Dominance of G_s in doubly G_s/G_i-coupled chimaeric A_1/A_{2A} adenosine receptors in HEK-293 cells

Amy L. TUCKER*†[1], LiGuo JIA*, Diane HOLETON*, Allen J. TAYLOR* and Joel LINDEN*†

*Department of Internal Medicine (Cardiovascular Division), Box 6012, MR4 Building, University of Virginia Health System, Charlottesville, VA 22908, U.S.A., and †Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, Charlottesville, VA 22908, U.S.A.

A_1 adenosine receptors inhibit adenylate cyclase by activating G_i/G_o, whereas A_{2A} receptors activate G_s. We examined how regions of A_1 and A_{2A} receptors regulate coupling to G-proteins by constructing chimaeras in which the third intracellular loops (3ICL or L) and/or the C-termini (or T) were switched. Pertussis toxin (PTX) was used in membrane radioligand binding assays to calculate the fraction of recombinant receptors coupled to G_i/G_o and in whole cells to differentially influence agonist-stimulated cAMP accumulation. Switching A_1/A_{2A} 3ICL domains results in receptors that maintain binding selectivity for ligands but are doubly coupled. Receptor chimaeras with an A_1 3ICL sequence (A_{2A}/A_1L or A_{2A}/A_1LT) respond to agonist stimulation with elevated cAMP despite being coupled predominantly to G_i/G_o. These chimaeras have basal cAMP levels lower than those of wild-type A_{2A} receptors, similar to wild-type A_1 receptors. The A_1 C-terminus modulates the coupling of receptors with A_1 3ICL such that A_{2A}/A_1LT is better coupled to G_i/G_o than A_{2A}/A_1L. The C-terminus has little impact on coupling to receptors containing A_{2A} 3ICL sequence. Our results show that the C-terminus sequence selectively facilitates coupling to G_i/G_o mediated by A_1 3ICL and not by other intracellular domains that favour G_i coupling. The C-terminus sequence has little or no effect on coupling to G_s. For doubly G_s/G_i-coupled adenosine receptors in HEK-293 cells, G_s-mediated stimulation predominates over G_i/G_o-mediated inhibition of adenylate cyclase. We discuss the signalling consequences of simultaneously activating opposing G-proteins within single cells.

Key words: cAMP, inhibitory G-protein, purinergic 1 receptors, signal transduction, stimulatory G-protein.

INTRODUCTION

The effects of adenosine are mediated by four G-protein-coupled cell-surface receptors, A_1, A_{2A}, A_{2B} and A_3 [1,2]. Adenosine receptors differ in both pharmacological properties and in selectivity for G-proteins: A_1 and A_3 adenosine receptors couple to G-proteins in the G_i/G_o subfamily [3–6]; A_{2A} receptors couple to G_s [7]; A_{2B} receptors are doubly coupled to G_s and G_q [8]. The selectivity of receptors for Gα subunits is thought to be due to structural differences between the receptors themselves, largely in the intracellular domains that are able to make physical contact with the Gα subunits. There are no consensus sequences in receptors that interact with a specific Gα subunit; rather, intracellular domains with conserved charge or secondary structure seem to govern receptor–G-protein interactions.

The most systematically studied of the family of rhodopsin-like receptors, to which adenosine receptors belong, are adrenergic and cholinergic muscarinic receptors. Mutational analyses have demonstrated that the third intracellular loop (3ICL) has a primary role in G-protein coupling selectivity [9–20]. The 3ICL is not always the exclusive determinant of selectivity; mutation of various receptors has revealed contributions by other intracellular domains that act in a co-ordinated fashion to confer optimal coupling. Within the family of biogenic amine receptors the first intracellular loop [21], the second intracellular loop (2ICL) [9,12,21] or the C-terminus [9,11] can modulate the predominant influence of the 3ICL.

Olah [7] has shown that the 3ICL, but not the C-terminus, of the A_{2A} adenosine receptor is important in regulating coupling to adenosine receptors. Here we show that the 3ICL and the C-terminus co-operatively influence coupling to the A_1 adenosine receptor.

EXPERIMENTAL

Materials

The Altered Sites Mutagenesis system was from Promega. [^{3}H]8-Cyclopentyl-1,3-dipropylxanthine ([^{3}H]CPX) and [^{3}H]CGS21680 were from Dupont NEN. N^6-(3-[^{125}I]Iodo-4-aminobenzyl)-adenosine ([^{125}I]ABA) and 2-[2-(4-amino-3-[^{125}I]iodophenyl)-ethylamino]adenosine ([^{125}I]APE) were synthesized and purified as described previously [22,23]. Pertussis toxin (PTX) was a gift from the laboratory of Dr Erik Hewlett (University of Virginia, Charlottesville, VA, U.S.A.). N^6-Cyclopentyladenosine (CPA) and forskolin were from Sigma. CGS 21680 and the phosphodiesterase inhibitor Ro-20-1724 were from RBI. Adenosine deaminase was from Boehringer Mannheim. Sequenase 2.0 was from U. S. Biochemical Corp. Lipofectamine was from Gibco BRL. Tissue culture supplies, including G418, were from Gibco BRL. CLDN10B was a gift from Dr M. Reff (SK&F Laboratories). The canine A_1 and A_{2A} receptor cDNA species were gifts from G. Vassart (Université Libre de Bruxelles, Bruxelles, Belgium).

Mutagenesis

The *Kpn*I/*Hin*dIII fragment of the canine A_1 adenosine receptor and the *Kpn*I/*Eco*RV fragment of the canine A_{2A} adenosine receptor cDNA species were subcloned into the pALTER

Abbreviations used: [^{125}I]ABA, N^6-(3-[^{125}I]iodo-4-aminobenzyl)adenosine; [^{125}I]APE, 2-[2-(4-amino-3-[^{125}I]iodophenyl)ethylamino]adenosine; CPA, N^6-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; GTP[S], guanosine 5′-[γ-thio]triphosphate; 2ICL, second intracellular loop; 3ICL, third intracellular loop; PTX, pertussis toxin; TM, transmembrane; XAC, xanthine amine congener, 8-{4-[(2-aminoethyl)aminocarbonylmethyloxy]phenyl}-1,3-dipropylxanthine; WT, wild-type.

[1] To whom correspondence should be addressed, at the Department of Internal Medicine (e-mail alt8t@virginia.edu).

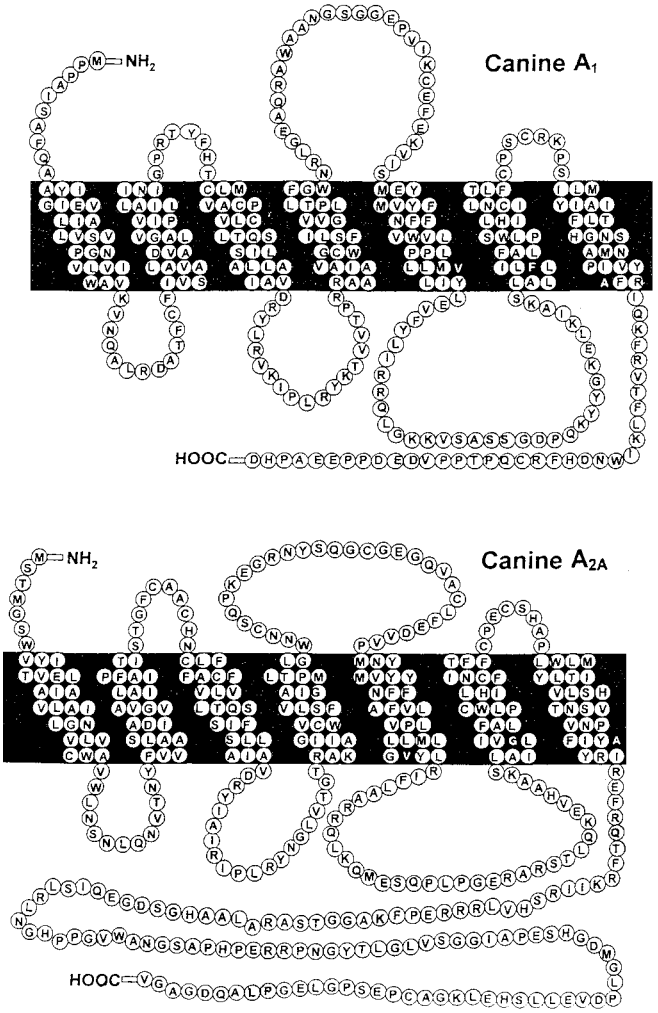

Figure 1 Diagrams of the A_1 and A_{2A} adenosine receptors

Each circle represents an amino acid residue designated by the indicated single letter code. The residues represented by black circles denote those into which the silent restriction sites were introduced.

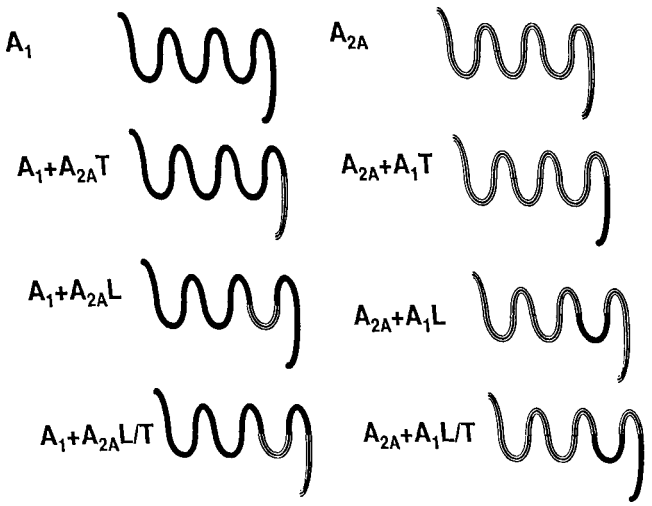

Figure 2 Diagram of the WT and chimaeric A_1 and A_{2A} adenosine receptors used in this study

The A_1 sequence is denoted in solid lines, the A_{2A} with striped lines. Beside each receptor is the nomenclature used to describe it in the text.

mutagenesis vector. Silent restriction sites were introduced into the cDNA species to be used as chimaeric receptor splice boundaries. *Spe*I sites were introduced at positions corresponding to Val-190 and Val-186 of the canine A_1 and A_{2A} receptors respectively. *Stu*I sites were placed at Phe-241 of the canine A_1 and at Gly-239 of the canine A_{2A} receptor. These sites flank the 3ICL (see Figures 1 and 2). *Nsi*I sites were introduced just proximal to the putative C-terminus at Ala-289 (A_1) and Ala-288 (A_{2A}). These sites were used to excise DNA fragments that were ligated together to create six chimaeric receptors in which sequences for the 3ICL or the C-terminus, or both, had been switched from one subtype to the other (see Figures 1 and 2). The chimaeric receptors were subcloned into the CLDN 10B expression vector and sequenced in their entirety on both strands by using Sequenase 2.0.

Cell culture and DNA transfection

HEK-293 cells were grown in DMEM (Dulbecco's modified Eagle's medium) F12 medium supplemented with 10% (v/v) fetal bovine serum and 1% (w/v) penicillin/streptomycin to 50% confluence on 60 mm plates and transfected with 20 μg of plasmid DNA by using Lipofectamine in accordance with the manufacturer's protocol. Transfected cells were grown for 48 h before beginning selection by the addition of 2 mg/ml of G418 to the medium. Resistant clones were screened by radioligand binding with [^{3}H]CPX and [^{3}H]CGS21680. Clonal lines with high specific binding were grown to confluence in 150 mm dishes in the presence of 0.8 mg/ml G418.

Treatment with PTX

For experiments with pertussis-intoxicated cells or membranes, plates at 80% confluence were treated with 200 ng/ml PTX for 18 h before whole cells were harvested for cAMP assays, or membranes for radioligand binding assays.

Membrane preparation

HEK-293 cells expressing recombinant adenosine receptors were harvested from 150 mm culture dishes in 25 ml of buffer A (10 mM Hepes/10 mM EDTA/0.1 mM benzamidine) at pH 7.4 and 4 °C, with mechanical agitation. Cells were homogenized with a Brinkmann Polytron on setting 5 for 30 s. The homogenates were centrifuged at 20000 ***g*** for 30 min. Pellets were resuspended in 25 ml of ice-cold HE buffer [10 mM Hepes/1 mM EDTA/0.1 mM benzamidine (pH 7.4)] and were washed twice by centrifugation. The final pellets were suspended in HE buffer supplemented with 10% (w/v) sucrose at a protein [24] concentration of 1 mg/ml, then aliquoted for freezing at −20 °C.

Radioligand binding

For binding studies, 0.01 mg of membrane protein was incubated with 2 units/ml (10 μg/ml) adenosine deaminase and with radioligand in HE buffer containing $MgCl_2$ at a final concentration of 4.9 mM, in the presence or absence of competing compounds. Binding reactions were incubated at 25 °C for 3 h and terminated by rapid filtration through Whatman glass fibre (GF/C) filters with a Brandel cell harvester. Filters were rinsed

three times with 3 ml of ice-cold buffer containing 10 mM Tris/HCl and 1 mM $MgCl_2$, pH 7.4. Saturation and competition binding studies were performed with the A_{2A} agonist [^{3}H]-CGS21680, the A_1 agonist [^{125}I]ABA or the A_1 antagonist [^{3}H]CPX as radioligands. CPX (2 μM) or CGS21680 (10 μM) was used for the determination of non-specific binding. Fractional coupling of receptors to G_i/G_o and G_s was determined with aliquots of membranes prepared from PTX-treated or untreated cells, and by the addition of 50 μM guanosine 5′-[γ-thio]triphosphate (GTP[S]) to membranes. The ratio of GTP[S]-sensitive binding that was prevented in membranes derived from PTX-treated cells was used to define the fraction of total G-protein-coupled receptor that was coupled to G_i/G_o.

cAMP assays

HEK-293 cells expressing recombinant adenosine receptors were pretreated with and without PTX and removed from 150 mm² culture dishes with mechanical disruption in 8 ml of DMEM. Cells were centrifuged at 500 ***g*** for 1 min and resuspended in DMEM containing adenosine deaminase buffered with 20 mM Hepes, pH 7.3, and aliquoted into assay tubes to make a final volume of 200 μl. Adenosine deaminase (5 units/ml; 25 μg/ml) was used to minimize receptor activation by endogenous adenosine released by the cells. Different cell densities were used because the nature of the expressed receptor influenced basal cAMP levels. Cells were allowed to recover at 25 °C for 1 h, after which various concentrations of CPA or CGS21680 were added to a final volume of 250 μl. Experiments were done in the presence and the absence of 10 μM isoprenaline (isoproterenol) or 5 μM forskolin to stimulate cAMP levels. All experiments were done in the presence of 25 μM Ro-20-1724 to block phosphodiesterase. After the addition of drug, cells were incubated for 15 min at 37 °C in a shaking water bath. Incubations were terminated with the addition of HCl to a final concentration of 0.1 M; cellular debris was removed by centrifugation for 15 min at 2000 ***g***. cAMP (500 μl) in the acid extract was acetylated by the addition of 22.5 μl of triethylamine/acetic anhydride (3.5:1, v/v) and the cAMP was measured by automated radioimmunoassay [25].

Data analysis

Saturation binding data were fitted to a single-site equation. In competition binding assays, IC_{50} and K_i values were calculated as described previously [26]. Differences in ligand binding and in maximal cAMP responses were compared using a Student's *t* test. Comparisons of the cAMP responses with forskolin stimulation in cells expressing wild-type (WT) or chimaeric receptors to one another or to untransfected HEK-293 cells were made by using an unpaired *t* test.

RESULTS

Stable expression of canine A_1/A_{2A} receptor chimaeras in HEK-293 cells

Figure 1 shows the splice sites for chimaeric receptors; Figure 2 shows a diagram of the chimaeric receptors constructed. Receptor expression levels varied between chimaeric receptors. Most of the receptors showed high levels of expression, with B_{max} values between 2.7 and 15 pmol/mg protein (Table 1). All clones of the A_1/A_{2A}T and the A_{2A}/A_1 LT chimaeric receptors were expressed poorly, with B_{max} levels of 0.6 and 0.2 pmol/mg protein respectively. Although expression did not differ significantly between the WT A_1 and A_{2A} receptors, all three types of receptor chimaera containing the A_{2A} 3ICL were more highly expressed than the corresponding receptors with the A_1 3ICL sequence (Table 1). This suggests that the 3ICL sequence influences expression.

Table 1 Parameters of radioligand binding to chimaeric A_1 and A_{2A} receptors

Results are means ± S.E.M. derived from equilibrium binding assays, each performed in triplicate.

Radioligand	Receptor	K_d (nM)	B_{max} (fmol/mg of protein)	*n*
^{3}[H]CPX	A_1	6.7 ± 0.7	5132 ± 1220	2
	$A_1 + A_{2A}$L	10.5 ± 1.4	15214 ± 1522	4
	$A_1 + A_{2A}$T	10.7 ± 2.5	600 ± 97	3
	$A_1 + A_{2A}$LT	7.2 ± 0.1	5581 ± 382	3
^{3}[H]CGS21680	A_{2A}	13.9 ± 2.5	4543 ± 1540	4
	$A_{2A} + A_1$L	7.9 ± 0.6	2735 ± 710	2
	$A_{2A} + A_1$T	24.1 ± 3.5	8990 ± 2216	3
	$A_{2A} + A_1$LT	12.2 ± 3.4	188 ± 36	2

Radioligand binding of subtype-selective ligands to chimaeric A_1/A_{2A} adenosine receptor

Exchange of the 3ICL and/or the C-terminus from A_1 receptors to A_{2A} receptors and vice versa did not alter the rank-order potencies for the A_1-selective and A_{2A}-selective ligands. Table 1 shows the results of saturation isotherms with the A_1-selective antagonist [^{3}H]CPX on membranes expressing WT A_1 receptors and chimaeric A_1/A_{2A} receptors retaining the A_1 sequence in the transmembrane (TM) regions. The receptors had similar affinities for [^{3}H]CPX, with K_d values ranging between 6 and 11 nM. This is typical for the affinity of [^{3}H]CPX reported for canine A_1 receptors [27]. All of the chimaeric receptors with A_1 TM sequences retained at least a 300-fold higher affinity for the A_1-selective agonist CPA than for the A_{2A}-selective agonist CGS21680 (results not shown). Conversely, all of the receptors with A_{2A} TM sequences had affinities for CGS21680 at least 20-fold greater than for CPA (results not shown). Table 1 also shows the results of saturation binding isotherms with the A_{2A}-selective agonist radioligand [^{3}H]CGS21680 on membranes expressing WT and chimaeric A_{2A}/A_1 receptors containing A_{2A} TM domains. The receptors had similar affinities for [^{3}H]-CGS21680, with K_d values ranging from 8 to 24 nM. There might have been some variation in the determination of these K_d values owing to some radioligand binding to uncoupled receptors [23].

cAMP responses to agonist stimulation in intact HEK-293 cells expressing WT and chimaeric receptors

Figure 3 shows the averaged results of multiple experiments all done in the presence of 10 μM isoprenaline or 5 μM forskolin. Isoprenaline or forskolin was used to stimulate cAMP accumulation for a better demonstration of inhibition for G_i/G_o-coupled receptors. Results were qualitatively similar for WT or chimaeric receptors in the absence of stimulation with isoprenaline or forskolin. CPA did not stimulate or inhibit cAMP accumulation in untransfected HEK-293 cells at concentrations less than 1 μM (Figure 3); however, at concentrations of 1 μM or above, both CPA and CGS 21680 stimulated cAMP through the activation of endogenous A_{2B} receptors in HEK-293 cells [8].

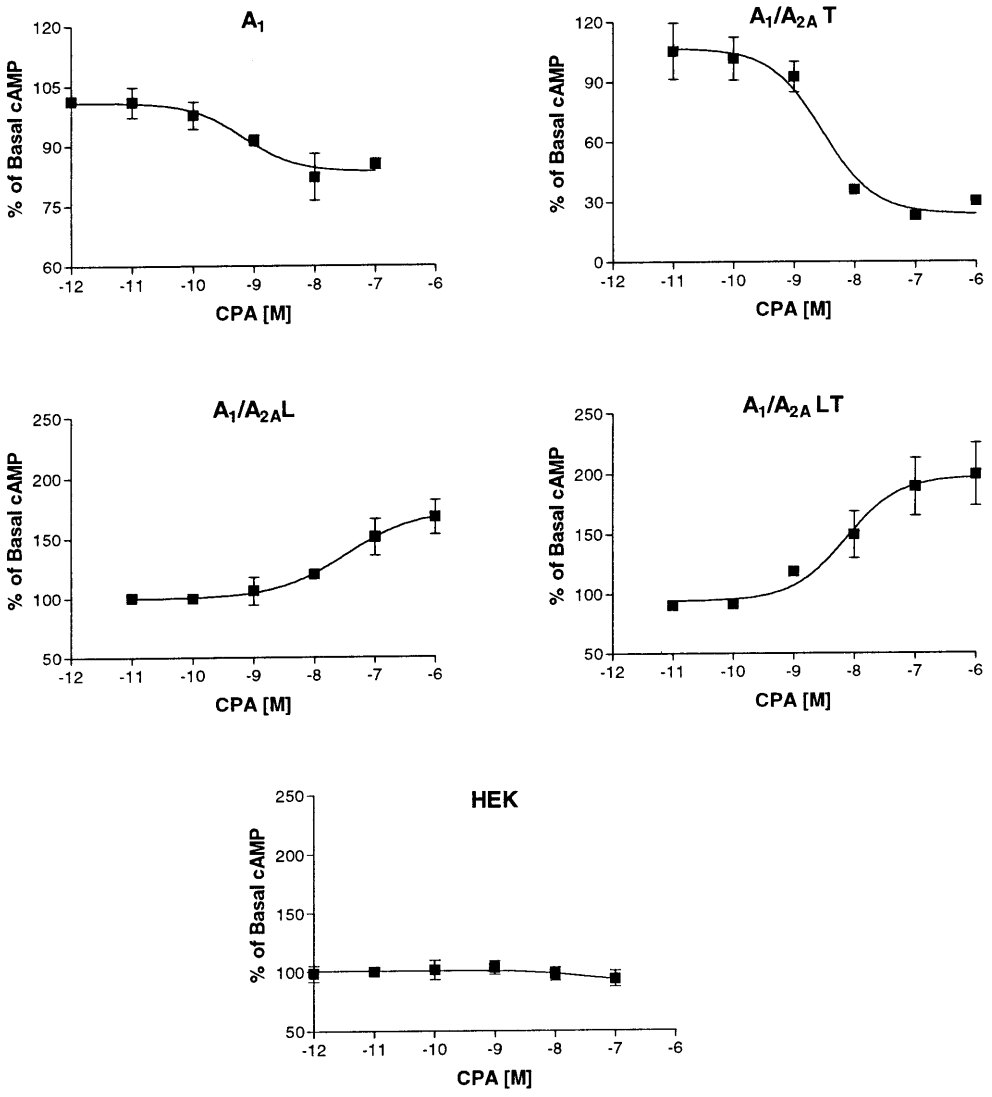

Figure 3 Effects of CPA on cAMP accumulation in HEK-293 cells transfected with WT A_1 and chimaeric A_1/A_{2A} adenosine receptors

Cumulative curves are shown from three or four independent assays performed in triplicate in the presence of forskolin or isoprenaline. cAMP values are expressed as percentage changes from basal levels. Basal cAMP levels per 20000 cells (means $\pm$ S.E.M.) were: A_1, 0.27 $\pm$ 0.06; A_1/A_{2A}T, 0.24 $\pm$ 0.4; A_1/A_{2A}L, 54.30 $\pm$ 15.95; A_1/A_{2A}LT, 13.45 $\pm$ 4.77 pmol/ml. The A_1 and A_1/A_{2A}T receptors were assayed at 20000 cells per tube, A_1/A_{2A}L at 50000 cells per tube and A_1/A_{2A}LT at 100000 cells per tube. There was no significant difference between the A_1/A_{2A}L and A_1/A_{2A}LT curves ($P = 0.148$).

Replacing the 3ICL of the A_1 receptor with the A_{2A} sequence changed the receptor from one effecting cAMP inhibition to one effecting stimulation (Figure 3). Switching only the C-terminus of the A_1 receptor to A_{2A} resulted in a receptor that remained inhibitory for adenylate cyclase. Despite a robust increase in cAMP accumulation with agonist stimulation, A_1/A_{2A}L receptors are doubly coupled, as demonstrated by a further 1.4-fold increase ($P = 0.05$) in maximal cAMP accumulation seen in response to PTX intoxication. The magnitude of the increase in cAMP levels seen both in the absence and in the presence of PTX was no greater for the A_1/A_{2A}LT chimaera than for the A_1/A_{2A}L chimaera, indicating that the C-terminus is not important for coupling the A_{2A} receptor to G_s.

None of the receptors that contained A_{2A} TM sequence was inhibitory for cAMP accumulation despite the substitution of the A_1 sequence from the 3ICL and/or the C-terminus (Figure 4). Figure 4 depicts experiments done in the absence of isoprenaline or forskolin; however, the results for those done in their presence were not qualitatively different, i.e. the A_{2A}/A_1L and A_{2A}/A_1LT receptors did not inhibit the accumulation of cAMP in isoprenaline-stimulated cells. Chimaeric receptors with an A_{2A} TM backbone and A_1 3ICL showed evidence for double coupling to G_s and G_i/G_o. Switching the 3ICL with or without the C-terminus from A_{2A} to A_1 substantially diminished basal cAMP levels and the maximal cAMP response to CGS21680 stimulation (Figure 4). These effects were significantly greater in the A_{2A}/A_1LT chimaera than in the A_{2A}/A_1L chimaera (Figure 4). The small increase in cAMP level observed in the presence of high concentrations of CGS21680 in cells expressing the A_{2A}/A_1LT receptor was similar in magnitude to the increase seen in untransfected HEK-293 cells and is attributable to endogenous A_{2B} receptors.

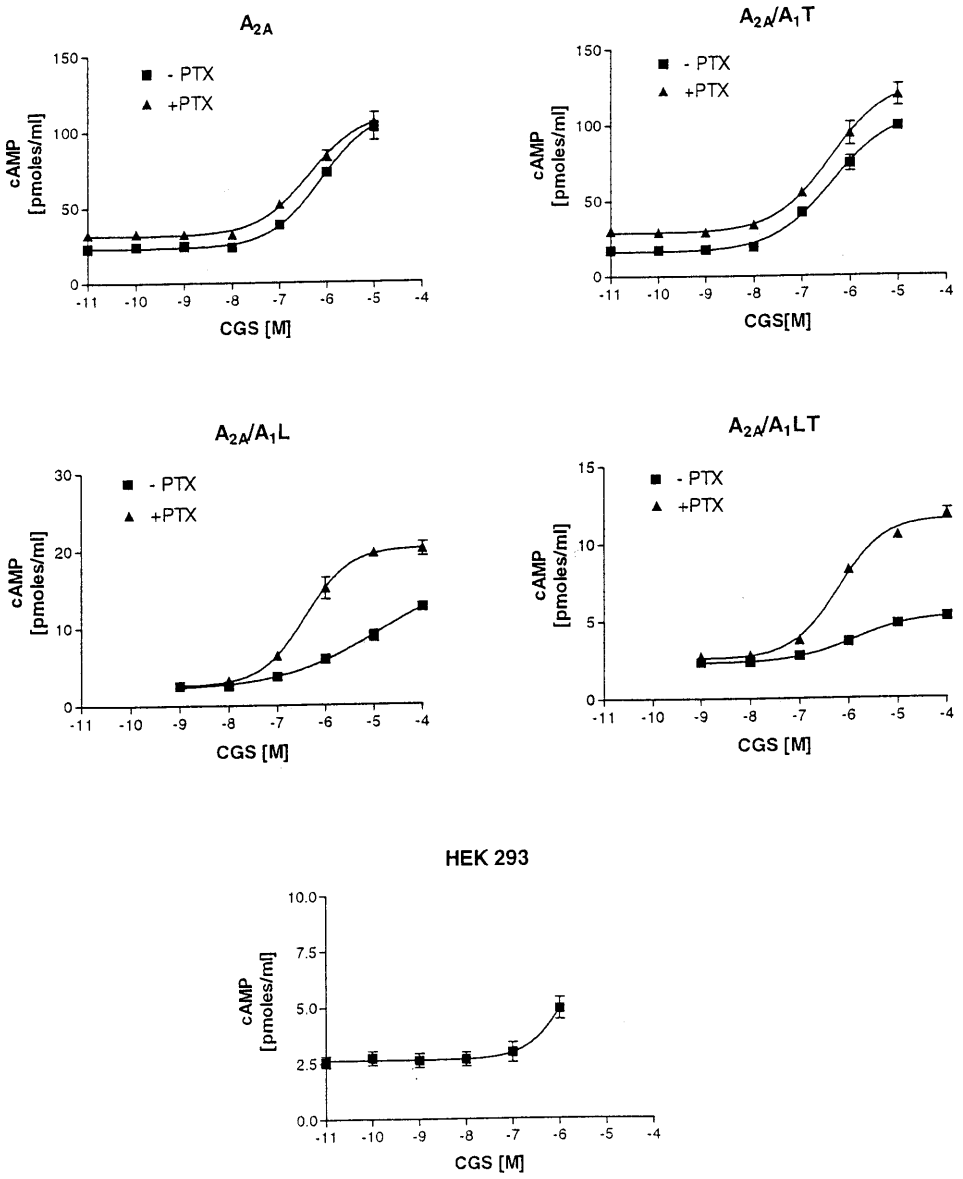

Figure 4 Effects of CGS21680 on cAMP accumulation in HEK-293 cells transfected with WT A_{2A} and chimaeric A_{2A}/A_1 adenosine receptors

To permit comparisons of absolute cAMP levels, all receptors were assayed at 20000 cells per tube. Basal levels of cAMP per 20000 cells (means ± S.E.M.) were: A_{2A}, 22.81 ± 0.22; A_{2A}/A_1T, 17.18 ± 0.60; A_{2A}/A_1L, 2.57 ± 0.22; A_{2A}/A_1LT, 2.36 ± 0.09 pmol/ml. Experiments were performed in the presence (▲) and in the absence (■) of PTX treatment. Results shown are representative curves from three or four independent assays performed in triplicate.

To investigate further the possibility of double coupling by receptor chimaeras, we examined the effects of PTX intoxication on cAMP accumulation in response to agonists (Figure 4). PTX had no significant effect on cells expressing the WT A_{2A} receptors or on cells expressing the A_{2A}/A_1T chimaeric receptor. In comparison with non-intoxicated cells, PTX intoxication significantly increased cAMP accumulation in the A_{2A}/A_1L chimaera (30%) and produced an even larger ($P = 0.02$) stimulation in the A_{2A}/A_1LT chimaera (2-fold), implying increased coupling efficiency to G_i/G_o in the double mutant.

It is noteworthy that the levels of cAMP accumulation seen by stimulating PTX-intoxicated cells expressing A_{2A}/A_1L and A_{2A}/A_1LT did not approach those seen by stimulating WT A_{2A} receptors. These results imply that chimaeric receptors have both an increased affinity for G_i and a decreased affinity for G_s relative to the WT A_{2A} receptor.

Determination of basal cAMP levels and responses to forskolin for WT and chimaeric receptors

The sequence of the 3ICL influenced the basal levels of cAMP and the maximal levels of cAMP accumulating in response to stimulation by forskolin in HEK-293 cells expressing recombinant adenosine receptors (Figures 5 and 6). Figure 5 shows the basal levels of cAMP in intact transfected cells. The basal levels of cAMP were correlated with the sequence of the 3ICL. The A_{2A}/A_1L and A_{2A}/A_1LT chimaeric receptor curves are superimposed. Cells expressing these receptors showed low basal

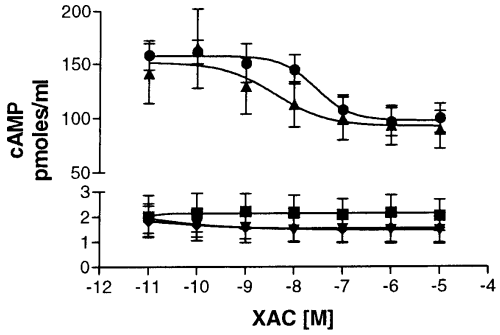

Figure 5 Effects of the non-selective inverse agonist XAC on basal cAMP levels in HEK-293 cells overexpressing WT and chimaeric adenosine receptors

Each receptor subtype was assayed at 100 000 cells per tube to permit comparisons of absolute cAMP values. The graph is representative of three experiments, each performed in triplicate. Symbols: ■, A_1; ▲, A_{2A}; ▼, A_{2A}/A_1L; ◆, A_{2A}/A_1LT; ●, A_{2A}/A_1T. Error bars indicate S.E.M.

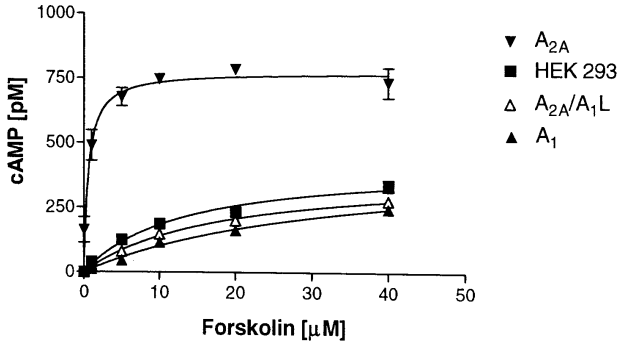

Figure 6 Forskolin-stimulated changes in cAMP levels in WT HEK-293 cells and cells expressing recombinant adenosine receptors

Each receptor subtype was assayed at 100 000 cells per tube to permit comparisons of absolute cAMP values. The graph is representative of duplicate experiments, each performed in triplicate. Symbols: ▼, A_{2A}; ■, HEK-293; Δ, $A_{2A}A_1L$; ▲, A_1. Error bars indicate S.E.M.

cAMP levels, similar to those seen in cells expressing the WT A_1 adenosine receptor, but agonists stimulated cAMP accumulation in these cells. The cAMP levels did not rise in response to the inverse agonist xanthine amine congener, 8-{4-[(2-aminoethyl)-aminocarbonylmethyloxy]phenyl}-1,3-dipropylxanthine (XAC) (Figure 5). HEK-293 cells expressing receptors containing A_{2A} 3ICL all had high basal cAMP levels. Figure 5 shows that XAC caused a dose-dependent decrease in basal cAMP levels for cells expressing WT A_{2A} and A_{2A}/A_1T receptors (other chimaeras containing the A_{2A} 3ICL were not tested). This suggests constitutive receptor activity in the absence of ligand, and/or receptor activation by endogenous adenosine that is not completely removed by added adenosine deaminase. The effect of XAC did not restore cAMP levels to values seen in untransfected cells. The magnitude of constitutive stimulation of adenylate cyclase gradually declined over weeks in culture for WT A_{2A} receptors (results not shown).

Figure 6 shows that the maximal cAMP response to stimulation by forskolin in cells expressing A_{2A}/A_1L was significantly lower than for untransfected HEK-293 cells ($P = 0.01$), but higher than for WT A_1 receptors ($P = 0.01$).

Table 2 One-point binding assays of chimaeric A_1 and A_{2A} receptors

Results are means ± S.E.M. derived from one-point assays, each performed in triplicate.

	Specific binding (% of total)	
Receptor	PTX	GTP + PTX
A_1	8 ± 2	4 ± 2
$A_1/A_{2A}T$	5 ± 3	1 ± 1
$A_1/A_{2A}L$	63 ± 9	36 ± 14
$A_1/A_{2A}LT$	62 ± 7	23 ± 7
A_{2A}	87 ± 19	67 ± 14
A_{2A}/A_1T	75 ± 8	55 ± 3
A_{2A}/A_1L	34 ± 13	11 ± 7
A_{2A}/A_1LT	27 ± 8	24 ± 9

Use of uncoupling agents in radioligand binding assay to quantify the fraction of receptors coupling to G_i/G_o and G_s

Radioligand binding experiments were performed with membranes prepared from cells pretreated with or without PTX to uncouple G_i/G_o. GTP[S] was added to uncouple all G-proteins (Table 2). [^{125}I]ABA detects mostly coupled WT A_1 receptors, with a decrease of over 90% in the specific binding to [^{125}I]ABA in the presence of either PTX or GTP[S] (Table 2). The binding of [^{125}I]APE to WT A_{2A} receptors was only partly inhibited by GTP[S], which is consistent with the previous observation that A_{2A} receptors are poorly coupled to any G-protein [23]. As expected for a G_s-signalling receptor, [^{125}I]APE binding to WT A_{2A} receptors was not significantly affected by PTX (Table 2).

Substitution of the A_{2A} C-terminus sequence into the A_1 receptor did not produce G_s coupling, as determined by binding measured in the presence of GTP[S] or PTX. Conversely, when the A_{2A} 3ICL sequence replaced A_1 sequence the fraction of PTX-sensitive [^{125}I]ABA binding to the chimaeric receptor fell to 40%, which is much lower than the 90% seen with the WT A_1 receptor. Binding to the $A_1/A_{2A}L$ receptor was also less sensitive to GTP[S], reflecting a lower overall coupling fraction than that seen with the G_s-coupled WT A_{2A} receptor. The coupling profiles of the A_1/A_{2A} L and $A_1/A_{2A}LT$ receptors were similar in these assays. The magnitude of cAMP stimulation in response to the agonist CGS21680 was similar. This suggests that the A_{2A} C-terminus has an insignificant role in G_s coupling, a conclusion also supported by Olah's analysis of a similar chimaeric receptor [7]. The 3ICL from the A_{2A} receptor seems to be important in G_s coupling, because substitution of the loop alone changes inhibitory receptors into stimulatory ones; however, although predominant, the 3ICL is not the sole determinant of G_s coupling. The substitution of only A_1 3ICL sequence into the A_{2A} receptor resulted in a receptor with a markedly attenuated cAMP stimulatory response and the suppression of basal cAMP levels, but nevertheless a stimulatory response to agonist.

Substitution of A_1 C-terminus sequence alone into the A_{2A} receptor did not significantly alter the coupling profiles to G_s and G_i from the WT A_{2A} receptor. There was some decrease in specific binding for each receptor in response to PTX in ligand binding assays but no functional correlate in cAMP assays. The fraction of specific binding sensitive to PTX was less than 25% for each receptor. The introduction of the A_1 3ICL sequence into the A_{2A} receptor caused coupling to G_i with the fraction of PTX-sensitive specific binding equalling 70% of the GTP[S]-sensitive binding. For the A_{2A}/A_1LT chimaeric receptor the fraction of GTP[S] binding sensitive to PTX increased to more than 95%.

Basal cAMP levels for the A_{2A}/A_1L and A_{2A}/A_1LT chimaeras were similar to those measured in WT A_1 receptor (Figure 5). Therefore the addition of the A_1 C-terminus to the A_1 3ICL sequence of A_{2A}/A_1 chimaeras increased the proportion of receptors coupled to G_i and decreased coupling to G_s. The A_{2A}/A_1LT chimaeric receptor did not inhibit adenylate cyclase despite having a very low coupling fraction to G_s. Binding in the presence of GTP[S] and PTX confirms the results of cAMP experiments by suggesting a receptor predominantly coupled to G_i, but with some double coupling to G_s.

DISCUSSION

This study establishes contributions by both the 3ICL and the C-terminus of the A_1 adenosine receptor in determining coupling selectivity for G_i. As with the A_{2A} receptor [7], the major determinant of coupling selectivity for the A_1 adenosine receptor is the 3ICL; however, unlike the A_{2A} receptor, the C-terminus domain of the A_1 receptor has a modulatory role. In contrast with A_{2A}/A_1L, stimulation of the A_{2A}/A_1LT chimaera showed no increase in cAMP above baseline, which is consistent with predominant coupling to G_i/G_o. This suggests a co-operative interaction between the A_1 3ICL and C-terminus in directing coupling to G_i/G_o. An agonist-stimulated increase in cAMP in the presence of PTX indicates that A_{2A}/A_1LT also couples to G_s. A_{2A} receptors with A_1 3ICL accumulated cAMP in response to agonists, but on the basis of binding studies they seem to be predominantly coupled to G_i/G_o. These results suggest that only a small fraction of adenosine receptors need to couple to G_s to stimulate adenylate cyclase.

Previous investigations have shown that both the 3ICL and the C-terminus of the β_2-receptor influence G_s coupling [9,11]. The C-terminus of the α_1-adrenergic receptor [28] is involved in the coupling efficiency of this receptor to G_q. The C-terminus of the bovine prostaglandin EP3 receptor has been shown to be involved in coupling to G_i : alternative splicing of the C-terminus determines G-protein selectivity for G_s, G_i and G_q [29]. The fact that the A_1 C-terminus alone has no effect on coupling but acts to increase coupling to G_i in the presence of the A_1 3ICL is reminiscent of the relationship between the 2ICL and 3ICL of the M1 muscarinic receptor [12], in which the 2ICL modulates coupling determined by the 3ICL.

The coupling of both A_1 and A_{2A} receptors is specified by determinants in addition to the 3ICL, as demonstrated by the observation that chimaeric receptors in which 3ICL sequences have been switched are doubly coupled. As with A_{2A} 3ICL substituted into A_1 receptors, the introduction of the 3ICL sequence from the β_2-adrenergic receptor into the G_i-coupled M2 muscarinic receptor results in a chimaeric receptor that couples to both G_s and G_i and is stimulatory to adenylate cyclase [30]. The A_{2A}/A_1L chimaeric receptor remains coupled to G_s in the absence of A_{2A} 3ICL sequence. Whereas the C-terminus of the A_{2A} receptor does not seem to influence coupling to G_s, Olah has shown that there are other receptor domains, including the 2ICL, that modulate coupling to G_s in A_{2A} adenosine receptor [7]. The interaction between receptors and G-proteins is complex and the intracellular domains might not solely account for it. Some studies suggest a role for TM amino acids in influencing G-protein selectivity [32,33]. Mapping the precise role of each domain contributing to G-protein selectivity for A_1 and A_{2A} adenosine receptors will require further investigation.

WT and chimaeric adenosine receptors with the A_{2A} 3ICL show constitutive activity. Receptors with the A_{2A} 3ICL sequence had an elevation of basal cAMP levels over untransfected HEK-293 cells and showed cAMP suppression in the presence of the non-selective inverse agonist XAC. Convincing evidence of constitutive activity of receptors with the A_1 3ICL sequence was not demonstrated, given that adding XAC did not produce an increase in cAMP levels. Although cells expressing receptors with the A_1 3ICL did not have a statistically significant suppression of basal cAMP levels in comparison with untransfected cells, the basal levels in untransfected cells were so low that further suppression would have been difficult to identify. Despite showing cAMP accumulation in response to agonist, chimaeric receptors with A_1 3ICL sequence had lower basal cAMP levels than receptors with A_{2A} 3ICL sequence. They also displayed a marked attenuation of maximal cAMP response to forskolin stimulation in comparison with both untransfected cells and cells transfected with receptors containing the A_{2A} 3ICL. An inhibitory modulatory effect on cAMP accumulation was seen in all cells expressing the A_1 3ICL sequence.

The binding results show a greater coupling to G_i/G_o than to G_s for A_{2A}/A_1L and A_{2A}/A_1LT chimaeric receptors; however, neither inhibited cAMP in response to agonist stimulation. It therefore seems that, for A_1 and A_{2A} adenosine receptors, the stimulation of G_s predominates over that of G_i functionally in the HEK-293 cell system. The results seen with A_1/A_{2A} adenosine receptor chimaeras were similar to those seen in chimaeric human β_2/α_{2A} adrenergic receptors expressed in CHW-112 cells [11] in which β_2 receptors containing the α_{2A} sequence in the 3ICL and/or the C-terminus showed an attenuated stimulation of adenylate cyclase but preserved a stimulatory response. When expressed in HEK-293 cells, the β_2 receptor is capable of coupling to either G_s or G_i but the effect on adenylate cyclase is stimulatory. The G_i coupling might occur only after receptor uncouples from G_s [34]. Our binding results to the WT A_{2A} and A_{2A}/A_1T chimaeric receptors suggest that a small fraction of binding to these receptors is PTX-sensitive. Although no functional effects of PTX were observed in cAMP assays, the results raise the possibility that WT A_{2A} receptors, like β-adrenergic receptors, might be able to couple to G_i/G_o under some circumstances. The dominance of the G_s effect for the β_2 adrenergic receptor is in contrast with that for the WT α_{2A} adrenergic receptor expressed in Chinese hamster ovary cells in which the receptor couples to both G_s and G_i but the G_i-mediated inhibition of adenylate cyclase is functionally predominant in the absence of PTX [35]. The same receptor expressed in Chinese hamster fibroblasts or COS-7 cells shows exclusively adenylate cyclase inhibition or stimulation respectively when challenged with agonist [35]. Results on several receptor subtypes indicate that for doubly coupled receptors the predominant functional effects depend on the receptor subtype, on the relative fraction of receptors coupled to a given G-protein and on the cell type in which the receptor is expressed. The predominant functional effect of one G-protein over another in a given cellular context might come into play when single cells express different receptors that activate G_s and G_i/G_o simultaneously. For example, in the myocardium, adenosine has been noted to activate G_s via A_{2A} receptors, and G_i/G_o via A_1 receptors [36].

The observation that A_{2A} receptors are poorly sensitive to GTP[S] is not new. It has been proposed that the A_{2A} is insensitive to guanine nucleotides because it is 'tightly coupled' [37]. However, our work suggests that the A_{2A} receptor is poorly coupled to G_s and binding is therefore only weakly sensitive to GTP[S] [23]. The A_{2A} agonist radioligands [^{125}I]APE and [^{3}H]CGS 21680 detect A_{2A} receptors in both high-affinity and low-affinity states, whereas [^{125}I]ABA detects mostly high-affinity binding to A_1 receptors. The fact that $A_1/A_{2A}L$ chimaeric receptors show much less GTP[S]-sensitive [^{125}I]ABA binding than do WT A_1

receptors, despite being able to cause the robust accumulation of cAMP in response to agonist, lends credence to the hypothesis that WT A_{2A} receptors are poorly coupled to G_s. $A_1/A_{2A}L$ and $A_1/A_{2A}LT$ receptors, which couple to both G_i/G_o and G_s, display more GTP[S] sensitivity than WT A_{2A} receptors, indicating a larger fraction of coupled receptors than of WT A_{2A}, which couples mainly to G_s. Overall, receptors predominantly coupled to G_s showed higher numbers of low-affinity or uncoupled receptors, suggesting that a small percentage of receptors need to be activated to cause a stimulatory effect and either that there is not much G_s present in the cells or that receptor access to G_s is somehow restricted.

In summary, the coupling of the A_{2A} receptor to G_s is determined largely by the 3ICL and not the C-terminus. Coupling of the A_1 receptor to G_i/G_o involves the 3ICL and the C-terminus. Chimaeric receptors with a 3ICL sequence discordant from the TM backbone display double coupling to G_i/G_o and G_s. When doubly coupled, G_s coupling predominates functionally for agonist-stimulated A_1 and A_{2A} adenosine receptors expressed in HEK-293 cells, even for receptors in which a larger fraction of receptors couple to G_i. The magnitude of cAMP accumulation in HEK-293 cells in the presence and in the absence of stimulation with forskolin are determined by the relative coupling to G_i and G_s for expressed receptors, which is in turn determined by the sequence of the 3ICL.

We acknowledge Anna S. Robeva and Heidi Figler for their experimental and graphical assistance and for helpful discussions. This work was supported by NIH Grant K08 HL03268 and by an American Heart Association–Virginia Affiliate Grant-In-Aid VA 95-G18, both to A. L. T.

REFERENCES

1 Olah, M. E. and Stiles, G. L. (1995) Adenosine receptor subtypes: characterization and therapeutic regulation. Annu. Rev. Pharmacol. Toxicol. **35**, 581–606
2 Tucker, A. L. and Linden, J. (1993) Cloned receptors and cardiovascular responses to adenosine. Cardiovasc. Res. **27**, 62–67
3 Olsson, R. A. and Pearson, J. D. (1990) Cardiovascular purinoceptors. Physiol. Rev. **70**, 761–845
4 Bellardinelli, L., Linden, J. and Berne, R. M. (1989) The cardiac effects of adenosine. Prog. Cardiovasc. Dis. **32**, 73–97
5 Zhou, Q-Y., Li, C., Olah, M. E., Johnson, R. A., Stiles, G. L. and Civelli, O. (1992) Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. Proc. Natl. Acad. Sci. U.S.A. **89**, 7432–7436
6 Palmer, T. M., Gettys, T. W. and Stiles, G. L. (1995) Differential interaction with and regulation of multiple G-proteins by the rat A_3 adenosine receptor. J. Biol. Chem. **270**, 16895–16902
7 Olah, M. E. (1997) Identification of A_{2a} adenosine receptor domains involved in selective coupling to G_s. Analysis of chimeric A_1/A_{2a} adenosine receptors. J. Biol. Chem. **272**, 337–344
8 Gao, Z., Chen, T., Weber, M. J. and Linden, J. (1999) A_{2B} adenosine and $P2Y_2$ receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 cells. J. Biol. Chem. **274**, 5972–5980
9 O'Dowd, B. F., Hnatowich, M., Regan, J. W., Leader, W. M., Caron, M. G. and Lefkowitz, R. J. (1988) Site-directed mutagenesis of the cytoplasmic domains of the human β_2-adrenergic receptor. J. Biol. Chem. **263**, 15985–15992
10 Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. and Lefkowitz, R. J. (1988) Chimeric α_2-,β_2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. Science **240**, 1310–1316
11 Liggett, S. B., Caron, M. G., Lefkowitz, R. J. and Hnatowich, M. (1991) Coupling of a mutated form of the human β_2-adrenergic receptor to G_i and G_s. J. Biol. Chem. **266**, 4816–4821
12 Wong, S. K. F., Parker, E. M. and Ross, E. M. (1990) Chimeric muscarinic cholinergic:β-adrenergic receptors that activate G_s in response to muscarinic agonists. J. Biol. Chem. **265**, 6219–6224
13 Hill-Eubanks, D., Burstein, E. S., Spalding, T. A., Brauner-Osborne, H. and Brann, M. R. (1996) Structure of a G-protein-coupling domain of a muscarinic receptor predicted by random saturation mutagenesis. J. Biol. Chem. **271**, 3058–3065
14 Wess, J., Brann, M. R. and Bonner, T. I. (1989) Identification of a small intracellular region of the muscarinic m3 receptor as a determinant of selective coupling to PI turnover. FEBS Lett. **258**, 133–136
15 Bluml, K., Mutschler, E. and Wess, J. (1994) Identification of an intracellular tyrosine residue critical for muscarinic receptor-mediated stimulation of phosphatidylinositol hydrolysis. J. Biol. Chem. **269**, 402–405
16 Burstein, E. S., Spalding, T. A. and Brann, M. R. (1996) Amino acid side chains that define muscarinic receptor/G-protein coupling: studies of the third intracellular loop. J. Biol. Chem. **271**, 2882–2885
17 Hogger, P., Shockley, M. S., Lameh, J. and Sadee, W. (1995) Activating and inactivating mutations in N-and C-terminal i3 loop junctions of muscarinic acetylcholine Hm1 receptors. J. Biol. Chem. **270**, 7405–7410
18 Lechleiter, J., Hellmiss, R., Duerson, K., Ennulat, D., David, N., Clapham, D. and Peralta, E. (1990) Distinct sequence elements control the specificity of G protein activation by muscarinic acetylcholine receptor subtypes. EMBO J. **9**, 4381–4390
19 Kubo, T., Bujo, H., Akiba, I., Nakai, J., Mishina, M. and Numa, S. (1988) Location of a region of the muscarinic acetylcholine receptor involved in selective effector coupling. FEBS Lett. **241**, 119–125
20 Liu, J., Blin, N., Conklin, B. R. and Wess, J. (1996) Molecular mechanisms involved in muscarinic acetylcholine receptor-mediated G protein activation studied by insertion mutagenesis. J. Biol. Chem. **271**, 6172–6178
21 Moro, O., Lameh, J., Hogger, P. and Sadee, W. (1993) Hydrophobic amino acid in the i2 loop plays a key role in receptor–G protein coupling. J. Biol. Chem. **268**, 22273–22276
22 Linden, J., Patel, A. and Sadek, S. (1985) [^{125}I]Aminobenzyladenosine, a new radioligand with improved specific binding to adenosine receptors in heart. Circ. Res. **56**, 279–284
23 Luthin, D. R., Olsson, R. A., Thompson, R. D., Sawmiller, D. R. and Linden, J. (1995) Characterization of two affinity states of adenosine A_{2a} receptors with a new radioligand, 2-[2-(4-amino-3-[^{125}I]iodophenyl)ethylamino]adenosine. Mol. Pharmacol. **47**, 307–313
24 Stowell, C. P., Kuhlenschmidt, T. G. and Hoppe, C. A. (1978) A fluorescamine assay for submicrogram quantities of protein in the presence of Triton X-100. Anal. Biochem. **85**, 572–580
25 Brooker, G., Terasaki, W. L. and Price, M. G. (1976) Gammaflow: a completely automated radioimmunoassay system. Science **194**, 270–276
26 Linden, J. (1982) Calculating the dissociation constant of an unlabeled compound from the concentration required to displace radiolabel binding by 50%. J. Cyclic Nucleotide Res. **8**, 163–172
27 Tucker, A. L., Robeva, A. S., Taylor, H. E., Holeton, D., Bockner, M., Lynch, K. R. and Linden, J. (1994) A_1 adenosine receptors. Two amino acids are responsible for species differences in ligand recognition. J. Biol. Chem. **269**, 27900–27906
28 Cotecchia, S., Exum, S., Caron, M. G. and Lefkowitz, R. J. (1990) Regions of the α_1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. Proc. Natl. Acad. Sci. U.S.A. **87**, 2896–2900
29 Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A. and Narumiya, S. (1993) Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. Nature (London) **365**, 166–170
30 Wong, S. K. and Ross, E. M. (1994) Chimeric muscarinic cholinergic:β-adrenergic receptors that are functionally promiscuous among G proteins. J. Biol. Chem. **269**, 18968–18976
31 Reference deleted
32 Hunyady, L., Bor, M., Balla, T. and Catt, K. J. (1995) Critical role of a conserved intramembrane tyrosine residue in angiotensin II receptor activation. J. Biol. Chem. **270**, 9702–9705
33 Negishi, M., Irie, A., Sugimoto, Y., Namba, T. and Ichikawa, A. (1995) Selective coupling of prostaglandin E receptor EP3D to G_i and G_s through interaction of α-carboxylic acid of agonist and arginine residue of seventh transmembrane domain. J. Biol. Chem. **270**, 16122–16127
34 Daaka, Y., Luttrell, L. M. and Lefkowitz, R. J. (1997) Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. Nature (London) **390**, 88–91
35 Eason, M. G. and Liggett, S. B. (1995) Identification of a G_s coupling domain in the amino terminus of the third intracellular loop of the α_{2A}-adrenergic receptor: evidence for distinct structural determinants that confer G_s versus G_i coupling. J. Biol. Chem. **270**, 24753–24760
36 Dobson, Jr, J. G. and Fenton, R. A. (1998) Cardiac physiology of adenosine. In Cardiovascular Biology of Purines (Burnstock, G., Dobson, Jr, J. G., Liang, B. T. and Linden, J., eds.), pp. 21–39, Kluwer, Dordrecht
37 Nanoff, C. and Stiles, G. L. (1993) Solubilization and characterization of the A_2-adenosine receptor. J. Recept. Res. **13**, 961–973

Received 13 April 2000/4 August 2000; accepted 11 September 2000