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CONSTITUTIVE ACTIVATION OF A_3 ADENOSINE RECEPTORS BY SITE-DIRECTED MUTAGENESIS

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Abstract

The objective of this study was to create constitutively active mutant human A_3 adenosine receptors (ARs) using single amino acid replacements, based on findings from other G protein-coupled receptors. A_3 ARs mutated in transmembrane helical domains (TMs) 1, 3, 6, and 7 were expressed in COS-7 cells and subjected to agonist radioligand binding and phospholipase C (PLC) and adenylyl cyclase (AC) assays. Three mutant receptors, A229E in TM6 and R108A and R108K in the DRY motif of TM3, were found to be constitutively active in both functional assays. The potency of the A_3 agonist Cl-IB-MECA (2–chloro-N^6-(3–iodobenzyl)adenosine-5′-*N*-methyluronamide) in PLC activation was enhanced by at least an order of magnitude over wild type (EC_{50} 951 nM) in R108A and A229E mutant receptors. Cl-IB-MECA was much less potent (>10-fold) in C88F, Y109F and Y282F mutants or inactive following double mutation of the DRY motif. The degree of constitutive activation was more pronounced for the AC signaling pathway than for the PLC signaling pathway. The results indicated that specific locations within the TMs proximal to the cytosolic region were responsible for constraining the receptor in a G protein-uncoupled conformation.

Keywords

purines; G protein-coupled receptor; phospholipase C; adenylyl cyclase; radioligand binding; nucleosides

Introduction

The most recently identified member of the adenosine receptor (AR) family, the A_3 AR, has already been shown to play a crucial role in some of the important physiological effects of adenosine. These include cardioprotection (1–4), eosinophil function (5), and neuroprotection (6). The local concentration of adenosine rises dramatically from the nanomolar to the micromolar range during ischemia and other stress to an organ, which has been shown to activate the A_3 and other AR subtypes and thus limit damage in both the

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heart and brain. In cardiac myocytes this protection is associated with activation of specific phospholipases, C or D (7). The A_3 AR couples to G_i and G_q proteins (8).

Recently, it has been established that many G protein-coupled receptors (GPCRs) may exist in a spontaneously active form in the absence of agonist (9,10). An inverse agonist acting at a constitutively active A_3 AR may be useful in modulating cell death in cancer, stroke and neurodegenerative diseases. This agonist-independent constitutive activity of receptors has been most readily observed in cell lines in which receptors were either overexpressed or mutated within the transmembrane helical domain (TM) or intracellular loops (11, 12). A number of amino acids or amino acid sequences were reported to be involved in regulating the active vs. inactive conformations of certain GPCRs (11). For example, constitutive activity was produced by mutation of the DRY motif, which is conserved among many GPCRs and occurs near the cytoplasmic end of TM3, in α_{1B}-adrenergic (13) and H_2 histamine receptors (14). Mutation of Ala293 (6.34) in the α_{1B}-adrenergic receptor (10) and the corresponding Cys322 in the 5-HT_{2A} receptor (15) also induced a constitutively active state of these receptors (10). The fact that various amino acid replacements at these particular homologous sites resulted in increased basal activity suggested that these regions function to constrain the unstimulated receptor in a conformation unfavorable for G protein coupling. However, in contrast to α_{1B} and 5-HT_{2A} receptors, the m_5 muscarinic receptor, when mutated in the same region, was devoid of constitutive activity (16). Thus, some of the above mentioned regions might not be critical determinants in receptor function among members of the rhodopsin-Hke GPCR family in general.

The constitutive activity of GPCRs was first demonstrated convincingly in the δ-opioid receptor (17), but was only extensively investigated after the report of the constitutive activity of mutant *am* receptors (18). As constitutive activity of G protein-coupled receptors is involved in a wide range of physiological and pathophysiological processes (19), intensive efforts are being directed towards this study (10, 20). A more complete understanding of the roles of constitutively active GPCRs in human disease and the elucidation at the molecular level of how these receptors could be inactivated may allow for reasonable development of drugs, such as inverse agonists. We were particularly interested in determining whether the A_3 AR could be rendered constitutively active using single amino acid replacements, the location of which was based on homology to other GPCRs.

EXPERIMENTAL PROCEDURES

Materials

Full-length cDNA encoding the human adenosine A_3 receptor was kindly provided by M. Atkinson, A. Townsend-Nicholson, and P.R. Schofield (Garvan Medical Institute, Sydney, Australia) and were subcloned in pcDNA3 as pcDNA3/hA_3R. The vector pcDNA3 was obtained from Invitrogen (Carlsbad, CA). All the enzymes used in this study were obtained from New England Biolabs (Beverly, MA). The QuickChange™ site-directed mutagesis kit was purchased from Stratagene (La Jolla, CA). Myo-[^{3}H]inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). The Sequenase Kit, version 2.0, DEAE-dextran, and [^{125}I]*N*^{6}-(4–amino-3-iodobenzyl)adenosine-5'-*N*-methyluronamide ([^{125}I]I-AB-MECA; 2000 Ci/mmol) were obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Fetal bovine serum (FBS) was from Life Technologies, Inc (Rockville, MD). All oligonucleotides were synthesized by Bioserve Biotechnologies (Laurel, MD). A monoclonal antibody (12CA5) against a hemagglutinin epitope (HA) and adenosine deaminase (ADA) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), and goat anti-mouse IgG antibody conjugated with horseradish peroxidase and 2-chloro-N^6-(3–iodobenzyl)adenosine-5'-N-methyluronamide (Cl-IB-MECA) were from Sigma (St. Louis, MO).

Transient expression of wild type (WT) and mutant ARs in COS-7 cells—4 X 10^6 COS-7 cells were seeded into 100-mm culture dishes containing 10 ml of DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100μg/ml streptomycin, and 2 μmol/ml glutamine. Cells were transfected approximately 24 h later with plasmid DNA (4 μg of DNA/dish) by the DEAE-dextran method (21) for 1 h, followed by treatment with 100 μM chloroquine for 3 h, and grown for an additional 24 h at 37 °C and 5% CO_2. Indirect cellular ELISA measurements using the HA-specific monoclonal antibody (12CA5) were carried out as described (36).

Inositol phosphate determination—The assay was carried out according to the general approach of Harden et al. (33). About 24 h after transfection, the cells were split into six-well plates (~0.75 X 10^6 cells/well; Costar, Cambridge, MA) in DMEM culture medium supplemented with 3 μCi/ml of myo-[^{3}H]inositol. After a 24 h labeling period, cells were preincubated in the presence of 3 U/ml ADA for 30 min at 37° with 10 mM LiCl and for 20 min at room temperature. The mixtures were swirled to ensure uniformity. Following the addition of the agonist Cl-IB-MECA, the cells were incubated for 30 min at 37° and 5% CO_2. The supernatants were removed by aspiration, and 750 μl of cold 20 mM formic acid was added to each well. Cell extracts were collected after a 30-min incubation at 4°C and neutralized with 250 μl of 60 mM NH_4OH. The inositol monophosphate fraction was then isolated by anion exchange chromatography (22). The content of each well was applied to a small anion exchange column (AG-1-X8; BioRad, Hercules, CA) that had been pretreated with 15 ml of 0.1 M formic acid/3 M ammonium formate, followed by 15 ml of water. The columns were then washed with 15 ml of a solution containing 5 mM sodium borate and 60 mM sodium formate. [^{3}H]Inositol phosphates (IP) were eluted with 4.5 ml of 0.1 M formic acid/0.2 M ammonium formate and quantified by liquid scintillation counting (LKB Wallace 1215 Rackbeta scintillation counter).

Pharmacological parameters were analyzed using the Prizm program (version 3.0, GraphPAD, San Diego, CA). Data were expressed as mean ± standard error for the number of experiments indicated.

Measurement of production of3',5'- cyclic AMP (cAMP)—The basal cAMP levels were measured by using a commercially available cAMP (low pH) Immunoassay Kit (R&D Systems, Inc., Minneapolis, MN), Briefly, COS-7 cells expressing WT and mutant human A_3 ARs were grown in 24 well plates to approximately 70% confluence. The medium was removed and replaced with 50 mM HEPES buffer at pH 7.4 in DMEM containing 3 U/ml ADA. After 30 min, the cells were then treated with 1 ml 0.1 N HC1, and cellular debris was removed by centrifugation for 5 min at 10,000X*g*. The level of cAMP was measured using a Bio-kinetics reader (Bio-TEK instruments Inc, VT).

Membrane Preparation—Transfected cells were washed twice with phosphate-buffered saline and scraped from the plate into ice-cold lysis buffer (50 mM Tris, pH 7.4, at it, containing 10 mM $MgCl_2$ and 1 mM EDTA). Harvested cells were homogenized using a Polytron homogenizer then centrifugated at 16,000X*g* for 20 min. The cell membrane pellet was resuspended in the same buffer. The protein concentration was measured using the Bradford assay (23). Membranes were aliquoted and stored at −80°C.

[^{125}I]I-AB-MECA Binding Assay—For competitive binding experiments, each tube contained 50 μl of membrane suspension, 25 μl of [^{125}I]I-AB-MECA (final concentration 1.0 nM), and 25 μl of increasing concentrations of Cl-IB-MECA in TrisHCl buffer (50 mM, pH 7.4) containing 10 mM $MgCl_2$, and 1 mM EDTA. Nonspecific binding was determined using 30 μM NECA in buffer. The mixtures were incubated at 37°C for 60 min. Binding

reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with ice-cold buffer (9 ml total). Radioactivity was determined using a Beckman 5500B γ-counter.

For saturation analysis of [^{125}I]I-AB-MECA binding, radioligand concentrations ranging from 0.1 to 12 nM were used. The K_d values of the radioligand were determined for all mutant ARs.

RESULTS

Expression of human WT and mutant A_3 ARs

A nonapeptide HA-tag, as an epitope for ELISA detection, was introduced between the initial and second amino acids of the WT A_3 AR using PCR techniques. We then used site-directed mutagenesis to create the following mutations (Table 1): N30A (TM1), C88F (TM3, near exofacial side), A229E (TM6), and Y282F (TM7). The DRY motif, located on the cytosolic end of TM3, was also mutated. Mutant and WT A_3 ARs were detected immunologically at the plasma membrane surface by virtue of the HA-tag using the 12CA5 monoclonal antibody (Table 1). This assay was specific for receptor proteins in which the amino-terminal sequence was readily accessible extracellularly. Except for the R108H mutant receptor with 29%, expression levels for the various mutants determined by this method generally ranged from 79% to 163% of the HA-tagged WT receptor.

Effect of the mutations on phosphoinositide hydrolysis

Basal IP levels of the various expressed receptors (Table 2) ranged from 71 % to 160 % of the level of WT. Three mutant ARs, A229E in TM6 and R108K and R108A in the DRY motif of TM3, were found to be constitutively active mutants (CAMs).

To measure the agonist-induced IP accumulation of the investigated mutants we used Cl-IB-MECA, because its binding affinity was not dramatically impaired by the mutations under study and because it was one of the most potent agonists for A_3 ARs. COS-7 cells transiently expressing the WT human A_3 AR displayed a substantial increase of IP in response to CI-IB-MECA (EC_{50} = 951 nM) that was not observed in control cells. Cl-IB-MECA potently induced accumulation of IP in both WT and most of the mutant A_3 ARs. The potency of Cl-IB-MECA in PLC activation was enhanced over WT by at least an order of magnitude in A229E (EC_{50} = 25.6 nM) and R108A (EC_{50}= 37.5 nM) mutant ARs. The potency of this agonist and the maximal IP levels achieved were greatly decreased (>10-fold) in the C88F, Y109F and Y282F mutant ARs. Following double mutation of the DRY motif (D107K/R108K and D107K/R108E), Cl-IB-MECA was completely inactive. Therefore, agonist activity at these two mutant ARs was not investigated further.

Basal cAMP production in COS-7 cells expressing WT and mutant human A_3 ARs

Basal cAMP accumulation was measured after transient expression of A_3 AR constructs in COS-7 cells (Table 3). Cells transfected with R108A, R108K and A229E mutant A_3 ARs showed 3.7-, 2.5- and 1.7-fold lower basal cAMP accumulation compared to the WT A_3 ARs, showing a more pronounced change of basal values than those obtained from the PLC assay. By comparison, the basal cAMP levels in cells transfected with other mutant ARs were similar to that of the WT AR. The AC results were generally consistent with the results from PLC assay.

Radioligand binding assay

To further characterize the AR mutants, ligand binding studies were performed on membranes from COS-7 cells expressing the WT or mutant ARs (Table 3). Saturation binding analysis of [^{125}I]I-AB-MECA indicated that the K_d values were similar at the WT and DRY motif mutant ARs, ranging from 0.55 to 2.0 nM. By contrast the decrease of agonist affinity was more pronounced in N30A, C88F and Y282F mutant ARs.

DISCUSSION

This was the first study to describe either single amino acid replacement or CAMs of the A_3 AR. Previously, ligand recognition and activation were studied using site-directed mutagenesis in A_1 A_{2A} and A_{2B}, but not A_3 adenosine receptors. In this study we have investigated both agonist-dependent and agonist-independent activity of mutant A_3 ARs. Three mutant ARs, A229E in TM6, R108A and R108K in the DRY motif of TM3, were found to be CAMs. The results indicated that specific locations within the TMs proximal to the cytosolic region were responsible for maintaining a conformation of the human A_3 AR that was able to couple to G proteins.

In contrast to the very different values for specific constitutive activity for other ARs and very different levels of receptor cell surface expression (24, 25), the basal IP accumulation determined in transfected COS-7 cells was relatively uniform for most of the mutants constructed in this study. Only three of the mutant ARs constitutively activated the phospholipase cascade. The cAMP assay was generally consistent with the PLC assay, except that the change in basal values by mutation was much larger than the change in basal IP production, suggesting the cAMP pathway played a major role in the constitutive activation of A_3 ARs.

An Asn residue in TM1 is highly conserved within the GPCR family (26). In the α_{1B}-adrenergic receptor, the mutation of the Asn residue to an Ala was found to produce constitutive activity (13). Mutation of the corresponding N30 to Ala in the A_3 AR did not lead to a constitutively active state of A_3 ARs, suggesting that this residue probably does not play as critical a role in the A_3 AR as in the α_{1B} adrenergic receptor. The initial finding that mutation of Ala293 in the α_{1B} adrenergic receptor by any other amino acid residue enhances agonist-independent activity has led to the hypothesis that a number of residues play critical roles in maintaining the receptor under an inactive conformation (18).

Only a limited, but increasing, number of mutant GPCRs have been found to be constitutively active to date. Constitutively activity of various GPCRs has been intensively studied through the mutation of residues in the DRY motif (TM3) (14, 25). It has been demonstrated that various substitutions of R143 resulted in α_{1B} adrenergic receptor mutants having increased constitutive activity, impairment, or complete loss of receptor-mediated response (13, 25). On the other hand, substitution in the positively charged R135 of the DRY motif in rhodopsin has led to a mutant receptor totally impaired in receptor-G protein coupling, although showing normal G_t binding (27). Thus, this highly conserved arginine within the DRY motif plays an important role in the activation process of these receptors. R108 of the A_3 AR in the DRY motif of TM3 is equivalent to R143 in the α_{1B} adrenergic receptor, which was also demonstrated to play an important role in receptor activation.

The originally proposed ternary agonist-receptor-G protein complex theory (28), as well as the allosteric ternary complex model (29), predicted that agonist binding increases binding affinity of the G protein for the receptor and vice versa. As a consequence, CAM receptors presumably simulate the agonist-activated state of the receptor and are likely to bind agonist ligands with higher affinity, in a so-called "high affinity state." In accordance with this

hypothesis, the three CAMs found in this study bound [^{125}I]I-AB-MECA with affinity equal to or greater than WT.

In conclusion, CAMs of the A_3 AR have been constructed. The degree of constitutive activation was more pronounced for the AC signaling pathway than for the PLC signaling pathway. It is hoped that the further exploration of constitutive activity in ARs may help to clarify the mechanisms of GPCR activation and highlight the specific agonist binding and activation processes of A_3 ARs.

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Table 1

Location of mutations in the TMs and detection by ELISA of HA-tagged A_3 human A_3 AR mutants expressed on the surface of COS-7 cells. Data are presented as means ± standard deviation of eight independent determinations.

Construct	Location (TM.residue)[a]	Expression (% of WT)[c]
N30A	1.50	110 ± 5
C88F	3.30	127 ± 18
D107N	3.49[b]	138 ± 32
D107K	3.49[b]	112 ± 16
D107R	3.49[b]	142 ± 39
R108K	3.50[b]	107 ± 5
R108A	3.50[b]	98 ± 4
R108N	3.50[b]	121 ± 23
R108E	3.50[b]	79 ± 26
R108H	3.50[b]	29 ± 22
Y109F	3.50[b]	137 ± 13
D107K/R108K	3.49/3.50[b]	133 ± 25
D107K/R108E	3.49/3.50[b]	126 ± 34
A229E	6.34	96 ± 7
Y282F	7.53	163 ± 41

[a] using notation of van Rhee and Jacobson (26). Homologous sites in other GPCRs were mutated resulting in CAMs (13, 24, 25, 34, 35).

[b] DRY motif.

[c] Expression level as percentage of HA-tagged A3 WT (100 %).

Table 2

Activation of PLC in WT and mutant human A_3 ARs. Maximal IP values are expressed as percentage increase above the basal levels of control IP formation set at 100% (n =3).

Construct	EC_{50} (nM)	Basal IP (% of WT)	Max. IP (% above basal)
WT	951 ± 35	100	502 ± 32
N30A	1574 ± 116	95 ± 3	375 ± 76
C88F	39,300±1,700	104 ± 7	273 ± 25
D107N	1,690 ± 230	103 ± 28	453 ± 44
D107K	187 ± 23	87 ± 21	465 ± 53
D107R	1,420 ± 130	71±17	424 ± 41
R108K	341 ± 39	130 ± 15*	454 ± 63
R108A	37.5 ± 8.6	150 ± 18*	431 ± 56
R108N	1,860± 150	90 ± 9	387 ± 28
R108E	279 ± 56	99 ± 6	425 ± 53
R108H	l,150 ± 110	88 ± 14	402 ± 52
Y109F	11,600 ± 400	106 ± 8	342 ± 23
D107K/R108K	NA	102 ± 7	115 ± 16
D107K/R108E	NA	89 ± 5	103 ± 13
A229E	25.6 ± 5.2	160 ± 17*	413 ± 47
Y282F	27,600 ± 2,300	94±11	287 ± 35

*indicates constitutive activity, basal value significantly higher than WT ($p < 0.05$).

NA: no activation detectable for 100 μM Cl-IB-MECA

Table 3

Agonist binding affinities determined in saturation and competition experiments and basal cAMP production in COS-7 cells that express WT and mutant human A_3 ARs (n = 3-4).

	[^{125}I]AB-MECA (K_d. nM)	Cl-IB-MECA (K_i binding, nM)	Basal cAMP (pmol/ml)
WT	1.2 ± 0.2	3.8 ± 0.6	256 ± 32
N30A	9.8 ± 2.3	137 ± 46	232 ± 28
C88F	8.3 ± 2.1	53.6 ± 7.8	240 ± 44
D107N	0.55 ± 0.13	1.3 ± 0.4	248 ± 34
D107K	0.90 ± 0.24	2.1 ± 0.5	220 ± 30
D107R	0.86 ± 0.11	1.8 ± 0.3	216 ± 52
R108K	0.95 ± 0.08	4.1 ± 1.0	149 ± 42*
R108A	0.70 ± 0.12	0.9 ± 0.1	101 ± 22*
R108N	0.68 ± 0.07	2.4 ± 0.4	238 ± 50
R108E	0.86 ± 0.09	5.4 ± 0.8	232 ± 58
R108H	0.85 ± 0.14	3.7 ± 1.1	252 ± 39
Y109F	2.0 ± 0.21	17 ± 4.6	236 ± 48
D107K/R108K	*a*		224 ± 43
D107K/R108E	*a*		242 ± 32
A229E	0.64 ± 0.08	2.7 ± 0.4	69 ± 12*
Y282F	9.3 ± 2.7	33.6 ± 4.7	250 ± 48

*a*specific binding of radioligand at 1 nM <20% of WT.

*basal value significantly lower than WT ($p < 0.05$).