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Agonist-Induced Desensitization of A_{2B} Adenosine Receptors

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ABSTRACT. Agonist-induced desensitization has been described for the A_1, A_{2A}, and A_3 adenosine receptor subtypes of the G protein-coupled receptor superfamily. Desensitization of the fourth adenosine receptor subtype, the A_{2B} adenosine receptor ($A_{2B}R$), has not been studied extensively. We sought to determine whether the $A_{2B}R$ is subject to agonist-induced desensitization. COS 7 cells, which exhibit endogenous expression of the $A_{2B}R$, and transfected CHO cells, which stably express a modified rat $A_{2B}R$ bearing a 5′ FLAG epitope tag, were studied. Cyclic AMP (cAMP) responsiveness to an acute challenge was measured after pretreating (desensitizing) cells with the adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA). Incubation with NECA resulted in hyporesponsiveness to acute agonist challenge in both COS 7 and transfected CHO cells. Desensitized cells exhibited restoration of cAMP responses after recovery for 24 hr in growth medium. Choleratoxin-induced cAMP responses were preserved in desensitized cells, and high concentrations of NECA were unable to overcome the desensitization. Membrane levels of the epitope-tagged $A_{2B}R$ were assessed by western blot in transiently transfected COS 7 cells. The expression of epitope-tagged $A_{2B}R$s was not different between control and desensitized cells. In northern blot analysis, levels of endogenous $A_{2B}R$ mRNA were similar in control and desensitized COS 7 cells. We conclude that the $A_{2B}R$ is subject to agonist-induced desensitization with preserved expression of $A_{2B}R$ mRNA and protein. Uncoupling of the A_{2B} adenosine receptor from the G protein complex may contribute to the mechanism of desensitization. BIOCHEM PHARMACOL **55**;6:873–882, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. adenosine A_{2B} receptors; desensitization; FLAG epitope tag; cAMP production; northern blot; western blot

Four subtypes of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3, comprise a subfamily of the G protein-coupled receptor superfamily [1, 2]. These receptors mediate the regulatory actions of adenosine in the central nervous system, the cardiovascular system, and the immune system. The four adenosine receptor subtypes share structural features including the presence of candidate phosphorylation, palmitoylation and glycosylation sites, as well as a high degree of amino acid sequence homology.

A feature common to many members of the G protein-coupled receptor superfamily is agonist-induced desensitization [3]. Agonist-induced desensitization has been described for three of the adenosine receptor subtypes: the A_1R† [4], the $A_{2A}R$ [5], and the A_3R [6]. Mechanisms of desensitization include uncoupling from G proteins, which may be mediated by receptor phosphorylation attributable to G protein receptor kinases or other second messenger kinases, and down-regulation [4–7]. Hyporesponsiveness to adenosine receptor stimulation may also result from alterations in signal transduction pathways [8, 9].

The $A_{2B}R$ is a widely distributed protein that exhibits relatively low affinity for adenosine [2, 10–12]. Although the precise physiologic functions of the $A_{2B}R$ remain undefined and are possibly dependent on species, roles for the receptor in regulating neuroglia function [13, 14], mast cell mediator release [15], myocardial contractility [16], vascular smooth muscle tone [17, 18], and intestinal chloride secretion [19] have been suggested. The low affinity of the receptor and a lack of highly selective $A_{2B}R$ agonists and antagonists limit the ability to study the receptor in intact animals [2]. Antibodies specific for the $A_{2B}R$ have not been described. With *in vitro* models, including transfection assay systems, enhanced formation of cAMP was identified as a signal transduction mechanism associated with the $A_{2B}R$ [12]. To our knowledge, agonist-induced

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† *Abbreviations:* A_1R, A_1 adenosine receptor; $A_{2A}R$, A_{2A} adenosine receptor; $A_{2B}R$, A_{2B} adenosine receptor; A_3R, A_3 adenosine receptor; NECA, 5′-*N*-ethylcarboxamidoadenosine; cAMP, cyclic AMP; DMEM, Dulbecco's Modified Eagle's Medium; CGS21680, 2-[4-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxyamidoadenosine; CSC, 8-(3-chlorostyryl) caffeine; CHO cells, Chinese hamster ovary cells; FBS, fetal bovine serum; TBS, Tris-buffered saline; PKA, protein kinase A; and PKC, protein kinase C.

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desensitization of the A_{2B} adenosine receptor subtype has not been reported.

We sought to determine whether the $A_{2B}R$, like other adenosine receptor subtypes, is subject to agonist-induced desensitization. Using a cell line that endogenously expresses the $A_{2B}R$ [10, 11] and transfected cells expressing an epitope-tagged rat $A_{2B}R$, we demonstrated that $A_{2B}R$-associated cAMP formation becomes desensitized in response to an adenosine receptor agonist. The findings suggest that uncoupling from the G protein complex may contribute to the mechanism of desensitization.

MATERIALS AND METHODS

Reagents

Trizol reagent and cell culture medium and supplies were obtained from Gibco-BRL. Calf serum, horse serum, FBS, chloroquin, theophylline [1,3-dimethylxanthine], cholera-toxin, and protease inhibitors were obtained from the Sigma Chemical Co. NuSerum was obtained from Fisher Scientific. The [^{3}H]cAMP assay kit was from Amersham Life Science, and DEAE-dextran was from Pharmacia. NECA, CGS21680, CSC, and Ro20-1724 were obtained from RBI. H-89 and bisindolylmaleimide-HCl were from Calbiochem. Liquiscint was from National Diagnostics. Radionucleotides were purchased from Dupont/NEN. An oligolabeling kit using the Klenow fragment of DNA polymerase was obtained from Pharmacia. Other reagents were of molecular biology grade.

Cell Culture

COS 7 cells were grown at 37° in an atmosphere of 5% CO_2 in DMEM supplemented with FBS [10% (v/v)], penicillin, and streptomycin. Cells were passaged by trypsinization and seeded at a density of 5×10^6 cells/150-mm plate for transfection the following day or 1×10^5 cells/well in Falcon 12-well tissue culture plates for experimental treatments the following day. CHO cells were grown at 37° in an atmosphere of 5% CO_2 in F-12 Nutrient Mixture supplemented with FBS [10% (v/v)], penicillin, and streptomycin. Cells were passaged by trypsinization, seeded at a density of 1×10^5/well in 12-well plates, and maintained in culture until the following day, when experimental treatments were administered. HEK293 cells were maintained in DMEM supplemented with horse serum [10% (v/v)] and plated at 2.5×10^5 cells/35-mm well for experiments 2 days later. Mouse 3T3-L1 cells were grown in DMEM supplemented with calf serum [10% (v/v)] and antibiotics and plated at 2×10^5 cells/35-mm well for experiments 2–3 days later.

cAMP Generation Studies

All dishes of cells received fresh medium 1–2 hr before experimental treatments were administered. Adenosine receptor antagonists were added 10 min before agonists.

FIG. 1. Deduced amino acid sequence of the epitope-tagged rat A_{2B} adenosine receptor protein. A spacer of 8 amino acids is introduced between the FLAG epitope tag and the native rat $A_{2B}R$ sequence.

Control dishes received an equal volume of vehicle alone (water, DMSO, or ethanol). The phosphodiesterase inhibitor Ro20-1724 (50 μM final concentration) was added immediately before the addition of control solutions or agonists. Incubations lasted for 10 min after which the medium was aspirated and the dishes were washed once with ice-cold phosphate-buffered saline. Then cells were scraped from the wells into an ice-cold solution of acetic acid (50 mM) and EDTA (5 mM). The cell extract was boiled for 2–3 min, chilled on ice, and centrifuged at 14,000 *g* for 2 min at 4°. The supernatant was collected, dried, and stored at −80° for cAMP assay using a competitive protein binding kit (Amersham Life Sciences). The pellet was saved for protein assay (BCA Protein Assay Reagent Kit; Pierce). Experiments were performed in triplicate or quadruplicate. Individual experiments were repeated 2–3 times. Representative experiments are presented.

Epitope-Tagged Receptor Construction

A vector consisting of the selectable expression plasmid pcDNA3 (Invitrogen) modified to contain the FLAG epitope tag sequence (Eastman Kodak) at the 5′ cloning site was provided by Dr. Vijaya Ramesh, Massachusetts General Hospital. The vector was digested with *Hin*dIII, and blunt ends were created with a fill-in reaction using Klenow. A plasmid containing the rat A_{2B} adenosine receptor cDNA (Genbank accession number m91466 [11, 12]) was provided by Dr. Steven M. Reppert, Massachusetts General Hospital. The insert was isolated by digestion with *Sma*I, which creates a blunt end in the 5′ untranslated region and a second blunt end in the 3′ cloning region of the plasmid. The ligation product of the epitope-tag vector with the receptor cDNA was predicted to encode the FLAG epitope and eight amino acids of additional sequence 5′ to, and in frame with, the native translation start site of the $A_{2B}R$ (Fig. 1). This structure was confirmed by dideoxy nucleotide DNA sequencing using standard methods.

Stable Transfections

The absence of endogenous functional A_{2B} receptors on CHO cells [12] was confirmed by treating the cells with NECA (1 μM) in the presence of Ro20-1724 for 10 min and assaying for cAMP formation. NECA did not stimulate cAMP formation in untransfected CHO cells. Cells were then seeded at a density of 100,000 cells/100-mm plate for transfection with the plasmid encoding the epitope-tagged $A_{2B}R$ the following day. Transfection was carried out with

the calcium phosphate method. Plasmid DNA was mixed with 35 μL of 2 M $CaCl_2$, and the volume was adjusted to 250 μL with water. The DNA/$CaCl_2$ solution was added in dropwise fashion to an equal volume of 2× HEPES-buffered saline solution, pH 7.12, and immediately applied to the cells. After 4 hr of incubation, the cells were subjected to glycerol shock [glycerol 15% (v/v) in HEPES-buffered saline] for 1 min, washed with DMEM, and returned to growth medium. The following day, the growth medium was supplemented with G418 (Gibco-BRL) at a final concentration of 500 μg/mL. Individual colonies were picked for amplification and characterization after 10–14 days.

Transient Transfections

One day after seeding, subconfluent COS 7 cells in 150-mm plates were transfected using the DEAE-dextran/chloroquin method. A transfection medium consisting of DMEM, NuSerum [10% (v/v)], and Tris–HCl (0.1 M) was prepared. After washing the cells with DMEM, transfection medium (4.5 mL) with plasmid DNA (11.25 μg) was added to the plates. After 1 min, transfection medium with DEAE-dextran (1.6 mg/mL) was added, and the plates were incubated at 37° for 3.5 hr. Then the dextran and DNA containing transfection medium was aspirated, and a solution of dimethyl sulfoxide [10% (v/v)] in PBS was applied to the plates for 1.5 min. The plates were incubated in growth medium containing chloroquin (51.6 mg/mL) for an additional 3.5 hr and subsequently returned to growth medium. After 48 hr cells were harvested for membrane protein.

Membrane Protein Preparation

Cells were washed twice in ice-cold PBS and then scraped into ice-cold Laemmli buffer [Tris base (0.24 M, pH 8.3), glycine (1.9 M) and SDS (35 mM)] supplemented with leupeptin (10 μg/mL), phenylmethylsulfonyl fluoride (PMSF) (0.1 mM), soybean trypsin inhibitor (10 μg/mL), and pepstatin A (1 μg/mL). Then cells were homogenized (10 strokes) on ice with a Wheaton 7 mL Dounce tissue grinder ("tight" pestle) and sonicated twice for 15 sec on ice at setting 7 with a Cell Disruptor W-350 (Branson Sonic Power Co.). The homogenates were centrifuged at 750 *g* for 5 min at 4°, after which the supernatant was saved and recentrifuged at 47,800 *g* for 35 min at 4°. The pellet was resuspended in Laemmli buffer with protease inhibitors (see above). An aliquot was saved for protein assay (BCA Protein Assay Reagent Kit; Pierce), and 5× SDS–PAGE sample buffer was added to the remainder of the sample. Samples were heated for 5 min at 55° and stored at −80°.

Western Blot

Electrophoresis and blotting of protein were performed with the Novex XCell II Mini-Cell and Blot Module under reducing and denaturing conditions. All samples were heated at 55–60° for 5 min before loading. Typically, 10–20 μg of protein was loaded in each lane of a 12% SDS–PAGE separating/5% stacking gel. Electrophoresis was carried out in Laemmli buffer (see above) without protease inhibitors for 90 min at 125 V. Proteins were transferred onto nitrocellulose membranes for 2 hr at 30 V in Tris base (12 mM) with glycine (96 mM) at pH 8.3. Membranes were blocked for 24 hr at 4° in TBS with nonfat dry milk [3% (w/v)]. Then they were washed three times for 10 min at room temperature in TBS-T (TBS with Tween 20 [0.05% (v/v)], Fisher Scientific) with milk [3% (w/v)]. Incubation with the primary antibody (Anti-FLAG M2 Monoclonal Antibody, 9 μg/mL, Eastman Kodak Co.) was carried out overnight at 4° in TBS-T with milk [3% (w/v)] and was followed by three 5-min washes at room temperature in TBS-T. Secondary antibody (alkaline phosphatase conjugated goat anti-mouse IgG, 1:10,000 dilution, Jackson Immuno Research Laboratories, Inc.) incubations were for 2 hr at room temperature in TBS-T with milk [3% (w/v)] followed by three 5-min washes at room temperature in TBS-T. The BCIP/NBT Phosphatase Substrate System (Kirkegaard & Perry Laboratories) was used to visualize immunoreactive protein.

Northern Blot Analysis

After treatment, cells were washed once with ice-cold PBS and collected by scraping in a small volume of PBS. Samples were pelleted by centrifugation for 2 min at 14,000 *g*, frozen in liquid nitrogen, and stored at −80°. Frozen cell pellets were thawed on ice for 5 min, and total cellular RNA was isolated by extraction with the Trizol reagent. RNA concentrations were determined by UV absorbency at 260 nm. The integrity of the RNA was checked by electrophoresis of an aliquot of each sample on an ethidium-stained agarose gel and inspection of the 28S and 18S ribosomal RNA bands.

RNA samples (10 μg) were lyophilized and resuspended in a solution of formaldehyde, formamide, water, and MOPS (3-[*N*-morpholino]propanesulfonic acid). Samples were heated for 15 min at 65°, size fractionated on a denaturing 1.1% agarose gel, and electroblotted onto GeneScreen nitrocellulose membrane (Dupont-NEN). The RNA was UV fixed to the membrane with a Stratalinker (Stratagene). Membranes were placed immediately into prehybridization solution consisting of 50% (v/v) formamide, 1% (v/v) SDS, 1 M NaCl with 10% (w/v) dextran and 10× Denhardt's solution buffered with Tris-HCl and supplemented with 100–200 μg/mL boiled, sheared salmon sperm DNA at 42° for 1–18 hr. Membranes were hybridized with ^{32}P-labeled cDNA probes in the same solution for 16–24 hr. After hybridization, membranes were washed twice for 5 min in 2× SSC at room temperature, followed by two washes in 2× SSC with 1% SDS at 65° (30 min each), and two washes in 0.2× SSC at room temperature (30 min each). SSC is 0.15 M NaCl and 0.015 M

sodium citrate, pH 7.0. Then membranes were sealed in bags and exposed to Kodak XAR film (Eastman Kodak) at −80° with one intensifying screen for 1–72 hr. Autoradiograms were scanned, and the images were analyzed by densitometry using the program NIH Image (v 1.59). The autoradiographic signal for the $A_{2B}R$ was normalized to the signal for β-actin.

The cDNA probe template for human β-actin was obtained from Clontech. The cDNA probe template (1600 bp) for the $A_{2B}R$ was obtained by digesting the rat $A_{2B}R$ cDNA with *Xho*I. cDNA probes were labeled by the random priming method with the large fragment of DNA polymerase I (Klenow fragment) to a specific activity of $0.5–1 \times 10^9$ cpm/μg. Labeled fragments were purified over a gel filtration column (NICK column, Pharmacia Biotech).

Statistical Evaluation

Representative individual experiments are presented. Responses were compared with an analysis of variance using the program Instat (Graphpad).

RESULTS

Adenosine Receptor Expression by COS Cells

To confirm that COS 7 cells express functional adenosine receptors positively coupled to adenylate cyclase, untransfected COS 7 cells were treated with adenosine receptor agonists and antagonists in the presence of the phosphodiesterase inhibitor Ro20-1724. cAMP levels increased in response to the nonselective agonist NECA. The effect was blocked by theophylline, but not by the $A_{2A}R$ selective antagonist CSC (Fig. 2). The selective $A_{2A}R$ agonist CGS21680 (1 μM final concentration) failed to elicit a cAMP response in similar assays (data not shown). These data are consistent with the endogenous expression of functional A_{2B} adenosine receptors by COS 7 cells, as suggested by previously reported radioligand binding and northern blot analysis with COS 6M cells [11].

Agonist-Induced Desensitization of Adenosine A_{2B}Rs

To test the hypothesis that the $A_{2B}R$, like the other three adenosine receptor subtypes, is subject to agonist-induced desensitization of cAMP production, COS 7 cells were pretreated (desensitized) with NECA (1 μM final concentration) for periods of 1–17 hr. The medium was changed, and the cells were acutely challenged with NECA or the water vehicle in the presence of Ro20-1724. Then the cAMP response was determined. Pretreatment with NECA for 1 hr resulted in a significant reduction in cAMP response to the acute agonist challenge. The desensitization effect was maximal by about 4 hr (Fig. 3A). A qualitatively similar response was obtained in CHO cells stably expressing the epitope-tagged $A_{2B}R$ (CHO-$A_{2B}R$ cells). However, maximal desensitization occurred more rapidly (Fig. 3B). In other experiments, mouse 3T3-L1 cells and human HEK293 cells were found to increase cAMP formation in response to NECA (0.01 to 10 μM), but not the $A_{2A}R$ selective agonist CGS21680 (1 μM). NECA pretreatment (1 μM, 2 hr) of 3T3-L1 cells resulted in decreased responsiveness to subsequent acute challenge with NECA (fold increase 5.3 vs 1.7), and HEK293 cells pretreated for 5.5 hr exhibited similar behavior (fold increase 39.5 vs 1.3) (not shown). Taken together, these observations suggest that desensitization of the $A_{2B}R$ is a property of the receptor itself, independent of the cell type or species in which the receptor is studied.

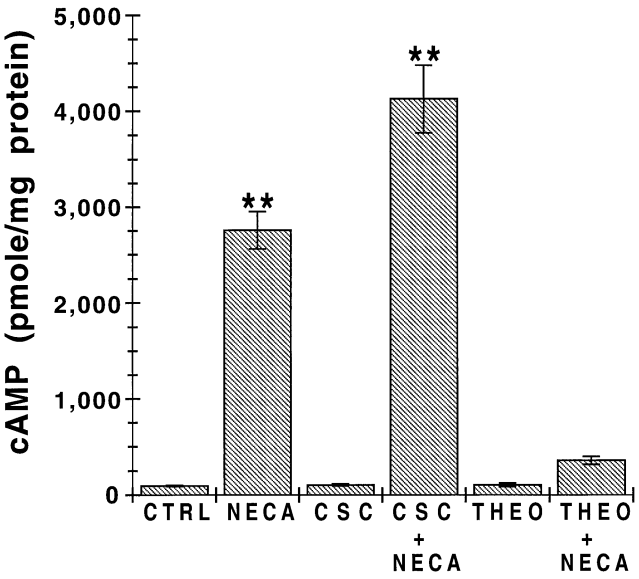

FIG. 2. cAMP production in COS 7 cells following treatment with NECA. COS 7 cells were treated in the presence of Ro20-1724 (50 μM final concentration) with water vehicle control, NECA (1 μM final concentration), or combinations of adenosinergic agents as indicated. The adenosine antagonists CSC (1 μM final concentration) and theophylline (THEO, 100 μM final concentration) were administered 10 min before water control or NECA. Data from one representative experiment were plotted as means ± SEM, N = 4 per treatment group. Key: (**) significantly different from control, $P < 0.01$. Other treatments were not significantly different from control ($P > 0.05$), as evaluated with Dunnett's test.

Recovery from Agonist-Induced Desensitization

Prolonged exposure to NECA could result in toxicity to cells, resulting in decreased cAMP production in response to subsequent acute agonist challenge. To evaluate this possibility, COS 7 cells were subjected to pretreatment for 17 hr with NECA (1 μM final concentration), followed by recovery for 24 hr in growth medium. Cells allowed to recover exhibited substantial restoration of their responsiveness to acute challenge with NECA (Fig. 4A), although the response magnitude was significantly less than in cells pretreated only with water. Qualitatively similar responses were obtained in parallel experiments with stably transfected cells expressing the $A_{2B}R$ (Fig. 4B). These data suggest that agonist-induced hyporesponsiveness to NECA

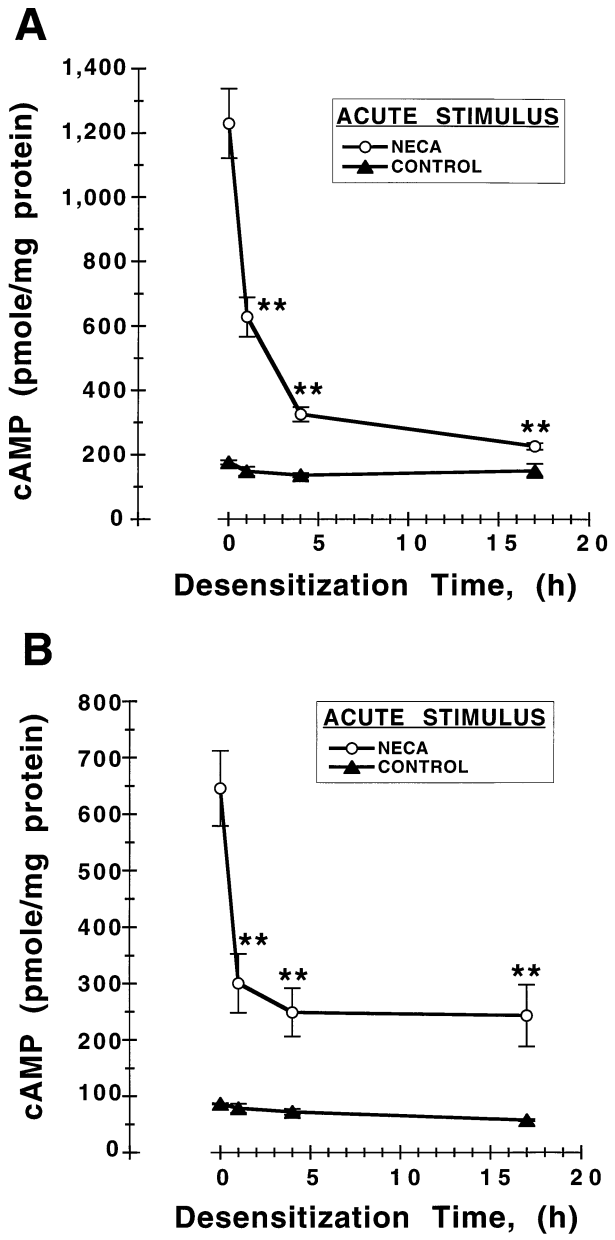

FIG. 3. Time-dependent desensitization of cAMP responses to NECA. Dishes of cells were pretreated (desensitized) with NECA (1 μM) for the indicated periods. The incubation medium was changed, and then cells were acutely challenged with (water control, ▲) or NECA (again at 1 μM, ○) in the presence of Ro20-1724 before harvesting for cAMP assay. Representative data from individual experiments (N = 3 per data point) are plotted as means ± SEM for (A) COS 7 cells, and (B) CHO-A_{2B}R cells. Key: (**) significantly different from acute NECA response with no desensitization ($P < 0.01$), as evaluated with Dunnett's test. cAMP production was not significantly different ($P > 0.05$) in the groups with the acute control challenge.

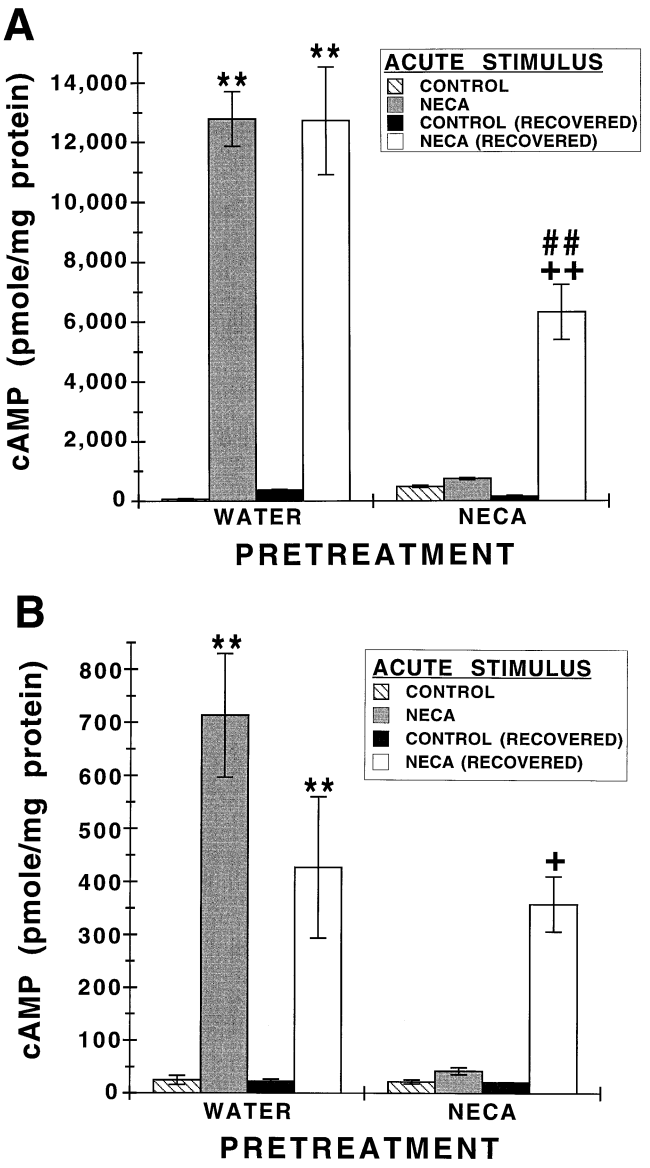

FIG. 4. Desensitization and recovery of cAMP response to NECA. Groups of cells were pretreated (desensitized) with NECA (1 μM) for 17 hr. Growth medium was replaced. Some groups were acutely challenged with water (control) or NECA (again 1 μM) in the presence of Ro20-1724. Other groups were allowed to recover for 24 hr before acute challenge. Then cells were harvested for cAMP assay. Representative data from individual experiments (N = 3 per data point) are plotted as means ± SEM for (A) COS 7 cells, and (B) CHO-A_{2B}R cells. Key: (**) significantly different from the control group (water pretreatment), $P < 0.01$; (++) significantly different from the control group (NECA pretreatment), $P < 0.01$; (+) significantly different from the control group (NECA pretreatment), $P < 0.05$; and (##) significantly different from the acute NECA response group (water pretreatment), $P < 0.01$. Other treatments were not significantly different from their respective pretreatment controls ($P > 0.05$), as evaluated by ANOVA with a Bonferroni comparison.

is substantially reversible and, therefore, not attributable to nonspecific toxicity alone.

Mechanisms Contributing to A_{2B}R Desensitization

A number of mechanisms that contribute to desensitization of G-protein coupled receptors have been described. Alterations in the signal transduction properties of the cell at the level of the G protein complex or in adenylate cyclase function could contribute to decreased production of cAMP. These possibilities were evaluated by desensitizing

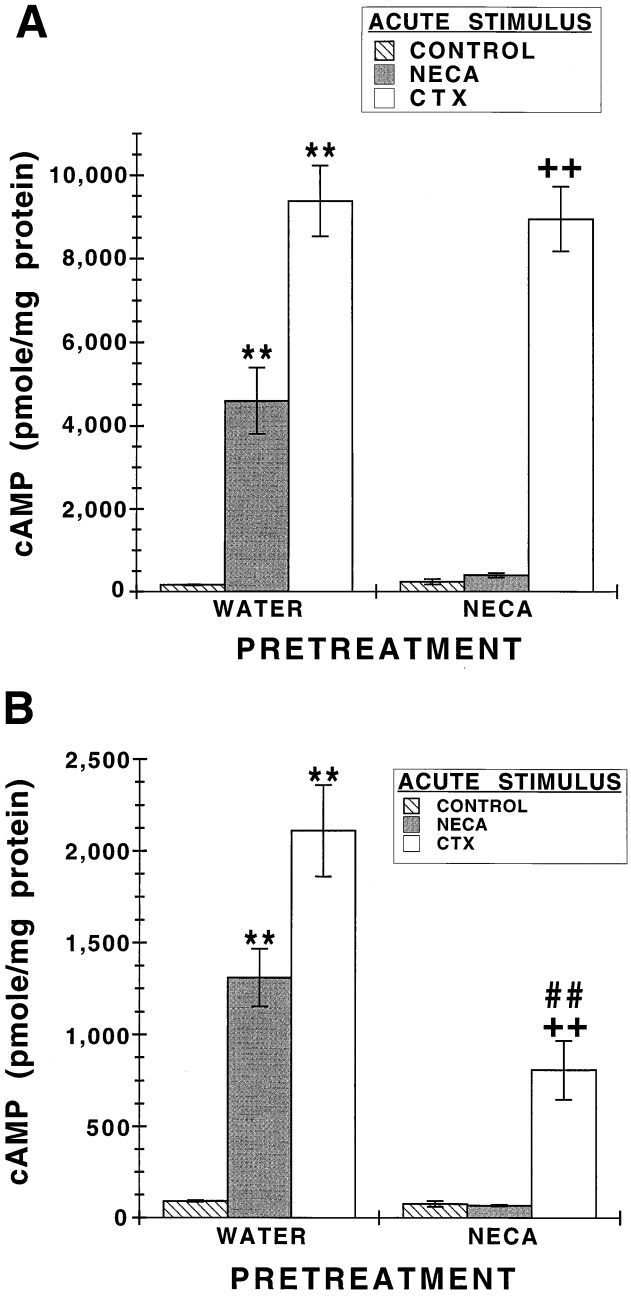

FIG. 5. Choleratoxin-induced cAMP production in desensitized cells. Dishes of cells were pretreated with water or NECA (1 μM) for 17 hr. Without changing the medium, some groups then received choleratoxin (CTX, 5 ng/mL final concentration). All groups were incubated for an additional 4 hr. Medium was changed, and cells were acutely challenged for 10 min with water or NECA in the presence of Ro20-1724. Cells were then harvested for cAMP assay. Representative data from individual experiments (N = 4 per data point) are plotted as means ± SEM for (A) COS 7 cells, and (B) CHO-A_{2B}R cells. Key: (**) significantly different from water pretreatment control, $P < 0.01$; (++) significantly different from NECA pretreatment control, $P < 0.01$; and (##) significantly different from the acute NECA response group (water pretreatment), $P < 0.01$. Other treatments were not significantly different from their respective pretreatment controls ($P > 0.05$), as evaluated by ANOVA with a Bonferroni comparison.

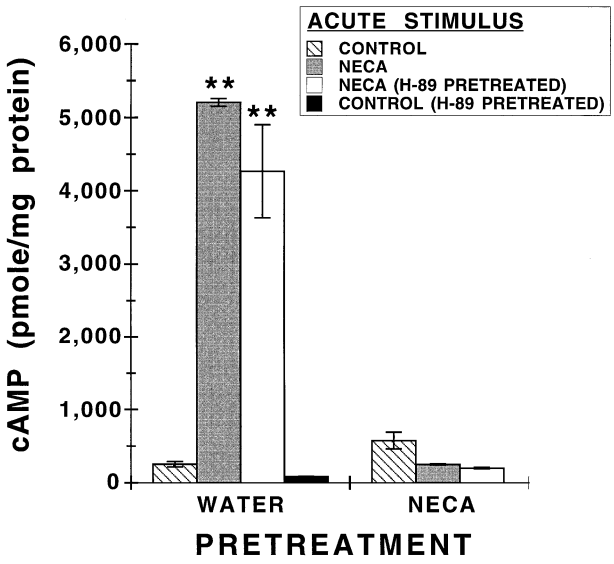

FIG. 6. cAMP production in desensitized COS 7 cells: Effect of PKA inhibition. Dishes of cells were pretreated with water or NECA (1 μM) for 17 hr. Some pretreatment groups received, in addition, the PKA inhibitor H-89 (10 μM), which was added 10–20 min before agents. Medium was changed, and cells were acutely challenged for 10 min with water or NECA in the presence of Ro20-1724. Then cells were harvested for cAMP assay. Representative data from one experiment (N = 4 per data point) are plotted as means ± SEM. Key: (**) significantly different from water pretreatment control, $P < 0.01$. Other treatments were not significantly different from their respective pretreatment controls ($P > 0.05$), as evaluated by ANOVA with a Bonferroni comparison.

cells and then incubating them with choleratoxin, a direct stimulator of the cAMP signal transduction pathway (Fig. 5, A and B). COS 7 cells exhibited preserved responsiveness to direct activation of the G protein complex. Stably transfected CHO cells retained significant responsiveness to stimulation of the G protein complex despite receptor desensitization. This suggests that cellular mechanisms for generating cAMP remain functional despite prolonged exposure to the adenosine receptor agonist.

Other possible mechanisms of desensitization were investigated in the COS 7 cell model. To identify a role for phosphorylation by PKA in agonist-induced desensitization, cells were desensitized with NECA in the presence of the selective PKA inhibitor H-89 (10 μM). H-89 did not prevent hyporesponsiveness to subsequent acute agonist challenge (Fig. 6). To identify a role for PKC, similar experiments were carried out in the presence of the selective PKC inhibitor bisindolylmaleimide (1 μM). Hyporesponsiveness to acute agonist challenge in desensitized cells was seen despite PKC inhibition (data not shown). To determine whether decreased receptor affinity for agonists accounts for hyporesponsiveness to NECA, desensitized cells were acutely challenged with high concentrations of NECA. Increasing the concentration of NECA in the acute challenge to 30 μM failed to overcome the reduction in cAMP production resulting from prolonged agonist exposure (Fig. 7).

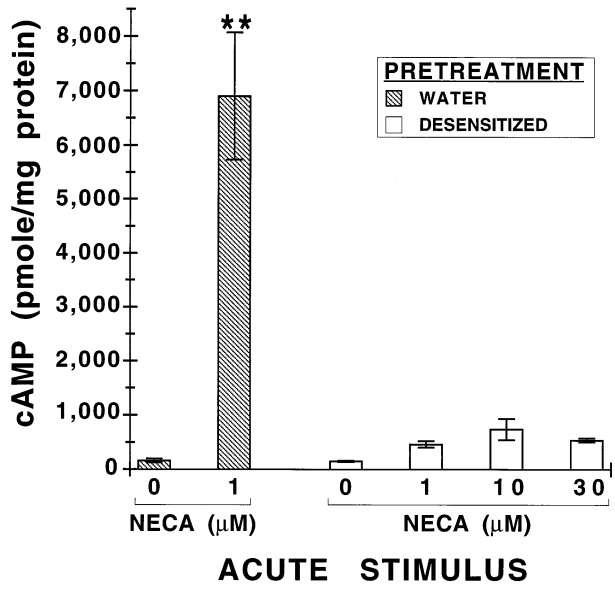

FIG. 7. cAMP production in response to high concentrations of NECA in desensitized COS 7 cells. Dishes of COS 7 cells were pretreated with water or NECA (1 μM) for 17 hr. Medium was changed, and cells were acutely challenged for 10 min with water or NECA at the indicated concentrations in the presence of Ro20-1724. Then cells were harvested for cAMP assay. Representative data from one experiment (N = 4 per data point) are plotted as means ± SEM. Key: (**) significantly different from water pretreatment control, $P < 0.01$. cAMP responses to acute challenge with NECA in NECA-pretreated cells were not significantly different from control ($P > 0.05$), as evaluated by ANOVA with a Bonferroni comparison.

Loss of receptor protein from cell membranes could also account for decreased responsiveness to agonist challenge. Decreased expression of the $A_{2B}R$ gene in the endogenously expressing COS 7 cell line was assessed by northern blot analysis of total cellular RNA extracted from control and NECA-treated cells. mRNA levels were similar in both groups (Fig. 8). To assess levels of the $A_{2B}R$ protein in cell membranes, western blot analysis of the epitope-tagged receptor was employed. Transfected CHO cells did not exhibit sufficient expression of the epitope-tagged $A_{2B}R$ for antibody detection. Because the desensitization characteristics of the epitope-tagged $A_{2B}R$ expressed in CHO cells resemble those of the native receptor expressed in COS 7 cells, we carried out western blot analysis of the epitope-tagged $A_{2B}R$ after transient transfection into COS cells. Membrane protein samples from mock transfected COS 7 cells typically exhibited a single, faint, immune reactive band of ~83 kDa. Following transfection, membrane samples from control (vehicle-treated) cells and NECA-desensitized cells exhibited three predominant bands of immunoreactivity and several lesser bands. Staining of the predominant bands of ~30 kDa, ~37 kDa, and ~70 kDa was of similar intensity in the desensitized and control cells (Fig. 9). Because there may be an effect of receptor number on measures of receptor modulation [20–22], similar experiments were carried out following transfection with one-

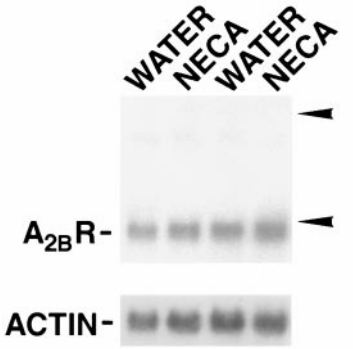

FIG. 8. Northern blot analysis of $A_{2B}R$ mRNA levels in COS 7 cells. COS 7 cells were pretreated with water or NECA (1 μM) for 17 hr, and total cellular RNA was isolated for analysis by northern blot. Each lane was loaded with 10 μg of RNA. The blot was initially hybridized to a probe for the $A_{2B}R$, then stripped and rehybridized to a probe for β-actin. Representative lanes from one experiment are depicted. Exposure for the $A_{2B}R$ probe was for 64 hr; exposure for the actin probe was for 19 hr. Arrowheads indicate the migration positions of the 28S and 18S ribosomal RNA species. The normalized data from two independent experiments were pooled (N = 8) and analyzed by the Mann–Whitney test. There was no significant difference (two-tailed P value = 0.4776) in the normalized hybridization intensities between the control (water) and NECA-pretreated (desensitized) groups.

fifth of the amount of plasmid encoding the tagged $A_{2B}R$. The immunoreactivity of membranes from NECA-pretreated and control cells was similar under these conditions of transfection, although the intensity of expressed bands was reduced (not shown). The observations from northern and western blot experiments suggest that NECA-desensitized COS 7 cells have preserved expression of $A_{2B}Rs$. Moreover, decreased agonist affinity, phosphorylation by PKC or PKA, and loss of receptors from membranes do not

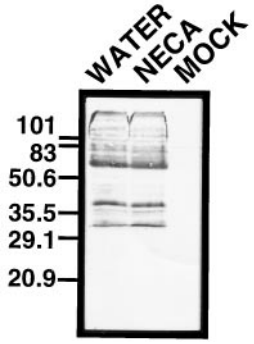

FIG. 9. Western blot analysis of the FLAG epitope-tagged $A_{2B}R$ in cell membranes. COS 7 cells were transiently transfected with the FLAG epitope-tagged $A_{2B}R$ or pcDNA3 (MOCK). $A_{2B}R$ transfected cells were pretreated for 17 hr with water or NECA (1 μM), and cell membranes were harvested for western blot analysis as described in Materials and Methods. Each lane was loaded with 10 μg of membrane protein. Representative lanes from one experiment assayed in duplicate are shown. The migration of size markers (kDa) is indicated. Identical results were obtained in two other, independent, experiments.

appear to account for hyporesponsiveness to subsequent acute agonist challenge.

DISCUSSION

Adenosine receptors mediate cellular responses to adenosine, an endogenous regulatory purine nucleoside. Intracellular catabolism of the high energy compound ATP provides a source of adenosine which then can be (1) recycled to reform high energy phosphates, (2) released from the cell, or (3) enzymatically degraded to inosine. Catabolism of extracellular ATP by ecto-ATPases is a mechanism to generate adenosine in the interstitium. High levels of adenosine result from the catabolism of ATP during periods of high energy utilization or during hypoxia when regeneration of ATP is hindered [23, 24]. Another potential source of extracellular adenosine is the ATP released in conjunction with neurotransmitters. Once in the extracellular space, adenosine can act as an extracellular regulator before being inactivated.

Adenosine released from hypoxic brain tissue causes vasodilation, increased blood flow, and decreased neural activity, which are neuroprotective [25]. Similarly, in the ischemic heart, adenosine contributes to vasodilation and bradycardia, both of which are protective in instances of unbalanced oxygen supply and demand [26, 27]. These findings suggest that adenosine integrates responses to ischemia by serving as a herald of cellular energy depletion, which facilitates oxygen and nutrient delivery, and also decreases energy utilization. Newby [28] proposed that adenosine is a "retaliatory metabolite" liberated by compromised cells in response to an insult. According to this concept, responses elicited by adenosine counteract external influences, such as a loss of nutrient and oxygen supply, which deplete energy resources and threaten cell survival.

Interestingly, receptors for the so-called "retaliatory metabolite" are subject to agonist-induced desensitization, a mechanism that terminates cellular responses to sustained stimulation by endogenous as well as exogenous agonists. Agonist-induced desensitization of adenosine receptor family members has been studied in several models including endogenously expressing [4, 9, 29] and transfected [5, 6] cell lines, brain slices [30], and *in vivo* [31, 32]. Like the well-characterized β-adrenergic family of receptors [3], the results suggest that A_1, A_{2A}, and A_3 adenosine receptors undergo agonist-induced functional desensitization. Multiple processes contribute to the loss of receptor responsiveness, including phosphorylation that can contribute to uncoupling, internalization of the receptor protein along with degradation, and alterations in the activities of enzymes in the signal transduction pathway of the receptors [2]. Agonist-induced desensitization of the A_{2B}R has not been reported, although heterologous desensitization of the A_{2B}R by activation of the A_{2A}R has been suggested [9] in cells where these receptors are co-expressed.

As suggested by studies in the chick myocardium, A_{2A}Rs and A_{2B}Rs coupled to the same functional responses may co-exist in some tissues [16]. Because A_{2B}Rs are relatively insensitive to adenosine, they may become activated, or remain activated, when other adenosine receptors have become desensitized by high levels of adenosine in pathophysiologic conditions. This would tend to preserve cell or organ functional responsiveness to adenosine, even in states of extreme adenosine overproduction. A similar concept has been proposed for neuroglia [14]. Desensitization of A_{2B}Rs would terminate even these responses and might protect cells from excessive adenosinergic stimulation in severely compromised tissues.

In testing the possibility that the A_{2B}R is subject to agonist-induced desensitization, we found that A_{2B}Rs expressed by COS 7 cells undergo a desensitization that is time dependent and reversible. The response to direct activation of G proteins with choleratoxin is largely preserved. Since the acute agonist challenges were carried out in the presence of the phosphodiesterase inhibitor Ro20-1724, loss of agonist responsiveness does not appear to result solely from changes in the signal transduction pathway, such as enhanced degradation of cAMP. We found that epitope-tagged A_{2B}Rs expressed in CHO cells behave in a fashion qualitatively similar to the endogenously expressed receptor. It is unlikely that activation by NECA of the adenosine receptors negatively coupled to adenylate cyclase, the A_1R and the A_3R, accounts for our observations because neither COS nor CHO cells appear to exhibit endogenous expression of these receptors [2, 33–36]. Because the affinity of A_{2B}Rs for NECA is relatively low [12], it is unlikely that membranes retain the agonist through washing and medium changes. Thus, retained agonist should not prevent subsequent agonist activation of the A_{2B}R. Furthermore, receptor responsiveness is restored in recovery experiments, which is consistent with reversible changes in the receptor protein accounting for loss of agonist responses.

By northern blot analysis, levels of A_{2B}R mRNA were preserved in COS 7 cells treated with NECA under conditions that produced maximum desensitization for cAMP production. Decreased receptor gene expression, with consequent decreased receptor protein synthesis, thus does not appear to account for diminished responses to agonists. These observations contrast with the finding in PC12 cells that adenosine receptor agonists cause decreased levels of A_{2A}R mRNA, which are associated with reduced receptor concentrations [37]. Cell type and species differences, along with the possibility of differential regulation of the two adenosine receptor subtypes, may account for the disparate observations.

The epitope tag system permits an initial assessment of the A_{2B}R protein, a task made difficult by the paucity of high affinity and selective ligands for the A_{2B}R. A complex immunoblotting pattern was obtained with membrane samples from COS cells transfected with the epitope-tagged receptor. One prominent band migrated at ~37 kDa, which is in reasonable agreement with the estimated molecular mass of the rA_{2B}R [11] plus the additional amino terminal

length of the tagged receptor. A second prominent band migrated at approximately twice this mass, possibly consistent with dimerization of the epitope-tagged receptor despite analysis in reducing and denaturing conditions. Apparent aggregation has been reported for hexahistidine-tagged and wild-type A_{2A}Rs in transfected cells [38], and also in brain membranes [38, 39]. The biochemical basis for these observations is not established. Our findings suggest that A_{2A} and A_{2B} adenosine receptor proteins may share structural features predisposing them to aggregate under commonly employed analytical conditions. As suggested for the transfected A_{2A}R [38], the third prominent immunoreactive band in the membranes from A_{2B}R transfected cells which migrates at ~30 kDa may represent a carboxyl-terminally truncated species resulting from degradation of the cytoplasmic tail.

Membrane levels of the epitope-tagged A_{2B}R are preserved in desensitized cells as determined by western blot analysis at two different concentrations of transfected plasmid and protein expression. Large scale loss (down-regulation) of the receptor protein is thus unlikely to account for the loss of agonist responsiveness in desensitized cells. Receptor number may influence the magnitude of measures of receptor modulation, such as down-regulation [20–22]. However, the absolute differences reported for modulatory effects in comparisons of cells expressing high and low numbers of receptors are relatively small and do not necessarily point to qualitatively different mechanisms of regulation. In conjunction with the northern blot data and the results of cAMP studies with choleratoxin and high concentration NECA stimulation, our observations with membrane proteins are consistent with the possibility that a mechanism contributing to agonist-induced desensitization of the A_{2B}R is uncoupling from the G protein complex.

Receptor uncoupling from G proteins is associated with phosphorylation [40]. Two classes of kinases, second messenger dependent kinases and G protein-coupled receptor kinases, have been implicated in the mechanisms leading to receptor desensitization. The rat A_{2B}R has multiple potential phosphorylation sites [11, 12]; thus, the substrate exists for the action of these enzymes. Our data with the PKA inhibitor H-89 and the PKC inhibitor bisindolylmaleimide suggest that these second messenger-dependent, serine-threonine kinases are not involved in the desensitization of the A_{2B}R. Although the role of other kinases is not excluded by our data, we postulate that a G protein-coupled receptor kinase contributes to regulation of the A_{2B}R. Additional work will be needed to elucidate the role of particular phosphorylation sites and further define the time sequence of desensitization. The development of receptor-specific antibodies and high affinity, specific A_{2B}R ligands would facilitate these studies.

In summary, we demonstrated that the A_{2B} subtype of adenosine receptor, like the related A_1, A_{2A}, and A_3 adenosine receptors, is subject to agonist-induced desensitization. The desensitized cell can recover functional responsiveness. The integrity of the cAMP signal transduction pathway is retained in the desensitized cell. We suggest that uncoupling of the A_{2B}R from the signal transduction pathway contributes to desensitization. Desensitization of the A_{2B}R is a potential mechanism to regulate adenosine responsiveness by immune, neural, gastrointestinal, or cardiovascular tissues.

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