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A $G\alpha_s$ Carboxyl-Terminal Peptide Prevents G_s Activation by the A_{2A} Adenosine Receptor

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ABSTRACT

The molecular mechanisms of interaction between G_s and the A_{2A} adenosine receptor were investigated using synthetic peptides corresponding to various segments of the $G\alpha_s$ carboxyl terminus. Synthetic peptides were tested for their ability to modulate binding of a selective radiolabeled agonist, [^{3}H]2-[4-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS21680), to A_{2A} adenosine receptors in rat striatal membranes. The $G\alpha_s$ peptides stimulated specific binding both in the presence and absence of 100 μM guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS). Three peptides, $G\alpha_s$(378–394)C^{379}A, $G\alpha_s$(376–394)C^{379}A, and $G\alpha_s$(374–394)C^{379}A, were the most effective. In the presence of GTPγS, peptide $G\alpha_s$(374–394)C^{379}A increased specific binding in a dose-dependent fashion. However, the peptide did not stabilize the high-affinity state of the A_{2A} adenosine receptor for [^{3}H]CGS21680. Binding assays with a radiolabeled selective antagonist, [^{3}H]5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine ([^{3}H]SCH58261), showed that the addition of the $G\alpha_s$ peptide modified the slope of the 5′-*N*-ethylcarboxamidoadenosine (NECA) competition curve, suggesting modulation of receptor affinity states. In the presence of GTPγS, the displacement curve was right-shifted, whereas the addition of $G\alpha_s$(374–394)C^{379}A caused a partial left-shift. Both curves were fitted by one-site models. This same $G\alpha_s$ peptide was also able to disrupt G_s-coupled signal transduction as indicated by inhibition of the A_{2A} receptor-stimulated adenylyl cyclase activity without affecting either basal or forskolin-stimulated enzymatic activity in the same membrane preparations. Shorter peptides from $G\alpha_s$ and $G\alpha_{i1/2}$ carboxyl termini were not effective. NMR spectroscopy showed the strong propensity of peptide $G\alpha_s$(374–394)C^{379}A to assume a compact carboxyl-terminal α-helical conformation in solution. Overall, our results point out the conformation requirement of $G\alpha_s$ carboxyl-terminal peptides to modulate agonist binding to rat A_{2A} adenosine receptors and disrupt signal transduction.

A large family of cell surface receptors, conforming to the heptahelical structure, elicit their physiological effects by first coupling to and activating a population of heterotrimeric GTP-binding proteins (G proteins), which then mediate the responses of a plethora of cellular effectors, including enzymes and ion channels. Heterotrimeric G proteins are composed of α-, β-, and γ-subunits and are classified by virtue of their α-subunit. In the resting state, the Gα-GDP subunit forms a high-affinity complex with the G$\beta\gamma$ heterodimer. Agonist binding to the receptor leads to conformational changes that promote a tighter interaction with specific heterotrimeric G proteins, catalysis of GDP release, and subsequent G protein activation. In the absence of guanine nucleotides, GDP or GTP, agonist binding to the receptor is stabilized by the bound G protein.

The structural basis of receptor-G protein interaction is an active area of study. The heterotrimeric G protein, rather than just the Gα or G$\beta\gamma$ subunit, is required for interaction with the receptor, but studies pointing out the importance of specific regions have been mainly focused to the Gα subunit

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ABBREVIATIONS: Gα and G$\beta\gamma$, the α and $\beta\gamma$ subunits of heterotrimeric G proteins; G_s, a G protein linked with the activation of adenylyl cyclase; $G\alpha_s$, the α subunit of G_s; $G\alpha_i$, the α subunit of a G protein (G_i) linked with the inhibition of adenylyl cyclase; $G\alpha_t$, the α subunit of the G protein (G_t or transducin) present in rod outer segments; $G\alpha_s$(374–394)C^{379}A, a synthetic peptide corresponding to those residues of $G\alpha_s$ with a cysteine substituted by an alanine (a Gα subunit followed by numbers refers to the corresponding peptide); GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); CGS21680, 2-[4-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; SCH58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HFA, hexafluoroacetone trihydrate; FSK, forskolin; DQF-COSY, double-quantum filter correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser spectroscopy; EM, energy minimization; RMS, root mean square.

(Hamm et al., 1988; Conklin et al., 1993; Rasenick et al., 1994; Lee et al., 1995; Mazzoni and Hamm, 1996; Bae et al., 1997). The most clearly defined contact site with the receptor includes the last 11 carboxyl-terminal amino acids of Gα subunits (Hamm et al., 1988; Conklin et al., 1993; Dratz et al., 1993; Gilchrist et al., 1998). However, there are also numerous evidences for the participation of other Gα regions (Hamm et al., 1988; Rasenick et al., 1994; Lee et al., 1995; Mazzoni and Hamm, 1996; Bae et al., 1997) as well as the Gβγ subunit (Taylor et al., 1996; Yasuda et al., 1996) in receptor interaction. Thus, the possibility exists that the molecular determinants of receptor-G protein coupling vary somewhat among specific subfamilies of receptors and G proteins.

The A_{2A} adenosine receptor belongs to the family of G protein-coupled receptors and activates adenylyl cyclase via coupling to G_s proteins (for review, see Palmer and Stiles, 1995; Ongini and Fredholm, 1996). This receptor is distributed in rat and human brain as well as in several peripheral tissues (Ongini and Fredholm, 1996). In brain, A_{2A} adenosine receptors are widely represented in the striatum where they are involved in dopaminergic pathways (Ongini and Fredholm, 1996) and elicit locomotor depression (Richardson et al., 1997). For this reason, new therapeutic strategies based on blockade of A_{2A} adenosine receptors are under investigation to treat Parkinson's disease (Richardson et al., 1997). It is now evident that striatopallidal neurons express both A_{2A} adenosine and D_2 dopamine receptors and these receptors act in an antagonistic manner (Ongini and Fredholm, 1996).

The A_{2A} adenosine receptor shows a partial insensitivity to modulation of agonist binding by guanine nucleotides (Nanoff et al., 1991; Mazzoni et al., 1993; Luthin et al., 1995). In a previous study on rat striatal membranes, we found that 100 μM GTPγS inhibits specific binding of a radiolabeled A_{2A}-selective agonist, [^{3}H]CGS21680, by only 60% and the inhibition depends on the presence of $MgCl_2$ (Mazzoni et al., 1993). Luthin et al. (1995) have reported that only a small fraction of A_{2A} adenosine receptors in rat striatal membranes are coupled to G proteins, suggesting a limited coupling exists between these receptors and G_s. A recent study (Gilchrist et al., 1998) has shown that the A_1 adenosine receptor G_i protein interface presents some peculiar aspects compared with other G protein-coupled receptors (Hamm et al., 1988; Rasenick et al., 1994). Thus, the molecular mechanisms involved in the interaction between A_{2A} adenosine receptors and G_s proteins are of great interest to define the G protein-coupling properties of adenosine receptors.

In this study, we have examined the ability of synthetic peptides corresponding to selected regions of the $G\alpha_s$ carboxyl terminus to affect agonist binding to A_{2A} adenosine receptors and to disrupt the receptor-mediated activation of G_s. Progressively longer segments of the $G\alpha_s$ carboxyl terminus were synthesized and tested for their effects on specific binding of [^{3}H]CGS21680 to rat striatal membranes. These peptides stimulated specific agonist binding. A 21-residue peptide also inhibited receptor-stimulated adenylyl cyclase activity, but it was not able to stabilize the receptor high-affinity state for agonist ligands. However, this $G\alpha_s$ peptide, which showed a marked propensity to form an α-helical structure in solution, influences the affinity state of the A_{2A} adenosine receptor. Our findings suggest that the carboxyl-terminal region of $G\alpha_s$ takes part in the formation of a complex binding site for the A_{2A} adenosine receptor.

Experimental Procedures

Materials. [^{3}H]CGS21680 (39.5 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA), and [α-^{32}P]ATP (30 Ci/mmol) and [^{3}H]cAMP (25 Ci/mmol) were from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). [^{3}H]SCH58261 (77 Ci/mmol) and SCH58261 were generous gifts of Dr. E. Ongini (Schering-Plough Research Institute, Milan, Italy). Myokinase, creatine kinase, leupeptin, GTP, and GTPγS were obtained from Roche Molecular Biochemicals (Mannheim, Germany). CGS21680 was from Research Biochemicals International (Natick, MA). Bacitracin, benzamidine, and phenylmethylsulfonyl fluoride (PMSF) were products of Fluka Chemie AG (Buchs, Switzerland). Adenosine deaminase, papaverine, and 5′-*N*-ethylcarboxamidoadenosine (NECA) were from Sigma Chemical Co. (St. Louis, MO). All other reagents were from standard commercial sources and of the highest grade available.

Membrane Preparation. Male Sprague-Dawley rats (150–200 g) were sacrificed by cervical dislocation, and striatal tissue was isolated from the brain by dissection. For radioligand binding assays, membranes were prepared essentially as described previously (Mazzoni et al., 1993). Membrane protein concentration was determined by the method of Lowry et al. (1951) using BSA as a standard. For the adenylyl cyclase assay, striatal tissue was suspended in 10 volumes of ice-cold buffer containing 10 mM HEPES/NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol (DTT) (buffer A) and homogenized with 12 strokes of a tight Teflon-glass homogenizer at 4°C. The homogenate was diluted 6-fold with buffer A and centrifuged at 46,000*g* for 20 min at 4°C. The pellet was resuspended in 10 volumes of ice-cold buffer A containing 1 mM benzamidine, 200 μg/ml bacitracin, and 2 U/ml adenosine deaminase, and incubated for 30 min at 30°C. The membrane suspension was centrifuged at 46,000*g* for 20 min at 4°C. The pellet was resuspended in 10 volumes of ice-cold buffer A containing protease inhibitors (as above) and centrifuged at 46,000*g* for 20 min at 4°C. This washing step was repeated. The final pellet was resuspended in 50 mM HEPES/NaOH, pH 7.5 (buffer B) and used immediately for the adenylyl cyclase assay. Protein concentration was determined using the Coomassie Blue binding method (Bradford, 1976) using BSA as a standard.

Peptide Synthesis. Peptides were synthesized by the continuous-flow solid phase method using Fmoc chemistry on an automatic synthesizer (Milligen 9050; Millipore, Bedford, MA). Crude peptides were purified by reversed HPLC on a preparative Vydac C_{18} column (2.2 × 25 cm) (Beckman System Gold, San Ramon, CA) using a 15 to 30% gradient of acetonitrile in 0.1% trifluoroacetic acid/distilled water (v/v). After lyophilization, purity was checked by analytical HPLC and electrospray mass spectrometry using a mass spectrometer (VG Quattro; Micromass, Altricham, UK) equipped with a standard Electrospray ion source. Molecular weight calculations were performed by deconvolution using MassLynx software, version 2.00 (Micromass). Table 1 shows the sequence of all peptides used in this study.

The $G\alpha_s$ and $G\alpha_{i1/2}$ peptides were dissolved in buffer C (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA) for radioligand binding assays or in buffer B for the adenylyl cyclase assay. In the assay mixtures, the pH ranged between 7.3 and 7.5. Peptide stock solutions were 2.5 mM. All peptide stock solutions were centrifuged at 11,000*g* for 4 min at room temperature, and supernatants were collected. The concentrations of the $G\alpha_s$ peptides in solutions were determined spectrophotometrically using the molar extinction coefficient for tyrosine at 280 nm (ϵ_{280nm} = 1400 M^{-1} cm^{-1}) as described by Rarick et al. (1994).

As we might face solubility problems with some peptides or degradation by tissue proteases, we performed either HPLC titration studies and spectrophotometric measurements to verify whether the

final concentration of the peptide in the test solution was affected by precipitation, aggregation, nonspecific adsorption to striatal membranes, or proteolysis. Accordingly, $G\alpha_s(374–394)C^{379}A$ (300 μM) was incubated in 0.5 ml of buffer C in the presence and absence of striatal membranes (~130 μg of proteins) for 10, 30, 60, and 120 min at 25 and 30°C. Samples were centrifuged at 11,000*g* for 4 min at room temperature, and supernatants were collected. As a control, the peptide was also incubated in buffer C in the absence of membranes without any final centrifugation. The concentrations of $G\alpha_s(374–394)C^{379}A$ in supernatant and control samples were compared by analytical HPLC or spectrophotometric measurement at 280 nm using the conditions described above. The peak areas or absorbances of supernatant and control samples, each assayed in triplicate, were found to vary within the range of ±5–10%, i.e., within the accuracy range of the methods.

Radioligand Binding Assays. Routine [^{3}H]CGS21680 binding assays were performed as described previously (Mazzoni et al., 1993). Striatal membranes (~130 μg of proteins) were incubated with 3 nM [^{3}H]CGS21680 in buffer C containing protease inhibitors (1 mM benzamidine, 100 μM PMSF, and 10 μM leupeptin) and adenosine deaminase (1 U/ml) in the presence and absence of $G\alpha_i(344–354)$ or $G\alpha_s$ peptides (~300 μM) for 2 h at 25°C. Equivalent experiments were performed in the presence of 100 μM GTPγS. Binding was terminated by vacuum filtration over Whatman GF/C glass fiber filters (Whatman Int. Ltd, Springfield Mill, Maidstone, UK), with five washes (3 ml each) of ice-cold buffer C. Nonspecific binding was determined in the presence of 100 μM NECA. In the presence and absence of GTPγS, specific binding represented 70% and 90% of total binding, respectively.

The dose-dependent effect of peptide $G\alpha_s(374–394)C^{379}A$ was evaluated by incubating membranes (~110 μg of proteins) in buffer C with 3.6 nM [^{3}H]CGS21680 in the presence of 100 μM GTPγS and seven different concentrations of the peptide ranging from 0 to 480 μM.

For saturation studies, membranes (~75 μg of proteins) were incubated in buffer C containing protease inhibitors (as above) with eight different concentrations of [^{3}H]CGS21680 (0.5–108 nM) in the presence or absence of $G\alpha_s(374–394)C^{379}A$ (350 μM). When experiments were carried out in the presence of GTPγS, the concentrations of [^{3}H]CGS21680 ranged between 0.5 and 250 nM.

[^{3}H]SCH58261 binding assays were performed essentially as described by Zocchi et al. (1996) with some modifications. Striatal membranes (~70 μg of proteins) were incubated with 0.2 nM [^{3}H]SCH58261 in buffer C containing protease inhibitors and adenosine deaminase (as above) for 1 h at 25°C. The binding reaction was terminated by vacuum filtration over Whatman GF/C glass fiber filters (Whatman Int. Ltd.) with three washes (5 ml each) of ice-cold buffer C. Nonspecific binding was determined in the presence of 100 μM NECA. Specific binding represented 90% of total binding.

To determine equilibrium binding parameters, membranes were incubated with seven different concentrations of [^{3}H]SCH58261 ranging from 0.03 to 5 nM. Saturation curves were fitted using the nonlinear regression equation of the GraphPad Prism program (GraphPad Software, San Diego, CA). The average values (n = 2) of dissociation constant (K_D) and maximal number of binding sites (B_{max}) were 0.6 nM and 750 fmol/mg of proteins, respectively.

Displacement experiments were performed by incubating membranes and [^{3}H]SCH58261 (0.2 nM) in the presence and absence of different concentrations of either SCH58261 (0.025–5 nM) or NECA (0.5–1000 nM). Control assays were carried out with and without GTPγS (100 μM), whereas the effect of $G\alpha_s(374–394)C^{379}A$ (300 μM) was evaluated in the presence and absence of the guanine nucleotide.

Adenylyl Cyclase Assay. Adenylyl cyclase activity was assayed by monitoring the conversion of [α-^{32}P]ATP to [α-^{32}P]cAMP, using a previously reported method (Johnson et al., 1994). The method involved addition of [α-^{32}P]ATP to membranes in the presence of an A_{2A} adenosine receptor agonist (NECA or CGS21680) and GTP or forskolin (FSK) to stimulate adenylyl cyclase and papaverine as a phosphodiesterase inhibitor. Briefly, to study G_s-mediated adenylyl cyclase activation, the enzymatic activity was routinely assayed in a 100-μl reaction mixture containing 50 mM HEPES/NaOH buffer, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 0.1 mg/ml creatine phosphokinase, 0.1 mg/ml bacitracin, 0.5 mg/ml creatine phosphate, 0.1 mM ATP, 0.05 mM cAMP, 2 U/ml adenosine deaminase, 0.9 μCi of [α-^{32}P]ATP, and 0.1 mM papaverine. The concentration of GTP and A_{2A} adenosine receptor agonists were 100 and 10 μM, respectively. Gα peptides were used at a concentration of 300 μM. The incubation was started by the addition of membranes (~25 μg of proteins) and was carried out at 30°C. To study FSK-mediated activation of adenylyl cyclase, membranes (~10 μg of proteins) were incubated at 23°C in 100 μl of medium containing the same buffer and reagents as above except that EGTA was omitted and $MgCl_2$ was 1 mM. The reactions were stopped after 10 min by placing assay tubes into an ice bath and adding 0.6 ml of a cold stop solution containing 120 mM $Zn(C_2H_3O_2)_2$/[^{3}H]cAMP (~12,000 cpm/sample) and then 0.5 ml of 144 mM Na_2CO_3. The total radiolabeled cAMP was isolated on columns of Dowex 50 ion-exchange resin and alumina as described previously (Johnson et al., 1994).

Data Analysis. A nonlinear multipurpose curve-fitting computer program (EBDA/LIGAND; Elsevier-Biosoft, Cambridge, UK) was used for analysis of saturation data. A partial F test was utilized to determine whether the binding data were best fitted by a one- or two-site model. Saturation curves were also fitted by a nonlinear regression analysis of the GraphPad Prism version 3.0 program (GraphPad Software). Data from concentration-response or displacement curves were analyzed by a least-squares curve-fitting computer program (GraphPad Prism, version 3.0), 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 (Cheng and Prusoff, 1973). Values represent the mean ± S.E. of at least three experiments except when otherwise stated. The statistical significance of the differences between means was determined by Student's t test using GraphPad Prism 3.0.

NMR and Structure Calculations. The sample for NMR spectroscopy was prepared by dissolving the appropriate amount of $G\alpha_s(374–394)C^{379}A$ in 0.5 μl of ^1H_2O phosphate buffer (pH 6.6) to obtain a 1 mM solution. The sample was lyophilized and redissolved

TABLE 1

Amino acid sequence of Gα synthetic peptides

The sequences were taken from Jones and Reed (1987). Peptides were synthesized and purified as described under *Experimental Procedures*. The identity of each peptide was monitored by electrospray mass spectroscopy.

Peptide	Amino Acid Sequence
$G\alpha_s(384–394)$	H_2N-Q R M H L R Q Y E L L-COOH
$G\alpha_s(382–394)$	H_2N-I I Q R M H L R Q Y E L L-COOH
$G\alpha_s(380–394)$	H_2N-R D I I Q R M H L R Q Y E L L-COOH
$G\alpha_s(378–394)C^{379}A$	H_2N-D **A** R D I I Q R M H L R Q Y E L L-COOH[a]
$G\alpha_s(376–394)C^{379}A$	H_2N-F N D **A** R D I I Q R M H L R Q Y E L L-COOH[a]
$G\alpha_s(374–394)C^{379}A$	H_2N-R V F N D **A** R D I I Q R M H L R Q Y E L L-COOH[a]
$G\alpha_{i1/2}(344–354)$	H_2N-I K N N L K D C G L F-COOH

[a] The bold letter indicates a Cys replaced by an Ala residue.

in aqueous solution with 50% (v/v) hexafluoroacetone trihydrate (HFA).

NMR spectra were recorded on a spectrometer (DRX-600; Bruker Instruments Inc., Billerica, MA). One-dimensional NMR spectra were recorded in the Fourier mode with quadrature detection, and the water signal was suppressed by a low-power-selective irradiation in the homogated mode. Double quantum filter correlation spectroscopy (DQF-COSY) (Piantini et al., 1982), total correlation spectroscopy (TOCSY) (Bax and Davis, 1985), and nuclear Overhauser spectroscopy (NOESY) (Jenner et al., 1979) experiments were run in the phase-sensitive mode using quadrature detection in ω_1 by time-proportional phase increase of initial pulse (Marion and Wüthrich, 1983). Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values. Before Fourier transformation, the time domain data matrices were multiplied by shifted $\sin^2$ functions in both dimensions. A mixing time of 70 ms was used for the TOCSY experiments. NOESY experiments were run at 275 K with mixing times in the range of 100 to 300 ms. The identification of spin systems in the DQF-COSY and TOCSY spectra, assignments of intra- and interresidual NOESY cross peaks, and their integration were obtained using the interactive program package XEASY (Bartels et al., 1995). The necessary pseudoatom corrections were applied for equivalent and nonstereospecifically assigned protons. Conversion of NOESY cross peak volumes into upper distance bounds was obtained using the CALIBA program incorporated into the program package DYANA (Guntert et al., 1997). Models of the $G\alpha_s$ peptide were generated with the structure calculation algorithm of DYANA (Guntert et al., 1997).

The conformer with the lowest value of the target function was subjected to a restrained energy minimization (EM) using the Amber-all force field (Weiner et al., 1984) with neglect of electrostatics available within the SYBYL software package (version 6.2; TRIPOS Inc., St. Louis, MO). A root mean square (RMS) derivative of 0.5 kcal mol^{-1} $Å^{-1}$ was adopted as a convergence criterion. The resulting conformation was fully relaxed through unrestrained EM (RMS deviation about nonhydrogen atoms ≅ 0.1 Å).

Results

Synthetic peptides of various length from the $G\alpha_s$ carboxyl terminus were used as probes of contact regions between the A_{2A} adenosine receptor and G_s. For other receptor-G protein systems, peptides corresponding to the last 11 residues of the $G\alpha$ carboxyl terminus can serve as inhibitors of signal transduction (Hamm et al., 1988; Rasenick et al., 1994; Gilchrist et al., 1998) and stabilize the high-affinity state of the receptor (Hamm et al., 1988). However, the ability of these peptides to stabilize receptor affinity for agonists is not observed for all receptor G protein systems (Gilchrist et al., 1998). We examined the importance of $G\alpha_s$ peptide size in relation to its competence to affect A_{2A} receptor-G_s protein interaction. Table 1 shows the amino acid sequences of the synthetic peptides used in this study. In $G\alpha_s$(378–394), $G\alpha_s$(376–394), and $G\alpha_s$(374–394), Cys^{379} was substituted with Ala to prevent peptide dimerization.

Effects of $G\alpha_s$ Carboxyl-Terminal Peptides on Agonist Binding to A_{2A} Adenosine Receptors. $G\alpha_s$ carboxyl-terminal peptides stimulated specific binding of [^{3}H]CGS21680 to A_{2A} adenosine receptors in rat striatal membranes (Fig. 1), whereas nonspecific binding was not altered. Both in the presence (Fig. 1b) and absence (Fig. 1a) of GTPγS, all peptides tested caused an evident increase of specific binding as compared with respective controls. However, in the presence of the GTP analog, this effect was generally more evident (Fig. 1b). The most effective peptides were $G\alpha_s$(378–394)C^{379}A, $G\alpha_s$(376–394)C^{379}A, and $G\alpha_s$(374–394)C^{379}A, whereas the shortest peptide, $G\alpha_s$(384–394), was less active. In accordance to the crystal structure of $G\alpha_s$ (Sunahara et al., 1997), the bioactive conformation of its carboxyl-terminal portion is an α-helix (α5) spanning from Asp^{368} to Leu^{394}. Thus, 17-, 19-, and 21-residue peptides may have a stronger propensity to assume an α-helical conformation than do the shortest $G\alpha_s$ peptides.

We also investigated whether a $G\alpha_{i1/2}$ carboxyl-terminal peptide, $G\alpha_{i1/2}$(344–354), modulated agonist binding to A_{2A} adenosine receptors in rat striatal membranes. The $G\alpha_{i1/2}$ peptide had no significant effect in the absence of the guanine nucleotide (Fig. 1a), but it was as effective as $G\alpha_s$(384–394), $G\alpha_s$(382–394), and $G\alpha_s$(380–394) in the presence of GTPγS (Fig. 1b).

The dose dependence of agonist binding stimulation was

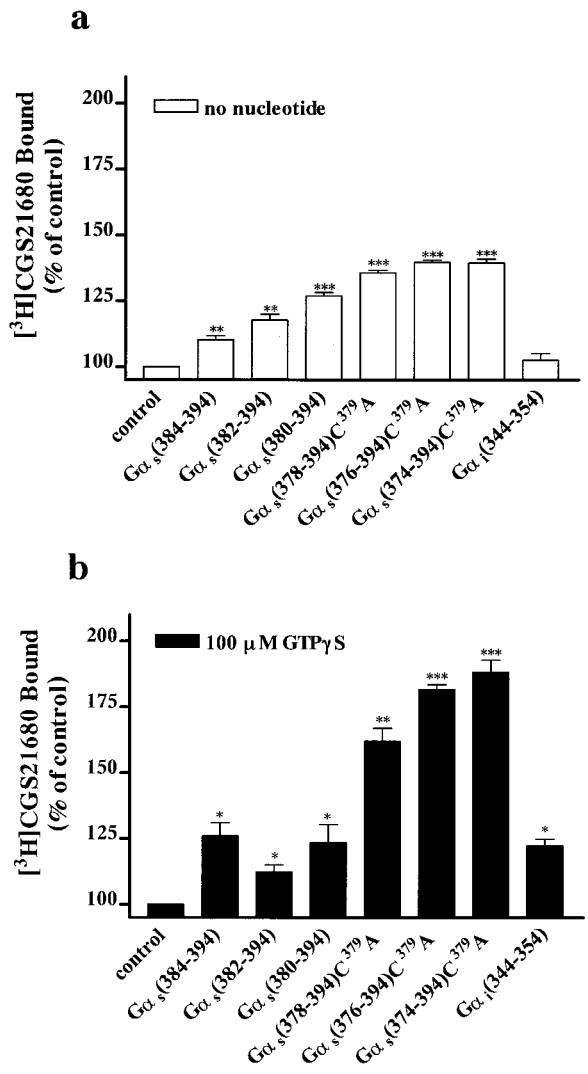

Fig. 1. Effects of $G\alpha_s$ synthetic peptides on [^{3}H]CGS21680 binding to rat striatal membranes. Membranes (130 μg of proteins) and [^{3}H]CGS21680 (3 nM) were incubated in 0.5 ml of buffer C containing protease inhibitors and adenosine deaminase in the presence and absence of $G\alpha_s$ or $G\alpha_{i1/2}$ peptides (300 μM) as described under *Experimental Procedures*. Peptide activity was tested in the presence (b) and absence (a) of 100 μM GTPγS. Control in the presence and absence of the guanine nucleotide was 39 ± 2 and 154 ± 6 fmol/mg of protein, respectively. Values are mean ± S.E. of four independent experiments, each performed in duplicate. Values that are significantly different from control values, as determined by paired Student's *t* test, are indicated (***$P < .001$; **$P < .01$; *$P < .05$).

examined for the most active peptide, $G\alpha_s(374–394)C^{379}A$. The effects of various concentrations of the peptide on [^{3}H]CGS21680 binding to rat striatal membranes were measured in the presence of 100 μM GTPγS. The dose-response curve is shown in Fig. 2. The derived EC_{50} value for stimulation of specific binding was 14.9 ± 1.6 μM (n = 3), whereas the maximal efficacy was 250 ± 5% of control at a concentration of 480 μM.

Effects of $G\alpha_s(374–394)C^{379}A$ on [^{3}H]CGS21680 Binding Parameters to A_{2A} Adenosine Receptors. To evaluate whether peptide $G\alpha_s(374–394)C^{379}A$ was able to stabilize the high-affinity state of the A_{2A} adenosine receptor and thus to mimic G_s, saturation binding studies were carried out in the presence and absence of a fixed concentration of the peptide. Analysis of data using nonlinear, curve-fitting programs revealed that both saturation curves were better represented by one-site models (Fig. 3a). In addition, Scatchard transformation of these data produced linear plots (Fig. 3b). This suggested the existence of a single class of binding sites in both conditions at the concentrations of ligand used. In the absence of the $G\alpha_s$ peptide, the K_D and B_{max} values were 10.3 ± 0.3 nM and 611 ± 77 fmol/mg of protein (n = 3), whereas in the presence of peptide $G\alpha_s(374–394)C^{379}A$ the K_D and B_{max} values were 9.1 ± 0.02 nM and 897 ± 4 fmol/mg of protein (n = 3), respectively. The addition of the $G\alpha_s$ peptide caused an increase of the maximal number of binding sites ($P < .05$) without significantly affecting the receptor affinity state.

In the presence of 100 μM GTPγS, $G\alpha_s(374–394)C^{379}A$ was responsible for a 2- to 3-fold increase of agonist binding affinity as compared with its control (K_D, 79.2 nM; n = 2), whereas the maximal number of binding sites showed a decrease (data not shown). However, the $G\alpha_s$ peptide was not able to shift back the receptor in a high-affinity state for agonist ligands.

Influence of $G\alpha_s(374–394)C^{379}A$ on Displacement of [^{3}H]SCH58261 Binding by NECA. To detect receptor affinity states and test the hypothesis that the 21-residue $G\alpha_s$ peptide stabilized an intermediate affinity state of rat A_{2A} adenosine receptors, we examined the effect of the peptide on the ability of an agonist, NECA, to compete specific binding of an A_{2A}-selective antagonist, [^{3}H]SCH58261, in the presence and absence of GTPγS.

In the absence of the guanine nucleotide, the NECA displacement curve was shallow and better represented by a two-site rather than one-site model ($P < .01$), indicating the presence of two affinity states of the receptor (Fig. 4a). The Hill coefficient was 0.7 ± 0.07 (n = 4) (i.e., significantly different from unity, $P < .005$). The K_i value for the high-affinity sites was 4.7 ± 0.8 nM, representing 40% of total binding sites, whereas the K_i value for the low affinity sites was 40.8 ± 1.7 nM. In the presence of 100 μM GTPγS, the

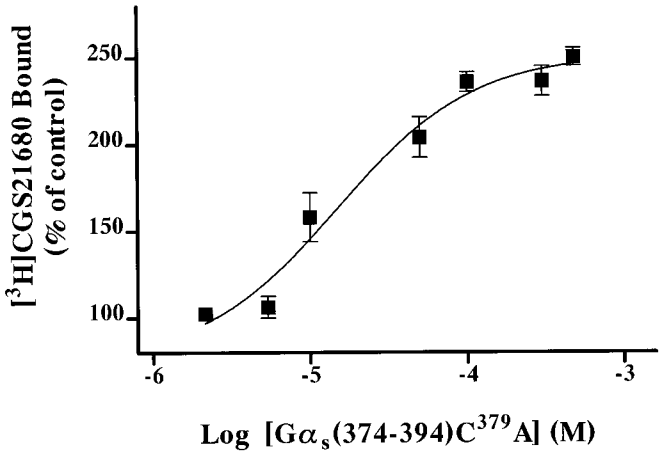

Fig. 2. Dose-dependent effect of peptide $G\alpha_s(374–394)C^{379}A$ on [^{3}H]CGS21680 binding to rat striatal membranes. Membranes (110 μg of proteins) were incubated with 3.6 nM [^{3}H]CGS21680 and various concentrations of $G\alpha_s(374–394)C^{379}A$ in the presence of 100 μM GTPγS as described under *Experimental Procedures*. Control was 31 ± 3 fmol/mg of protein. The EC_{50} value for stimulation of specific binding was 14.9 ± 1.6 μM. Values are mean ± S.E. of three independent experiments, each performed in duplicate. The EC_{50} value was determined by fitting the data as a sigmoidal dose-response curve with the GraphPad Prism version 3.0 computer program.

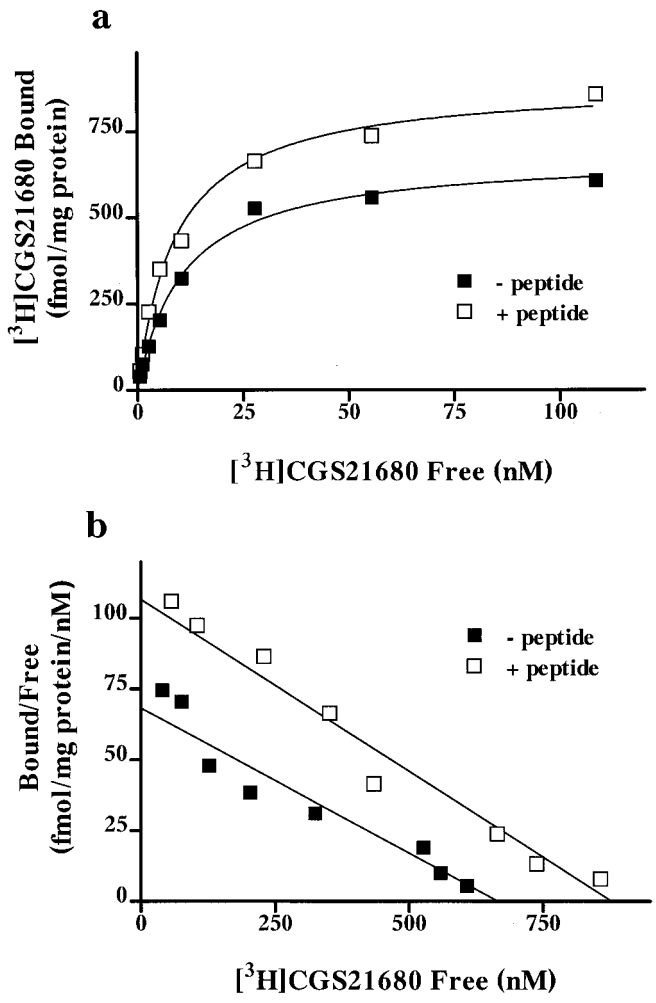

Fig. 3. Saturation isotherms (a) and Scatchard plots (b) of specific [^{3}H]CGS21680 binding to rat striatal membranes in the presence and absence of peptide $G\alpha_s(374–394)C^{379}A$. Membranes (75 μg of proteins) were incubated with eight different concentrations of [^{3}H]CGS21680 ranging from 0.5 to 108 nM in the presence and absence of $G\alpha_s(374–394)C^{379}A$ as described under *Experimental Procedures*. In the absence of the $G\alpha_s$ peptide, the derived K_D and B_{max} values were 10.9 nM and 680 fmol/mg of protein, whereas in the presence of the peptide, the K_D and B_{max} values were 9.1 nM and 890 fmol/mg of protein, respectively. Nonlinear, curve-fitting computer programs (EBDA/LIGAND and GraphPad Prism version 3.0) were used to analyze equilibrium binding data and to calculate binding parameters. The illustrated data are taken from single experiments carried out in duplicate. The results are representative of experiments repeated two additional times.

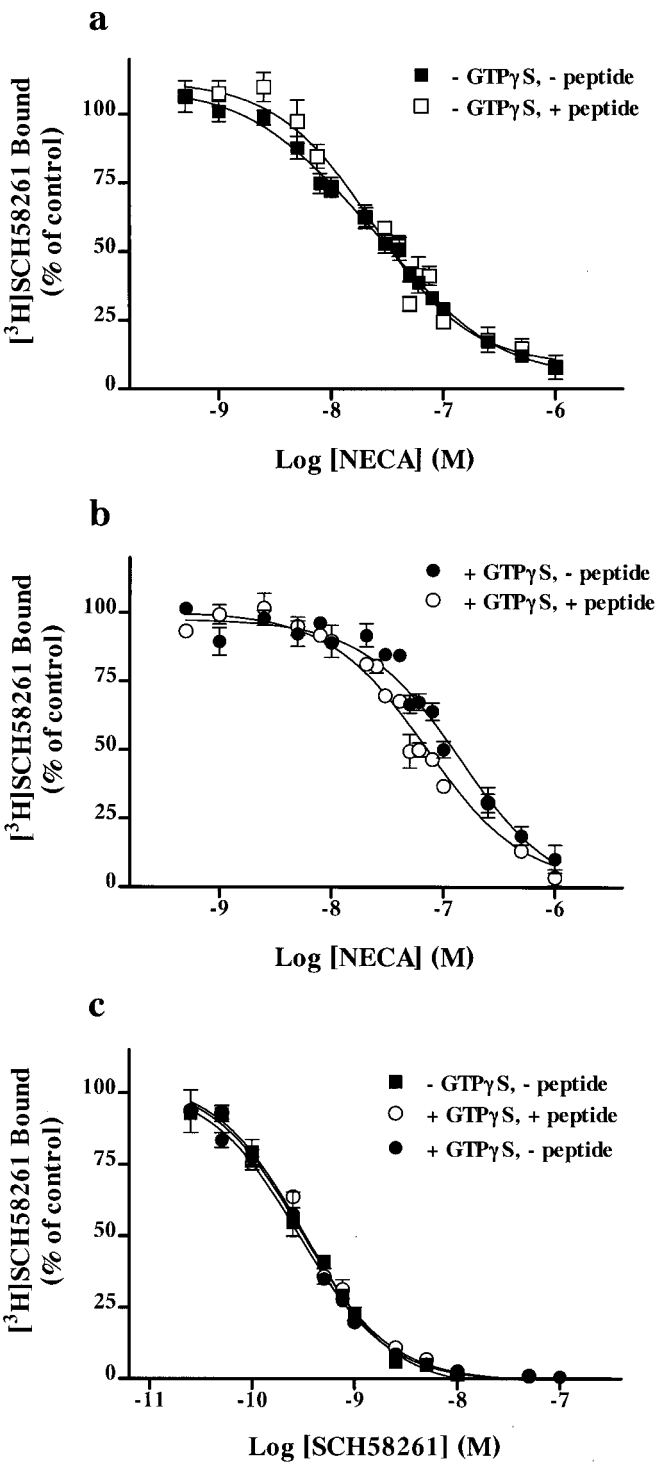

Fig. 4. Effect of $G\alpha_s(374–394)C^{379}A$ on displacement of [^{3}H]SCH58261 binding by NECA or SCH58261. Rat striatal membranes (70 μg of proteins) were incubated with 0.2 nM [^{3}H]SCH58261 and various concentrations of either NECA (0.5–1000 nM) (a and b) or SCH58261 (0.025–5 nM) (c) in the presence (b and c) and absence (a and c) of GTPγS (100 μM) with and without $G\alpha_s(374–394)C^{379}A$ (300 μM) as described under *Experimental Procedures*. In the absence of both the guanine nucleotide and $G\alpha_s$ peptide, control was 106 ± 4 fmol/mg of protein (n = 5), whereas in the presence of the peptide, control was 114 ± 2 fmol/mg of protein (n = 3). In the presence of GTPγS with and without the $G\alpha_s$ peptide controls were 111 ± 4 (n = 4) and 96 ± 5 (n = 4) fmol/mg of protein, respectively. Values are mean ± S.E. of at least three independent experiments, each per formed in duplicate. The EC_{50} values were determined by fitting the data as one- or two-site competition curves with the GraphPad Prism version 3.0 computer program. The K_i values were calculated from the EC_{50} values by the Cheng and Prusoff equation (Cheng and Prusoff, 1973). a, displacement of [^{3}H]SCH58261 binding by NECA in the absence of GTPγS with and without the $G\alpha_s$ peptide. In the absence of the peptide, data were significantly better represented by a two-site model ($P < .01$), whereas data obtained in the presence of the peptide were fitted by a one-site model. b, displacement of [^{3}H]SCH58261 binding by NECA in the presence of GTPγS with and without the $G\alpha_s$ peptide. Both curves were represented by one-site models. c, displacement of [^{3}H]SCH58261 binding by SCH58261. In all three conditions, data were fitted by one-site models.

NECA competition curve was right-shifted and better fitted by a one-site model (Hill coefficient, 1.5 ± 0.3, n = 3) (Fig. 4b). The K_i value was 100.7 ± 0.9 nM, which was significantly different ($P < .001$) from the values calculated for the low and high-affinity sites in basal displacement conditions.

In the absence of GTPγS the addition of peptide $G\alpha_s(374–394)C^{379}A$ did not shift the displacement curve (Fig. 4a) but modified the curve slope. A one-site model represented better the curve than a two-site model (Hill coefficient, 0.9 ± 0.1, n = 3), whereas a K_i value of 17.9 ± 0.9 nM was obtained. This K_i value was significantly different ($P < .001$) from the values derived for the low and high-affinity sites in the absence of the peptide. In the presence of GTPγS, the addition of the $G\alpha_s$ peptide caused a left-shift of the displacement curve (Fig. 3b), which was still better represented by a one-site rather than a two-site model (Hill coefficient, 1.2 ± 0.2, n = 3). The K_i value (48.1 ± 0.8 nM) was 2-fold lower than that obtained in the presence of GTPγS alone ($P < .001$) but similar to the value for the low affinity state of the receptor in the absence of the nucleotide. Thus, the peptide appeared to modulate receptor affinity states for agonist ligands.

Because modulation of NECA displacement curves could result as a consequence of a direct effect on [^{3}H]SCH58261 binding to the receptor, the influence of the $G\alpha_s$ peptide on SCH58261 competition was evaluated. In the absence of both GTPγS and peptide, SCH58261 displaced [^{3}H]SCH58261 binding with a monophasic pattern that was fitted by a one-site model (K_i = 0.2 ± 0.06 nM, n = 3). Neither GTPγS nor GTPγS plus the peptide caused any shift or modification of SCH58261 competition curves (Fig. 4c). This result proved that $G\alpha_s(374–394)C^{379}A$ did not change or influence the receptor affinity for antagonist ligands.

Functional Effects of $G\alpha_s(374–394)C^{379}A$ on A_{2A} Adenosine Receptor Signal Transduction. The effects of peptides $G\alpha_s(374–394)C^{379}A$, $G\alpha_s(384–394)$, and $G\alpha_{i1/2}(344–354)$ on adenylyl cyclase stimulated by agonist activation of A_{2A} adenosine receptors were evaluated (Fig. 5a). The addition of peptide $G\alpha_s(374–394)C^{379}A$ decreased the basal cAMP production, whereas $G\alpha_s(384–394)$ and a control peptide, $G\alpha_{i1/2}(344–354)$, had no effect. However, statistical analysis showed that, in the presence of $G\alpha_s(374–394)C^{379}A$, cAMP production was not significantly different from basal values.

The addition of 100 μM GTP caused a significant increase of cAMP production over the basal activity. Both CGS21680 (10 μM) and NECA (10 μM) in the presence of GTP significantly stimulated adenylyl cylcase activity as compared with basal and GTP-stimulated enzymatic activity (Fig. 5a). The addition of peptide $G\alpha_s(374–394)C^{379}A$ inhibited agonist-stimulated adenylyl cyclase activity by approximately 35% (Fig. 5a). In fact, the production of cAMP decreased to the same level of that obtained incubating membrane with GTP alone. On the other hand, peptides $G\alpha_s(384–394)$ and

$G\alpha_{i1/2}$(344–354) had no major effects on NECA-stimulated adenylyl cyclase activity leading to 7% inhibition and 9% stimulation of cAMP production, respectively.

Because peptide $G\alpha_s$(374–394)C^{379}A inhibited basal adenylyl cyclase activity, we tested its effect on FSK-stimulated enzymatic activity to verify a possible direct modulation of the catalytic unit. Neither $G\alpha_s$(374–394)C^{379}A, $G\alpha_s$(384–394), nor $G\alpha_{i1/2}$(344–354) significantly modified FSK-induced cAMP production (Fig. 5b). Overall, these results suggest that peptide $G\alpha_s$(374–394)C^{379}A disrupted the signal transduction mechanism, which leads from agonist-activated A_{2A} receptor to increase of cAMP production.

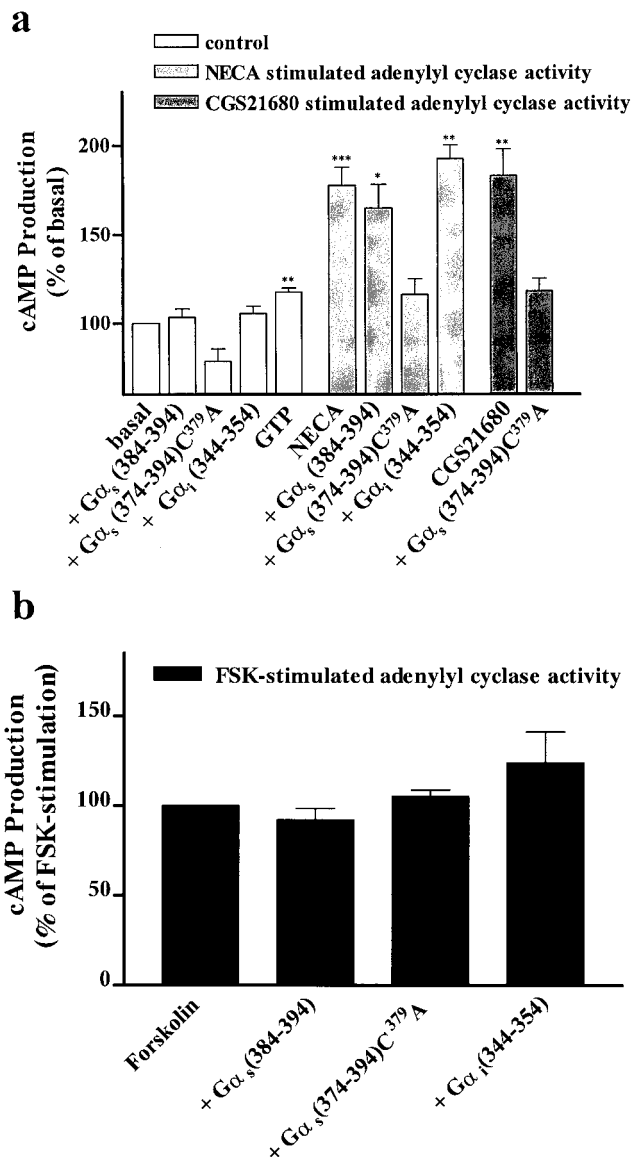

Fig. 5. Modulation of A_{2A} agonist- and FSK-stimulated adenylyl cyclase by $G\alpha_s$ and $G\alpha_{i1/2}$ peptides in rat striatal membranes. For basal adenylyl cyclase activity (a) rat striatal membranes (25 μg of proteins) were incubated with [α-^{32}P]ATP (0.9 μCi) in the absence of GTP with and without $G\alpha_s$ or $G\alpha_{i1/2}$ peptide (300 μM), whereas for G_s-mediated stimulation of adenylyl cyclase activity (a), membranes (25 μg of proteins) were incubated with [α-^{32}P]ATP in the presence of GTP (100 μM) or GTP plus an agonist (10 μM NECA or CGS21680) with and without $G\alpha$ peptides (300 μM) as described under *Experimental Procedures*. The basal adenylyl cyclase activity was 72 ± 5 pmol/10 min/mg of protein (n = 7). Values are mean ± S.E. of at least three independent experiments, each performed in triplicate. Values that are significantly different from control values, as determined by paired Student's t test, are indicated ($^{***}P < .001$; $^{**}P < .01$; $^{*}P < .05$). For basal and FSK-stimulated adenylyl cyclase activity (b) rat striatal membranes (10 μg of proteins) were incubated with [α-^{32}P]ATP (0.9 μCi) in the presence and absence (basal) of FSK (10 μM) with or without $G\alpha$ peptides (300 μM) as described under *Experimental Procedures*. The basal adenylyl cyclase activity was 2 ± 0.8 pmol/10 min/mg of protein (n = 3), whereas FSK-stimulated activity was 18 ± 0.3 pmol/10 min/mg of protein (n = 3). Values are mean ± S.E. of at least three independent experiments each performed in triplicate.

NMR and Structure Calculations. The conformational properties of $G\alpha_s$(374–394)C^{379}A were extensively studied by NMR spectroscopy and molecular modeling. The usual high conformational freedom of linear short peptides in water solution leads to inextricable mixtures of isoenergetic confomers. The use of a solvent system having suitable viscosity and polarity properties allows the most energetically stable confomers to prevail, thus making the NMR spectra more interpretable (Amodeo et al., 1991). Therefore, we recorded spectra in a mixture of water:HFA (50:50, v/v). HFA, which is a typical structure stabilizing cosolvent, acts by favoring the intramolecular hydrogen bonds and consequently folded conformations (Rajan et al., 1997).

NMR analysis was made using one- and two-dimensional protonic homonuclear techniques. DQF-COSY (Piantini et al., 1982), TOCSY (Bax and Davis, 1985), and NOESY (Jenner et al., 1979) experiments were recorded on a Bruker 600 MHz at 300 K. The complete ^{1}H chemical shift assignment of the 21-residue peptide was achieved using the interactive program package XEASY (Bartels et al., 1995).

The low region of the NOESY spectrum is shown in Fig. 6. The unusually high number of cross peaks indicated a clear tendency of $G\alpha_s$(374–394)C^{379}A to assume a folded conformation. In particular, the qualitative evaluation of sequential and medium connectivities was suggestive of a helical conformation in the carboxyl-terminal region.

Structure calculations were performed using the DYANA software package (Guntert et al., 1997). The NOE effects were translated into interprotonic distances, which were used as constraints in subsequent annealing procedures to produce 20 conformations. The geometry whose interprotonic distances best fitted NOE-derived distances was then refined through successive steps of restrained and unrestrained EM calculations to yield a conformer consistent with the NOE distance constraints (deviation less than 1.2 Å) and an RMS deviation from the starting geometry (backbone of nonhydrogen atoms) of 0.12 Å. As expected from the qualitative analysis of the NOESY spectrum, $G\alpha_s$(374–394)C^{379}A exhibited a pattern of backbone torsion angles consistent with an α-helical secondary structure. The α-helix involved residues from Arg^{380} to Leu^{393} with small deviations from canonical patterns around Gln^{384}. In contrast, the lack of well defined patterns of NOE connectivity for 374–380 residues indicated that the amino terminus was structurally disordered.

Recently, we have reported NMR analysis and structure calculation of $G\alpha_s$(384–394) (Albrizio et al., 2000). In Fig. 7a a superimposition of $G\alpha_s$(374–394)C^{379}A and $G\alpha_s$(384–394) structures is shown. In accordance with its longer length, the 21-residue peptide presented a more folded conformation than that of the 11-residue peptide. The most significant alignment was possible for the five carboxyl-terminal Cα atoms. In both peptides, this region displayed an α-helical torsion angle pattern. The degree of folding depended on the peptide size, but both fragments retained greater compact-

ness peculiarities in the carboxyl terminus independently from their length. Figure 7b shows an overlay of NMR-derived conformation of $G\alpha_s(374–394)C^{379}A$ and the 374–393 fragment extracted from the crystallographic structure of $G\alpha_s$ parent protein retrieved from the Bookhaven Protein Data Bank (Sunahara et al., 1997).

Discussion

The structural determinants of receptor-G protein interactions have recently received significant attention. Because the A_{2A} adenosine receptor appears to be loosely coupled to G_s in rat striatal membranes (Luthin et al., 1995), a study of their interaction mechanisms is important in understanding certain peculiar characteristics of this G protein-coupled receptor system. Therefore, we synthesized and used a series of peptides corresponding to various segments of the $G\alpha_s$ carboxyl terminus in an attempt to identify specific requirements for interaction of the subunit with the A_{2A} adenosine receptor.

This study shows that synthetic peptides corresponding to progressively longer segments of the $G\alpha_s$ carboxyl terminus modulate agonist binding to the A_{2A} adenosine receptor in rat striatal membranes. The ability of these peptides to stimulate agonist binding is related to their size, i.e., amino acid numbers. Thus, the 11-residue peptide, $G\alpha_s(384–394)$, is one of the least effective, whereas peptides containing 17 to 21 residues, $G\alpha_s(378–394)C^{379}A$, $G\alpha_s(376–394)C^{379}A$, and $G\alpha_s(374–394)C^{379}A$, are the most active. The specificity of longer peptide effects, at the level of the receptor-G_s interface, is supported by the evidence that they show a greater ability to stimulate agonist binding in the presence of GTPγS.

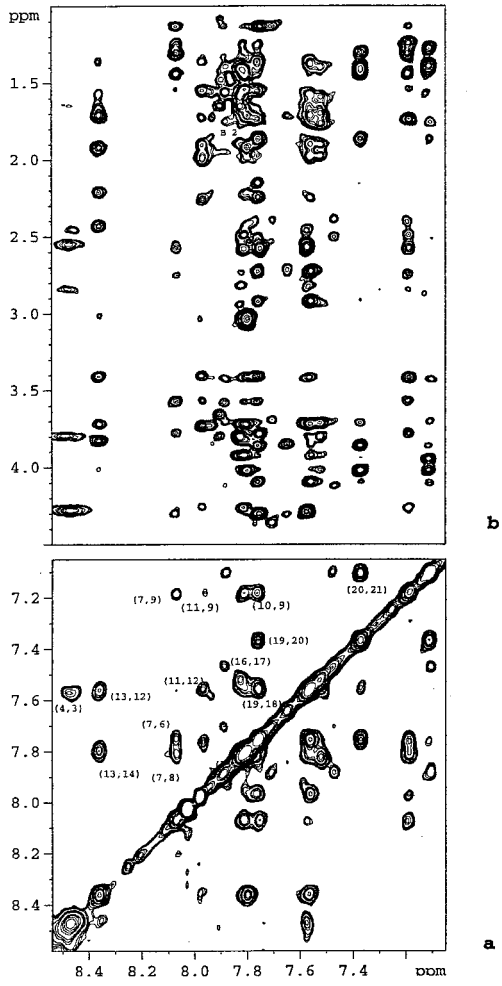

Fig. 6. NOESY spectrum of $G\alpha_s(374–394)C^{379}A$. Backbone amidic-NH (i,i + 1) (NH/NH) (a) and NH/alkyl side chain (b) regions of the 150-ms NOESY spectrum of the $G\alpha_s$ peptide in water:HFA (50:50, v/v) at 300 K. Sequential NH/NH connectivities are labeled.

A role of the carboxyl-terminal α-helix (α5) in specific receptor recognition has been indicated by the crystal structure resolution of $G\alpha_s$·GTPγS showing a continuous helix from Asp^{368} to Leu^{394} (Sunahara et al., 1997). Our NMR analysis demonstrates a marked propensity of the 11- (Albrizio et al., 2000) and 21-residue peptides to form an α-helical structure in solution. Although for the shortest peptide, a defined structure is represented by a turn of α-helix between Arg^{389} and Leu^{394}, for the longest peptide, the α-helical structure spans from Asp^{381} to Leu^{394}. This shows good overlapping with α-5 of the $G\alpha_s$ subunit (Sunahara et al., 1997). These results are in agreement with our observation that the longest peptides have a higher ability to stimulate [^{3}H]CGS21680 binding and support the important role of carboxyl-terminal conformation for $G\alpha_s$ interaction with the A_{2A} adenosine receptor. Indeed, the importance of this α-helix is also pointed out by the notion that $G\alpha_s$ is the only Gα subunit so far crystallized that shows a defined structure at the level of the last carboxyl-terminal residues (Noel et al., 1993; Coleman et al., 1994; Sunahara et al., 1997). An 11-residue synthetic peptide from the $G\alpha_t$ carboxyl terminus does not acquire an α-helical structure, but it displays a β-turn around Gly^{348} (Glu^{392} in $G\alpha_s$) in the presence of the inactive receptor, dark-rhodopsin (Rh) (Dratz et al., 1993). This conformation is lost when the peptide is bound to light-activated rhodopsin (metarhodopsin II or Rh*) (Dratz et al., 1993). Thus, an α-helix appears to be a specific bioactive determinant for interaction of $G\alpha_s$ with its cognate receptors.

In the presence of GTPγS, which uncouples G proteins from receptors, a $G\alpha_{i1/2}$ carboxyl-terminal peptide, $G\alpha_{i1/2}(344–354)$, is as effective as the shortest $G\alpha_s$ peptides in stimulating agonist binding. This finding may suggest that in some conditions the A_{2A} adenosine receptor is able to interact with G_i proteins. Daaka et al. (1997) have shown that a classical G_s coupled receptor, the β_2-adrenergic receptor, switches to activate G_i proteins after phosphorylation by protein kinase A. A similar event may also occur for agonist-activated A_{2A} adenosine receptors in rat striatum.

In rat striatal membranes, agonist-activated A_{2A} adenosine receptors interact tightly with heterotrimeric G_s proteins in the presence of 5 to 10 mM $MgCl_2$ and absence of guanine nucleotides (Mazzoni et al., 1993). This interaction stabilizes the high-affinity state of A_{2A} adenosine receptors. However, it is now evident (Luthin et al., 1995) that in this condition there are still some A_{2A} adenosine receptors uncoupled from G_s. The $G\alpha_s$ carboxyl-terminal peptides may bind to these receptors in the low affinity state and stimulate [^{3}H]CGS21680 binding (Fig. 1a). In the presence of GTPγS (Mazzoni et al., 1993), most receptors are uncoupled from G_s and are, therefore, in the low affinity state. The addition of the $G\alpha_s$ peptide may restore the high-affinity state, mimicking the effect of G_s. This possibility has been tested for the longest $G\alpha_s$ peptide, $G\alpha_s(374–394)C^{379}A$, which shows dose

dependence and saturability of its stimulatory activity. Indeed, $G\alpha_s(374–394)C^{379}A$ evokes a larger stimulation of specific agonist binding in the uncoupling condition, but it does not stabilize the high-affinity state of the receptor either in the presence or absence (Fig. 3) of the guanine nucleotide. An intermediate receptor affinity state for the agonist may be reached.

When the effect of $G\alpha_s(374–394)C^{379}A$ on agonist displacement of [^{3}H]SCH58261 binding has been examined, a stabilization of an intermediate affinity state of A_{2A} adenosine receptors is observed. The addition of the $G\alpha_s$ peptide appears to modulate the low and high-affinity states of the receptor leading to a single intermediate affinity state. In the presence of GTPγS, the agonist competition curve is shifted to the right and fitted by a one-site model as compared with that in the absence of the nucleotide (Fig. 4b), whereas the addition of the $G\alpha_s$ peptide causes a left shift of the displacement curve. The peptide is responsible for a 2-fold decrease of the K_i value. This K_i value is similar to that calculated for the low affinity state of the receptor in basal displacement conditions. The peptide appears to affect the receptor conformation in a subtle way. The observation also implicates the existence of multiple distinct receptor conformations, which differ in their interaction with G_s proteins. A similar possibility has been suggested for rhodopsin (Arnis and Hofmann, 1995), the human thyroid-stimulating hormone (Gudermann et al., 1996), and A_1 adenosine receptors (Gilchrist et al., 1998). Our data suggest that G_s has an important role in modulating receptor affinity for agonist ligands. The A_{2A} adenosine receptor affinity states seem to be regulated by the type of interaction with the $G\alpha_s$ subunit. However, an alternative or complementary explanation is possible. Disruption of G_s activation by the 21-residue peptide (see below) compromises agonist-induced GTPγS binding to $G\alpha_s$ and, consequently, the shift of receptor affinity without affecting receptor-G_s coupling. The indirect effect is that the binding affinity for agonist ligands does not decrease while A_{2A} receptors are still coupled to heterotrimeric G_s proteins. In support of this interpretation is a recent observation (Wade et al., 1999) that basic residues within the carboxyl-terminal portion of the third intracellular loop of the α_{2a}-adrenergic receptor are important for G_i activation but not required for receptor-G_i coupling. Similarly, different determinants of the $G\alpha$ subunits can be involved in receptor-G protein coupling and activation.

The $G\alpha_s(374–394)C^{379}A$ peptide effectively inhibits receptor-mediated adenylyl cyclase activation in rat striatal membranes. The inhibition appears to be specific because the peptide shows no significant effect on basal and FSK-stimulated cAMP production. This observation supports the notion that the carboxyl-terminal region of $G\alpha_s$ is critical for signal transduction from the activated A_{2A} adenosine receptor to G_s, but it is not so decisive for mimicking the effects of G_s on the receptor. In addition, disruption of signal transduction appears to require a 21-residue segment rather than the extreme carboxyl terminus. Thus, the peptide secondary structure is a determinant factor in supporting its activities. Recently, Gilchrist et al. (1998) have examined the effects of 11-residue $G\alpha_i$ carboxyl-terminal peptides and analogs on agonist binding to A_1 adenosine receptors and activation of

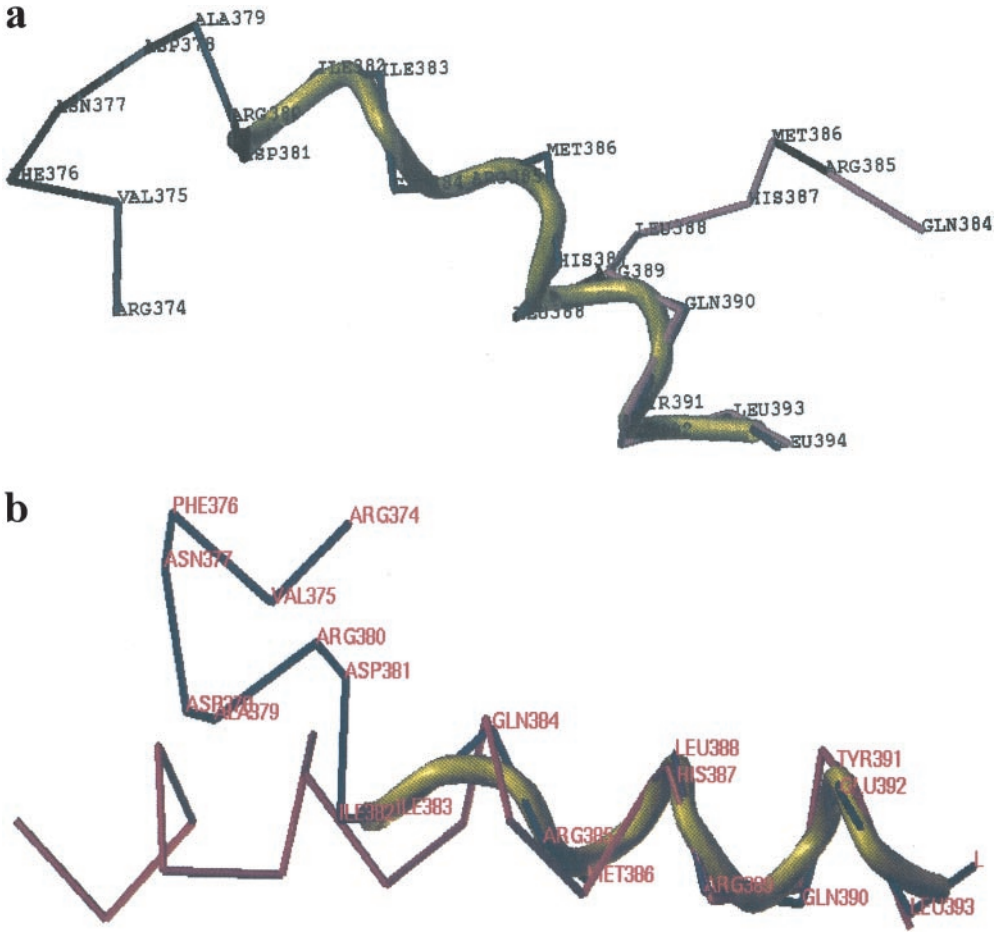

Fig. 7. Alignment of Cα atoms of $G\alpha_s(374–394)C^{379}A$ with corresponding atoms of $G\alpha_s(384–394)$ and the $G\alpha_s$ fragment Arg^{374}-Leu^{393}. a, $G\alpha_s(374–394)C^{379}A$ is represented by a blue ribbon, whereas $G\alpha_s(384–394)$ is shown as a red ribbon. The $G\alpha_s(384–394)$ structure was obtained as reported by Albrizio et al. (2000). The helical arrangement of Arg^{380} to Leu^{393} residues is highlighted by a yellow tube ribbon. b, $G\alpha_s(374–394)C^{379}A$ is represented by a blue ribbon, whereas the $G\alpha_s$ fragment Arg^{374} to Leu^{393} is shown as a red ribbon. The α-helical structure of the $G\alpha_s$ carboxyl terminus (red) was retrieved from the X-ray structure of the parent protein (Sunahara et al., 1997). The yellow tube ribbon represents the helix exhibited by both $G\alpha_s(374–394)C^{379}A$ and $G\alpha_s$ carboxyl-terminal fragment.

the effector pathway. Although the $G\alpha_i$ peptide and some analogs completely block receptor-activated K^+ current, they do not stabilize the high-affinity state of the receptor. The similarity is obvious and may underlie a specific molecular mechanism of interaction between adenosine receptors and G proteins.

Some studies have pointed out the importance of other regions of the $G\alpha$ subunits beside the extreme carboxyl terminus in directing the G protein coupling to their cognate receptors. Both tryptic digestion experiments (Mazzoni and Hamm, 1996) and a site-directed mutagenesis investigation (Onrust et al., 1997) have demonstrated that the α4-β6 loop of $G\alpha_t$ is a point of rhodopsin contact. Furthermore, using various $G\alpha_t/G\alpha_{i1}$ chimeric proteins, Bae et al. (1997) have shown that the α4-helix and α4-β6 loop are critical for specific 5-HT_{1B} receptor-G_{i1} interaction and are required for G protein activation by the receptor. In addition, this study has also involved a secondary role for the amino-terminal half of $G\alpha_{i1}$ in 5-HT_{1B} receptor coupling.

Our study provides additional evidence that the molecular mechanisms of interaction have similar features in different receptor-G protein systems, but the role and importance of each contact site change depending on the receptor type. In the case of the A_{2A} adenosine receptor, the α-helix conformation of the $G\alpha_s$ carboxyl terminus seems to be important for signal transduction. However, other parts of the $G\alpha_s$ molecule are probably involved in determining the allosteric modulation of receptor affinity for agonists. Thus, as suggested for the A_1 adenosine receptor, the interacting site on $G\alpha$ subunits may be a mosaic with each piece playing a distinct role depending on the type of receptor and G protein. Whereas the $G\alpha_s$ carboxyl terminus is pivotal for receptor-mediated activation of G_s, multiple interactions may be required to stabilize the high-affinity state of the receptor.

In conclusion we have found that the α-helical conformation of the $G\alpha_s$ carboxyl terminus is involved in supporting its ability to interact with the A_{2A} adenosine receptor, but this part of the molecule does not stabilize the high-affinity state of the receptor. The 21-residue carboxyl-terminal peptide, which is able to disrupt signal transduction, causes a direct conformational change of the receptor with stabilization of an intermediate affinity state or induces an indirect effect by preventing GTPγS binding to $G\alpha_s$.

Acknowledgments

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