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Random Mutagenesis of the Human Adenosine A_{2B} Receptor Followed by Growth Selection in Yeast. Identification of Constitutively Active and Gain of Function Mutations

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ABSTRACT

To gain insight in spontaneous as well as agonist-induced activation of the human adenosine A_{2B} receptor, we applied a random mutagenesis approach in yeast to create a large number of receptor mutants and selected mutants of interest with a robust screening assay based on growth. The amino acid sequence of 14 mutated receptors was determined. All these mutated receptors displayed constitutive activity. In particular, single-point mutations at T42A, V54L, and F84S and a triple-point mutation at N36S, T42A, and T66A resulted in high constitutive activity. In addition, a C-terminally truncated (after Lys269) mutant, Q214L I230N V240M V250M N254Y T257S K269stop, was highly constitutively active. The T42A, V54L, and F84S mutants showed a considerable decrease, 4.9- to 6.9-fold, in the EC_{50} value of 5′-*N*-ethylcarboxamidoadenosine (NECA), an adenosine analog. Combined mutation of I242T, K269R, V284A, and H302Q, as well as F84L together with S95G, resulted in an even greater potency of NECA of 10- and 18-fold, respectively. In fact, all constitutively active mutants had an increased potency for NECA. This suggests that the wild-type (wt) human A_{2B} receptor itself is rather silent, which may explain the low affinity of agonists for this receptor. To verify the ability of the mutant receptors to activate mammalian second messenger systems, cAMP experiments were performed in CHO cells stably expressing the wt and T42A receptors. These experiments confirmed the increased sensitivity of T42A for NECA, because the EC_{50} values of T42A and the wt receptor were 0.15 ± 0.04 and 1.3 ± 0.4 μM, respectively.

Extracellular adenosine is an endogenous agonist that activates membrane-bound proteins, the adenosine receptors. Four subtypes of adenosine receptors have been identified, the A_1, A_{2A}, A_{2B} and A_3 receptors. They all belong to the class A or rhodopsin-like family of G protein-coupled receptors (GPCRs). These GPCRS constitute one of the larger protein classes, consisting of approximately 650 members in humans (Lander et al., 2001; Venter et al., 2001). Information on these proteins is collected in a target-specific database, the GPCRDB (www.gpcr.org).

Although all four adenosine receptors are activated by adenosine and several adenosine analogs, the adenosine A_{2B} receptor has a markedly lower affinity for these agonists compared with the other three subtypes (Fredholm et al., 2001). Despite the synthesis and biological testing of a very large number of adenosine analogs, no selective, high-affinity adenosine A_{2B} receptor agonists were obtained (De Zwart et al., 1998, 1999a). The prototypic, albeit nonselective, agonist for this receptor is 5′-*N*-ethylcarboxamidoadenosine (NECA). In contrast, several high-affinity antagonists have been developed for this receptor (Kim et al., 1998, De Zwart et al., 1999b). The most selective and potent antagonist currently available is MRS1754 (Kim et al., 2000).

Previously, we applied information from the GPCRDB to select amino acids of the human adenosine A_{2B} receptor for site-directed mutagenesis (Beukers et al., 2000). The aim of that study was to gain more insight in the interaction of agonists with the human adenosine A_{2B} receptor. Our bioinformatics approach yielded five amino acids that we mutated into the corresponding amino acids of the closely related, high-affinity, human adenosine A_{2A} receptor. One of the mutated adenosine A_{2B} receptors, the N273Y mutant, had 61-fold improved affinity for an adenosine analog, 2-(1-hexynyl) adenosine.

In the current study, we developed a random mutagenesis approach to identify amino acids of the human adenosine A_{2B}

ABBREVIATIONS: GPCR, G protein-coupled receptor; NECA, 5′-*N*-ethylcarboxamidoadenosine; MRS1754, 8-[4-[((4-cyanophenyl)carbamoylethyl)oxy]phenyl]-1,3-di(*n*-propyl)xanthine; GPCRDB, G protein-coupled receptor database; PCR, polymerase chain reaction; wt, wild-type; DMEM, Dulbecco's modified Eagle's medium; 3-AT, 3-aminotriazole; CHO, Chinese hamster ovary; TM, transmembrane.

receptor involved in receptor activation. We combined this random mutagenesis approach with a robust screening assay in yeast to identify amino acids involved in constitutive activation of the receptor as well as amino acids that increase the susceptibility of the receptor for agonists. An engineered pheromone signaling pathway in yeast allowed us to select mutations of interest based on growth. cAMP measurements in stably transfected CHO cells were carried out to verify the ability of the mutant receptors to activate a mammalian second messenger system.

Materials and Methods

DNA Constructs. The human adenosine A_{2B} receptor clone in pcDNA3 (Beukers et al., 2000) was transferred to the pDT-PGK plasmid kindly provided by Dr. S. J. Dowell from GSK (Stevenage, UK). This plasmid was used to transfect yeast cells. The mutagenic PCR that we developed to introduce random mutations worked with DNA fragments up to 400 bp in length. To obtain such fragments, we introduced a silent mutation in the human adenosine A_{2B} gene, resulting in a KpnI site at the border between the second intracellular loop and the fourth transmembrane domain, as shown in Fig. 1, a and b. The primer used to introduce this silent mutation was 5′-CTT GCT CGG GTA CCC GGT ACC AAA CTT TTA TAC C3′; the resulting KpnI site is underlined. A second endogenous BglII site located at the border between the fifth transmembrane domain and the third intracellular loop was exploited as well. Through restriction with EcoRI and KpnI, we obtained the ATG-KpnI fragment; through restriction with BglII and HindIII, we obtained the BglII-stop fragment (see Fig. 1b). Both fragments were subjected to random mutagenesis.

Random Mutagenesis. The method we used to introduce random mutations by PCR was adapted from Fromant et al. (1995). The mutagenic PCR contained an excess of one of the four nucleotides and an altered ratio of [$MgCl_2$] and [$MnCl_2$]. The mutagenic reaction contained 10 ng of template DNA, 0.1 μM concentrations of each primer, 0.2 mM concentrations of dNTPs as well as 3.4 mM concentrations of the dNTP in excess, 0.5 mM $MnCl_2$, 4.7 mM $MgCl_2$, and 0.5 units of Super *Taq* polymerase in 100 μl of buffer consisting of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, and 0.1% Triton X-100. The number of cycles was set to 10. The ATG-KpnI and the BglII-stop fragments were mutagenized separately with mutagenic PCR. For the ATG-KpnI fragment, the 5′-CAT GAA TTC CAT ATG CTG CTG

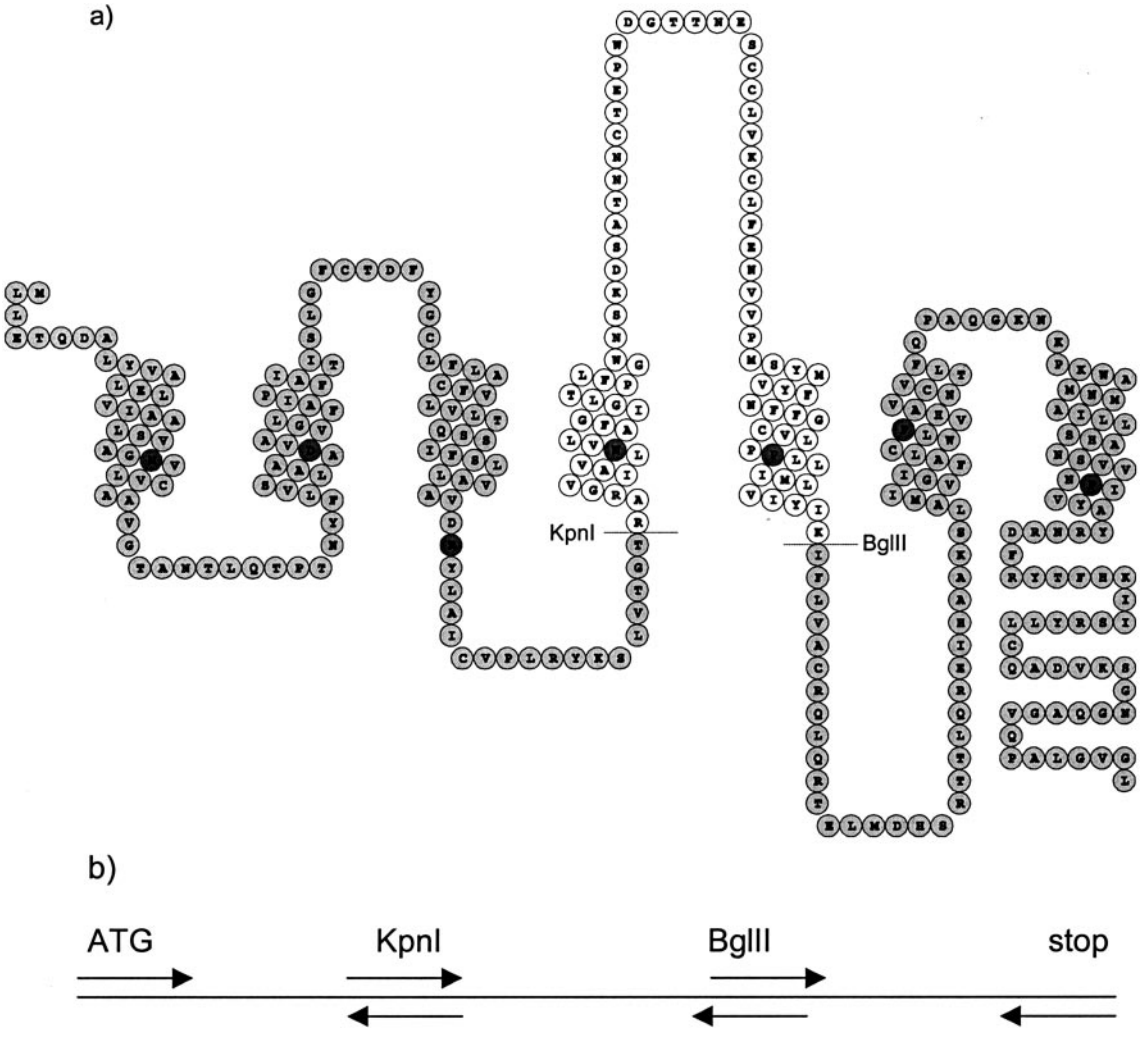

Fig. 1. a, snake-plot of the human adenosine A_{2B} receptor, with gray fragments representing amino acids that are the subject of random mutagenesis in this study. The horizontal lines represent the equivalent position of the restriction enzyme sites on the DNA level. In addition, the highly conserved amino acids that are used to align the class A GPCRs are marked (Ballesteros and Weinstein, 1995). These residues are present in the following motifs TM1, N in $G(X)_3N$ or in GN; TM2, D in $L(X)_3D(X)_7P$ or in $L(X)_3D(X)_8P$; TM3, R in $S(X)_3L(X)_2I(X)_2DR$ or $S(X)_3L(X)_2I(X)_2HR$; TM4, W in $W(X)_8P$ or in $W(X)_9P$; TM5, P in $F(X)_2P(X)_7Y$; TM6, P in $F(X)_2C(X)_2P$; TM7, P in $L(X)_7D,NP(X)_2Y$, in which X can be any amino acid and the subscript stands for the number of X present. b, schematic representation of the human adenosine A_{2B} receptor construct. Indicated are the restriction enzymes that were used to obtain the ATG-KpnI and the BglII-stop fragments.

CAG ACA CAG GA-3′ primer and the previously mentioned primer used to introduce the silent KpnI site were used. To obtain the BglII-stop fragment, the primers 5′-CAT TAA GAT CTT CCT GGT GGC CTG CAG GCA GCT TC-3′ and 5′-GAT AAG CTT CTA GAT CAT AGG CCC ACA CCG AGA G-3′ were used (the restriction site for BglII is underlined).

The mutagenic PCR products were submitted to agarose gel electrophoresis and the gel bands containing the mutated fragments were isolated from the gel and purified. Subsequently, the mutated fragments were amplified further with 10 cycles of a regular PCR with the same primer sets.

Transformation in Yeast. The mutated fragments were ligated in the pDT-PGK plasmid containing the wt human adenosine A_{2B} receptor with the KpnI site after restriction with the appropriate restriction enzymes (EcoRI/KpnI or BglII/HindIII). To obtain mutant libraries, the plasmids were transformed to XL-10 gold *Escherichia coli* bacteria via electroporation.

DNA from these bacteria was isolated and used to transform yeast according to the method described by Gietz et al. (1995). Dr. S. J. Dowell kindly provided the yeast *Saccharomyces cerevisiae* strain we used. This strain is derived from the MMY11 strain (Olesnicky et al., 1999) and was further adapted to communicate with mammalian GPCRs through the introduction of a chimeric G protein (Brown et al., 2000). The genotype of this strain is: *MAT***a***his3 leu2 trp1 ura3 can1 gpa1*Δ::Gα_{i3} *far1*Δ::ura3 sst2Δ::ura3 Fus1::FUS1-HIS3 LEU2::FUS1-lacZ ste2Δ::G418^R. To measure signaling of GPCRs, the pheromone signaling pathway of this strain was coupled via the FUS1 promotor to HIS3, a gene encoding the key enzyme in histidine production, imidazole glycerol-phosphate dehydrase.

Growth Selection. The growth selection plates contained minimal agar medium to which tryptophan and adenine were added. The lack of leucine allowed selection for the strain, whereas uracil omission was used to select for the plasmid. To select for the presence of human adenosine A_{2B} receptors, histidine was omitted and 7 mM 3-AT was added to the medium. 3-AT is a competitive inhibitor of imidazole glycerol-phosphate dehydrase and is used to suppress the basal growth that occurs in histidine-deficient medium. This optimal concentration of 3-AT was achieved through careful titration to suppress growth. In some experiments, we added 1.3 IU/ml adenosine deaminase to the agar plates, to address a potential role of endogenous adenosine (Price et al., 1996).

The initial screen was carried out in the presence of 4 nM NECA, a concentration at which the wt human adenosine A_{2B} receptor barely grows. Plasmids from yeast colonies that did grow under these conditions were isolated and used in a second round of transformation. A few cells of the resulting yeast colonies were precultured for 16 h in minimal yeast nitrogen base without amino acids supplemented with tryptophan, adenine, and leucine. The cell suspensions thus obtained were adjusted to a concentration of 400,000 cells/ml and droplets of 1.5 μl were spotted on growth selection plates containing 7 mM 3-AT and either 0, 4, 40, or 400 nM NECA. Growth was quantified with Quantity One imaging software from Bio-Rad (Hercules, CA). Growth was corrected for local background on the plate and expressed as a ratio with respect to the basal growth of the wt receptor, in the absence of NECA.

Stable Transfection of CHO Cells. To transfect CHO cells with the wt and the mutated human adenosine A_{2B} receptor, the DNA encoding these receptors was transferred from the yeast pDT-PGK to the mammalian pcDNA3 vector. CHO cells were cotransfected with peYFP-N1, a plasmid encoding enhanced yellow fluorescent protein, to identify transfected cells. peYFP-N1 was obtained from BD Biosciences Clontech (Etten Leur, The Netherlands).

The day before transfection, cells were seeded at 50,000 cells per well in a 24-well plate. For each well, 2.3 μl of *N*-(2,3-dioleoyloxy-1-propyl)trimethylammonium methyl sulfate (1 mg/ml) was carefully added to 0.7 μg of each of the DNAs in a total volume of 50 μl of DMEM without serum. This mixture was left at room temperature for 20 min to form liposomes. Cells were washed twice with DMEM without serum; next, 50 μl of DMEM without serum was added followed by the transfection mix. Cells were left for 2 h at 37°C, 5% CO_2. Subsequently, the cells were washed with phosphate-buffered saline and treated with 5% dimethyl sulfoxide in phosphate-buffered saline for 3 min. The dimethyl sulfoxide was removed, and 500 μl of a 1:1 mixture of DMEM and Ham's F12 medium, containing 10% newborn calf serum, 2 mM glutamax, 50 IU/ml penicillin, and 50 μg/ml streptomycin, was added to each well. After 48 h, the cells were harvested and the medium was replaced with fresh medium containing 0.8 mg/ml G418 to select for cells that had taken up the plasmid. The cells were transferred from the 24-well plate to a 5-ml Petri dish, and the medium was replaced every other day. After 10 days, individual colonies were selected and transferred to separate wells in a 24-well plate. Clones with sufficiently high expression levels to perform cAMP measurements were used in the cAMP assays.

cAMP Assay. cAMP experiments were performed as described previously (Beukers et al., 2000). In short, cells were seeded at 300,000 cells/well in a 24-well plate 24 h before cAMP generation. Cells were washed twice with 50 mM HEPES-buffered DMEM, pH 7.4. Cells were incubated for 30 min at 37°C in 300 μl of HEPES-buffered DMEM, pH 7.4, containing 50 μM rolipram and 50 μM cilostamide to prevent breakdown of cAMP as well as 1 IU/ml adenosine deaminase. Subsequently, either DMEM (basal), 10 μM forskolin (maximal stimulation), or varying concentrations of NECA were added in a volume of 100 μl. After 15-min incubation at 37°C, the medium was removed and 200 μl of ice-cold 0.1 N HCl was added. The samples were stored at −20°C. cAMP production was measured with a radioligand binding assay based on the interaction of cAMP with protein kinase A as described previously (Beukers et al., 2000).

Bioinformatics. The G protein-coupled receptor database, GPCRDB, is a target-specific database that contains, among others, the amino acid sequences of the GPCRs (http://www.gpcr.org; Horn et al., 1998). In addition, computationally derived data are available, such as amino acid sequence alignments. The adenosine receptors belong to the class A or rhodopsin-like family of GPCRS. The alignment of these class A receptors is based on the presence of highly conserved amino acids in each of the seven transmembrane domains and is visualized in so-called snake plots (see Fig. 1). Moreover, mutation data of the GPCRs are available in tinyGRAP (http://www.grap.fagmed.uit.no/GRAP/homepage.html; Beukers et al., 1999), a subdatabase of the GPCRDB, that can be accessed via the snake plots. Amino acid residues for which mutation data are available are colored white. Next to snake plots for individual receptors, the GPCRDB also contains snake plots for receptor families. In this study, we used the snake plot for class A receptors and investigated whether point mutation data were available in other class A receptors for the amino acids that were involved in constitutive activity of the human adenosine A_{2B} receptor.

Results

Mutant Constructs. To screen the human adenosine A_{2B} receptor for amino acids that are involved in receptor activation, we developed a random mutagenesis assay. This assay is based on a mutagenic PCR method. The PCR conditions were optimized to generate a large number of mutated receptors with a relatively low mutation frequency. Indeed, many mutated receptors contained point mutations, whereas the mutated receptor with the largest mutation frequency contained seven mutated codons. This PCR method allowed the introduction of random mutations in fragments up to 400 bp long. To obtain fragments of the human adenosine A_{2B} receptor amenable to random mutagenesis, we used two restriction sites. One site, the BglII site, was endogenous, whereas the KpnI site was introduced via a silent mutation.

The protein fragments that arise upon restriction, are shown in Fig. 1. Because literature data show that the ATG-KpnI fragment and the BglII-stop fragment are most frequently involved in receptor activation, we focused on these two highlighted fragments (Pauwels and Wurch, 1998; Parnot et al., 2002).

Mutations in the ATG-KpnI as well as the BglII-stop fragment were introduced at random with mutagenic PCR with either excess dTTP, dCTP, or dGTP. The mutations were in accordance with the nucleotide excess present. A mutagenic PCR in the presence of an excess dATP resulted in early termination of the PCR reaction. This phenomenon might be caused by excessive build-in of dATP leading to extensive misalignment of the DNA template in the active site of DNA polymerase. An average of 4000 mutants was screened for every construct.

Screening in Yeast. To screen for receptor activation, we used a yeast assay based on growth. Human adenosine A_{2B} receptors have been demonstrated to couple to the endogenous signal transduction pathway of yeast (Brown et al., 2000). After transformation of the yeast strain with the wt human adenosine A_{2B} receptor, we determined the sensitivity of the strain for the agonist NECA. As shown in Fig. 2, growth of the yeast strain is NECA-dependent.

Growth is a very suitable selection criterion, because only mutants of interest show up on the selection plate. The selection plate contained 4 nM NECA, a concentration at which only very little growth of yeast strains expressing the wild-type receptor occurs. Strains showing increased growth were picked and their plasmids were isolated and used to transform another yeast cell. These cells were then transferred to plates containing NECA at concentrations of 0, 4, 40, and 400 nM. As a control, the wt human adenosine A_{2B} receptor was transferred in triplicate to these plates. The concentration-response curve of growth of the wt receptor is shown in Fig. 3. The EC_{50} value of NECA to stimulate growth was 97 ± 36 nM for the wt receptor.

Constitutively Active and Gain of Function Mutations. From our combined random mutagenesis and screening method, we selected 14 mutated receptors. Growth of these receptors in the absence of NECA was quantified and related to the growth of the wt human adenosine A_{2B} receptor (Fig. 4). As can be seen in this graph, all mutated receptors are constitutively active although the degree of constitutive activity varies. Growth of the wt receptor is the mean of the three transformations as presented in Fig. 3. Because not all the dose-response curves show full saturation, we will comment only on those curves that show a shift in NECA responsiveness of ≥4-fold. Three point-mutated receptors were obtained twice independently: V54L (a and b), F84L (a and b), and S91G (a and b). The constitutive activity of each of the copies of the mutants was quite similar demonstrating the reproducibility of the assay.

After constitutive activity in the absence of NECA, we determined the growth concentration-response curves of these mutants for NECA. Again, the two copies of the V54L, F84L, and S91G mutants behaved similarly (see Fig. 5). The EC_{50} values of V54La and V54Lb were 14 and 17 nM, respectively; of F84La and F84Lb, 44 and 24 nM, respectively; and of S91Ga and S91Gb, 56 and 66 nM, respectively (Table 1).

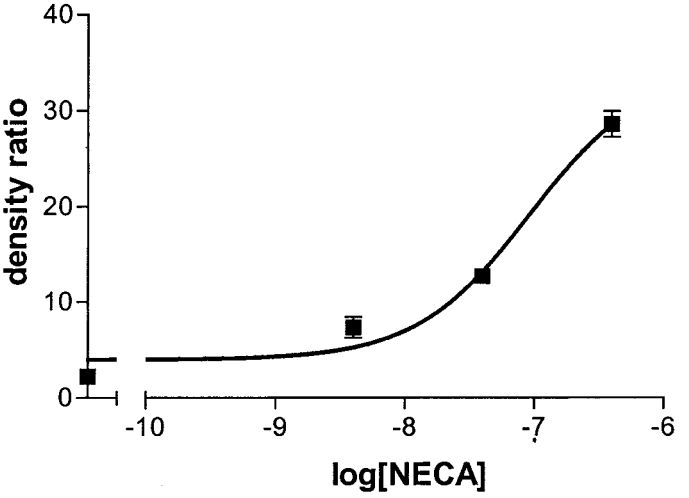

Fig. 3. Growth of yeast strains transformed with the wt human adenosine A_{2B} receptor in the presence of NECA. Experiment performed in triplicate. The intercept with the *y*-axis represents the growth, expressed as the density ratio with respect to the basal growth of the wt receptor in the absence of NECA.

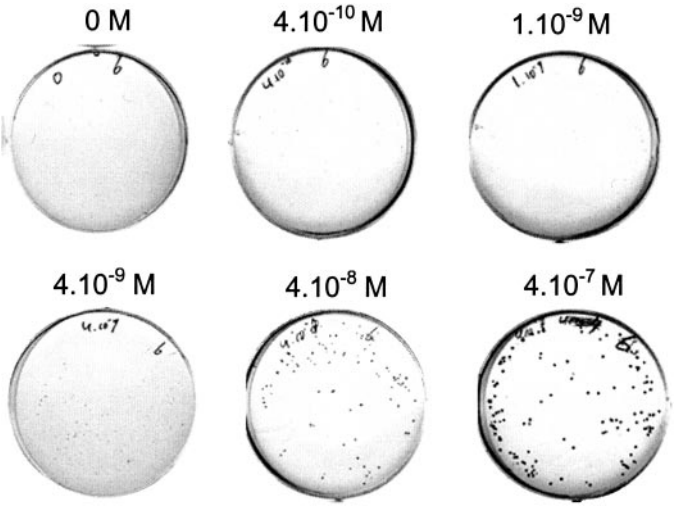

Fig. 2. Growth of a yeast strain expressing the wt human adenosine A_{2B} receptor in the presence of varying concentrations of NECA as indicated.

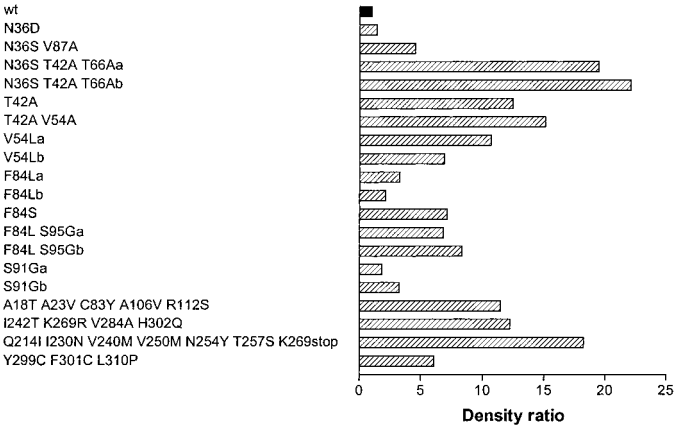

Fig. 4. Growth of the mutated receptors and the wt human adenosine A_{2B} receptor in the absence of NECA. The horizontal bars represent the growth, expressed as the density ratio with respect to the basal growth, of the wt receptor in the absence of NECA. The growth of the wt receptor was 2.25 ± 0.06 in arbitrary units and was tested in triplicate.

Thus, these three mutations result not only in constitutive activity but also in gain of function with respect to NECA.

As was already shown in Fig. 4, all mutated receptors demonstrate constitutive activity. The most constitutively active mutant, 20- to 22-fold over wt, contained three mutations: N36S, T42A, and T66A. A reliable EC_{50} value of NECA could not be determined for this mutant because of the high level of constitutive activity. The point mutant T42A itself was also constitutively active at 12-fold. In addition, the T42A mutant had a 4.9-fold decreased EC_{50} value for NECA. A double mutation at both T42A and V54A had no additional effect on the EC_{50} value of NECA but did increase the constitutive activity somewhat (15- versus 12-fold). These effects on constitutive activity and the EC_{50} value of NECA are also depicted in Fig. 6a. A point mutation at N36D had little effect on constitutive activity and resulted in a slight gain of function (Fig. 6b). The double mutant N36S V87A was slightly constitutively active and the EC_{50} value of NECA for this mutant was decreased 3.2-fold (Fig. 6b). Interestingly, the triple mutant N36S T42A T66A obtained twice was highly constitutively active (Fig. 6b).

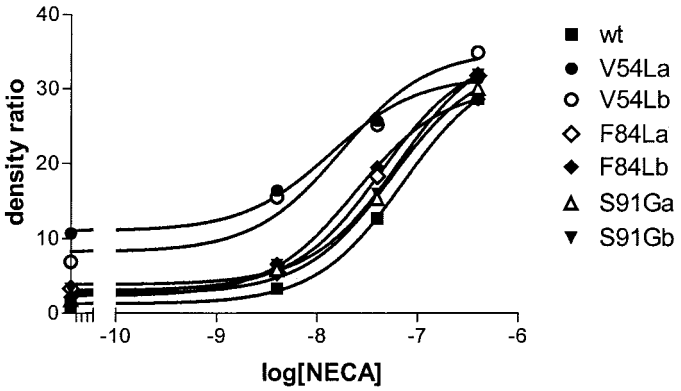

Fig. 5. Growth concentration-response curves of the wt (■) human adenosine A_{2B} receptor versus both copies of V54L (●, ○), both copies of F84L (◆, ◇), and both copies of S91G mutant adenosine A_{2B} receptors (▼, △). The intercept with the *y*-axis represents the growth expressed as the density ratio with respect to the basal growth of the wt receptor in the absence of NECA.

Another interesting position in the human adenosine A_{2B} receptor was F84 (see Fig. 6c). This residue was mutated in three different receptor mutants. On two occasions, this mutation was a single-point mutation. As stated, the F84La and F84Lb mutants were slightly constitutively active (3.3- and 2.1-fold, respectively) and the EC_{50} value of NECA was decreased moderately (2.2- and 4.0-fold, respectively). Mutation of the same residue to serine (F84S) resulted in more constitutive activity (7.1-fold) and a stronger decrease of the EC_{50} value of NECA (6.5-fold). A double mutant that was found twice and contained both F84L and S95G was more constitutively active than the single F84L mutant and had an 18- to 19-fold lower EC_{50} value for NECA. A mutant with five mutations, A18T A23V C83Y A106V R112S, was considerably constitutively active, 12-fold compared with wt, and its EC_{50} value for NECA was 2.8-fold lower compared with wt.

The above-mentioned mutations were generated in the ATG-KpnI fragment of the human adenosine A_{2B} receptor. The mutated receptors mentioned below resulted from at random mutagenesis of the BglII-stop fragment of the receptor (see Fig. 6d). Interestingly, this fragment did not yield any single-point mutations.

Mutation of four residues, I242T K269R V284A H302Q, resulted in a mutant with a 12-fold increased constitutive activity that in addition had a 9.8-fold lower EC_{50} value for NECA. Mutation of three residues in the C-terminal tail of the receptor, Y299C F301C L310P, resulted in a mutant with moderate constitutive activity (6.1-fold) as well as a moderate decrease in EC_{50} (4.6-fold).

In contrast to all the other mutants generated, the Q214L I230N V240M V250M N254Y T257S K269stop mutant no longer responded to stimulation with NECA. Despite the fact that this mutant effectively misses the seventh transmembrane domain and the C-terminal tail, it was highly constitutively active (18-fold) compared with the wt receptor.

In Table 1, an overview is presented of the mutated recep-

TABLE 1

Overview of mutated human adenosine A_{2B} receptors that were selected based on their ability to produce increased growth in the presence of 4 nM NECA.

In addition, the EC_{50} values of NECA to induce growth via these mutated receptors were determined.

Mutation	-Fold Constitutive Activity	EC_{50} Value NECA	-Fold Difference versus wt EC_{50}
		nM	
wt	1.0	97	1.0
N36D	1.4	42	2.3
N36S V87A	4.5	30	3.2
N36S T42A T66Aa	20	N.D.	N.D.
N36S T42A T66Ab	22	N.D.	N.D.
T42A	12	20	4.9
T42A V54A	15	23	4.2
V54La	11	14	6.9
V54Lb	6.9	17	5.7
F84La	3.3	44	2.2
F84Lb	2.1	24	4.0
F84S	7.1	15	6.5
F84L S95Ga	6.8	5.3	18
F84L S95Gb	8.3	5.1	19
S91Ga	1.8	56	1.7
S91Gb	3.2	66	1.5
A18T A23V C83Y A106V R112S	12	35	2.8
I242T K269R V284A H302Q	12	9.9	9.8
Q214L I230N V240M V250M N254Y T257S K269stop	18	—	—
Y299C F301C L310P	6.1	21	4.6

N.D., could not be accurately determined because of the high level of constitutive activity; —, no effect of NECA observed.

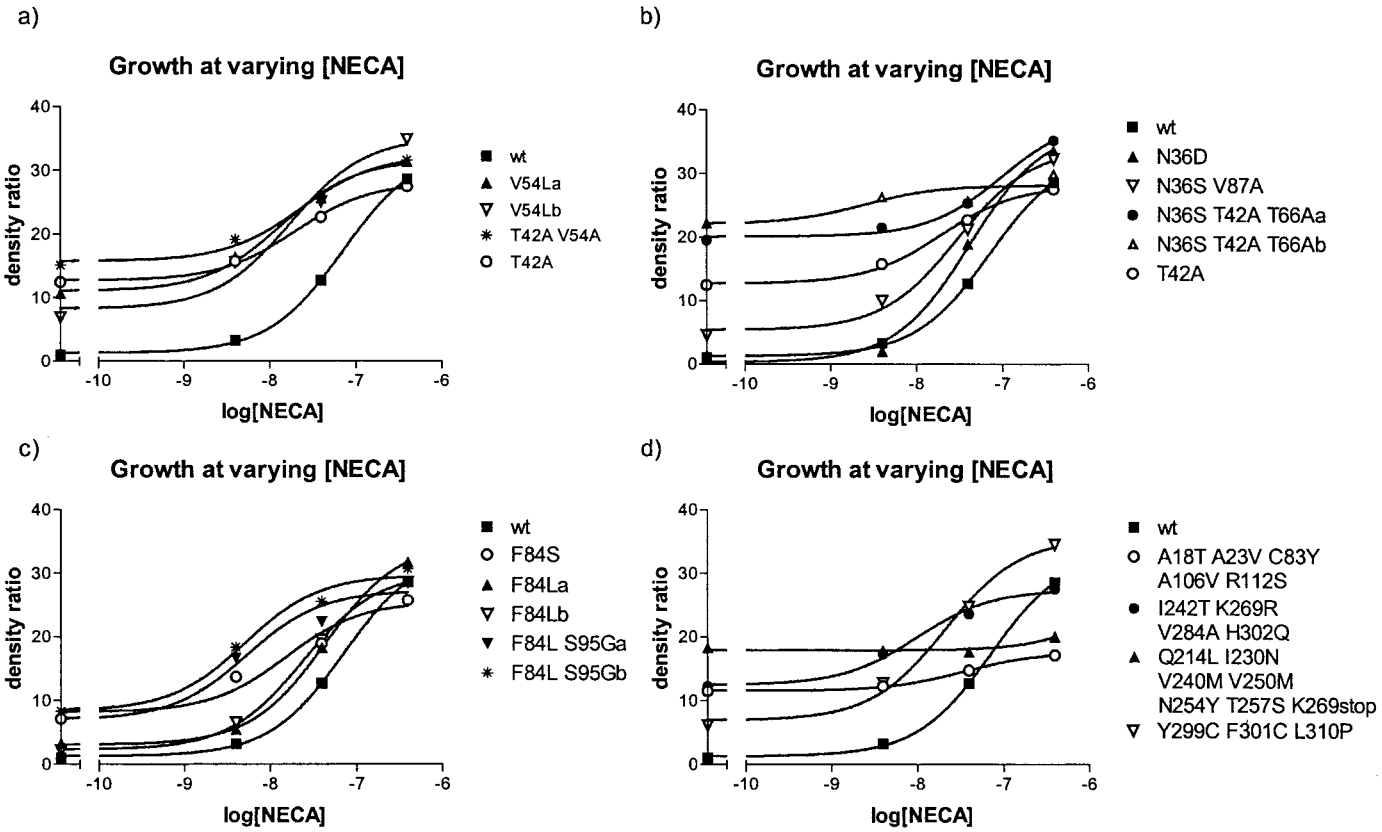

Fig. 6. Constitutive activity and NECA-dependent growth of mutated receptors. Mutant receptors that contained mutations in the ATG-KpnI fragment of the receptor are shown for mutations at Val54 and Thr42 (a), for mutations at Asn36 and/or at Thr42 (b), and for mutations at Phe84 (c). Mutant receptors containing mutations in the BglII-stop fragment of the receptor are shown in d. The intercept with the *y*-axis represents the growth expressed as the density ratio with respect to the basal growth of the wt receptor in the absence of NECA.

TABLE 2

Growth in the presence and absence of adenosine deaminase

For each mutant, 600 cells were cultured on agar and the effect of 1.3 IU/ml adenosine deaminase in the presence or absence of 3-AT (7.0 mM) was determined in triplicate. Results are expressed as the density ratio of the growth in the presence and in the absence of adenosine deaminase. Data are presented as mean ± S.D.

	Without 3-AT	With 3-AT
F84L S95G	1.0 ± 0.2	N.D.
Q214L I230N V240M V250M N254Y T257S K269 stop	1.0 ± 0.1	1.2 ± 0.2
A18T A23V C83Y A106V R112S	1.0 ± 0.2	0.9 ± 0.2
V54L	1.0 ± 0.1	0.6 ± 0.9

N.D., no ratio could be determined because hardly any growth was observed in the presence of 3-AT in both the presence and the absence of adenosine deaminase.

tors that were selected and studied in this report. Their fold constitutive activity versus the wt human adenosine A_{2B} receptor as well as their EC_{50} value for NECA are shown.

To address a potential role of endogenous adenosine we selected four representative mutants, V54L; A18T A23V C83Y A106V R112S; Q214L, I230N, V240M, V250M, N254Y T257S K269stop; and F84L, S95G, and tested their growth in the presence and absence of 1.3 IU/ml adenosine deaminase (Table 2). Adenosine deaminase had no effect on the growth of these mutants. To investigate whether an effect of endogenous adenosine might be masked by the presence of 3-AT, we also tested the growth of these four mutants in the absence of 3-AT. Again, adenosine deaminase had no effect. Hence, these data demonstrate that the growth in the presence of 3-AT is caused by the constitutive activity of the receptor and is not caused by binding of endogenous adenosine to the receptor.

cAMP Measurements in CHO Cells. To verify whether the mutant human adenosine A_{2B} receptors were able to activate a second-messenger system of mammalian cells, DNA encoding the point mutants T42A, F84S, the double mutant containing F84L and S95G, and the wt human adenosine A_{2B} receptor was transferred to pcDNA3 to allow transfection of CHO cells. Unfortunately, human embryonic kidney 293 and COS-7 cells, two other mammalian cell systems, express endogenous adenosine A_{2B} receptors. Because the expression levels of transiently transfected cells were too low, stable transfections were carried out. The various clones were tested for their ability to generate cAMP upon activation with varying concentrations of NECA. For the wt adenosine A_{2B} receptor and the T42A mutant receptor, cAMP levels were sufficiently high to allow analysis. The EC_{50} values of NECA for cAMP stimulation were 1.3 ± 0.4 μM and 0.15 ± 0.04 μM for the wt and the T42A mutant receptor, respectively (see Fig. 7). Thus, the responsiveness of the T42A receptor for NECA was increased 8.7-fold. Basal cAMP production was slightly higher (2.3-fold) for the T42A receptor compared with the wt receptor.

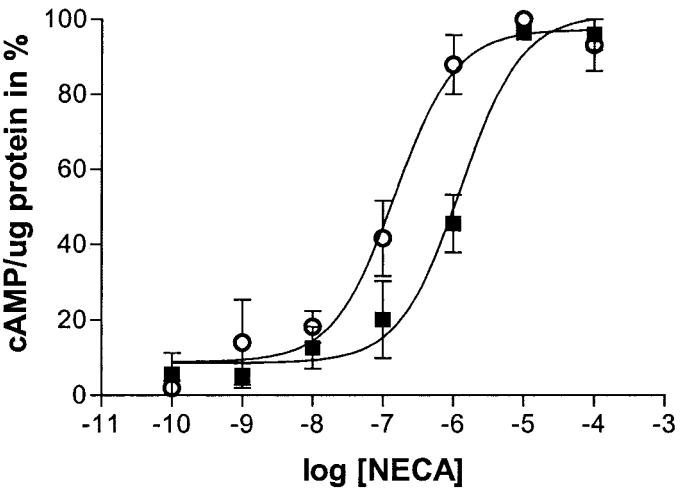

Fig. 7. NECA-stimulated cAMP production in CHO cells stably transfected with the wt (■) and the T42A mutant adenosine A_{2B} receptor (○). cAMP levels are expressed as a percentage, with basal cAMP levels (produced in the absence of NECA) set to 0%. Curves are the means of three independent experiments performed in triplicate.

Discussion

In this study, we describe a successful method to generate random mutations in the human adenosine A_{2B} receptor. Moreover, through the screening assay in yeast, we were able to select mutated receptors with constitutive activity as well as mutated receptors for which NECA has higher potency. This screening assay is based on an engineered yeast strain that was used to functionally express a wide variety of GPCRs (Brown et al., 2000). The pheromone signaling pathway of the MATa mating type yeast uses the Ste2p receptor, a G protein-coupled receptor. Upon activation of this receptor, a mitogen-activated protein kinase cascade is activated through the endogenous Gpa1p G protein, resulting in the induction of mating genes such as FUS1. The engineered yeast strain lacks the endogenous Ste2p receptor and expresses a humanized G protein instead of the Gpa1p G protein. Moreover, in this engineered yeast strain, the HIS3 gene encoding an enzyme crucial for the production of the essential amino acid histidine is placed behind the FUS1 promoter. Finally, a human GPCR, in our case the adenosine A_{2B} receptor or one of its mutants, is expressed in these cells. As a result, in the absence of histidine, the only yeast cells that can grow are those in which the human adenosine A_{2B} receptor or one of its mutants is active.

The strength of the screening assay is best illustrated by the fact that we obtained 14 highly interesting receptor mutants. Quantification of the amount of growth turned out to be very reproducible, as illustrated by the three transformations of the wt receptor (see Fig. 3) as well as by the reproducibility of the data obtained for all the mutants that we found twice independently (see Figs. 5 and 6, a–c). Moreover, the EC_{50} value for NECA we obtained for the wt receptor, 97 ± 36 nM, agreed very well with the EC_{50} value of 87 ± 8 nM reported in the literature (Brown et al., 2000). Not all the dose-response curves showed full saturation. Therefore, we confined our comments to shifts in responsiveness to NECA of ≥4-fold. In addition, the four NECA concentrations allow for a rough estimate of the EC_{50} values only.

To determine whether the yeast assay was predictive of the functional properties of these mutant receptors in mammalian cells, cAMP measurements were performed on stably transfected CHO cells. Three mutants were selected for this purpose, T42A, F84S, and a double mutant F84L S95G. Only for the T42A mutant and the wt receptor were clones obtained with high enough expression levels to perform cAMP studies. The screening assay in yeast proved predictive of the increased NECA responsiveness. In the yeast assay, NECA had a 4.9-fold lower EC_{50} for the T42A receptor than for the wt receptor, which was mimicked in the CHO cells, in which the EC_{50} for cAMP production was decreased 8.7-fold compared with the wt receptor (Fig. 7). Basal cAMP production in the CHO cells was slightly higher (2.3-fold) for the T42A receptor compared with the wt receptor. In a previous study, we introduced point mutations in the human adenosine A_{2B} receptor based on the criterion that the equivalent amino acid was different but conserved among the other adenosine receptor subtypes (Beukers et al., 2000). In our current study, none of the mutant receptors fulfilled this criterion. In fact, the mutated amino acids that yielded a ≥4-fold increased potency for NECA were either conserved among all four adenosine receptors (T42) or were present in at most two of the three other human adenosine receptors (see Table 3). The latter amino acids were V54, T66, F84, and S95.

Unfortunately, no experimentally determined three dimensional structure is available for the adenosine A_{2B} receptor. To get some indication of the location of the four amino acids mentioned above, we used the receptor model for the most closely related human adenosine A_{2A} receptor. This model is present in the protein database (PDB code 1mmh; http://www.rcsb.org/pdb) and has NECA as a ligand in it. We evaluated the location of the four mutated amino acids with respect to the homologous amino acids of the human adenosine A_{2A} receptor. Although we are aware of the limitations of the receptor models, we were struck by the fact that all four

TABLE 3

Mutations in the human adenosine A_{2B} receptor that yield a ≥4-fold decrease in EC_{50} value as indicated for NECA versus the amino acids present in the other adenosine receptors

Amino acids that differ from the human adenosine A_{2B} receptor are in bold type. In addition, amino acids that are identical to the mutation present in the human adenosine A_{2B} receptor are underlined. The K_i or K_d value of NECA for the human receptors, as determined in radioligand binding studies, is given in parentheses (Fredholm et al., 2001).

hA_{2B} Receptor (330 nM)	-Fold Decrease (EC_{50})	hA_1 Receptor (14 nM)	hA_{2A} Receptor (20 nM)	hA_3 Receptor (6.2 nM)
T42A	4.9	Thr	Thr	Thr
V54L	6.9/5.7	Val	**Ile**	**Ile**
T42A V54A	4.2	Thr, Val	Thr, **Ile**	Thr, **Ile**
F84S	6.5	**Pro**	Phe	**Leu**
F84L S95G	18/19	**Pro**, **Ala**	Phe, Ser	**Leu**, Ser
I242T K269R V284A H302Q	9.8	**Leu**, **Ser**, **Ala**, **Leu**	**Leu**, **Leu**, Val, **Arg**	**Leu**, **Gln**, **Met**, **Leu**
Y299C F301C L310P	4.6	**Val**, Phe, **Arg**	**Gln**, Phe, Leu	**Glu**, **Tyr**, **Val**

amino acids turned out to be located far away from NECA (≥9 Å).

Several studies have been published in which the equivalent amino acid of the human adenosine A_{2B} receptor was mutated in other adenosine receptors. For example, mutation of Ser90 to Ala of the human adenosine A_{2A} receptor, the equivalent of S91G in this study, resulted in a 4.7-fold increase in affinity for NECA (Jiang et al., 1996). Pro86 in the human adenosine A_1 receptor is the equivalent of Phe84. Mutation of Pro86 to Phe resulted in a >10-fold decrease in the affinity of NECA (Rivkees et al., 1999). Apparently, the phenylalanine residue hampers the interaction with NECA in both receptors. Finally, the equivalent of Ser95 in the human adenosine A_1 receptor was subjected to mutation. Mutation of this residue, Ser94 to Ala, resulted in a complete loss of affinity for NECA, whereas mutation to Thr resulted in a 2-fold decrease in NECA affinity (Barbhaiya et al., 1996).

We compared our results not only with data on other adenosine receptors but also with literature data on constitutive activity in other class A receptors. To make this comparison we used data from the GPCRDB and its subdatabase tinyGRAP. Within the GPCRDB, a numbering system has been introduced to align more distantly related class A GPCRs. This numbering system is based on the presence of several highly conserved amino acids in each of the seven transmembrane domains (Ballesteros and Weinstein, 1995). These conserved residues are highlighted in the snake-like plot of the human adenosine A_{2B} receptor (Fig. 1). In Table 4, the results of this literature search are presented. Indeed, many of the amino acids that showed up in our screen as being important for constitutive activity of the human adenosine A_{2B} receptor were also involved in constitutive activity of other class A GPCRs. In addition, these data suggest that the amino acids Ile242 and Val284 might be responsible for the constitutive activity obtained in the quadruple mutant I242T, K269R, V284A, and H302Q. The amino acids Ala106, Arg112, Tyr299, Phe301, and Leu310 could not be taken into account because no reliable alignment can be made for these residues as a result of the absence of highly conserved amino acids in the intracellular domains in which they are located.

However, mutations in the C terminus of class A GPCRs have frequently been shown to be involved in constitutive activity. For example, truncation of the C-terminal tail of the $5HT_4$ receptor (Claeysen et al., 1999), the prostaglandin E2 receptor EP4 subtype (Bastepe and Ashby, 1997), the TRHR receptor (Zaltsman et al., 2000), and the rat SSTR2 receptor (Schwartkop et al., 1999) resulted in constitutive activity. The Q214L, I230N, V240M, V250M, N254Y, T257S, K269 stop mutant is very interesting as well. A random mutagenesis study in yeast on the human C5a anaphylatoxin receptor revealed several truncated receptors that displayed constitutive activity. One of them, referred to as R92, resembled to a very large degree the mutant we obtained (Baranski et al., 1999). Next to various other amino acids that were mutated, the stop codon was present in the equivalent position; moreover, this mutated receptor contained mutations at the equivalent positions of Ile230, Val240, Val250, Asn254, and Thr257. These data provide support for our finding that a truncated receptor lacking both TM7 as well as the C terminus can be constitutively active. A recent report on the human adenosine A_{2A} receptor showed that truncation of this receptor at position 311 results in a nonconstitutively active receptor, whereas the wt receptor, as well as a receptor truncated at amino acid 360, are constitutively active (Klinger et al., 2002).

As stated, many reports in the literature confirm our finding that truncation at the C terminus results in constitutive activation of GPCRs. Hence, this approach may be a way to introduce constitutive activity in orphan receptors to enable the search for inverse agonists for these receptors.

Interestingly, all selected mutated human adenosine A_{2B} receptors turned out to be constitutively active despite the fact that our selection screen was based on increased growth in the presence of 4 nM NECA. Experiments in the presence of adenosine deaminase proved that the constitutive activity was not compromised by the presence of endogenous adenosine (Table 2).

Constitutive activity is generally thought to represent a shift in the population of receptors from R toward R* (Leff, 1995). Hence, the affinity and potency of agonists for constitutively active receptors are increased, compared with wt receptors. Indeed, constitutive activity of the mutant receptors was accompanied by an increased potency for NECA. This finding may suggest that the human adenosine A_{2B} receptor itself is relatively silent, and consequently agonists will have a low affinity and potency for this receptor. In line with this hypothesis are the findings that 1) no agonists have been found to date with increased affinity for the receptor, and 2) the selected mutant receptors contained mutations in amino acids located far away from the putative binding site

TABLE 4

Point mutations resulting in constitutive activity of amino acids in other class A receptors at the equivalent position of the amino acids that are involved in constitutive activity of the human adenosine A_{2B} receptor

Mutation in hA_{2B} Receptor	Constitutively Active Mutation of Equivalent Amino Acid	Literature Reference
T42A	M71K in mMC1	Vage et al. (1999)
	M73K in sMC1	Vage et al. (1999)
V54L	L91D in viral KSHV[a]	Rosenkilde et al. (2000)
S95G	L126A in hC5a	Baranski et al. (1999)
V240M	I630L in hTSH	Tonacchera et al. (1998)
I242T	T577I in hLHCG	Kosugi et al. (1995)
	T632I in hTSH	Porcellini et al. (1994)
V250M	Y282F in mTRHR	Colson et al. (1998)
	Y282A in mTRHR	Colson et al. (1998)
N254Y	M292L in $r\alpha_{1A}$	Hwa and Perez (1996)
V284A	C672Y in hTSH	Duprez et al. (1994)

h, human; m, mouse; s, sheep; r, rat; C5a anaphylatoxin receptor; KSHV, Karposi's sarcoma-associated herpes virus; LHCG, lutropin-choriogonadotropin hormone receptor; MC1, melanocortin-1 receptor; TRHR, thyrotropin-releasing hormone receptor; TSH, thyrotropin receptor.

[a] wt receptor is constitutively active, mutant is not.

of NECA. The mutations apparently increase the activity of the receptor (causing constitutive activation), and are probably not improving the binding of the agonist NECA.

In our experiments, we have been unable to determine the receptor expression levels because they were too low to enable either radioligand binding or immunofluorescence studies (data not shown). Hence, we cannot completely rule out the possibility that our data may have been affected by differences in receptor expression levels (Lutz and Kenakin, 1999). Milligan et al. (2002) reported in their review article that constitutively active mutant receptors are generally expressed at lower levels than wt receptors. If this were also the case in our experiments, we might have under- rather than overestimated the constitutive activity as well as the increased potency of the mutant receptors for NECA versus the wt receptor.

In conclusion, our random mutagenesis approach has yielded both highly constitutively active as well as strong gain of function mutant adenosine A_{2B} receptors. The pharmacological analysis of the wt and T42A mutant receptors in mammalian CHO cells corroborated our findings in yeast. The latter assays, however, are rather cumbersome, particularly on adenosine A_{2B} receptors. Evaluation of GPCRs with the screening assay in yeast overcomes such problems.

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