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Inverse Agonists at A_1 Adenosine Receptors in Rat Cerebral Cortex[δ]

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ABSTRACT

According to the traditional receptor model, competitive antagonists share with agonists the ability to bind to a common site on receptors, but they are different from agonists in that they cannot trigger the biological response-*i.e.*, they lack intrinsic efficacy. Recent findings extend the model by indicating that not all antagonists display an intrinsic efficacy of zero but that some display 'inverse agonism'. In the present study we studied the inverse agonism at A_1 adenosine receptors in membranes prepared from rat cerebral cortex. Eight commercially available A_1 adenosine receptor antagonists (CGS-15943, ADPX, CPT, DPCPX, DPX, N-0840, PACPX and 8-PT) were screened for inverse agonism by measuring the extent of [^{35}S]guanosine-5'-(γ-thio) triphosphate ([^{35}S]GTPγS) binding to G proteins. The agonist-induced stimulation of [^{35}S]GTPγS bindings was completely blocked in the presence of A_1 adenosine receptor antagonists. Under optimal conditions, two types of antagonists could be distinguished. Seven antagonists including DPCPX decreased the basal [^{35}S]GTPγS binding in the absence of agonist, displaying inverse agonist activity. One (CGS-15943) had no effect on the basal bindings. N-ethylmaleimide treatment reduced the basal bindings as well as agonist-mediated stimulation of [^{35}S] GTPγS bindings, indicating that a substantial amount of this binding reflects an activated state of the G proteins. In good agreement with these findings, 0.1 mM GTP decreased the apparent affinity of the receptors for the agonist PIA, increased that for DPCPX, and had no effect on that for CGS-15943.

Key Words: Inverse agonist, A_1 Adenosine receptor, Cerebral cortex, [^{35}S]GTPγS binding

Abbreviations: CGS-15943, 9-Chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine; ADPX, 1-Allyl-3,7-dimethyl-8-phenylxanthine; CPT, 8-Cyclopentyltheophylline; DPCPX, 8-Cyclopentyl-1,3-diprophylxanthine; DPX, 1,3-Diethyl-8-phenylxanthine; N-0840, N^6-Cyclopentyl-9-methyladenine; PACPX, 1,3-Dipropyl-8-(2-amino-4-chloro-phenyl)-xanthine; 8-PT, 8-Phenyltheophylline; BSA, Bovine serum albumin; [^{35}S]GTPγS, [^{35}S]Guanosine-5, (γ-thio)triphosphate; NEM, N-ethylmaleimide; DTT, dithiothreitol

INTRODUCTION

According to the classical receptor theory, competitive antagonists share with agonists the ability to bind to a common site on receptors, but they are different from agonists in that they cannot trigger the biological responses-*i.e.*, they lack intrinsic efficacy. Recent findings extend the theory by indicating that not all antagonists display an intrinsic efficacy of zero but that some display 'inverse agonism' (Costa *et al.*, 1991; Schutz and Freissmuth, 1992; Milligan *et al.*, 1995; Leff, 1995). These antagonists called 'inverse agonists' or 'negative antagonists' may have biological effects op-

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posite to those of an agonist because of the ability to stabilize the inactive state of receptors. This was first established for the action of carbolines on the GABAa/benzodiazepine receptor complex (Braestrup *et al.*, 1982). They compete for binding at benzodiazepine receptors, but produce effects that are opposite to those of the benzodiazepines, including inhibition of GABA-induced Cl^- channel opening and promotion of seizures.

There is considerable evidence that antagonists not only bind to the receptors, but also induce a conformational change, which favors uncoupling of the receptor from G proteins. Thus, if a guanine nucleotide is added, it binds to the vacant site on G proteins, the association between receptor and G protein is weakened. In contrast, antagonists were initially thought to bind equally well to empty and G protein-coupled receptors. Several recent reports have indicated that agonist and antagonist binding to receptors are reciprocally modulated by guanine nucleotides in the muscarinic receptors (Burgisser *et al.*, 1982), the β_2-adrenoceptors (Freissmuth *et al.*, 1991; Chidiac *et al.*, 1993; Samama *et al.*, 1993) and A_1 adenosine receptor (Green, 1984).

Studies by Costa and coworkers showed some δ-opioid receptor antagonists inhibit basal GTPase activity in NG108-15 cell membranes (Costa and Herz, 1989; Costa *et al.*, 1989 and 1991). Two types of antagonists were found. One type has no intrinsic activity, since it neither stimulates nor inhibits the GTPase activity of G proteins and its apparent affinity for the receptor is not altered by pertussis toxin-mediated uncoupling of receptors and G proteins. The second type, however, can inhibit GTPase and thus shows negative intrinsic activity, and its affinity for receptors is increased following uncoupling from G proteins. In the present study we show that two types of antagonists can be distinguished for A_1 adenosine receptors in rat cerebrocortical membranes by measuring the extent of [^{35}S]GTPγS bindings to G proteins: those that lack any, and those that have some inverse agonism.

MATERIALS AND METHODS

Chemicals

[^{35}S]Guanosine 5'-(γ-thio)triphosphate (1000~1500 Ci/mmol) were obtained from DuPont NEN® (Boston, MA, U. S. A.); CGS-15943, ADPX, CPT, DPCPX, DPX, N-0840, PACPX, and 8-PT from Research Biochemicals International (Natick, MA, U. S. A.); BSA, adenosine deaminase from calf intestine, GDP, Tris-HCl and EDTA from Sigma Chemicals (St. Louis, MO, U. S. A.); GF/B glass microfiber filters from Whatman International Ltd. (Maidstone, UK). All other chemicals were of analytical grade.

Animals

Male Sprague-Dawley rats weighing 170 to 230 g, which had been acclimatized in the animal care facilities of the university for more than a week, were used in all experiments. Rats were allowed free access to food and tap water, under a light-dark cycle with the light on from 6 a.m. to 6 p.m.

Preparation of crude brain membranes

Crude brain membranes were prepared as described by Lorenzen *et al.* (1993). Briefly, rats were sacrificed by decapitation around 10 a.m. The cerebrocortical layers were dissected out and homogenized in 20 volumes of ice-cold 0.32 M sucrose buffer using a Potter-Elvejehm homogenizer. The resulting homogenates were then centrifuged at 3,500 rpm (1,000×g) for 10 min in a Sorvall RC-5B centrifuge. The supernatant was centrifuged at 18,000 rpm (20,000×g) for 30 min. The supernatant was discarded, and the pellet was washed twice in water. The final pellet was resuspended in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, treated with adenosine deaminase (0.5 units/ml) at 37℃ for 15 min, and frozen in −70℃.

Determination of [^{35}S]GTPγS bindings

[^{35}S]GTPγS bindings were determined by the method of Lorenzen *et al.* (1993). The incubation mixture contained in a total volume of 200 μl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 10 μM GDP, 1 mM DTT, 100 mM NaCl, 0.2 units/ml adenosine deaminase, 0.3~0.5 nM [^{35}S]GTPγS (about 50,000 cpm) and 0.5% BSA. The incubation was carried out for 30 min at 25℃. Incubations were terminated by rapid filtration of the samples through glass fiber filters (Whatman GF/B), followed by two 5 ml washes of the same buff-

er. After tranferring the filters into vial containing 13 ml of scintillation cocktail, the radioactivity was determined in a Beckman scintillation counter.

Treatment of membranes with NEM

Membranes at a protein concentration of approxi. 1 mg/ml were incubated with 1 mM NEM for 15 min on ice. The incubation was then terminated by adding 10 mM DTT (final concentration), and membranes were centrifuged at 14,000 RPM (20,000×g) for 30 min 4℃ in an Eppendorf centrifuge. Finally, the membranes were resuspended in 50 mM Tris-HCl (pH 7.4) for determination of [^{35}S]GTPγS binding.

Determination of protein concentrations

Protein concentrations were determined by the method of Bradford using BSA as standard (Bradford, 1976).

Data analysis

K_i values for antagonists were calculated from the dose-response curves with GraphPad Prism® (GraphPad Software Inc., San Diego, CA, U. S. A.). Comparisons between groups were carried out using the Student *t*-test.

RESULTS

Effects of various A_1 adenosine antagonists on the concentration-[^{35}S]GTPγS binding curves of PIA

Agonist-mediated activation of GTPase activity in membranes for G protein-coupled receptors including A_1 adenosine receptors can be studied by measuring the modulation of [^{35}S]GTPγS bindings to G proteins (Lorenzen *et al.*, 1993). The extent of stimulation of [^{35}S]GTPγS bindings is dependent on the presence of high concentration of Mg^{2+} (1～10 mM), GDP (10 μM), and NaCl (100 mM). Under optimal conditions, the agonist R-PIA stimulated [^{35}S]GTPγS bindings to G proteins in a concentration-dependent manner and maximal stimulation was 2 to 2.5-fold (Fig. 1). This stimulation was completely blocked in the presence of A_1 adenosine antagonists. As shown in Fig. 1, DPCPX also inhibited the basal bindings. The inhibition of the basal bindings was small (10 to 20%), but repro-

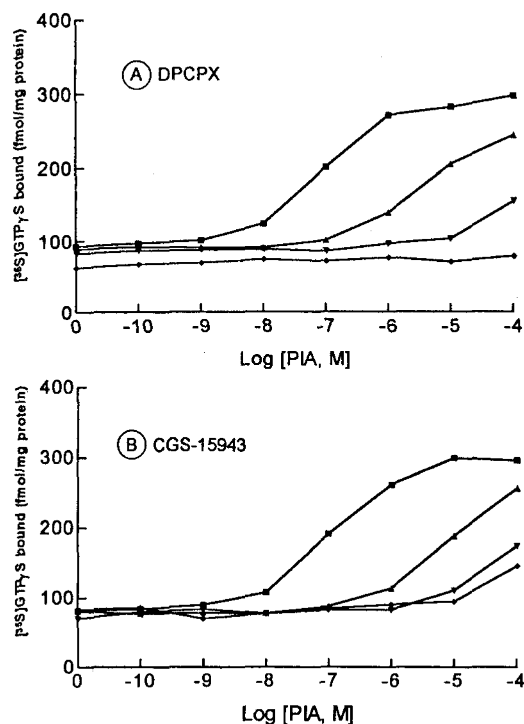

Fig. 1. Effect of DPCPX and CGS-15943 on the concentration-[^{35}S]GTPγS bindings curves of PIA in membranes prepared from rat cerebral cortex. [^{35}S]GTPγS bindings were determined in various concentrations of DPCPX(Ⓐ) and CGS-15943 (Ⓑ) (■, none; ▲, 10^{-6} M; ▼, 10^{-5} M; ◆, 10^{-4} M).

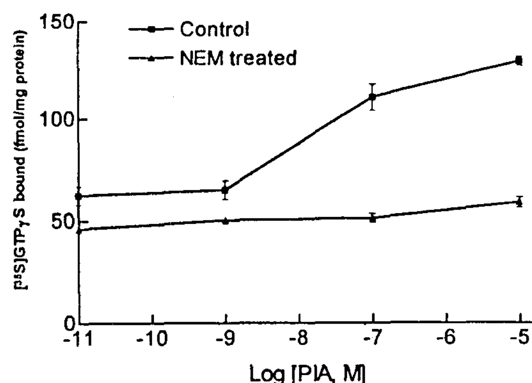

Fig. 2. Effect of NEM on the concentration-[^{35}S]GTPγS bindings curves of PIA in membranes prepared from rat cerebral cortex. [^{35}S]GTPγS bindings were determined in membranes treated with 1 mM N-ethylmaleimide.

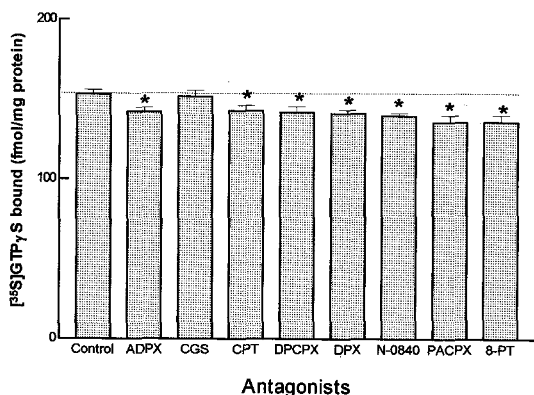

Fig. 3. Effect of various adenosine A_1 receptor antagonists on basal [^{35}S]GTPγS bindings in membranes prepared from rat cerebral cortex. [^{35}S]GTPγS bindings were determined in the presence and absence of 10^{-4} M of antagonists.

ducible and statistically significant. In contrast, CGS-15943 had no inhibitory effect on basal binding, although they completely blocked the PIA-stimulated [^{35}S]GTPγS bindings. N-ethylmaleimide treatment almost completely inhibited PIA-mediated stimulation of [^{35}S]GTPγS bindings, and the basal bindings were reduced by 26%, indicating that a substantial amount of this binding reflects an activated state of the G proteins (Fig. 2). When eight commercially available A_1 adenosine agonists were screened for the inhibition of basal bindings under these conditions, all but CGS-15943 decreased the basal [^{35}S]GTPγS bindings (Fig. 3).

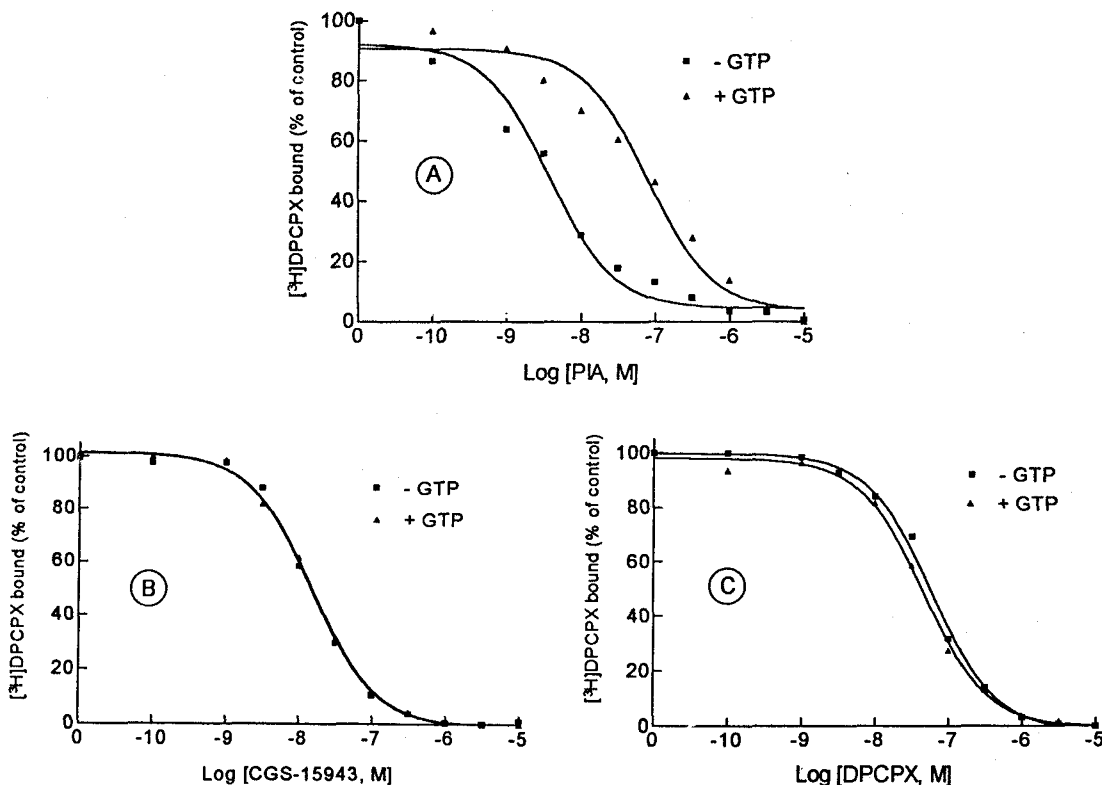

Fig. 4. Effect of GTP on the binding isotherms of PIA, CGS-15943 and DPCPX for A_1 adenosine receptors in membranes prepared from rat cerebral cortex. Bindings of PIA (Ⓐ), CGS-15943 (Ⓑ) and DPCPX(Ⓒ) were measured as competition for the binding sites labeled by [^{3}H]DPCPX (approxi. 0.4 nM) in the presence(▲) or absence (■) of 0.1 mM GTP.

Effects of GTP on binding isotherms of PIA, CGS-15943 and DPCPX for A_1 adenosine receptors

Antagonists which neither promote nor oppose the formation of the ternary complex between agonist-occupied receptor and G proteins do not discriminate between high-and low-affinity forms of receptors, and their affinities are not expected to be changed by GTP. However, if an antagonist had greater affinity for the uncoupled form of the receptor than the coupled form, its apparent binding would be increased by the presence of GTP. We studied the effect of GTP on the binding isotherms of PIA, CGS-15943 and DPCPX for the competition for the A_1 adenosine binding sites labeled [^{3}H]DPCPX. As shown in Fig. 4, GTP (0.1 mM) decreased the apparent affinity of the receptor for the agonist PIA, increased that for DPCPX, and had no effect on that for CGS-15943.

DISCUSSION

The defining property of inverse agonists is their ability to inhibit the spontaneous activity of receptors (Costa *et al.*, 1991; Schutz and Freissmuth, 1992; Milligan *et al.*, 1995; Leff, 1995). *In vivo,* however, this hormone-independent activity is masked by the activity stimulated by low levels of hormones. Thus, the blockade of a tonic hormonal activation of the receptor by neutral antagonists might mimic inverse agonists. Therefore, identification of inverse agonists requires careful control of the experimental system *in vitro* (Chidiac *et al.*, 1991; Samama *et al.*, 1993).

The results of the present study show that antagonists for A_1 adenosine receptors can display a spectrum of intrinsic efficacy that ranges from null to negative values, as determined by their ability to inhibit basal [^{35}S]GTPγS bindings to rat cerebrocortical membranes. One potential coveat in studying the effects of inverse agonist is that contaminating agonist, in this case adenosine, may have been present in membranes or generated during incubation. However, several observations in this study indicate that the inhibition of [^{35}S]GTPγS bindings by the inverse agonists are independent of the possible influence of such contaminants.

1) It is estimated that the endogenous adenosine were diluted at least 10^5-fold during the routine preparation of washed membranes.

2) At the preparation step the membranes were pretreated with 0.5 unit/ml of ade-nosine deaminase at 37℃ for 15 min, and 0.2 units/ml of adenosine deaminase was also present in the assay mixture for the determination of [^{35}S]GTPγS bindings.

3) Treatment of membranes with NEM reduced the basal [^{35}S]GTPγS bindings by 26%, indicating a substantial amount of this binding reflects an activated state of the G proteins. Furthermore, the effects of these inverse agonists were abolished in parallel to that of the agonist when loss of receptor responsiveness was caused by alkylation by NEM (Lorenzen *et al.*, 1993).

The degree of inverse agonist activity for [^{35}S] GTPγS bindings is correlated with the ability of the antagonists to discriminate between the activated and inactive states of receptors, when determined by competition of binding sites labeled [^{3}H]DPCPX in the presence and absence of 0.1 mM GTP. These data support the two-state model, which predict that inverse agonists may be active by preferentially stabilizing the inactive state of receptors (Leff, 1995). The existence of antagonists with negative intrinsic efficacy is likely to be a general phenomenon for most, if not all, G protein-coupled receptors. Guanine nucleotides-mediated effects on antagonist binding have been reported for the muscarinic receptors (Burgisser *et al.*, 1982), the β_2-adrenoceptors (Freissmuth *et al.*, 1991) and A_1 adenosine receptor (Green, 1984).

In the present study the extents of inhibition of [^{35}S]GTPγS bindings by these inverse agonists were small but reproducible. In order to further characterize the inverse agonist properties of these antagonists, detailed studies are needed to find out the optimal conditions by maximizing the extent of inhibition in the rat cerebral cortex.

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=국문초록=

흰쥐의 뇌의 A_1 아데노신 수용체에 작용하는 역효현제에 관한 연구

연세대학교 원주의과대학 약리학교실과 기초의학 연구소[1] 및 연세대학교 의과대학 약리학교실[2]

박 경 선[1]·양 완 숙[1]·김 경 환[2]

전통적인 수용체 이론에 따르면 상경적 길항제는 효현제와 수용체의 같은 부위에 작용하지만, 효능(efficacy)이 없기 때문에 생물학적 반응을 일으키지는 않는다. 그러나 최근에 발표되는 자료들에 따르면 모든 길항체의 효능(efficacy)이 0가 아니라 음수도 될 수가 있다고 생각된다. 이러한 음수의 효능을 갖는 약물을 역효현제라 부른다. 본 연구에서는 쥐의 cerebral cortex에서 얻은 membranes을 사용하여, A_1 아데노신 수용체에 작용하는 역효현제를 연구하였다. 8개의 길항제로 알려진 약물들이 G단백에 대한 $[^{35}S]GTP\gamma S$ 결합을 감소시키는 정도를 측정함으로써 역효현제의 특성을 검색하였다. 효현제에 의한 $[^{35}S]GTP\gamma S$ 결합의 증가는 이들 길항제들에 의해 완전히 억제되었지만, 검색한 8개의 길항제는 두 군으로 구분되었다. DPCPX를 포함한 7개 길항제는 효현제 부재시의 basal $[^{35}S]GTP\gamma S$ binding을 통계적으로 의의있게 감소시켜 역효현제의 특성을 나타내는 반면, CGS-15943은 basal $[^{35}S]GTP\gamma S$ binding에 아무런 영향을 주지 않았다. NEM을 membranes에 처치하면 PIA에 의한 $[^{35}S]GTP\gamma S$ binding이나 basal binding 둘다 감소하는데 이는 $[^{35}S]GTP\gamma S$ binding의 상당부분이 G단백의 activated state를 나타내는 것을 알 수 있다. 또한 $[^{3}H]DPCPX$를 이용한 competitive binding assay에서 0.1 mM GTP는 효현제인 PIA의 apparent affinity를 감소시켰으며, DPCPX의 apparent affinity는 증가시키고, CGS-15943에는 아무런 영향을 미치지 않았다. 이것은 상기의 $[^{35}S]GTP\gamma S$ bindings의 결과를 뒤받침해 주는 결과라고 생각된다.