panel A, saturation isotherms of [^{3}H]CHA binding to rat cortical membranes in the indicated conditions. Panel B, scatchard plots derived from saturation data. This is representative of four independent experiments performed in duplicate. In this experiment, the K_D and B_{max} values were 0.82 nM and 396 fmol/mg protein for untreated membranes and 2.44 nM and 313 fmol/mg protein for membranes treated with SIN-1, respectively.

of Graph Pad (Version 3.00) non-linear regression analysis. Membrane treatment with SIN-1 influenced the K_i value of high affinity state (K_H) causing a 2-fold increase (Fig. 5, and Table 1).

To investigate $ONOO^-$ targets, we evaluated the influence of two A_1 agonists, CHA and R-PIA, and an antagonist, DPCPX, on SIN-1 effects. While DPCPX did not significantly interfere with SIN-1 activity, CHA and R-PIA potentiated its effects (Fig. 6). In the presence of CHA or

Table 1
Inhibition of [^{3}H]DPCPX binding to rat cortical membranes by CHA

	K_H (nM)	K_L (nM)	R_H (%)
Control	0.54 ± 0.17	144.70 ± 71.40	64.08 ± 3.09
SIN-1	0.95 ± 0.33	186.90 ± 52.30	50.54 ± 4.17

Control membranes and membranes treated with SIN-1 (0.5 mM) were incubated with [^{3}H]DPCPX in the presence and absence of CHA concentrations ranging between 0.01 nM and 1 μM. The K_i values for the high (K_H) and low (K_L) affinity sites were calculated from the EC_{50} values by the Cheng and Prusoff equation. R_H is the percentage distribution of high affinity sites. All values are means ± SEM from three independent experiments performed in duplicate.

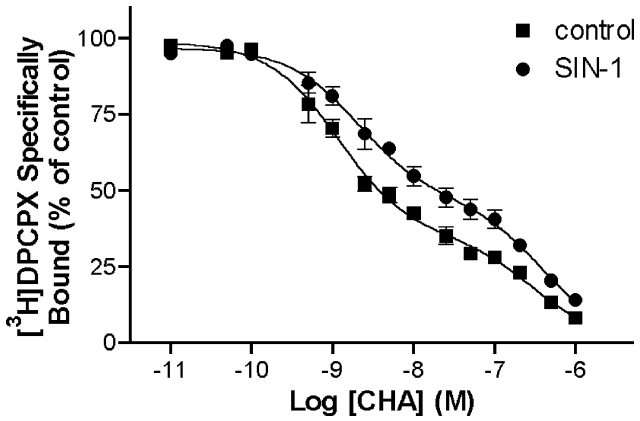

Fig. 5. Competition of [^{3}H]DPCPX binding to rat cortical membranes by CHA in basal conditions (control) or after SIN-1 treatment for 120 min. Membrane treatment was carried out as described under Section 2. Control and treated membranes (80 μg of protein) were incubated with 0.8 nM [^{3}H]DPCPX in the presence and absence of increasing concentrations of CHA, ranging from 0.01 nM to 1 μM. Unspecific binding was measured in the presence of 15 μM R-PIA. Data points represent means ± SEM of three independent experiments performed in duplicate.

R-PIA, agonist binding inhibition increased from 39 to 63 and 62%, respectively.

3.3. Modulation of [^{35}S]GTPγS binding by SIN-1

In order to study the influence of SIN-1 on receptor mediated G_i protein activation, we analyzed agonist stimulated [^{35}S]GTPγS binding to rat cortical membranes. Our initial experiments showed that [^{35}S]GTPγS binding to rat cortical membranes increased in a time-dependent

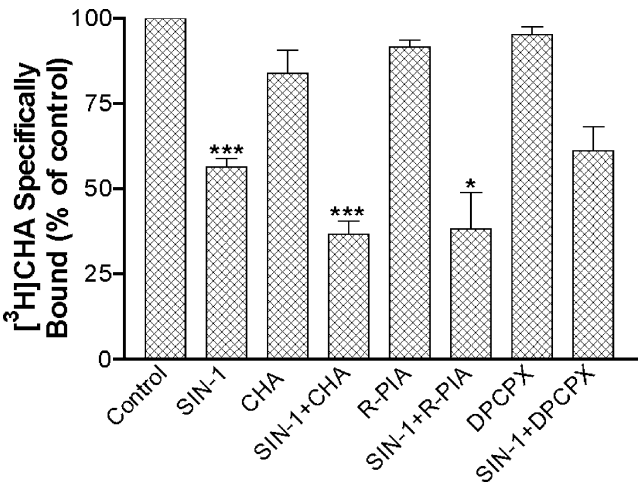

Fig. 6. Influence of rat cortical membranes pre-incubation with A_1AR agonist or antagonist ligands on SIN-1 inhibition of [^{3}H]CHA binding. Membranes were incubated with 50 nM CHA, R-PIA or 35 nM DPCPX for 10–15 min followed by SIN-1 (0.5 mM) treatment for additional 120 min. Specific binding of 1.2 nM [^{3}H]CHA to membranes was measured and compared to the appropriate controls. Data points are means ± SEM of three to four independent experiments performed in duplicate. SIN-1 treated membranes: $^{***}P < 0.001$ vs. untreated membranes (control). SIN-1 treated membranes in the presence of CHA: $^{***}P < 0.001$ vs. SIN-1 treated membranes. SIN-1 treated membranes in the presence of R-PIA: $^{*}P < 0.01$ vs. SIN-1 treated membranes.

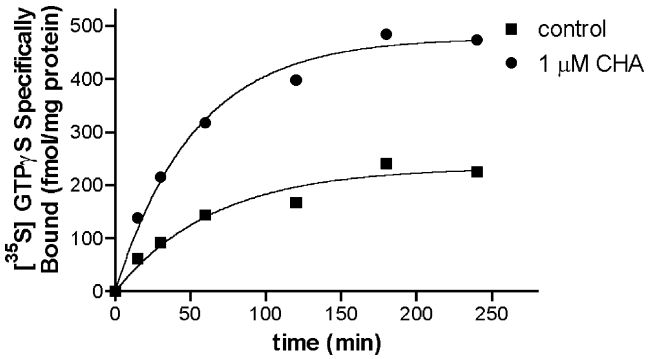

Fig. 7. Time course of [^{35}S]GTPγS specific binding to rat cortical membranes in the presence and absence of 1 μM CHA. Membranes (5 μg of protein) were incubated with 0.3 nM [^{35}S]GTPγS for various periods of time (0–240 min) as described under Section 2. Data points are means ± SEM of three independent experiments performed in triplicate.

fashion reaching a steady-state after 2 hr in both basal and stimulated conditions (Fig. 7). Analysis of time course data in the presence and absence of 1 μM CHA revealed that both curves were better fitted according to a one-phase exponential association. However, neither the K_{ob} nor half-life value showed any significant changes.

CHA and R-PIA (0.1 nM–50 μM) increased [^{35}S]GTPγS binding to membranes in a concentration-dependent manner (Fig. 8) with EC_{50} values of 16.31 ± 3.21 nM (N = 3) and 27.45 ± 9.77 nM (N = 3), respectively. A 2 hr treatment with SIN-1 ranging between 0.1 and 5 mM caused a concentration-dependent reduction of agonist stimulated [^{35}S]GTPγS binding, without affecting basal binding (Fig. 9).

Dilution experiments of [^{35}S]GTPγS with GTPγS identified two binding sites or affinity states in both basal and receptor-stimulated conditions. In the presence of CHA (1 μM), the K_D value (K_{D1}) for the high-affinity sites showed a significant 2-fold decrease as compared to the basal value while other binding parameters were unchanged (Table 2). Membrane treatment with 1 mM SIN-1 for 2 hr caused modest changes of K_{D1} but the

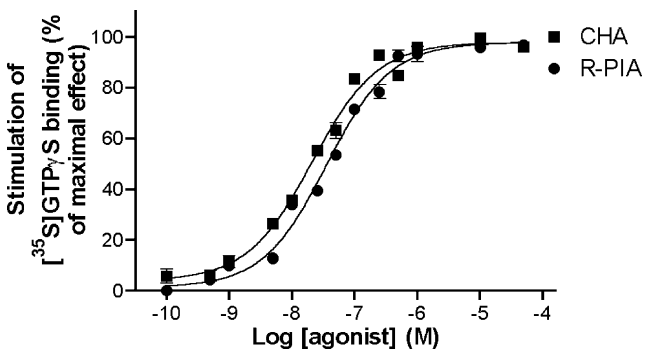

Fig. 8. Concentration–response curves of CHA and R-PIA stimulated [^{35}S]GTPγS binding to rat cortical membranes. Membranes (5 μg of protein) were incubated with 0.3 nM [^{35}S]GTPγS in the presence and absence of increasing concentrations of CHA or R-PIA, ranging from 0.1 nM to 50 μM as described under Section 2. Data points represent the means ± SEM of three independent experiments performed in triplicate.

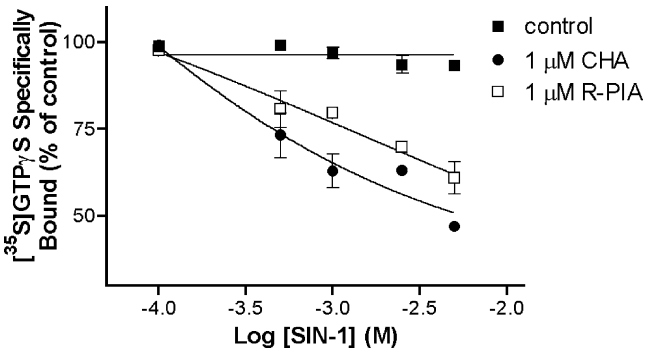

Fig. 9. SIN-1 inhibits agonist stimulated [^{35}S]GTPγS binding to rat cortical membranes. Membranes were treated with five different concentrations of SIN-1 (0.1–5 mM) for 120 min as described under Section 2. Then, aliquots of membranes were incubated with 0.3 nM [^{35}S]GTPγS in the presence and absence of 1 μM CHA or R-PIA. Data points represent means ± SEM of at least three independent experiments performed in triplicate.

observed difference between basal and stimulated conditions was not detectable anymore. However, in stimulated conditions the K_D value (K_{D2}) for the low-affinity sites showed a 3-fold increase as compared to the value obtained for corresponding basal conditions. At the same time, a 2-fold decrease of the B_{max} value (B_{max2}) for the low-affinity sites was also observed.

4. Discussion

The present study demonstrates that $ONOO^-$ produced using the chemical NO donor, SIN-1, affects both agonist binding to A_1AR and G_i protein activation in rat brain cortical membranes. Our data show that $ONOO^-$ is the main actor in this process since other NO donors which do not release $O_2^{\bullet -}$ are not very effective and pre-treatment with SOD abolishes SIN-1 effect on agonist binding.

$ONOO^-$ which is the product of the near diffusion-limited reaction between $O_2^{\bullet -}$ and NO is believed to be partially responsible for toxic and detrimental effects of NO in biological systems [5]. Indeed, $ONOO^-$ has been implicated in the pathology of several neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, multiple sclerosis and ischemia/reperfusion injury [28,29]. This strong but selective oxidant may be a critical agent contributing to impaired function caused by excessive oxidative stress. Immediate biological effects of $ONOO^-$ involve several different molecules including proteins with efficient modification of cysteine, methionine, tryptophan and tyrosine residues [30–35]. Thus, $ONOO^-$ has been shown to affect the sarcoplasmic reticulum Ca^{2+}–ATPase pump functions through modification of its thiol groups [36] and inactivate the enzyme tyrosine hydroxylase by nitration of tyrosine residues [37,38]. While in various studies, the influence of NO and ROS on GPCRs and G_i/G_q proteins signaling has been addressed [19,20,39] the effect of $ONOO^-$ on signal transduction has not been extensively investigated. However, in guinea pig airways $ONOO^-$ inhibits the bronchoprotective effects of β_2-adrenergic receptor agonists [40].

Our results demonstrate that exposition of rat cortical membranes to $ONOO^-$ causes a 2-fold decrease of A_1AR affinity for agonist ligands affecting the high-affinity state of the receptor without modifying the total number of binding sites. In addition, membrane pretreatment with A_1 receptor agonists increases the inhibitory effect of $ONOO^-$ on agonist ligand binding while an antagonist does not induce any change. This observation strongly indicates that the activated forms of either receptor, coupled G_i protein or both are favorable substrates for modification. Thus, we investigated the influence of $ONOO^-$ on A_1AR-mediated activation of G_i proteins by measuring [^{35}S]GTPγS binding to membranes. $ONOO^-$ reduces agonist-stimulated [^{35}S]GTPγS binding in a concentration-dependent fashion without affecting basal binding. Dilution experiments show that [^{35}S]GTPγS binds to rat cortical membranes to two binding sites, defined as high and low affinity sites. Stimulation with an A_1 adenosine receptor agonist causes a significant 2-fold decrease of the K_{D1} value for the high affinity site while the K_{D2} value for the low affinity site does not change. Membrane treatment with SIN-1 blunts the difference between K_{D1} values for high affinity sites in basal and stimulated conditions. However, addition of 1 μM CHA to treated membranes induces a 2-fold decrease of both K_{D2} and B_{max2} values for the low affinity sites. These findings suggest that $ONOO^-$ exerts a repressive influence on receptor-mediated GTP exchange on $G\alpha_i$ subunits possibly by affecting receptor–G_i coupling.

$ONOO^-$ targets may be represented by amino acid residues located on contact interfaces of the A_1AR and/or $G\alpha_i$ subunit. $ONOO^-$ can cause nitration of tyrosine residues as well as oxidation and nitrosylation of cysteine

Table 2
Binding parameters for [^{35}S]GTPγS to rat cortical membranes under different experimental conditions

	K_{D1} (nM)	K_{D2} (nM)	B_{max1} (pmol/mg protein)	B_{max2} (pmol/mg protein)
Control	12.78 ± 1.88	371.80 ± 116.72	12 ± 4	50 ± 10
1 μM CHA	6.01 ± 1.01*	388.50 ± 73.00	10 ± 4	64 ± 7
1 mM SIN-1	11.02 ± 2.14	598.70 ± 199.60	10 ± 2	56 ± 15
SIN-1 + CHA	7.11 ± 1.90	241.85 ± 134.85	7 ± 3	25 ± 6

K_D and B_{max} values for [^{35}S]GTPγS binding to rat cortical membranes in basal conditions (control) and after treatment with 1 mM SIN-1. All values are means ± SEM from three independent experiments performed in triplicate. *$P < 0.01$ vs. control K_{D1} value.

residues. Indeed, Miyamoto *et al*. [20] have reported that a NO donor, *S*-nitrosoglutathione (GSNO), can modulate bradykinin signaling pathways by selectively inhibiting G_i and G_q proteins. Recently, ROS including NO have been also shown to activate G_i proteins targeting $G\alpha_i$ subunits [39].

In summary, our study demonstrates that $ONOO^-$ is responsible for attenuation of A_1AR responsiveness and signaling by functional uncoupling the receptor from the G_i protein. We cannot discriminate whether the $ONOO^-$ effect is mainly exerted on the receptor and/or $G\alpha_i$ subunits. Interestingly, $ONOO^-$ causes inhibition of receptor G_i protein signaling while ROS and NO prevalently induce increased GDP/GTP exchange in G_i proteins [39] and $p21^{ras}$ [41]. Our findings may have important implications for regulation of A_1AR signaling during conditions of increased cellular release of adenosine.

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