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Pharmacological Characterization of the Rat A_{2a} Adenosine Receptor Functionally Coupled to the Yeast Pheromone Response Pathway

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SUMMARY

The rat A_{2a} adenosine receptor, a G protein-coupled receptor, was functionally expressed in the yeast *Saccharomyces cerevisiae*. High affinity binding sites for A_{2a} adenosine agonists were detected in yeast membranes containing the endogenous G_α protein Gpa1. Agonist saturation binding isotherms using [^{3}H]5′-*N*-ethylcarboxamidoadenosine indicated that the A_{2a} adenosine receptor expressed in yeast cell membranes displays pharmacological properties equivalent to those observed when the receptor is expressed in human embryonic kidney 293 cell membranes. The rank order of potency of various agonists in [^{3}H]5′-*N*-ethylcarboxamidoadenosine competition binding assays performed with yeast cell membranes was comparable to that seen for the receptor expressed in mammalian cell membranes. Adenosine agonist-dependent growth response of yeast strains expressing the A_{2a} adenosine receptor was elicited via activation of the yeast pheromone-response pathway. Induction of a pheromone-responsive *FUS1-HIS3* reporter gene in *far1 his3* cells permits cell growth in medium lacking histidine. The sensitivity of the bioassay was increased by deletion of the *STE2* gene, which encodes the yeast α-mating pheromone receptor. The growth response was dose dependent, and agonists of varying affinities displayed a rank order of potency comparable to that observed in competition binding assays. Agonist-activated growth assays performed in liquid culture gave ED_{50} values for various adenosine agonists consistent with reported K_d values. Yeast strains expressing a single receptor/G protein complex will be useful as a model system for the study of receptor/G protein interactions *in vivo*.

Adenosine receptors are members of the seven-transmembrane domain G protein-coupled receptor superfamily (1). Many physiological effects are influenced by activation of adenosine receptors, including cardiovascular homeostasis, immune responses, and lipid metabolism, as well as many different central nervous system responses (1). In most cases, autacoid regulation of adenosine receptors by endogenous adenosine is potentiated under conditions of increased stress such as hypoxia or ischemia. To date, four subtypes of adenosine receptors, classified as A_1, A_{2a}, A_{2b}, and A_3, have been identified by molecular cloning (2–5). A_1 and A_3 adenosine receptors couple to inhibition of adenylyl cyclase via pertussis toxin-sensitive G proteins (1, 5, 6). The A_{2a} and A_{2b} subtypes are coupled to stimulation of adenylyl cyclase via association with the heterotrimeric G protein G_s (1, 4). The A_{2a} and A_{2b} subtypes can be distinguished pharmacologically using C2-substituted adenosine analogs such as CGS-21680. CGS-21680 binds with high affinity to the A_{2a} subtype but binds with poor affinity to the A_{2b} subtype (7).

Adenosine receptor agonists selective for the A_2 subtypes are purine based and are, in general, expensive and difficult to synthesize (8, 9). In addition, many mammalian cells express multiple subtypes of endogenous adenosine receptors as well as multiple G proteins. High-throughput screening and studies of ligand/receptor and receptor/G protein interactions could be simplified by an expression system that expresses one receptor and one heterotrimeric G protein in the absence of endogenous receptors or G proteins. The yeast *Saccharomyces cerevisiae* is a model organism for such molecular biological and genetic studies (10). Yeast requires the action of G protein-coupled pheromone receptors and heterotrimeric G proteins to regulate the pheromone-response pathway (11). Haploid yeast cells detect the presence of peptide-mating pheromones through activation of endogenous G protein-coupled pheromone receptors (Ste2 and Ste3) and heterotrimeric G proteins (Gpa1, Ste4, and Ste18), stimulating a signal transduction pathway that results in cell cycle arrest and transcriptional induction of pheromone-responsive genes (11). Each of these genes can be deleted by homologous recombination and replaced with constructs expressing

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ABBREVIATIONS: SSTR2, somatostatin receptor subtype 2; HEK, human embryonic kidney; NECA, 5′-*N*-ethylcarboxamidoadenosine; PIA, N^6-phenylisopropyladenosine; CHA, N^6-cyclohexyladenosine; AT, 3-amino-1,2,4-triazole; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

receptors and G proteins of interest. We previously reported that the G_i-coupled SSTR2 can be functionally coupled to the yeast pheromone-response pathway (12). Yeast strains were genetically modified by deletion of the *far1* gene (*FAR1* encodes a negative regulator of G1 cyclins) to respond to agonist activation of the receptor by cell growth instead of cell cycle arrest. Growth of strains expressing SSTR2 was shown to be dependent on somatostatin agonists (12). In this report, we demonstrate the functional coupling of a G_s-coupled receptor, the rat A_{2a} adenosine receptor, to the yeast pheromone-response pathway.

Materials and Methods

Reagents and molecular biological manipulations. All adenosine analogs were purchased from Research Biochemicals (Natick, MA). Molecular biological manipulations were performed by standard methods (13). All other reagents were of the highest grade possible.

Cloning of the rat A_{2a} adenosine receptor. First-strand cDNA was synthesized from rat brain mRNA using Superscript Reverse Transcriptase and oligo$(dT)_{16}$ (Life Technologies, Grand Island, NY) following the protocol provided with the kit. The cDNA template was then used to amplify the rat adenosine A_{2a} receptor (14) by polymerase chain reaction in two overlapping halves using the following synthetic oligonucleotides: primer 1, 5′-AAACAAGCTTGCCATGGGCTCCTCGGTGTACA-3′; primer 2, 5′-TGCAGCGTGGACCGAGTCCGCTC-3′; primer 3, 5′-ATGCTGGCCATCTACCTACGGATT-3′; and primer 4, 5′-AAAATCTAGATCAGGAAGGGGCAAACTCTGA-3′.

Primers 1 and 2 and primers 3 and 4 were used in pairs to amplify the 5′ and 3′ halves of the receptor, respectively. Primers 1 and 4 encode *Hin*dIII and *Xba*I sites. Primers 2 and 3 were designed to include the unique *Pvu*II restriction site within the receptor. The amplified fragments were digested with the appropriate enzymes, purified, and ligated together. The ligated halves were then amplified with primers 1 and 4 to create the complete coding region of the receptor. After digestion with *Hin*dIII and *Xba*I, the full-length receptor DNA was cloned into pCRII (InVitrogen, San Diego, CA). The final construct was confirmed by automated DNA sequence analysis.

Yeast expression vectors. A *Xho*I/*Kpn*I polymerase chain reaction fragment of the *ADH1* gene (15) encoding its transcriptional terminator (nucleotides 1798–1815) was amplified using primers CGAATTTCTTATGATTT (5′) and GGACGGATTACAACAGGT (3′). Plasmid pLP99 was created by the insertion of this fragment after digestion with the appropriate enzymes into corresponding sites in pRS426 (multicopy, *URA3*). A *Sac*I/*Bam*HI fragment encoding the *ADH1* promoter (nucleotides 226–750) was amplified using primers TGATGGTACATAACG (5′) and TGTATATGAGAGTTGA (3′), digested with appropriate enzymes, and inserted into pLP99, creating the expression vector pLP100. The rat A_{2a} adenosine receptor was amplified using primers AAAGATCTAAAATGGGCTCCTCGGTGTAC (5′) and AAGTCGACTCAGGAAGGGGCAAACTC (3′) introducing a *Bgl*II site at the −3 position and a *Sal*I site immediately after the stop codon at position +1234 of the coding sequence. The fragment was digested with the appropriate enzymes and inserted into corresponding sites in pLP100, forming pLP116. The yeast expression vector pLP83 harboring the yeast G_α gene, *GPA1*, has been described previously (12).

Construction of yeast strains. Yeast cell culture and media conditions were according to standard conditions, and DNA-mediated transformation was performed using the lithium acetate method (16). Construction of the parent yeast strain LY296 (Table 1) as used in the current study has been described in detail (12). Yeast strains expressing the rat A_{2a} adenosine receptor were constructed by transforming LY296 with the receptor expression vector pLP116, creating LY574. LY574 was transformed with the yeast G_α protein expression plasmid pLP83 (12), creating LY594. LY594 was transformed with the *ste2ΔLEU2* allele found in plasmid pAB506 (17) to create LY764. At least four individual transformants from each strain were chosen for analysis.

TABLE 1
Genotypes of yeast strains

Strain	Receptor		Genotype
	Ste2	A_{2a} Adenosine	
LY296	+	−	MATa gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3Δ200 leu2Δ1
LY574	+	+	LY296 pLP116
LY594	+	+	LY296 pLP116, pLP83
LY764	−	+	LY594 *ste2ΔLEU2*

Mammalian cell expression. The *Hin*dIII/*Xba*I-digested full-length receptor DNA was inserted into pRC/CMV (InVitrogen) and subsequently used to transfect HEK 293 cells (American Type Culture Collection, Rockville, MD) using a calcium phosphate transfection kit from Stratagene (La Jolla, CA). Stable, G-418 resistant (2 mg/ml) clones were identified and examined for their abilities to bind [^{3}H]NECA in the presence of 20 nM DPCPX. DPCPX, an A_1 adenosine receptor-selective antagonist, binds the A_1 receptor subtype with ~1000-fold higher affinity than the A_{2a} adenosine receptor subtype (18). DPCPX (20 nM) blocks A_1 receptor sites that may be present in HEK 293 cells without affecting [^{3}H]NECA binding to A_{2a} adenosine receptor sites. One clone expressing high levels of [^{3}H]NECA binding was chosen for further studies.

Radioligand binding assays. All radioligand binding assays were performed in 96-well microtiter plates using binding buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.25% bovine serum albumin) containing protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 100 μg/ml bacitracin, and 100 μg/ml benzamidine). All components were diluted in binding buffer containing protease inhibitors and added to the microtiter plate wells in the following order: binding buffer, nonradiolabeled competitor, and [^{3}H]NECA (25–37 Ci/mmol, Amersham, Arlington Heights, IL). Membranes were diluted in binding buffer containing protease inhibitors and treated with 2 units/ml adenosine deaminase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) at 37° for 20 min. The A_1 adenosine receptor-selective antagonist DPCPX was added at a final concentration of 20 nM. Binding reactions were initiated by adding 5–70 μg of membrane protein in a 170-μl volume. Final reaction volume was 200 μl/well. All incubations were carried out at room temperature for 2 hr. Free radioligand was separated from bound by rapid filtration through a glass-fiber filter using an Inotech cell harvester as described previously (19). The filter disks were washed several times with cold (4°) binding buffer lacking bovine serum albumin before counting in an ICN Biochemicals (Cleveland, OH) liquid scintillation counter (60% efficiency). Radioligand binding studies of the rat A_{2a} adenosine receptor expressed in yeast were performed exactly as described for the receptor expressed in mammalian cells.

Agar plate bioassays. Overnight liquid cultures of LY594 and LY764 in SC-glucose (2%) lacking tryptophan and uracil were centrifuged and resuspended in sterile H_2O. Cells (2×10^5) were plated in 30 ml of SC-galactose (2%) agar medium lacking tryptophan, uracil, and histidine (adjusted to pH 6.7 with concentrated KOH and equilibrated to 50°). Adenosine deaminase (1.3 units/ml) was added to the media. The indicated amounts of selected adenosine agonists were applied to the surface of the agar, and the plates were incubated at 30° for 48–72 hr (12).

Liquid bioassays. LY594 and LY764 cells were cultured overnight in SC-glucose (2%) lacking tryptophan and uracil. The cells were diluted 1000-fold in SC-glucose lacking tryptophan, uracil, and histidine, pH 6.8, and containing adenosine deaminase (0.13 unit/ml). LY764 cells were cultured in the presence of 5 mM AT to

decrease basal growth rate. AT is a competitive inhibitor of the *HIS3* gene product imidazoleglycerol-phosphate dehydratase. Samples of the cell suspension (180 μl) were dispensed to wells of sterile 96-well microtiter dishes containing 20 μl of serially diluted samples (10^{-4} to -10^{-11} M) of adenosine receptor agonists. The plates were incubated at 30° for 18 hr with agitation (600 rpm). Growth was monitored by recording increases in absorbance at 620 nm using a microplate reader. Assays were conducted in duplicate, and growth rate measurements were obtained during the logarithmic phase of yeast cell growth.

Data analysis. All data were analyzed using GraphPAD Prism (GraphPAD Software, San Diego, CA) and are presented as the mean ± standard deviation unless noted. K_i values were determined using the equation of Cheng and Prusoff (20).

Results

The A_{2a} adenosine receptor was functionally expressed in yeast to facilitate a detailed examination of the ligand-binding properties and protein/protein interactions of the receptor. Receptor expression was achieved by placing the rat A_{2a} adenosine receptor under the control of the alcohol dehydrogenase promoter (*ADH1*, Ref. 15) in plasmid pLP116 (Fig. 1). Coupling of the A_{2a} adenosine receptor to downstream elements of the pheromone-response pathway requires G_α protein. The parent yeast strain used in this analysis, LY296, lacks a functional chromosomal *GPA1* gene (12). Complementation of the *GPA1* deficiency with various plasmid-borne G_α protein-encoding genes allows considerable flexibility in examining receptor/G_α protein interactions. In preliminary experiments, either $G_{\alpha s}$ or a chimeric Gpa1-$G_{\alpha s}$ protein were expressed under the control of the *GPA1* promoter along with the A_{2a} adenosine receptor. Under these conditions, neither $G_{\alpha s}$ nor Gpa1-$G_{\alpha s}$ fully suppressed the pheromone-response pathway. The resulting receptor- and agonist-independent growth observed made determination of the agonist-dependent growth response difficult. In a previous report, the rat SSTR2 was found to couple efficiently to the yeast G_α protein, Gpa1 (12). Thus, in the present study, G_α protein was supplied by the *GPA1* allele present in pLP83 (12). The genotypes of the yeast strains used in the present study are displayed in Table 1.

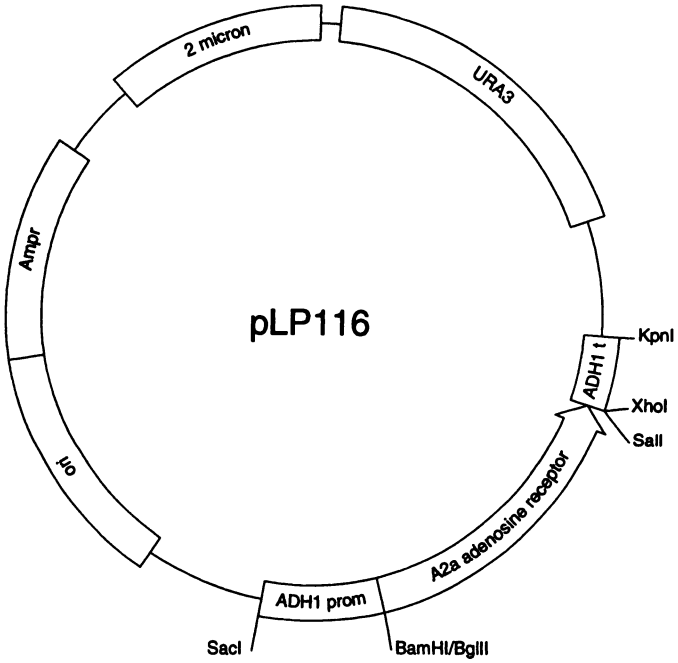

Fig. 1. Plasmid pLP116 confers expression of the rat A_{2a} adenosine receptor in yeast. The yeast expression plasmid pLP116 for expression of the rat A_{2a} adenosine receptor under the control of the *ADH1* promoter was constructed as described in Materials and Methods.

The ligand-binding properties of the rat A_{2a} adenosine receptor expressed in yeast were examined by [^{3}H]NECA binding to crude membrane fractions prepared from both yeast and HEK 293 cells transformed with rat A_{2a} adenosine receptor expression plasmids. Membranes prepared from yeast strains expressing the rat A_{2a} adenosine receptor (LY594) and HEK 293 cells stably transfected with the rat A_{2a} adenosine receptor cDNA exhibited saturable binding of the adenosine agonist [^{3}H]NECA (Fig. 2 and Table 2). Saturation binding isotherm analysis indicated that the calculated affinities of [^{3}H]NECA were equivalent for the mammalian and the yeast-expressed receptor (Table 2). The total number of [^{3}H]NECA binding sites observed was consistent with values obtained for the yeast α-mating pheromone receptor (200 fmol/mg, Ref. 21). There was no detectable specific or saturable binding of [^{3}H]NECA in membranes prepared from wild-type HEK 293 cells (in the presence of the A_1 adenosine receptor antagonist DPCPX) or in yeast cells lacking pLP116 (LY296) (Table 2).

Previous reports have demonstrated that tritiated ligands such as [^{3}H]NECA or [^{3}H]CGS-21680 discriminate poorly between high and low affinity states for A_{2a} adenosine receptors (22, 23). Saturation binding of [^{3}H]NECA in membranes from LY574, a strain lacking Gpa1, revealed no significant difference in binding affinity (K_d = 43 ± 22 nM) compared with membranes prepared from strains containing Gpa1 (LY594). However, for many G protein-coupled receptors, high and low affinity agonist binding sites can be identified by their sensitivity to nonhydrolyzable GTP analogs such as GTPγS. In membranes prepared from either HEK 293 cells or LY594 cells, 1 μM GTPγS decreased [^{3}H]NECA (20 nM) binding by 25%, indicating the presence of A_{2a} adenosine receptors coupled to G_α. These data suggest the presence of high affinity [^{3}H]NECA-binding sites in LY594 membranes.

Examination of [^{3}H]NECA competition binding with several agonists of varying affinities yielded results consistent with those observed with the saturation binding analysis (Fig. 3 and Table 3). The pharmacological profile of the receptor expressed in LY594 cells was equivalent to that observed for the receptor expressed in HEK 293 cells and in previous reports (9). The rank order of potency and binding affinities of the agonists tested were the same in both the yeast and mammalian systems. The rank order of potency for the agonists was found to be NECA > *R*-PIA > CHA ≥ *S*-PIA. *R*-PIA bound to the yeast-expressed receptor with a 10-fold-higher affinity than *S*-PIA, indicating that stereoselectivity for this ligand is unaltered when the receptor is expressed in yeast.

In addition to the rat A_{2a} adenosine receptor, LY594 cells express the G protein-coupled α-mating pheromone receptor Ste2 in their plasma membranes (12). To examine the effects of Ste2 on the binding properties of the rat A_{2a} adenosine receptor, the *STE2* gene was deleted from LY594 to create strain LY764. As measured by [^{3}H]NECA saturation binding and competition binding, no significant differences were observed in the affinity of ligand binding to LY594 and LY764

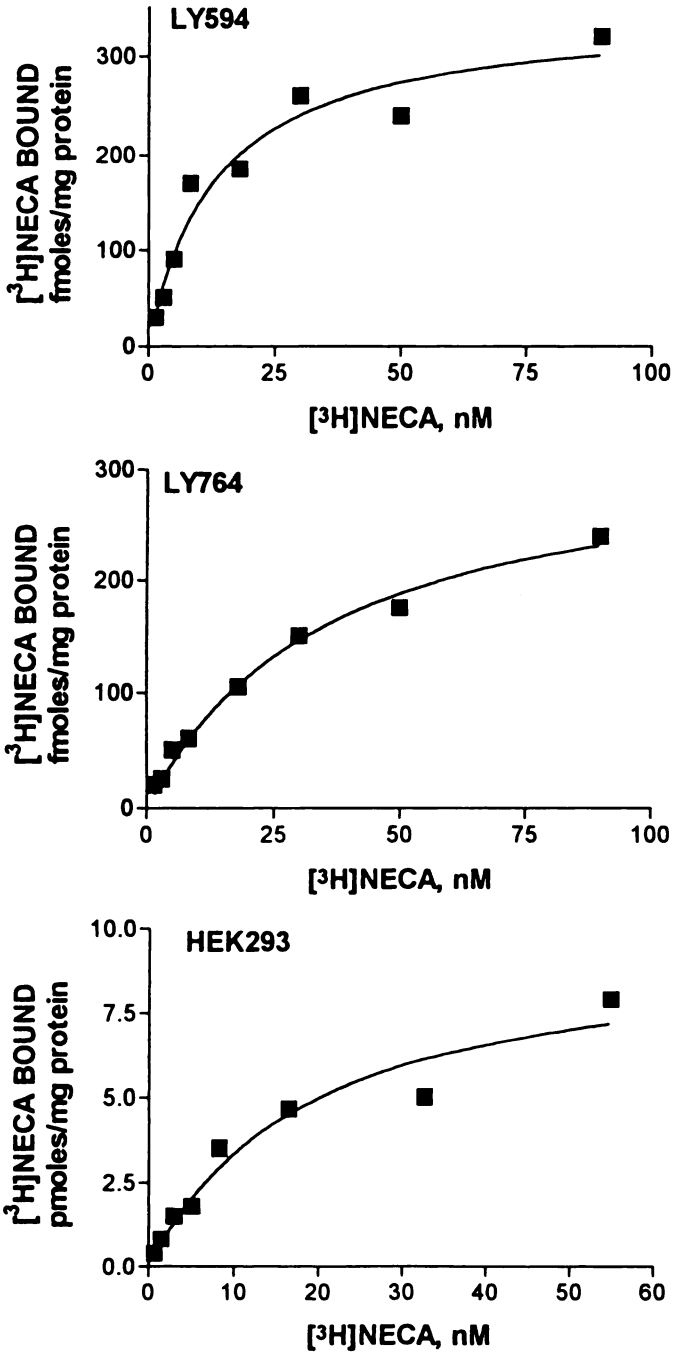

Fig. 2. [^{3}H]NECA saturation radioligand binding to the rat A_{2a} adenosine receptor expressed in yeast and HEK 293 cells. *Top* and *middle*, crude membrane fractions from yeast strains LY594 and LY764 (Table 1) were prepared, and saturation binding analysis was performed with [^{3}H]NECA as described in Materials and Methods. *Bottom*, crude membrane fractions from HEK 293 cells expressing the rat A_{2a} adenosine receptor were prepared, and saturation binding was performed as described in Materials and Methods. Data are representative of experiments performed in duplicate. Nonspecific binding ranged from 5% to 20%.

membranes. The expression level of the A_{2a} adenosine receptor measured by [^{3}H]NECA saturation binding in strain LY594 (*STE2*) was approximately twice that observed in LY764 (*ste2ΔLEU2*) cells (Fig. 2 and Table 2).

The ability of the A_{2a} adenosine receptor to couple to the pheromone-response pathway was examined in agar plate

TABLE 2

Summary of radioligand binding data of mammalian and yeast strains expressing the rat A_{2a} adenosine receptor

Cell line	Expression system	[^{3}H]NECA binding	
		K_d	B_{max}
		nM	*fmol/mg of protein*
Wild-type[a]	HEK 293		NB
Transfected[b]	HEK 293	42 ± 18	18,000 ± 4,400
LY296[a]	Yeast		NB
LY594[b]	Yeast	26 ± 12	450 ± 128
LY764[b]	Yeast	36 ± 24	262 ± 66

NB, no detectable binding.
[a] Data from two independent determinations.
[b] Data from three independent determinations.

bioassays conducted as described in Materials and Methods. In preliminary analyses of the yeast cell response to adenosine agonists, an unexpectedly high level of agonist-independent growth was observed that interfered with the interpretation of results. We surmised that the observed growth might be due to receptor activation by adenosine released into the growth medium by the yeast cells themselves. In mammalian cells, secreted adenosine often forms an autacoid pathway, activating endogenous adenosine receptors (1). Therefore, adenosine deaminase was added to the agar medium to degrade adenosine present in the medium, resulting in decreased background growth and increased signal-to-noise ratio. Adenosine deaminase catalyzes the deamination of adenosine at the C6 position to inosine, inactivating the ligand (24). Under these conditions, a dose-dependent growth response of strains expressing the A_{2a} adenosine receptor (LY594) was observed in response to four different amounts of NECA (Fig. 4). The growth response was proportional to the amount of NECA applied to the plates. Detectable growth of the yeast cells was produced by as little as 50 pmol of NECA in strain LY594 and 5 pmol of NECA in strain LY764. The addition of adenosine deaminase to the media virtually eliminated background growth without altering the size of the zone of growth in response to applied adenosine agonists (Fig. 4). The adenosine deaminase inhibitor *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride produced a very small zone of growth, and when adenosine was applied with *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride, a large zone of growth was observed (data not shown). The addition of the adenosine receptor antagonist 8-(3-chlorostyryl) caffeine to the agar plates also decreased background growth (data not shown). Strains that do not express the A_{2a} adenosine receptor and a strain in which the A_{2a} adenosine receptor was coexpressed with the chimeric G_α protein, Gpa1-$G_{\alpha i2}$ (12), exhibited no detectable growth response to any of the adenosine receptor agonists tested. These results are consistent with A_{2a} adenosine receptor mediated activation of the pheromone-response pathway by coupling to the endogenous heterotrimeric G protein complex.

The role of the *STE2* gene product in regulating the response to the agonist-activated adenosine receptor was examined by comparing the growth response of cells in which *STE2* was deleted (LY764) with those that express a wild-type complement of the pheromone receptor (LY594). Growth in response to NECA was greatly enhanced in LY764 cells relative to LY594 cells (Fig. 4). The time required to detect the growth response for LY764 (48 hr) was significantly less

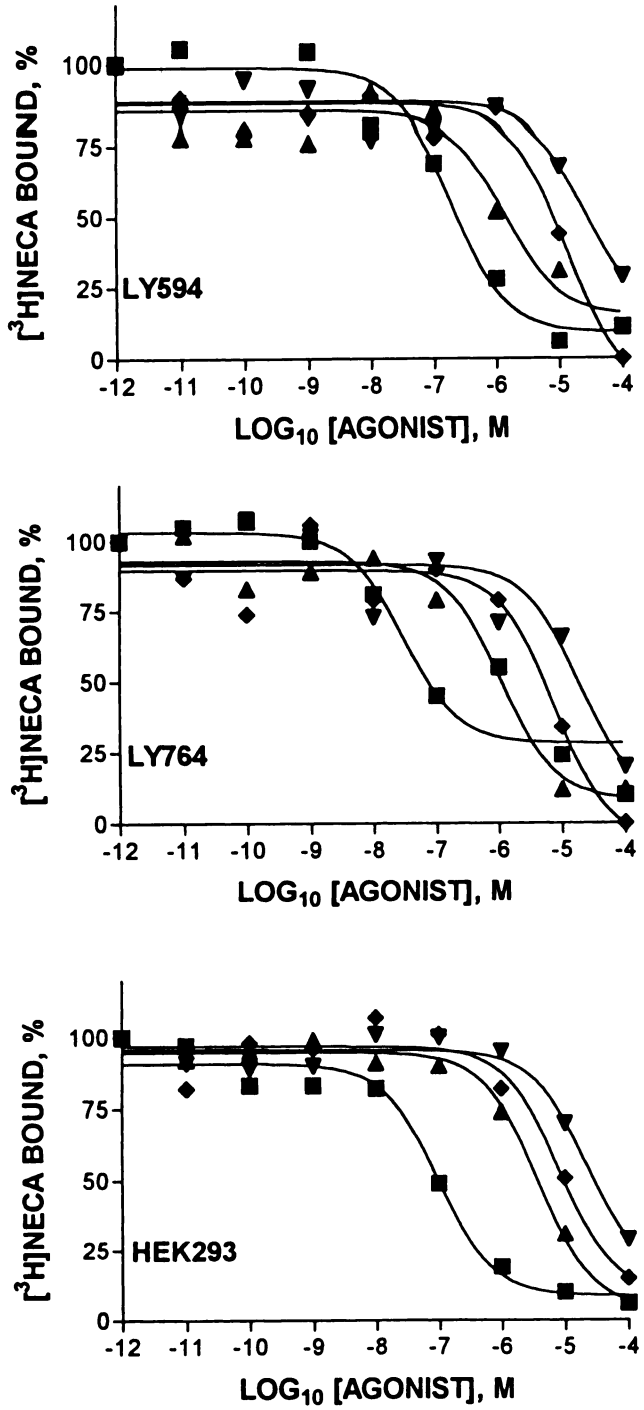

Fig. 3. Competition of [^{3}H]NECA binding by adenosine receptor agonists. The ability of increasing concentrations of adenosine receptor agonists (10^{-12} to 10^{-4} M) to displace [^{3}H]NECA (20 nM) was examined in crude membrane fractions prepared from yeast strains and HEK 293 cells expressing the rat A_{2a} adenosine receptor. Data are representative of experiments performed in duplicate. ■, NECA; ▲, *R*-PIA; ▼, *S*-PIA; ◆, CHA.

than that observed for LY594 (72 hr), although LY764 exhibited higher background growth than LY594. The diameters of the zones of growth produced by agonist treatment were also much larger in the LY764 strain lacking *STE2*. In an effort to reduce the assay incubation time, glucose replaced galactose as the carbon source. The response to agonist of cells plated in media containing glucose was reduced to 18–24 hr. However, the signal-to-noise ratio was decreased when cells were grown in media containing glucose (data not shown). This effect was due to increased background rather than decreased signal.

TABLE 3

Competition binding of adenosine agonists to HEK 293 cells and yeast strains LY594 and LY764 expressing the rat A_{2a} adenosine receptor

Agonist	K_i		
	HEK 293	LY594	LY764
		nM	
NECA	35 ± 5	48 ± 3	12 ± 5
R-PIA	901 ± 88	469 ± 10	241 ± 45
S-PIA	5564 ± 170	5867 ± 1670	4857 ± 105
CHA	3397 ± 777	4366 ± 825	2845 ± 358

Binding data from three independent determinations for all except *R*-PIA and *S*-PIA in strain LY764, for which there were two independent determinations.

To further characterize the A_{2a} adenosine receptor growth response, agonists of varying affinities were examined. The calculated binding affinities ranged from low nanomolar to low micromolar (Table 3), a 500-fold range in affinities. In strains LY594 and LY764, agonists of varying affinities produced a halo of growth proportional to the affinity of the various ligands for the A_{2a} adenosine receptor (Fig. 5). In agreement with the radioligand binding data, the rank order of potency was NECA > *R*-PIA > *S*-PIA ≥ CHA. Comparison of the response to equivalent molar amounts of NECA and α-factor in LY594 suggests that these agonists may activate the pathway with similar efficacy (Fig. 5). As with the dose response to NECA, the halo of growth in response to these agonists was larger in LY764 cells than in LY594 cells.

In mammalian cells, A_{2a} adenosine receptors modulate the activity of an endogenous cAMP-dependent signal transduction pathway through activation of adenylyl cyclase (1). Each adenosine agonist exhibits characteristic potency in stimulation of its effector enzyme. In yeast cells expressing the A_{2a} adenosine receptor, the pheromone response signal transduction pathway is activated by adenosine agonists. To assess the validity of activation of yeast growth as a quantitative measure of the potency of adenosine agonists and to determine whether these values may be reliably related to their ability to stimulate adenylyl cyclase activity, both LY594 and LY764 cells were cultured in liquid medium in the presence of several concentrations of agonists of varying potency. Yeast cell growth was induced by all four agonists tested, and the potencies agreed well with their respective affinities (Table 4 and Fig. 6). The concentration of the agonists at which half-maximal yeast cell growth was achieved agrees well with the rank order of the calculated K_i values (NECA > *R*-PIA > *S*-PIA ≥ CHA) for each compound (Table 4). The rank order of potency was the same in LY594 and LY764; however, the potency of agonist-stimulated growth was slightly improved in LY764 cells compared with LY594 cells despite the fact that the LY764 cells were grown in the presence of 5 mM AT.

Discussion

In this report, we describe a yeast expression system that responds to adenosine agonists by rat A_{2a} adenosine receptor-dependent activation of the pheromone-response path-

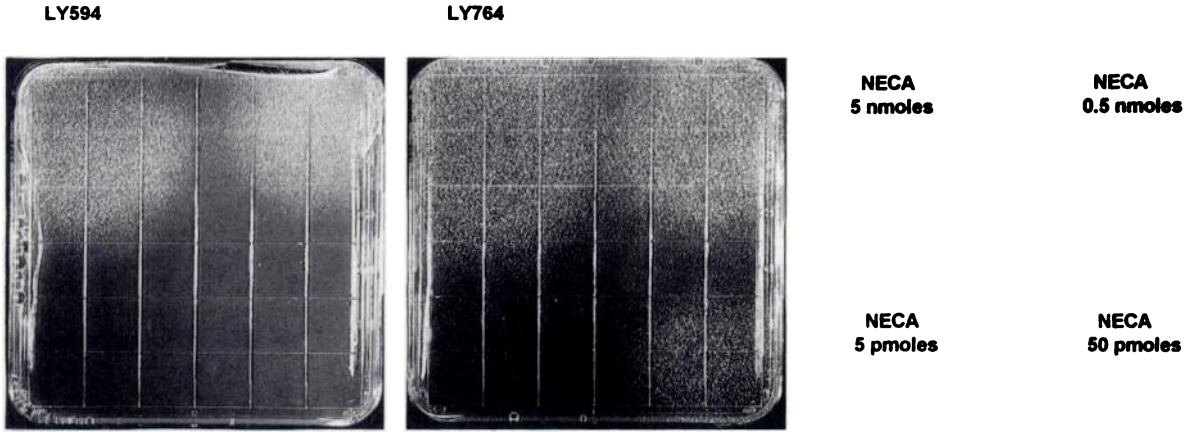

Fig. 4. Growth of yeast in response to increasing concentrations of the adenosine receptor agonist NECA. Samples of overnight cultures of LY594 (*left*) and LY764 (*middle*) cells (Table 1) were plated in agar medium, and NECA was applied as described in Materials and Methods. The plates were incubated at 30° for 48 hr.

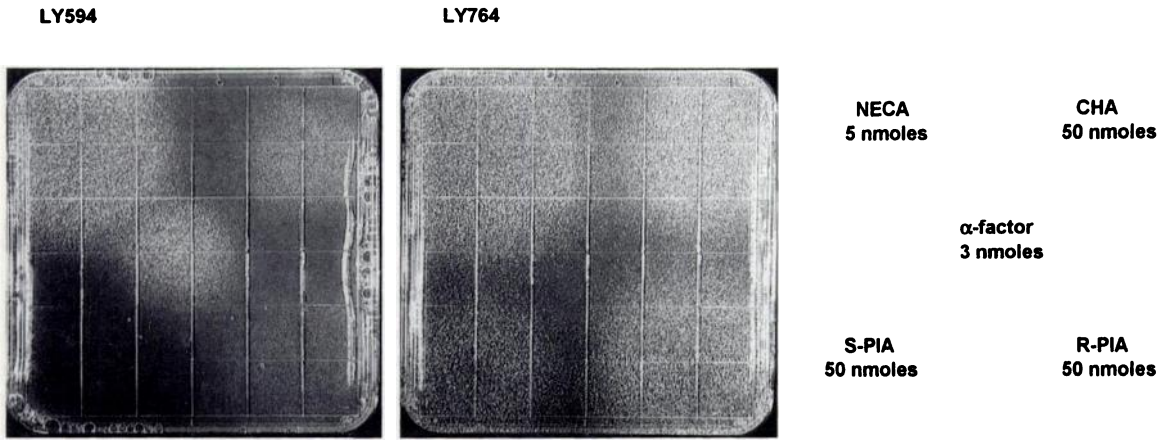

Fig. 5. Growth of yeast in response to adenosine agonists of varying affinities. Samples of overnight cultures of LY594 (*left*) and LY764 (*middle*) cells (Table 1) were plated in agar medium, and each of the agonists (*right*) was applied as described in Materials and Methods. The plates were incubated at 30° for 48 hr.

TABLE 4

ED_{50} values of adenosine agonist-stimulated growth in yeast strains LY594 and LY764

Agonist	ED_{50}	
	LY594	LY764
	nM	
NECA	46 ± 6	28 ± 4
R-PIA	475 ± 187	259 ± 113
S-PIA	2048 ± 371	1158 ± 583
CHA	4775 ± 633	1694 ± 453

Data from four independent determinations.

way. Pharmacological analysis of LY594 and LY764 revealed that the A_{2a} adenosine receptor expressed in yeast displays agonist-binding affinities and rank order of potency equivalent to those observed in mammalian systems. Examination of the signaling capabilities of the A_{2a} adenosine receptor expressed in yeast revealed that the rank order of potency as measured in both plate and liquid assays is the same as that observed in mammalian cells. The ED_{50} values for agonist-stimulated growth are comparable to the calculated agonist binding affinities determined in competition binding assays. In addition, the ED_{50} values for NECA-stimulated growth in yeast are comparable to those reported for NECA-stimulated adenylyl cyclase in rat striatum and PC12 cells (25). These results indicate that the A_{2a} adenosine receptor expressed in yeast assembles in an appropriate agonist-binding conformation, efficiently couples to the yeast heterotrimeric G protein, and transduces the signal inherent in agonist binding via the pheromone-response pathway.

The modifications made to the expression system in this report offer some advantages over our previous results (12). Functional expression of the rat A_{2a} adenosine receptor was accomplished by placing the cDNA under the control of the *ADH1* promoter in pLP116 (Fig. 1). The *ADH1* promoter permits high level expression of heterologous gene products with either glucose or galactose as the carbon source. In our previous report of a mammalian G protein-coupled receptor (SSTR2) expressed in yeast, growth in galactose was required for induction of receptor expression under the control of the *GAL1,10* promoter. Control of receptor expression via the *ADH1* promoter eliminates the need for this induction step and allows for overnight results, whereas growth in galactose required 48 hr. Despite the fact that growth in glucose decreases the signal-to-noise ratio, receptor expression under these conditions enabled detection of agonist binding sites pharmacologically equivalent to those observed in native tissues.

A profound increase in sensitivity to adenosine agonists in the yeast bioassay was observed as a result of deletion of the

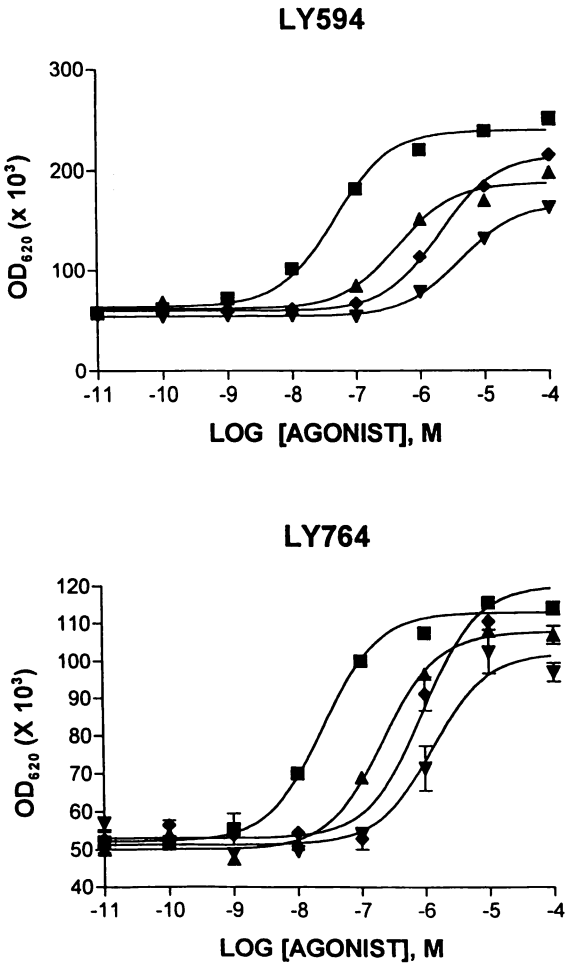

Fig. 6. Adenosine agonist-stimulated growth of yeast strains LY594 and LY764 shown by quantification in liquid bioassays. LY594 and LY764 cells (Table 1) expressing the rat A_{2a} adenosine receptor were cultured in the presence of increasing concentrations of adenosine agonists (10^{-10} to 10^{-4} M). Growth was measured by determining the absorbance at 620 nm. The dose-response curve was plotted using values obtained during the logarithmic phase of growth as described in Materials and Methods. ■, NECA; ▲, *R*-PIA; ▼, *S*-PIA; ◆, CHA. Data are from four independent determinations.

STE2 gene. This effect cannot be accounted for by changes in expression of the A_{2a} adenosine receptor and was not simply due to coexpression of Ste2 blocking expression of the A_{2a} adenosine receptor. The absence of Ste2 had little effect on the total number of detectable A_{2a} adenosine receptor-binding sites. In fact, the B_{max} value determined by [^{3}H]NECA saturation binding isotherms was lower in strain LY764 than in strain LY594 cells. One explanation for the increase in sensitivity may be that Ste2 competes directly with the A_{2a} adenosine receptor for a limited amount of heterotrimeric G protein. The G protein complex is an important regulatory step in the pheromone-response pathway with the pheromone receptors expressed in stoichiometric excess (26). G protein-coupled receptors are thought to interact with the heterotrimeric G protein with low affinity in the absence of agonist; agonist binding confers on the receptor the high affinity agonist binding state. Binding of agonist to receptor in reconstituted systems increases the affinity of receptor for the heterotrimeric G protein, enabling this complex to more efficiently catalyze the G protein-mediated exchange of GDP for GTP (27). Expression of the A_{2a} adenosine receptor in MATa cells establishes a competition with Ste2 receptor for the heterotrimeric G protein complex. By deleting *STE2* in LY764, the only receptor capable of coupling to the G protein is the A_{2a} adenosine receptor. In this model, a greater proportion of available G protein would be coupled to the A_{2a} adenosine receptor, leading to increased sensitivity to agonist. Our data indicate little or no effect of Ste2 on the agonist binding (affinity or B_{max}) of A_{2a} adenosine receptor binding sites, yet the growth response induced by NECA-mediated activation of the A_{2a} adenosine receptor is greatly increased by deletion of *STE2*. In the presence of an adenosine agonist, the A_{2a} adenosine receptor may actually displace Ste2 from its low affinity, agonist-independent interaction with the heterotrimeric G protein.

Alternatively, Ste2 may be exerting a desensitization effect *in trans* in cells responding to agonist-bound A_{2a} adenosine receptor. Mating pheromone receptors unoccupied by agonist have been shown to inhibit the pheromone-response pathway in a G protein-independent manner (28). Inappropriate expression of the a-factor receptor, Ste3, in MATa cells confers dominant resistance to the cell cycle arrest phenotype of MATa cells exposed to a-factor. The effect does not require a-factor, indicating that the unoccupied receptor mediates the activity of an as-yet-uncharacterized adaptation pathway independent of agonist activation of the pheromone-response pathway. Thus, in the absence of Ste2, reassembled G protein complexes may be activated repeatedly by the A_{2a} adenosine receptor.

Reports from other laboratories have yielded limited information on the pharmacology of heterologous G protein-coupled receptors in yeast. King *et al.* (29) demonstrated agonist-stimulated induction of the *S. cerevisiae* pheromone-response pathway mediated by β_2-adrenergic receptors. The receptor expression levels required for the response were extremely high, >100 pmol/mg of protein. In contrast, the A_{2a} adenosine receptor expression levels observed in the current study are well within the expected physiological range in both mammalian and yeast cells. A recent report of expression of the human β_2-adrenergic receptor in the fission yeast *Schizosacharomyces pombe* failed to demonstrate high affinity agonist binding or functional coupling to the pheromone-response pathway (30). In addition, a yeast/mammalian receptor fusion protein was required for efficient expression in *S. pombe*, a modification that is unnecessary for functional receptor expression in *S. cerevisiae* (12). Agonist binding affinities were indicative of receptor uncoupled from its G protein. These results suggest that the *S. pombe* expression system may be useful only for pharmacological analysis of uncoupled receptors or that other manipulations may be required to achieve functional coupling of the receptor to the *S. pombe* G protein. Muscarinic and dopamine receptors expressed in *S. cerevisiae* exhibited neither functional coupling of receptor to the pheromone-response pathway nor high affinity agonist binding (31–33). As reported here, functional A_{2a} adenosine agonist-binding sites in yeast are pharmacologically equivalent to those observed in native tissues. In support of the agonist binding data, growth of yeast cells in response to adenosine receptor agonists displays a rank order of potency equivalent to that observed for mammalian cells. In addition to the G_s-coupled A_{2a} adenosine receptor, we succeeded in obtaining both functional coupling and high

affinity agonist binding of the G_i-coupled SSTR2 (12). For both receptors, the pharmacological properties are similar to those observed in native tissues, indicating that these receptors are functionally coupled to the yeast heterotrimeric G protein. Thus, yeast expression of heterologous G protein-coupled receptors seems to be a generally useful means of rapidly and conveniently determining their pharmacological properties.

Expression of G protein-coupled receptors in yeast confers many advantages over other heterologous expression systems. Heterologous expression in mammalian cells or in the baculovirus system is limited by the presence of endogenous G protein-coupled receptors that may confuse determination of receptor pharmacological properties. For example, studies of specific individual adenosine receptor subtypes are frequently hampered by the endogenous expression of other subtypes. A_1, A_{2a}, and A_{2b} adenosine receptors are expressed together in many tissues and cell lines (34). To obtain an accurate pharmacological profile of the A_{2a} adenosine receptor, binding to contaminating receptor subtypes must be blocked. In yeast, a single receptor and one heterotrimeric G protein complex may be expressed (12). In this manner, yeast expression permits pharmacological analysis in the complete absence of other adenosine receptor subtypes, removing the requirement for the use of adenosine receptor subtype-selective antagonists that mask the presence of endogenously expressed receptors in mammalian cell membrane preparations. Coexpression of a single receptor and a single G protein allows for the formation of an intact signal transduction pathway that generates a distinct and clear phenotype, growth. This arrangement allows for simple analysis of protein/protein interactions of the A_{2a} adenosine receptor and G protein and is made more convenient by the observation that the A_{2a} adenosine receptor seems to interact efficiently with the yeast heterotrimeric G protein complex. In addition, *S. cerevisiae* is more amenable to rapid genetic manipulation than many other eukaryotic cell expression systems, allowing other components of mammalian G protein-coupled receptor signal transduction pathways to be coexpressed.

Several important aspects of receptor/G protein coupling are apparent in the current study. Sufficient conservation of function is retained despite the evolutionary distance between mammals and yeast to permit functional coupling of a mammalian receptor to a yeast heterotrimeric G protein. Although the A_{2a} adenosine receptor shares predicted topological configuration (seven putative transmembrane domains) with the yeast receptors, Ste2 and Ste3, little primary sequence homology is present, particularly within the intracellular loops. In this light, it is notable that Ste2 and Ste3 also share little amino acid sequence homology although they couple to the same heterotrimeric G protein. Nevertheless, the structural aspects required to allow coupling of the A_{2a} adenosine receptor (current study) and the SSTR2 (12) to the yeast heterotrimeric G protein have been conserved.

The intracellular loops of G protein-coupled receptors play a major role in determining the specificity of the interaction with and activation of G proteins (35). Given the large phylogenetic distance between yeast and mammals, coupling of the rat A_{2a} adenosine receptor to the yeast G protein complex seemed, at first, surprising. However, several reports of G protein-coupled receptors capable of interaction with multiple G protein subtypes have appeared (36), indicating that preference for a single G protein is not absolute. In addition, previous results demonstrated that the SSTR2 couples efficiently to the yeast G_α protein, Gpa1, as well as to the chimeric G_α protein, Gpa1-$G_{\alpha i2}$ (12). The fact that the A_{2a} adenosine receptor couples to the yeast G protein indicates that this receptor may also interact with a variety of G proteins in native tissues.

Several lines of evidence suggest that the A_{2a} adenosine receptor couples to the endogenous yeast G_α, Gpa1. Functional G protein-coupled receptor-mediated signal transduction requires a heterotrimeric G protein; neither the α nor β/γ subunits alone are sufficient to transduce a signal in response to receptor activation (37, 38). Strains expressing the chimeric G_α protein, Gpa1-$G_{\alpha i2}$ do not mediate signaling by the A_{2a} adenosine receptor; however, Gpa1-$G_{\alpha i2}$ efficiently couples to the SSTR2 (12). This suggests that the presence of a G_α protein is not sufficient for signal transduction; the G protein must also functionally couple to the receptor. [^{3}H]NECA binding demonstrates GTPγS sensitivity in membranes prepared from LY594, indicating that some fraction of A_{2a} adenosine receptors is coupled to Gpa1. No growth in response to adenosine agonists is observed in strains lacking the A_{2a} adenosine receptor. These data imply that signal transduction occurs as the result of A_{2a} adenosine receptor activation of the Gpa1/Ste4/Ste18 heterotrimeric G protein.

The apparent flexibility in G_α protein/G protein-coupled receptor interactions might be explained by the extensive amino acid similarity between members of the G protein superfamily. Unfortunately, this analysis reveals that although Gpa1 and $G_{\alpha i2}$ share ~60% homology in their carboxyl-terminal regions, the major contact domain for receptors, Gpa1 and $G_{\alpha s}$, share little obvious homology in this region. Interestingly, two independent reports have demonstrated that a member of the G_q family, mouse $G_{\alpha 15}$, and its human homolog, $G_{\alpha 16}$, are capable of interacting with a variety of G protein-coupled receptors, indicating that under some conditions, G proteins can be promiscuous in their interactions with receptors (39, 40). Cotransfection of several different G_s- and G_i-coupled receptors, including the A_{2a} adenosine receptor, with $G_{\alpha 15}$ or $G_{\alpha 16}$ allowed coupling of the receptors to activation of phospholipase C. These G proteins exhibit no more similarity to $G_{\alpha s}$ than does Gpa1. Some discrete aspect of three-dimensional structure common to these three G_α proteins may account for their ability to interact with G protein-coupled receptors in a promiscuous fashion.

G protein-coupled receptor expression in yeast provides a powerful genetic tool for the determination of the structural determinants of ligand/receptor interactions as well as receptor/G protein interactions, including ligand stereoselectivity, partial agonist properties, and agonist and antagonist binding domains. In addition, the effects of G protein interactions on agonist binding to wild-type and constitutively active receptors can be examined in strains lacking a G protein α subunit. Chimeric adenosine receptors could be expressed in this system to determine structural aspects that confer subtype-selective ligand binding. Indeed, the A_1 adenosine receptor has been functionally expressed in yeast and exhibits pharmacological properties corresponding to this subtype.[2] Finally, this system will be useful for the discovery of agents

[2] L. A. Price, J. Strnad, M. H. Pausch, and J. R. Hadcock, manuscript in preparation.

that interact with receptors and downstream signaling and regulatory elements (e.g., mammalian Sst2 homologs, RGS proteins, arrestins, receptor kinases) of therapeutic importance.

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