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Desensitization of the Canine A_{2a} Adenosine Receptor: Delineation of Multiple Processes

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SUMMARY

Stable cell lines that express the canine-derived A_{2a} adenosine receptor (A_{2a}AR) have been generated. Using a previously characterized anti-A_{2a}AR antibody probe, we have identified the recombinant receptor protein and examined the desensitization process of this G protein-coupled receptor. Agonist exposure induced a rapid desensitization of A_{2a}AR-stimulated adenylyl cyclase activity. This was associated with reduced affinity of the receptor for the A_{2a}AR-selective agonist [^{3}H]CGS21680 and agonist-stimulated phosphorylation of the receptor protein. Agonist-stimulated A_{2a}AR sequestration into a light membrane fraction was also detected over the same time frame but, whereas inhibition of this process did not affect the extent of desensitization, the rapid recovery normally observed after short term agonist exposure was dramatically reduced. Long term agonist treatment resulted in the down-regulation of A_{2a}ARs and up-regulation of $G_{i\alpha2}$ and $G_{i\alpha3}$, as determined by immunoblotting. Recovery of A_{2a}AR function after agonist removal required several hours and was associated with the return of receptor levels to control values. In contrast, inactivation of G_i proteins by pertussis toxin treatment did not alter the extent of agonist-induced desensitization observed. Neither short nor long term desensitization could be mimicked by elevation of intracellular cAMP levels alone. Therefore, these data suggest that A_{2a}AR desensitization is mediated by multiple, temporally distinct, agonist-dependent processes. Agonist-stimulated phosphorylation of the receptor may induce short term desensitization by impairing receptor-G_s coupling, whereas long term down-regulation of receptor number and up-regulation of inhibitory G proteins mediate long term adaptation.

The ability of the ubiquitous nucleoside adenosine to modulate important physiological processes such as platelet aggregation, lipolysis, and neurotransmission has been known for many years (1). Only recently, however, has the molecular nature of the cell surface ARs responsible for initiating these events been elucidated (2). Functional and molecular cloning studies have demonstrated that ARs can be divided into at least three groups; A_1ARs mediate inhibition of adenylyl cyclase activity via interaction with one (or more) G_i proteins, whereas A_2ARs stimulate adenylyl cyclase activity by coupling to G_s (2). Expression of the rat A_3AR confers adenosine-mediated inhibition of adenylyl cyclase activity, although whether this is its only signaling function *in vivo* is uncertain (3). Each of the cloned ARs exhibits the predicted seven-membrane-spanning domain topography characteristic of most G protein-coupled receptors (4, 5).

A_2ARs can be further divided into A_{2a} and A_{2b} subtypes, which have been distinguished by pharmacological and molecular biological studies (6–8). Although both receptors are capable of stimulating adenylyl cyclase activity, only the A_{2a}AR is capable of binding the agonist CGS21680 with high affinity (7, 8). Furthermore, *in situ* hybridization and Northern blotting experiments have demonstrated that these receptors exhibit distinct patterns of expression (7).

Agonist-induced desensitization or refractoriness is a universal feature of G protein-coupled receptors, although only the β_2-adrenergic receptor and rhodopsin have been studied extensively. These studies suggest that, after short term agonist exposure, agonist-occupied β_2-adrenergic receptors uncouple from G_s due to phosphorylation events catalyzed by receptor-specific kinases (e.g., βARK-1 and -2) and/or kinases regulated by levels of intracellular second messengers (e.g., PKA). Phosphorylation of the receptor by βARK(s) increases the affinity

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ABBREVIATIONS: AR, adenosine receptor; ADA, adenosine deaminase; βARK, β-adrenergic receptor kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; NECA, 5′-*N*-ethylcarboxamidoadenosine; PIA, phenylisopropyladenosine; PAPA-APEC, (−)-N^6-[(*R*)-1-methyl-2-phenylethyl] adenosine; PMSF, phenylmethylsulfonyl fluoride; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PTx, pertussis toxin; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary.

of the receptor for cytosolic factors ('arrestin proteins'), thereby competitively inhibiting receptor binding to G_s, whereas phosphorylation by PKA directly impairs the ability of the receptor to interact with G_s, as well as increasing its ability to couple to G_i (9, 10). Receptor sequestration, a process whereby a receptor translocates to an ill defined 'light membrane' fraction, has been described in several cell lines, although the mechanisms by which this occurs are poorly understood (11, 12). The mechanisms by which long term desensitization occurs are also unclear, although studies on many receptors have shown that prolonged exposure to agonists can regulate expression of both a receptor and/or its associated G protein (13–16). Moreover, phenomena whereby long term activation of the stimulatory pathway of adenylyl cyclase results in the enhanced functioning of other G protein-coupled pathways have also been described (16, 17).

Several studies in rat kidney cells (18), vascular smooth muscle cells from rat aorta (19), NG108–15 cells (21), and DDT_1 MF-2 cells (20) have shown that the A_2AR response undergoes a rapid desensitization after short term exposure (several minutes) to agonists such as 2-chloroadenosine, NECA (18, 19, 21), or the more A_{2a}AR-selective ligand PAPA-APEC (20). Moreover, this desensitization is homologous, inasmuch as stimulatory hormone-, fluoride-, and forskolin-stimulated adenylyl cyclase activities are unaffected under these conditions (18–21). Nevertheless, study of A_2AR desensitization in established cell lines has several limitations. First, the A_2AR-stimulated adenylyl cyclase response in many cell types is relatively small, compared with that of other G_s-coupled receptors. Second, it is possible that a given cell line may express both A_{2a}ARs and A_{2b}ARs, as well as other A_2AR subtypes that have yet to be isolated; indeed, we have noted that A_{2a}ARs from liver and brain exhibit different reactivities to two polyclonal antibody preparations raised against distinct regions of the canine-derived A_{2a}AR protein (22). As a result of these technical difficulties, the mechanisms by which the observed desensitization occurs have remained unknown. To circumvent these problems, we have transfected CHO cells with the canine thyroid-derived RDC8 cDNA, which codes for an A_{2a}AR (23), and isolated clonal cell lines that exhibit both high levels of specific agonist binding and robust stimulation of adenylyl cyclase activity, to begin to characterize mechanisms of short and long term A_{2a}AR desensitization at the molecular level.

Experimental Procedures

Materials. [^{32}P]Phosphoric acid, [α-^{32}P]ATP, [α-^{32}P]NAD$^+$, [^{3}H]cAMP, [^{3}H]CGS21680, and ^{125}I-Protein A were from DuPont-New England Nuclear. Protein A-agarose, soybean trypsin inhibitor, leupeptin, PMSF, benzamidine, pepstatin A, chloramine T, ATP, dATP, GTP, creatine phosphokinase, concanavalin A, and HEPES (sodium salt) were from Sigma. Cyanogen bromide-activated Sepharose 4B was from Pharmacia. Phosphocreatine, restriction enzymes, and T4 ligase were from Boehringer-Mannheim. All electrophoresis reagents were from Bio-Rad. Cell culture reagents were from GIBCO. PTx was from List Biochemicals. The plasmid pSVNeo was from Pharmacia. All other chemicals were of the highest grade commercially obtainable.

Cell culture and transfections. Transfected CHO cells were grown as monolayers in 75-cm^2 flasks, in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml G418 (to maintain selection pressure). Approximately 3×10^5 cells in 25-cm^2 flasks were co-transfected with 30 μg of pBC12-BI expression vector (described in Ref. 24 and kindly provided by Dr. Bryan Cullen, Duke University) containing wild-type A_{2a}AR cDNA (described in Ref. 23 and kindly provided by Dr. Gilbert Vassart, Universite Libre de Bruxelles, Belgium) and 1.5 μg of pSVNeo (conferring G418 resistance), by a modified calcium phosphate precipitation/15% glycerol shock procedure (24). Cells were maintained in the presence of 400 μg/ml G418 until ~14 days after transfection, when isolated colonies were selected and replated. Identification of receptor-expressing clones was by radioligand binding using 20 nM [^{3}H]CGS21680, as described below.

Crude membrane preparation and radioligand binding. Membranes from CHO cells were prepared as follows. After aspiration of medium from the flasks, monolayers were rapidly washed three times with 20 ml of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 5 mM EDTA). Four milliliters of fresh lysis buffer were then added, into which the cells were scraped before Dounce homogenization (20 strokes) on ice. After centrifugation at 42,000 × g for 15 min at 4°, membranes were resuspended in a minimal volume of 50 mM HEPES, pH 6.8, 10 mM $MgCl_2$ (50/10 buffer), with 0.15 unit/ml ADA, for immediate use in binding assays as described previously (25). Protein concentrations were determined by the method of Bradford (26). Typically, experiments using CHO cells employed 20–80 μg of protein/tube.

Desensitization conditions. Cells in monolayers nearing confluence were washed once with 20 ml of PBS, and fresh Ham's F-12 medium containing 5% fetal bovine serum was added. NECA and ADA (included to remove adenosine released by the cells) were then added (to the final concentrations specified in the figure legends) and incubations were carried out for the indicated times at 37° in a cell culture incubator. Incubations were terminated by placing flasks on ice, aspirating off the medium, and rapidly washing the monolayer three times with 20 ml of ice-cold lysis buffer. Cells were then detached by scraping into 4 ml of lysis buffer and membranes were made as described previously.

Photoaffinity labeling. This was performed as described previously, using 0.8 nM ^{125}I-azido-PAPA-APEC (27); membranes from one 75-cm^2 flask were used for each condition.

Assay of adenylyl cyclase activity. Membranes prepared from one flask of transfected CHO cells were immediately resuspended by Dounce homogenization (20 strokes) in 1 ml of 75 mM Tris·HCl (pH 7.4 at 30°) containing 200 mM NaCl and 12.5 mM $MgCl_2$. After incubation with 6 units/ml ADA for 15 min at 30° to remove endogenous adenosine, 40 μl of membrane suspension (~40 μg of protein) were added to 40 μl of reaction mixture (0.14 mM dATP, 5 mM phosphocreatine, 30 units/ml creatine phosphokinase, 12 μM GTP, and 1.5 μCi of [α-^{32}P]ATP) and 20 μl of water or drugs. Papaverine (100 μM) was also included to inhibit low-K_m phosphodiesterases. For experiments in which NaF or forskolin was included in the assay, GTP was omitted from the reaction mixture. All incubations were for 15 min at 30°. Reactions were terminated by placing the tubes on ice and adding 1 ml of stop solution (10×10^3 cpm/ml [^{3}H]cAMP, 0.3 mM cAMP, and 0.4 mM ATP) to each tube. [^{32}P]cAMP was purified by sequential chromatography with Dowex-50 and alumina columns, as described by Salomon *et al.* (28).

Preparation of a light membrane fraction. Three flasks of CHO-A_{2a}AR cells were used for each light membrane preparation. After treatment with vehicle or agonist, monolayers were rapidly washed with ice-cold PBS and scraped into 4 ml of PBS containing 0.25 mg/ml concanavalin A to block further receptor redistribution (12). Cells were collected by centrifugation and were resuspended in 5 ml of lysis buffer supplemented with protease inhibitors previously shown to prevent A_{2a}AR degradation (25); these were soybean trypsin inhibitor (100 μg/ml), leupeptin (5 μg/ml), pepstatin A (1 μg/ml), and PMSF (0.1 mM). After Dounce homogenization (20 strokes), a pellet was sedimented by centrifugation at 42,000 × g for 20 min. Four milliliters of the supernatant were collected, and light membranes were pelleted by centrifugation at 140,000 × g for 70 min. The resulting 'glassy' pellets were resuspended and equivalent amounts were prepared for electrophoresis and immunoblotting.

SDS-PAGE and immunoblotting. For immunoblotting of G protein subunits, the appropriate amounts of membrane protein were collected by centrifugation and prepared for electrophoresis by resuspension in 10% (w/v) SDS electrophoresis sample buffer and boiling for 5 min before loading for SDS-PAGE. For immunoblotting of A_{2a}ARs, ~1 mg of membrane protein was collected by centrifugation and solubilized with 100 μl of 0.8% (v/v) Triton X-100 in 100 mM Na_2HPO_4, pH 6.5, 5 mM EDTA, containing 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 100 μM benzamidine, and 0.1 mM PMSF. After incubation on ice for 1 hr to solubilize membrane proteins, soluble fractions were collected by centrifugation at 100,000 × g for 1 hr at 4°, in a benchtop ultracentrifuge. Protein concentrations of soluble fractions were then determined and 300 μg were added to an equal volume of 16% (w/v) SDS electrophoresis sample buffer before boiling for 2 min and electrophoresis. Discontinuous electrophoresis was performed as described by Laemmli (29), using 10% (w/v) polyacrylamide resolving gels. Transfer of proteins to nitrocellulose and immunodetection with primary antibodies and ^{125}I-Protein A were performed as described previously (22).

The following primary antibodies were used for immunoblotting at the concentrations indicated in parentheses: TP/2 (4 μg/ml affinity-purified IgG) for detection of A_{2a}ARs (22), TG982 (1/4000 dilution of serum) for detection of $G_{i\alpha 2}$ (30), RM/1 (1/1000 dilution of serum) for detection of $G_{s\alpha}$ (31), TG977 (1/8000 dilution of serum) for detection of $G_{i\alpha 3}$ (30), and TG987 (1/4000 dilution of serum) for detection of G protein β subunits (30). TP/2 was affinity purified from whole serum as described previously (22). Blots were exposed to Kodak XAR film with dual intensifying screens for 12–48 hr. Quantitation of immunoblots was by excision and counting of γ-radiation from bands of interest, with suitable correction for background radiation. For each antibody concentration used, initial experiments were performed to determine the range in which the relationship between the amount of protein loaded and the ^{125}I-Protein A detected was linear; subsequent comparative immunoblots used protein loadings within these ranges.

Labeling of CHO cells with [^{32}P]orthophosphate. Cell monolayers nearing confluence were washed twice with PBS, and fresh Ham's medium minus serum, supplemented with $^{32}P_i$ (5 mCi/treatment), 30 μM sodium phosphate, pH 7.2, and 0.3 unit/ml ADA, was added. After 2.5 hr at 37° in a cell culture incubator, agonist, forskolin, or vehicle was added directly to this medium and the incubation was continued for an additional 30 min.

Immunoprecipitation of A_{2a}ARs. After labeling, flasks were placed on ice, the medium was removed, and the monolayers were rapidly washed five times with ice-cold PBS. Cells were then scraped into 4 ml of HPEN (50 mM HEPES, pH 7.2, 10 mM $Na_4P_2O_7$, 100 mM NaCl, 4 mM EDTA). This buffer also included protease inhibitors (10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.1 mM PMSF) and phosphatase inhibitors (10 mM NaF, 1 mM phosphoserine, 1 mM phosphothreonine, 1 mM β-glycerophosphate, and 0.1 mM sodium vanadate). After Dounce homogenization (20 strokes), membrane pellets were collected by centrifugation. These pellets were washed twice by rehomogenization in HPEN plus inhibitors and further centrifugation. The final membrane preparation was solubilized in 0.8% Triton X-100/HPEN plus inhibitors by pipette trituration and passage through a 20-gauge needle. After solubilization on ice for 2 hr, soluble fractions were collected by centrifugation at 100,000 × g for 1 hr. SDS was then added to a final concentration of 0.08% and the mixture was precleared of nonspecifically binding proteins by overnight incubation at 4° with preimmune serum and 150 μl of Protein A-agarose. After centrifugation, the supernatant was incubated with 20 μg/ml affinity-purified TP/2 antibodies for 3 hr on ice; nonspecific immunoprecipitation was assessed by the inclusion of antigenic peptide at 20 μg/ml. After the addition of 30 μl of Protein A-agarose and further incubation for 2 hr, immunoprecipitates were collected by centrifugation and extensively washed with HPEN plus

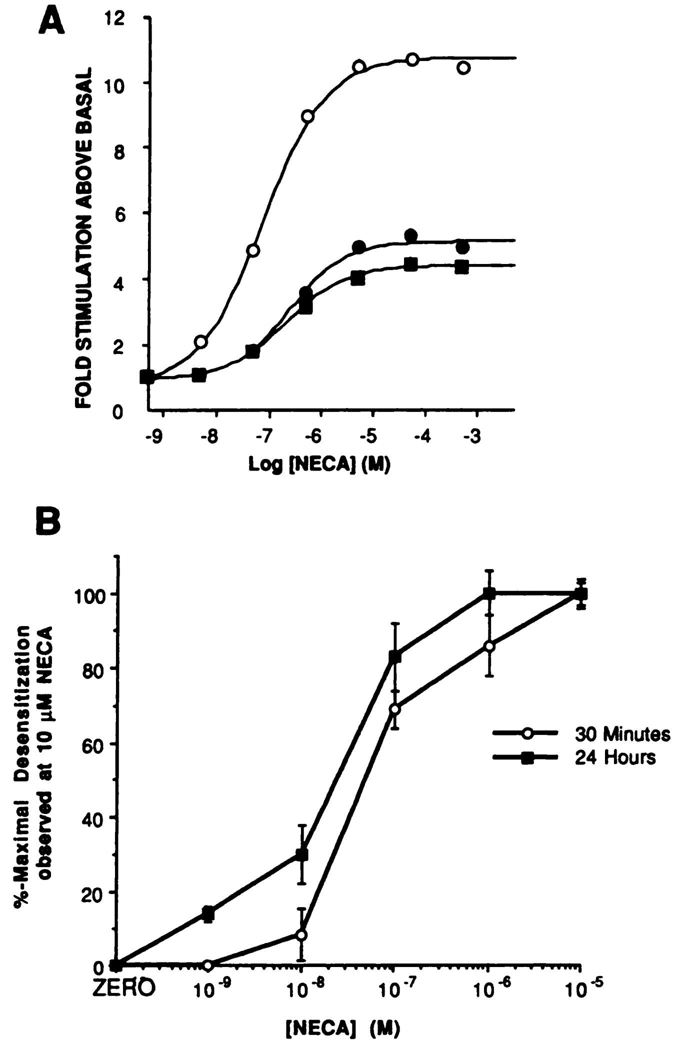


Fig. 1. Desensitization of the A_{2a}AR-stimulated adenylyl cyclase response. A, Dose dependence of NECA-stimulated adenylyl cyclase activity in membranes prepared from cells treated in the absence (○) or presence of 10 μM NECA for either 30 min (●) or 24 hr (■) at 37°. This experiment is representative of 10 that produced quantitatively similar results; basal activities were 7.9 ± 2.2 pmol/min/mg (control), 8.0 ± 0.6 pmol/min/mg (30-min treated), and 7.0 ± 1.0 pmol/min/mg (24-hr treated). B, Dose dependence of A_{2a}AR desensitization to increasing concentrations of NECA at 30 min and 24 hr. Data are presented as a percentage of the desensitization observed with 10 μM NECA (set at 100%); the mean desensitization produced by exposure to 10 μM NECA in these experiments was to 59% (30 min) and 45% (24 hr) of the 10 μM NECA-stimulated adenylyl cyclase activity produced in membranes from ADA-treated cells. Each *data point* is the mean ± standard error of three separate determinations.

inhibitors containing 1% Triton X-100 and 0.1% SDS, followed by two washes with HPEN and inhibitors without detergent. Phosphoproteins were eluted by the addition of 30 μl of SDS-PAGE sample buffer and incubation at room temperature for 60 min. After centrifugation to sediment the Protein A-agarose, equal volumes of supernatant were analyzed by SDS-PAGE and autoradiography.

PTx-catalyzed [^{32}P]ADP-ribosylation. This was performed on isolated membrane preparations as described by Ribeiro-Neto *et al.* (32).

Data analysis. Scatchard plots (33) of radioligand binding data were fitted by least squares analysis. Adenylyl cyclase dose-response curves were analyzed by a computer-assisted curve-fitting program previously validated (34). Data in the text and in tables are presented as mean ± standard errror values for the number of experiments indicated, unless otherwise stated. Graphs depict representative experiments, which were performed at least twice. Statistical analyses were performed using Student's t test (two-tailed), with an α probability of 0.05.

Results

Characterization of CHO cells expressing A_{2a}ARs. Cotransfection of CHO cells with pSVNeo and pBC-A_{2a}AR cDNAs resulted in the isolation of several clonal cell lines that exhibited at least 50% specific binding of 20 nM [^{3}H]CGS21680; one of these clones was chosen for further analysis. Scatchard analysis of data from saturation isotherms performed on membrane preparations showed that [^{3}H]CGS21680 bound to a single, saturable, high affinity site with a K_d of 7.4 ± 1.7 nM (10 experiments) and B_{max} values ranging from 0.88 to 2.20 pmol/mg in 10 experiments. The existence of a single class of binding sites for [^{3}H]CGS21680 and the K_d value observed are consistent with previous observations of endogenous A_{2a}ARs in rat, bovine, and canine striatum, as well as those from PC-12 cells (35, 36). In parallel experiments, no specific binding of [^{3}H]CGS21680 was detected in membranes from nontransfected CHO cells (data not shown).

The expressed A_{2a}AR was functional, inasmuch as membranes from transfected cells displayed a ligand-stimulated adenylyl cyclase activity that exhibited the expected A_{2a}AR pharmacology, i.e., a potency order of NECA > CGS21680 > (*R*)-PIA > (*S*)-PIA, with Hill coefficients for each of these ligands of 0.9 or greater, indicative of interaction of each ligand with a single class of receptor binding sites. The response of membranes from nontransfected cells to either 10 μM CGS21680 or 10 μM NECA was barely detectable (~5% above basal). Therefore, the adenylyl cyclase response observed in membranes from transfected cells was due to the expression of the A_{2a}AR cDNA.

Agonist-induced desensitization of A_{2a}AR function. Fig. 1 and Table 1 show the desensitization pattern of A_{2a}AR function after exposure of cells to 10 μM NECA and 0.3 unit/ml ADA. Desensitization was first detected after a 15-min agonist exposure, and after 30 min the maximal stimulation elicited by NECA was ~50% of that observed in membranes

TABLE 1

Time course of onset of A_{2a}AR desensitization

Monolayers of CHO-A_{2a}AR cells were treated either with 10 μM NECA and 0.3 unit/ml ADA or with ADA alone (controls) for the indicated times before extensive washing, membrane preparation, and assay of adenylyl cyclase activity with increasing concentrations of NECA, as described in Experimental Procedures. Dose-response curves were generated by a computer modeling program, to give EC_{50} and maximal fold stimulation values. For these experiments, the maximal fold stimulation above basal activity obtained with NECA in membranes from ADA-treated cells (controls) was 10.3 ± 1.4 (mean ± standard error, 15 experiments).

	Stimulation	EC_{50}	n^a
	% of control	*μM*	
Control	100	0.09 ± 0.01	15
15 min	86.0 ± 0.5	0.18 ± 0.03	3
30 min	52.9 ± 3.3	0.15 ± 0.07	3
60 min	57.8 ± 1.6	0.16 ± 0.06	3
120 min	65.4 ± 1.2	0.16 ± 0.10	3
24 hr	42.0 ± 3.7	0.19 ± 0.07	4

[a] *n*, number of experiments.

TABLE 2

NaF- and forskolin-stimulated adenylyl cyclase activities in membranes from CHO-A_{2a}AR cells after agonist treatment

Cells were incubated with 0.3 unit/ml ADA in the absence (control) or presence of 10 μM NECA for either 30 min or 24 hr at 37°. Membranes were then prepared for assay of adenylyl cyclase activity with 10 mM NaF and 10 μM forskolin, as described in Experimental Procedures. The values for fold stimulation above basal are means ± standard errors for three experiments. Basal activities in these experiments were 8.0 ± 3.3 pmol/min/mg (control), 6.5 ± 2.2 pmol/min/mg (30 min treated), and 4.8 ± 1.1 pmol/min/mg (24-hr treated).

	Adenylyl cyclase activity			
	10 mM NaF		10 μM Forskolin	
	30 min	24 hr	30 min	24 hr
	pmol/min/mg			
Control	15.8 ± 2.9	14.7 ± 3.7	9.9 ± 1.3	9.4 ± 0.5
+10 μM NECA	14.6 ± 0.7	13.6 ± 2.8	9.2 ± 2.2	10.1 ± 0.2

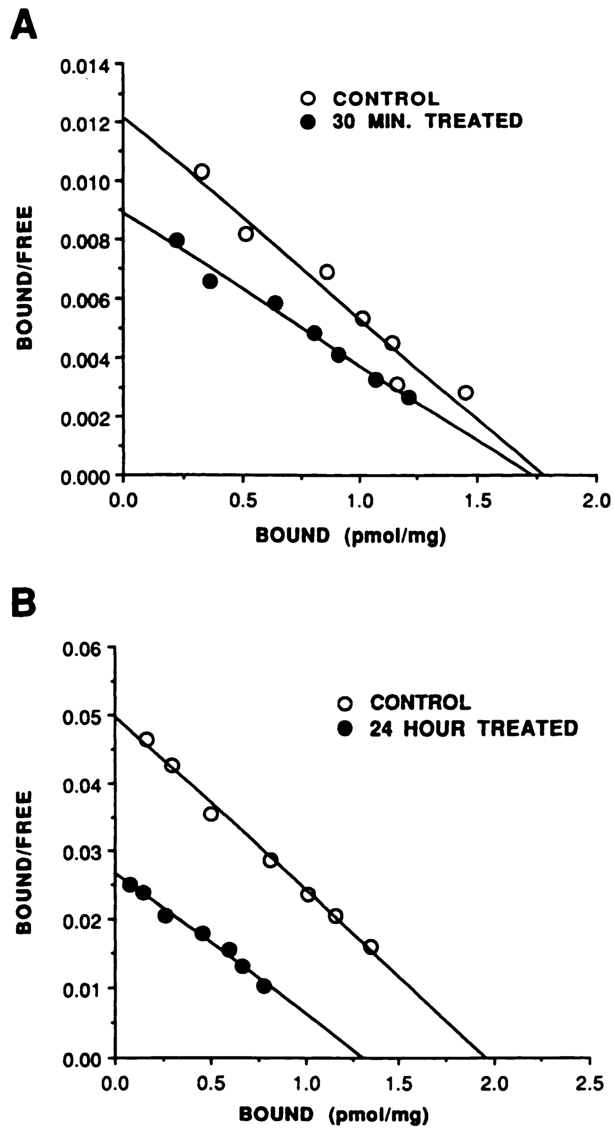
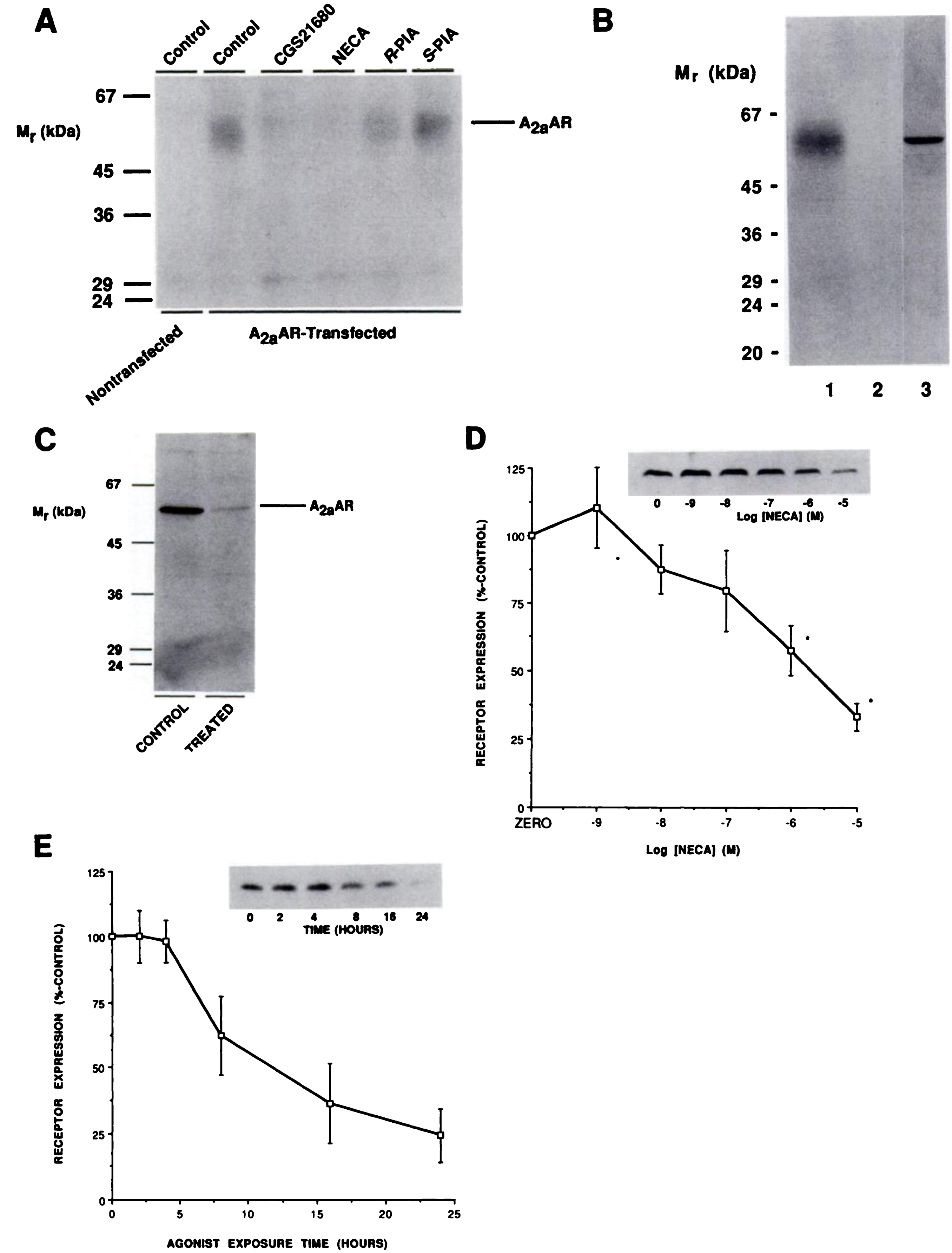


Fig. 2. Scatchard analysis of [^{3}H]CGS21680 binding after short and long term desensitization. CHO-A_{2a}AR cells were incubated with 0.3 unit/ml ADA, in the absence or presence of 10 μM NECA, for either 30 min (A) or 24 hr (B) before membrane preparation for [^{3}H]CGS21680 saturation binding experiments, as described in Experimental Procedures. ○, Binding in membranes from ADA-treated cells (controls); ●, binding in membranes from 10 μM NECA-treated cells. Data are presented from single experiments, which are representative of at least three performed for each time point.


A
Control
Control
CGS21680
NECA
R-PIA
S-PIA
67
Mr (kDa)
45
36
29
24
A2aAR
Nontransfected
A2aAR-Transfected
B
Mr (kDa)
67
45
36
29
24
20
1
2
3
C
67
Mr (kDa)
45
36
29
24
A2aAR
CONTROL
TREATED
D
RECEPTOR EXPRESSION (%-CONTROL)
125
100
75
50
25
0
ZERO
-9
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-6
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Log [NECA] (M)
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-9
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-5
Log [NECA] (M)
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RECEPTOR EXPRESSION (%-CONTROL)
125
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75
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25
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0
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15
20
25
AGONIST EXPOSURE TIME (HOURS)
0
2
4
8
16
24
TIME (HOURS)

prepared from cells that had been treated with ADA alone (Fig. 1A; Table 1). Incubation with NECA for 60 min or 120 min did not produce any further reduction in maximal stimulation; indeed, after incubation with agonist for 24 hr, the maximal stimulation produced by NECA fell only slightly further, to ~40% of that observed in membranes from control cells (Fig. 1A; Table 1). Desensitization of the A_{2a}AR response was associated with large reductions in the maximal adenylyl cyclase stimulation produced by NECA but with only a small increase in its EC_{50} value (Fig. 1A; Table 1). The abilities of 30-min or 24-hr exposure of cells to NECA to elicit desensitization exhibited similar dose dependencies (IC_{50} for 30-min exposure, ~40 nM; for 24-hr exposure, ~20 nM) (Fig. 1B).

Short term (30-min) or long term (24-hr) desensitization produced by treatment with 10 μM NECA did not result in any changes in the efficacies with which sodium fluoride or forskolin was able to stimulate adenylyl cyclase activity, suggesting that the G_s-catalytic unit interaction is unaffected by the desensitization process (Table 2). Therefore, the desensitization process selectively diminishes productive interaction between the A_{2a}AR and G_s.

Agonist-induced changes in [^{3}H]CGS21680 binding to the A_{2a}AR. After an agonist exposure time sufficient to cause functional desensitization, the number of binding sites for [^{3}H]CGS21680 did not change but the K_d increased by ~2-fold (K_d increased from 10.0 ± 1.5 nM to 20.5 ± 3.1 nM, three experiments, $p < 0.05$; B_{max} values were 1.46 ± 0.30 pmol/mg and 1.71 ± 0.44 pmol/mg for control and 30-min-treated samples, respectively, $p > 0.1$); Scatchard analysis of the data demonstrated that the whole population of [^{3}H]CGS21680 binding sites were converted to the lower affinity state, because binding to agonist-treated cells was still consistent with a single class of binding sites (Fig. 2A). Because agonists display a higher affinity for receptors that are coupled to their appropriate G protein, the increase in K_d observed after agonist exposure is consistent with reduced coupling efficiency between the A_{2a}AR and G_s.

[^{3}H]CGS21680 saturation binding experiments on membranes prepared from cells that had been treated with agonist for 24 hr exhibited additional alterations in agonist binding (Fig. 2B). Hence, whereas binding in membranes from treated cells was still of lower affinity (K_d increased from 10.3 ± 1.1 nM to 17.5 ± 2.8 nM, three experiments, $p < 0.05$), the total number of agonist binding sites was also reduced by approximately 40% (B_{max} was reduced from 1.91 ± 0.31 pmol/mg to 1.25 ± 0.20 pmol/mg, three experiments, $p < 0.05$).

Therefore, changes in agonist binding appear to occur in two stages; short term agonist exposure induces an apparent impairment of the coupling between the A_{2a}AR and G_s, manifested as reduced affinity of the receptor for [^{3}H]CGS21680. Long term agonist exposure also induces down-regulation of the total number of agonist binding sites.

Quantitation of A_{2a}ARs in membranes from control and desensitized cells. The identity of the expressed A_{2a}AR was determined by two methods. Photoaffinity labeling of CHO cell membranes with the agonist photoaffinity probe ^{125}I-azido-PAPA-APEC identified a single band, of 60 kDa, which was not present in nontransfected CHO cells (Fig. 3A). The labeling of this protein was blocked by AR agonists in a manner consistent with an A_{2a}AR pharmacology, i.e., CGS21680 = NECA > (*R*)-PIA > (*S*)-PIA (Fig. 3A). Immunoblotting of membranes from transfected cells with affinity-purified antibody TP/2 identified a single immunoreactive band, at 60 kDa, which was absent in nontransfected cells and which comigrated exactly with the labeled protein (Fig. 3B). We have previously characterized the ability of this antibody to recognize endogenous canine A_{2a}ARs, by immunoblotting and immunoprecipitation of photoaffinity-labeled receptors (22).

To determine changes in the total A_{2a}AR population, rather than just those receptors identified in agonist radioligand binding studies, we used antibody TP/2 in comparative immunoblotting studies. Immunoblotting of membranes from cells that had been treated for 24 hr in the presence or absence of 10 μM NECA demonstrated that a dramatic reduction in the level of immunoreactive receptors was associated with conditions under which the A_{2a}AR response underwent functional desensitization (87 ± 6% reduction, compared with levels in control cells, four experiments) (Fig. 3C). The loss of immunoreactive A_{2a}ARs was dose dependent, although relatively high doses were required (Fig. 3D). Time-course experiments showed that receptor number remained steady until 4 hr. Receptor loss was detected at 8 hr of treatment, and after 24 hr levels of receptor reached their minimum level (Fig. 3E).

A_{2a}AR sequestration after short term desensitization. Immunoblotting studies demonstrated that receptor levels did not decline until after several hours of agonist exposure. Therefore, mechanisms other than receptor loss must have been responsible for the short term uncoupling and desensitization. One possible explanation was that receptor sequestration, analagous to that described for other G protein-coupled receptors (11, 12, 37–41), occurred. However, preincubation with 0.45 M sucrose, an inhibitor of receptor internalization in CHO cells (37), did not affect the subsequent ability of NECA to cause desensitization after a 30-min agonist exposure (the response to 10 μM NECA was reduced to 61 ± 12% of control stimulation, three experiments). These observations suggested either that the A_{2a}AR did not undergo sequestration or that it underwent sequestration but this process was not involved in mediating

Fig. 3. Quantitation of A_{2a}ARs by immunoblotting after long term desensitization. A, Photoaffinity labeling of A_{2a}ARs with ^{125}I-azido-PAPA-APEC. Membranes from nontransfected or A_{2a}AR-transfected CHO cells were subjected to photoaffinity labeling as described in Experimental Procedures. The indicated agonists were present at a final concentration of 1 μM. B, Co-migration of A_{2a}ARs identified by photoaffinity labeling and immunoblotting. Membranes from A_{2a}AR-transfected CHO cells were identified by photoaffinity labeling in the absence (*lane 1*) or presence (*lane 2*) of 1 μM NECA or immunoblotting with 4 μg/ml affinity-purified TP/2 (*lane 3*), as described in Experimental Procedures. C, Treatment of CHO-A_{2a}AR cells with 0.3 unit/ml ADA for 24 hr at 37° in the absence (*CONTROL*) or presence (*TREATED*) of 10 μM NECA before membrane preparation and solubilization for immunoblotting with 4 μg/ml affinity-purified TP/2, as described in Experimental Procedures. This experiment is one of four performed, which produced quantitatively similar results. D, Dose dependence of A_{2a}AR loss with increasing concentrations of NECA. Cells were incubated with the indicated concentrations of NECA for 24 hr at 37° before membrane preparation, immunoblotting with TP/2, and quantitation as described in Experimental Procedures. Data are presented as means ± standard errors for three experiments. *Inset*, autoradiograph from one such experiment. *, Statistically significant ($p < 0.05$) reduction in receptor levels. E, Time course of loss of immunoreactive A_{2a}AR. Cells were incubated at 37° with 10 μM NECA for the indicated times before membrane preparation and immunoblotting with 4 μg/ml affinity-purified TP/2. The data are presented as mean ± half the range of values from two experiments, one of which is depicted in the *inset*.

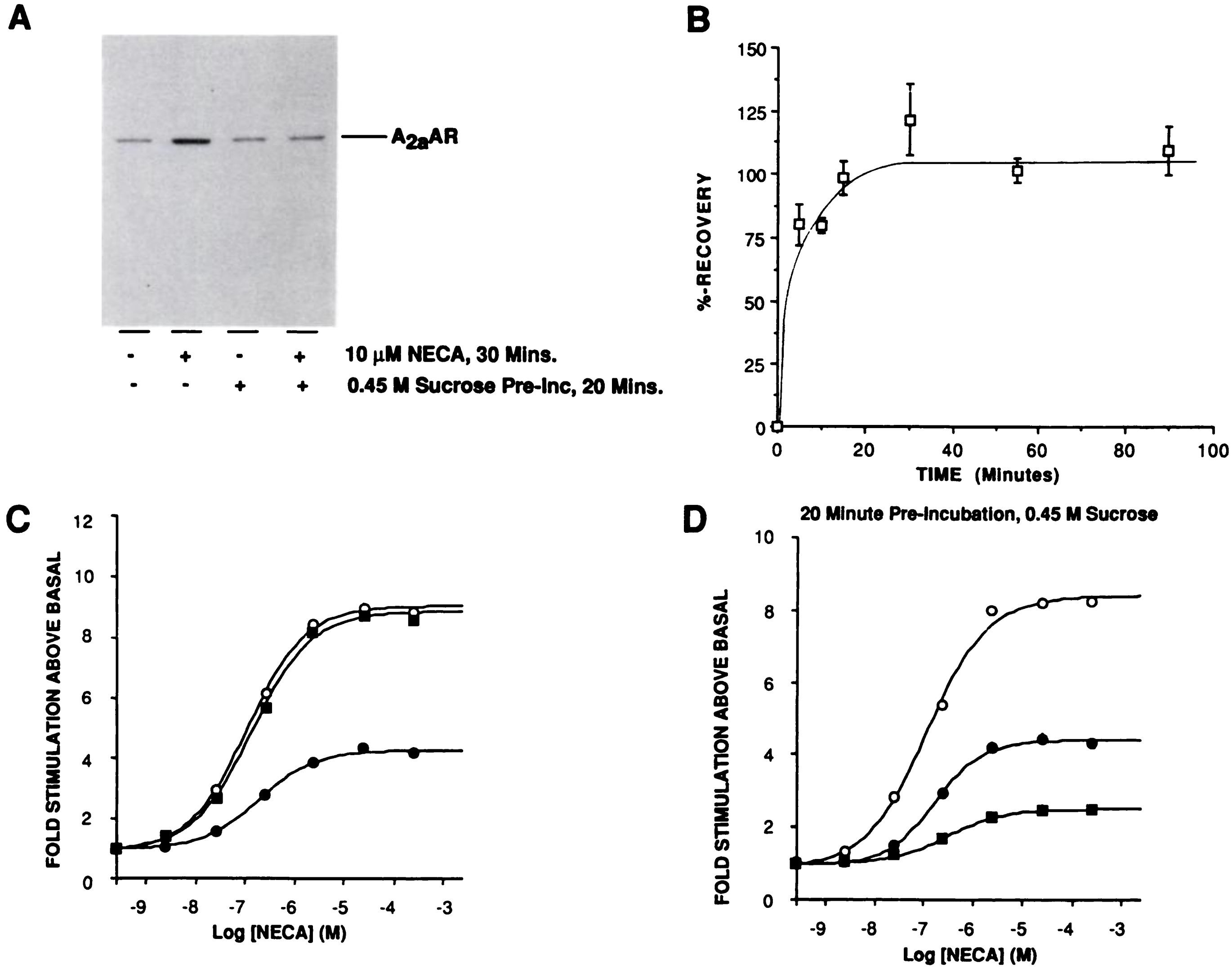

Fig. 4. $A_{2a}AR$ sequestration and recovery after short term desensitization. A, Cells were preincubated in medium with or without 0.45 M sucrose for 20 min at 37° before the addition of ADA alone (control) or with NECA to a final concentration of 10 µM and incubation for an additional 30 min. Light membrane fractions were then prepared and equal amounts were analyzed by SDS-PAGE and immunoblotting with affinity-purified anti-$A_{2a}AR$ antibodies, as described in Experimental Procedures. This experiment is one of three performed, which produced similar results. B, For recovery of $A_{2a}AR$ function after short term desensitization, cells were treated with or without 10 µM NECA and 0.3 unit/ml ADA for 30 min at 37°. Treated cells were then washed three times with warm PBS and incubated in agonist-free medium for the indicated times at 37°. Membranes were then simultaneously prepared for assay of adenylyl cyclase activity in the absence or presence of 10 µM NECA. The mean desensitization in these experiments was to 58% of untreated controls. Data points are presented as means ± standard errors from three separate determinations. Percentage of recovery was calculated as 100 × [fold stimulation (recovering cells) − fold stimulation (treated cells)/fold stimulation (control cells) − fold stimulation (treated cells)]. C, Cells were treated with (●) or without (○) 10 µM NECA for 30 min at 37°. One batch of treated cells were then washed and incubated in agonist-free medium, as described in B, for an additional 30 min (■). Membranes were then prepared simultaneously for assay of adenylyl cyclase activity with increasing concentrations of NECA. This experiment is one of three performed, which produced similar results. D, Cells were preincubated for 20 min at 37° with medium containing 0.45 M sucrose before the addition of ADA, with or without NECA, and assay of adenylyl cyclase activity as described for C. This is one of three experiments, which produced similar results.

short term desensitization. To discriminate between these possibilities, light membrane fractions were prepared, for immunoblotting with TP/2, from cells to which agonist was added after preincubation with or without sucrose (Fig. 4A); these experiments demonstrated that agonist treatment caused a rapid accumulation of receptors in light vesicles, consistent with a sequestration event (240 ± 30% increase, three experiments). However, sucrose pretreatment completely inhibited agonist-stimulated $A_{2a}AR$ accumulation in light membranes (4 ± 6% increase, three experiments) without drastically affecting basal levels of receptor in this membrane fraction (13 ± 7% increase in basal levels of receptor due to sucrose treatment, three experiments) (Fig. 4A).

Intriguingly, although sucrose pretreatment did not block short term desensitization, it altered the ability of the cells to recover from this state. The ability to regain adenylyl cyclase responsiveness was studied after exposure of the cells to 10 µM NECA for 30 min. After agonist removal, maximal adenylyl cyclase stimulation was regained very rapidly ($t_{1/2} < 5$ min) and remained stable for at least 90 min; analysis of NECA dose-response curves demonstrated that both potency and efficacy of $A_{2a}AR$ stimulation were completely restored (Fig. 4, B and C). Preincubation for 20 min with 0.45 M sucrose before agonist addition drastically reduced the resensitization observed 30 min after agonist washout (Fig. 4D). Hence, whereas untreated cells recovered to 90 ± 7% (four experiments) of the control stimulation 30 min after agonist removal, sucrose-pretreated cells either recovered only marginally or, as shown in Fig. 4D, exhibited further desensitization (pretreated cells recovered to 44 ± 15% of the control stimulation, four experiments).

Agonist-stimulated phosphorylation of the A_{2a}AR. Because inhibition of receptor sequestration did not affect the ability of the A_{2a}AR to desensitize after 30-min agonist exposure, we wished to ascertain whether the receptor was modified during the desensitization process. Phosphorylation was an obvious candidate for such a modification, because this has been implicated in mediating short term desensitization of both rhodopsin and the β_2-adrenergic receptor, both of which bear structural similarities to the A_{2a}AR (4). Hence, we labeled CHO-A_{2a}AR cells with [^{32}P]orthophosphate and incubated the cells with or without NECA before cell lysis and solubilization of membranes for immunoprecipitation of A_{2a}ARs with antibody TP/2. After a 30-min agonist exposure, a 60-kDa phosphoprotein was immunoprecipitated by TP/2; immunoprecipitation of this protein was completely abolished by the inclusion of antigenic peptide in the immunoprecipitation reaction (Fig. 5A). Despite the presence of several nonspecific bands in the immunoprecipitation, only the 60-kDa protein exhibited the necessary properties of the A_{2a}AR. First, phosphorylation was observed only in transfected cells (Fig. 5A). Second, the precipitated protein had the same molecular mass as the expressed A_{2a}AR. Finally, immunoprecipitation of this protein was completely abolished by the inclusion of antigenic peptide, whereas the labeling of the other bands was merely altered with the background labeling. The increased background labeling observed in lanes containing immunoprecipitated receptors is commonly observed when low abundance, phosphorylated, transmembrane receptors are enriched by either immunoprecipitation or purification by column chromatography (42, 43).

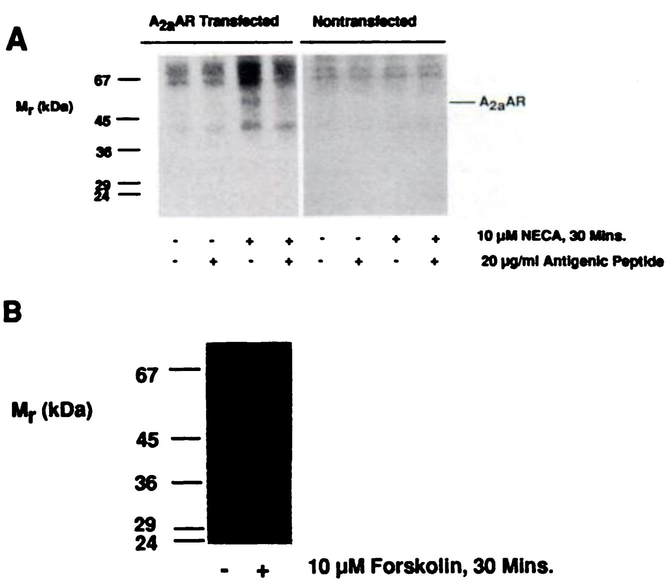

Fig. 5. Agonist-stimulated *in vivo* phosphorylation of the A_{2a}AR. A, Either untransfected (CHO) or A_{2a}AR-transfected (CHO-A_{2a}AR) cells were incubated with [^{32}P]orthophosphate and ADA for 2.5 hr before the addition of fresh ADA alone (control) or with NECA to a final concentration of 10 µM for 30 min at 37°. Membranes were then prepared for solubilization and immunoprecipitation with 20 µg/ml affinity-purified TP/2, in the absence or presence of antigenic peptide, as described in Experimental Procedures. B, CHO-A_{2a}AR cells were preincubated with [^{32}P]orthophosphate, as described for A, before incubation with 10 µM forskolin or 0.1% ethanol vehicle and immunoprecipitation as described.

Treatment of CHO-A_{2a}AR cells with 10 µM forskolin before solubilization and immunoprecipitation failed to stimulate the phosphorylation of the A_{2a}AR (Fig. 5B). This is consistent with the lack of consensus sites for PKA phosphorylation (4) and also eliminates the possibility that PKA indirectly mediates A_{2a}AR phosphorylation via activation of other kinases.

Effects of cAMP generation and PKC activation on A_{2a}AR responsiveness. To determine whether cAMP generation was responsible for either the rapid onset of desensitization or the long term reduction in responsiveness, cells were treated for 30 min or 24 hr with either 10 µM forskolin or 0.1% ethanol vehicle (Table 3). Treatment of cells with these compounds reduced neither the efficacy nor the potency with which NECA was capable of stimulating adenylyl cyclase activity in isolated membranes. Moreover, reducing the Mg^{2+} concentration in the adenylyl cyclase assay by 10-fold, to detect subtle changes in receptor-G protein coupling, failed to unmask any cAMP-mediated reduction in A_{2a}AR function (Table 3). Similarly, 30-min exposure of CHO-A_{2a}AR cells to 1 µM phorbol 12-myristate 13-acetate, a potent activator of PKC enzymes, did not result in a consistent reduction in A_{2a}AR-stimulated adenylyl cyclase activity under either assay condition, despite the presence of consensus PKC phosphorylation sites on the A_{2a}AR (Table 3). Hence, in this system, activation of either PKA or PKC could not account for the observed desensitization.

Recovery of A_{2a}AR function after long term desensitization. As shown in Table 1 and Fig. 1A, long term exposure to agonist resulted in only a slightly greater inhibition of A_{2a}AR function, compared with that observed after 30 min. However, after 24-hr exposure to 10 µM NECA, removal of agonist resulted in a much slower recovery of adenylyl cyclase responsiveness, which occurred over a period of several hours (Fig. 6A); analysis of NECA dose-response curves after 16 hr of recovery in agonist-free medium showed that both the efficacy and potency of the adenylyl cyclase response were restored (Fig. 6B). Immunoblotting of membranes demonstrated that recovery of the adenylyl cyclase response was associated with a complete recovery of receptor levels, suggesting that the two phenomena are related (Fig. 6C).

Quantitation of G protein subunits in membranes from control and desensitized cells. One possible mode of regulation whose importance in the desensitization process has been demonstrated in other systems involves regulation of the quantity of G protein subunits (14–17). Therefore, comparative immunoblotting experiments were performed using membranes from cells that had been treated in the presence or absence of 10 µM NECA for 24 hr (Fig. 7; Table 4). Immunoblotting with antisera specific for $G_{i\alpha2}$ or $G_{i\alpha3}$ demonstrated that long term exposure to agonist was associated with increased levels of both of these proteins in membranes from transfected cells (Fig. 7, A and B; Table 4). Long term agonist exposure did not consistently alter the expression of G_s α or β subunits (Fig. 7, C and D; Table 4). Interestingly, the ability of NECA to increase expression of these proteins was not solely due to its ability to elevate intracellular cAMP levels, because parallel treatment with 10 µM forskolin did not significantly alter the expression of either G_i α subunit (<10% difference, compared with controls, in three experiments) (Fig. 7, A and B). Similar treatment

TABLE 3

Effects of PKA and PKC activation on A_{2a}AR-stimulated adenylyl cyclase activity

Cells were treated for either 30 min or 24 hr in the presence of 0.3 unit/ml ADA and either 10 μM forskolin or 0.1% (v/v) ethanol vehicle (control). Cells were also treated for 30 min with ADA and either 1 μM phorbol 12-myristate 13-acetate (PMA) or 1 μM 4α-phorbol, an inactive phorbol ester (PKC control). Membranes were then prepared for assay of adenylyl cyclase activity in the absence or presence of 10 μM NECA, with $MgCl_2$ present at final concentrations of either 10 mM (high [Mg^{2+}]) or 1 mM (low [Mg^{2+}]), as described in Experimental Procedures. Data are presented as means ± standard errors for three experiments.

	30 min				24 hr			
	High [Mg^{2+}]		Low [Mg^{2+}]		High [Mg^{2+}]		Low [Mg^{2+}]	
	Stimulation[a]	EC_{50}	Stimulation	EC_{50}	Stimulation	EC_{50}	Stimulation	EC_{50}
	Fold	μM	fold	μM	fold	μM	fold	μM
Control	8.7 ± 0.6	0.13 ± 0.04	11.5 ± 0.3	0.21 ± 0.03	11.6 ± 0.2	0.21 ± 0.06	11.5 ± 0.3	0.28 ± 0.05
10 μM Forskolin	9.7 ± 0.7	0.19 ± 0.04	11.2 ± 0.2	0.40 ± 0.11	11.5 ± 1.3	0.23 ± 0.08	11.2 ± 0.3	0.50 ± 0.12
PKC control	8.3 ± 1.2	—[b]	14.3 ± 0.5	—	—	—	—	—
1 μM PMA	8.1 ± 1.1	—	15.9 ± 0.5	—	—	—	—	—

[a] Stimulation above basal.
[b] —, not determined.

with 100 μM 8-bromo-cAMP also failed to increase expression of G_i α subunits (data not shown).

Therefore, to ascertain whether functional G_i activity is necessary to observe A_{2a}AR desensitization, cells were treated for 24 hr with or without agonist, in the presence or absence of 20 ng/ml PTx to catalyze the ADP-ribosylation and inactivation of G_i α subunits (Fig. 8A). However, treatment with PTx did not alter the desensitization pattern observed, suggesting that the elevated expression of $G_{i\alpha2}$ and $G_{i\alpha3}$ is not directly involved in mediating long term desensitization; the response to 10 μM NECA after 24-hr agonist treatment was reduced to 41 ± 11% (PTx treated) or 50 ± 14% (untreated) of controls (set at 100%) (three experiments) (Fig. 8B).

Discussion

The isolation of a cDNA clone for the canine A_{2a}AR has facilitated the generation of stable cell lines expressing this protein in the absence of any other A_2ARs, thereby providing an ideal system to examine the roles of different processes in mediating the functional desensitization of the A_{2a}AR. As with any study utilizing expression of a 'foreign' receptor in a cell type in which the receptor is naturally deficient, any phenomena observed should serve as a basis from which to determine the desensitization mechanisms used by cells expressing the receptor endogenously.

Treatment of A_{2a}AR-transfected cells with agonist induced a rapid reduction in NECA-stimulated adenylyl cyclase activity in subsequently isolated membranes. After 30 min of agonist exposure, the reduced A_{2a}AR-stimulated adenylyl cyclase activity was not accompanied by any diminution in stimulation mediated by sodium fluoride or forskolin, suggesting that the functioning of G_s and the catalytic unit of adenylyl cyclase was unaffected by the desensitization process. These data are consistent with the 'homologous' nature of A_{2a}AR desensitization previously reported in rat kidney cells (18), smooth muscle cells from rat aorta (19), NG108–15 cells (21), and DDT$_1$ MF-2 cells (20) and support a model whereby desensitization selectively diminishes A_{2a}AR-G_s interaction. Furthermore, agonist radioligand binding studies demonstrated that a 30-min agonist treatment induced formation of a receptor population that bound agonist with reduced affinity, without altering the maximal A_{2a}AR binding capacity. Because agonists bind with higher affinity to receptors that are coupled to their appropriate G protein, the shift of the whole population of receptors to lower affinity suggested that, whereas the number of A_{2a}AR-G_s complexes was not altered, the efficiency of the A_{2a}AR-G_s interaction was reduced. Inhibition of receptor sequestration did not affect the ability of the A_{2a}AR functional response to desensitize, suggesting that internalization of the receptor was not responsible for the observed desensitization. It was therefore possible that the receptor protein was modified such that its ability to interact with G_s was impaired. Consistent with this hypothesis was the finding that the ability of NECA to induce short term desensitization was associated with the agonist-stimulated phosphorylation of immunoprecipitable A_{2a}ARs. Neither receptor phosphorylation nor desensitization could be mimicked by elevation of intracellular cAMP levels alone, although it is possible that simultaneous elevation of cAMP levels and agonist occupation of receptors are required for these effects to be manifested.

By analogy with other G protein-coupled receptor systems, including rhodopsin and the α_{2A}- and β_2-adrenergic receptors (42, 44), we suggest that the observed agonist-induced phosphorylation of the A_{2a}AR may be responsible for the rapid loss of receptor function. The inability of PKA activation alone to induce desensitization and receptor phosphorylation might suggest that a receptor-specific kinase analogous or identical to the βARK enzymes may be responsible (45). This is also suggested by secondary structural comparisons between the A_{2a}AR, the β_2-adrenergic receptor, and rhodopsin. Each of these receptors has a cytoplasmic carboxyl-terminal domain containing many serine and threonine residues in an acidic milieu, which studies using peptide substrates have shown to be an important factor in determining susceptibility to phosphorylation by βARK (46).

The ability of sucrose pretreament to inhibit both A_{2a}AR sequestration and the rapid recovery normally observed after short term agonist exposure is consistent with the results of similar studies performed with the human β_2-adrenergic receptor in CHO cells (37). Taken together with work on β-adrenergic receptors in frog erythrocytes (47), this suggested that sequestration of receptors may provide the means by which phosphorylated receptors may be concentrated in phosphatase-enriched vesicles for dephosphorylation and recycling back to the plasma membrane. Inhibition of this process before agonist exposure would therefore result in an accumulation of phosphorylated receptors that could not be dephosphorylated after agonist removal (37). In the case of the A_{2a}AR, it remains to be

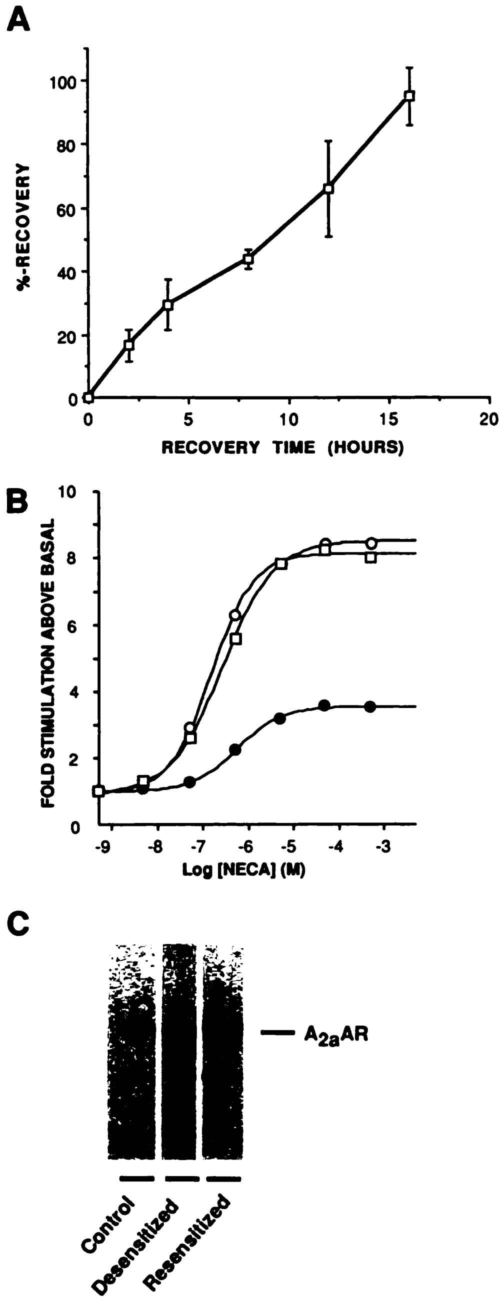

Fig. 6. Recovery of A_{2a}AR function after long term desensitization. CHO-A_{2a}AR cells were either exposed for 24 hr at 37° to 0.3 unit/ml ADA in the absence or presence of 10 μM NECA or exposed for 24 hr to 10 μM NECA and 0.3 unit/ml ADA before extensive washing with PBS, addition of agonist-free medium containing 0.3 unit/ml ADA, and further incubation at 37° for the indicated times. Membranes were then prepared simultaneously for assay of adenylyl cyclase activity, as described in Experimental Procedures. A, Time course of recovery of the 10 μM NECA-stimulated adenylyl cyclase response in isolated CHO-A_{2a}AR cell membranes after 24-hr desensitization. Data are pooled from four experiments, with each *data point* representing the mean ± standard error of three determinations. B, Dose-response curves for NECA-stimulated adenylyl cyclase activity in membranes from ADA-treated controls (O), 24-hr desensitized cells (●), or 24-hr desensitized cells allowed to recover in agonist-free medium for 16 hr (□). The EC_{50} values for NECA in this experiment were 0.28 μM (control), 0.56 μM (desensitized), and 0.33 μM (resensitized). This experiment is one of two performed, which produced identical results. C, Resensitization detected by immunoblotting. CHO-A_{2a}AR cells were treated as described in B and subjected to immunoblotting with affinity-purified TP/2 as described in Experimental Procedures. In this experiment, agonist treatment down-regulated receptors to 28% of the control level. Subsequent recovery in the absence of agonist increased receptor levels to 110% of the control value. This is one of three immunoblots performed.

proven whether the phosphorylation of the receptor is indeed responsible for the agonist-stimulated diminution in signaling capacity of the A_{2a}AR, although the similarity between our data and those reported in Ref. 37 might suggest that similar processes occur.

Long term treatment of CHO-A_{2a}AR cells with elevated concentrations of NECA for up to 24 hr produced a slightly larger reduction in A_{2a}AR function, compared with that seen at 30 min. However, unlike the short term desensitization process, this second phase of A_{2a}AR desensitization was associated with a dose- and time-dependent reduction in the levels of immunoreactive A_{2a}ARs and, by 24 hr, a reduction in the total number of agonist binding sites, as measured with [^{3}H] CGS21680. Moreover, unlike reversal from short term agonist treatment, recovery from desensitization caused by long term agonist exposure occurred over a period of several hours, rather than a few minutes, further suggesting that the desensitization mechanisms operative at 30 min and 24 hr are distinct. Like short term desensitization, adenylyl cyclase activation by fluoride and forskolin was unaffected, suggesting a defect at the level of the A_{2a}AR-G_s interaction. Interestingly, the reduced affinity of the A_{2a}AR for [^{3}H]CGS21680 observed after 30-min agonist exposure, which was associated with receptor phosphorylation, persisted after 24 hr. However, at the latter time point receptor down-regulation appeared to be the dominant mechanism, because recovery after long term agonist exposure occurred over several hours, rather than the few minutes necessary after short term treatment. Nevertheless, the disparity between the NECA dose dependencies for receptor down-regulation and desensitization is most likely due to the fact that multiple mechanisms appear to be responsible for desensitization, such that when certain mechanisms are not fully manifested (e.g., down-regulation) the contribution of others may be sufficient to induce maximal desensitization. In this regard, receptor sequestration, although not involved at early time points, may play an increasingly significant role as total receptor number decreases. However, this phenomenon is difficult to investigate, because treatment for long periods with inhibitors of sequestration adversely affects adenylyl cyclase responsiveness in these cells (40). It was also for these reasons that we could not determine whether receptor sequestration was required to observe down-regulation; more elegant cell biological studies involving immunofluorescence and immunoelectron microscopic techniques will be necessary to answer this question.

The difference in the extent of receptor down-regulation as determined by agonist binding versus immunoblotting is not entirely unexpected. Previous studies on α_2- and β-adrenergic receptor subtypes have similarly produced anomalous results when comparing agonist and antagonist binding (48, 49). Hence, whereas the β-adrenergic receptor agonist hydroxybenzylisoproterenol is a full agonist capable of completely and competitively blocking antagonist binding, saturating concentrations of [^{3}H]hydroxybenzylisoproterenol recognize only 60% of the receptor complement identified in antagonist binding studies (48). An analogous situation may be occurring in transfected CHO cells, with [^{3}H]CGS21680 being capable of identi-

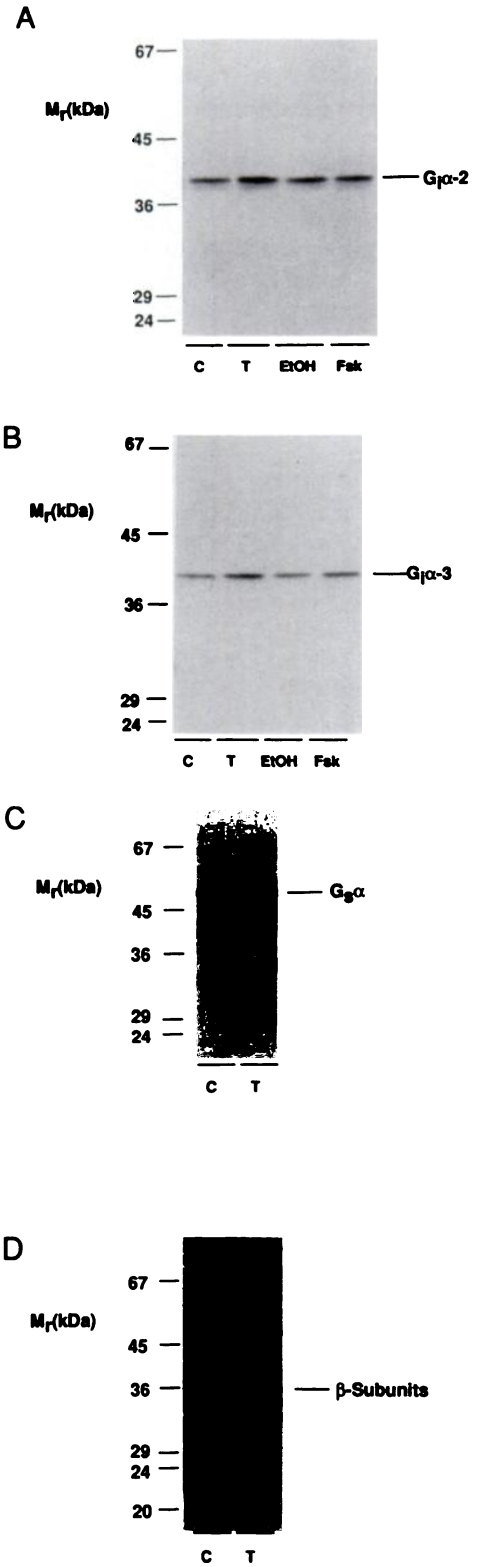

Fig. 7. Regulation of G protein subunits after long term desensitization. CHO-A_{2a}AR cells were treated with 0.3 unit/ml ADA alone (*C*) or with 10 μM NECA (*T*), 10 μM forskolin (*Fsk*), or 0.1% (v/v) ethanol vehicle (*ETOH*), for 24 hr at 37°. Membranes were then prepared for SDS-PAGE and immunoblotting with the antisera described in Experimental Procedures for detection of $G_{i\alpha2}$ (A), $G_{i\alpha3}$ (B), $G_{s\alpha}$ (C), and β (D) subunits. For A and B, 100 μg of membrane protein were loaded in each lane; for C and D, 75 μg were used in each lane. These are representative comparisons; composite data from several experiments are given in Table 4.

TABLE 4

Expression of G protein subunits in membranes prepared from cells after long term agonist exposure

CHO-A_{2a}AR cells were treated with 0.3 unit/ml ADA in the absence (control) or presence of 10 μM NECA for 24 hr. Membrane preparations were then subjected to immunoblotting with the primary antibodies described in Experimental Procedures. The results are expressed as means ± standard errors for *n* experiments.

G protein subunit	Expression	*n*
	% of control	
$G_{s\alpha}$	115 ± 12	4
$G_{i\alpha2}$	162 ± 30[a]	5
$G_{i\alpha3}$	161 ± 28[a]	5
β subunits	90 ± 10	4

[a] Significantly different from control value ($p < 0.05$).

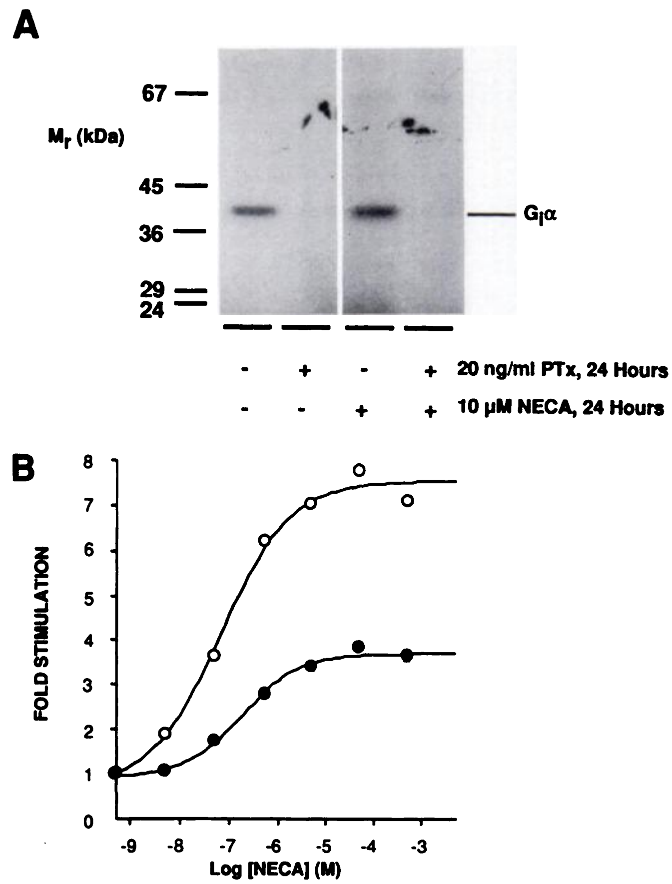

Fig. 8. Effect of PTx treatment on A_{2a}AR desensitization. CHO-A_{2a}AR cells were treated for 24 hr at 37° with or without 10 μM NECA, in the presence or absence of 20 ng/ml PTx, before membrane preparation as described in Experimental Procedures. A, After treatment of CHO-A_{2a}AR cells with or without PTx, 30 μg of membranes from control and 24-hr 10 μM NECA-treated cells were subjected to PTx-catalyzed [^{32}P]ADP-ribosylation *in vitro* and labeled proteins were visualized by SDS-PAGE and autoradiography. Using this approach, it was estimated that >90% of G_i α subunits were ADP-ribosylated by a 24-hr incubation with 20 ng/ml PTx. B, Dose dependence of NECA-stimulated adenylyl cyclase activity in membranes prepared from cells treated with 20 ng/ml PTx and 0.3 unit/ml ADA in the absence (○) or presence (●) of 10 μM NECA for 24 hr at 37°. This is one of three quantitatively similar experiments.

fying only a fraction of the total number of A_{2a}ARs expressed. Therefore, the extent of down-regulation measured by agonist binding may underestimate the total quantity lost. Alternatively, it is possible that a desensitization-induced conformational change in the A_{2a}AR diminishes the immunoreactivity of the receptor with the antibody. This would be unlikely, however, because the membranes are solubilized and electrophoresed under denaturing conditions. The resolution of these questions awaits the development of a selective, high affinity, radiolabeled, A_{2a}AR antagonist.

An interesting phenomenon associated with long term A_{2a}AR desensitization was the agonist-dependent up-regulation of $G_{i\alpha 2}$ and $G_{i\alpha 3}$. These changes in G protein expression did not directly affect the ability of either fluoride or forskolin to stimulate adenylyl cyclase activity under conditions in which the A_{2a}AR was desensitized. Also, treatment with PTx to inactivate G_i proteins during agonist treatment did not diminish the agonist-induced desensitization observed. Nevertheless, increased expression of G_i α subunits provides a potential mechanism by which the A_{2a}AR can modulate the signaling capacity of receptors coupled to the inhibition of adenylyl cyclase activity; analogous cross-regulation of the stimulatory and inhibitory pathways of adenylyl cyclase has been described by us (15) and others (17) in various model systems. Attempts to directly determine whether the increased expression of G_i α subunits enhanced their ability to inhibit adenylyl cyclase were unsuccessful, because we could not measure any detectable inhibition of forskolin-stimulated activity by either GTP or nonhydrolyzable analogues (data not shown). Therefore it seems that, in the absence of an activated G_i-coupled receptor, "tonic," GTP-dependent, receptor-independent functioning of G_i in CHO cells is very low, unlike in adipocytes, where it is readily observed under our assay conditions (50). This would also explain why we did not observe a decrease in fluoride- or forskolin-stimulated adenylyl cyclase activity after long term agonist treatment.

In conclusion, we have demonstrated for the first time that multiple, temporally distinct, processes are associated with the phenomenon of A_{2a}AR desensitization. Short term agonist exposure causes a rapid impairment of the receptor/G protein interaction, leading to reduced A_{2a}AR-stimulated adenylyl cyclase activity. This is associated with reduced affinity of the receptor for agonist, receptor phosphorylation, and sequestration of receptors into a light vesicle population. Long term treatment leads to receptor down-regulation, recovery from which takes several hours, and the elevated expression of inhibitory G protein α subunits, which could potentially modulate the functioning of receptors coupled to other signal transduction pathways.

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