

Induction of Multiple Effects on Adenylyl Cyclase Regulation by Chronic Activation of the Human A₃ Adenosine Receptor

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SUMMARY

The A₃ adenosine receptor (A₃AR) contributes to several cardiovascular effects of adenosine, including antihypertensive and cardioprotective effects. Although several studies have detailed the mechanisms underlying agonist-mediated desensitization of the rat A₃AR, the regulation of the human A₃AR, which displays only a 70% amino acid identity with the rat homologue, has not been addressed. Using a Chinese hamster ovary cell line stably expressing a recombinant human A₃AR, we demonstrated that prolonged treatment with the AR agonist 5'-N-ethylcarboxamidoadenosine induces uncoupling of the A₃AR from G proteins and functional desensitization. In addition to A₃AR desensitization, a 1.5–2.5-fold increase was noted in the adenylyl cyclase (AC) activity achieved in the presence of GTP with or without forskolin. This sensitization of AC activity was not a consequence of the down-regulation of G_i proteins

induced by NECA treatment and was not associated with sustained or transient increases in the expression of G_s. Time course experiments revealed that the onset of sensitization was half-maximal between 2 and 3 hr but was not due to the synthesis of new proteins because cycloheximide treatment failed to inhibit sensitization. The inability of the sensitization process to alter the AC activity obtained in the presence of manganese chloride suggests that prolonged A₃AR activation increases the coupling efficiency between G_s and AC catalytic units. This phenomenon has implications for long term cellular adaptation to agonist because in agonist-treated cells, the extent to which a suboptimal concentration of forskolin could increase phosphorylation of the cAMP-responsive element binding protein was elevated compared with vehicle-treated controls.

Despite its recent identification, the involvement of the A₃AR in several physiological effects of adenosine has been proposed; these include cardioprotection and neuroprotection from prolonged ischemia, bronchoconstriction, mast cell and eosinophil activation, and induction of hypotension (1–3). These phenomena are initiated by agonist binding to A₃AR proteins whose genes have been isolated from rat, sheep, rabbit, and humans (4–7). Despite the classification of these proteins as A₃ARs, the rat protein exhibits only a 70% identity with the other species homologues. This is reflected in notable pharmacological differences, in particular, a 100–1000-fold-lower affinity of the rat A₃AR for certain xanthine compounds compared with the recombinant human and sheep receptors (1, 5–7). A recent report demonstrated that

the rat A₃AR mRNA is subject to an alternative splicing event within the coding sequence, resulting in the insertion of a 17-amino acid segment within the second intracellular loop (8). This report also stated that the human A₃AR message did not seem to undergo similar processing (8).

We demonstrated previously that prolonged agonist exposure of CHO cells expressing a recombinant rat A₃AR results in a desensitization of receptor function that is associated with the down-regulation of specific G protein subunits (9). Given the structural and pharmacological differences displayed by the rat and human A₃ARs, it is important to determine whether the desensitization mechanisms induced by agonist occupation of the rat A₃AR are unique to this species homologue or are characteristic of the other A₃ARs. In this study, we describe the effects of prolonged agonist exposure on CHO cells expressing a recombinant human A₃AR and demonstrate that prolonged agonist exposure not only results in receptor desensitization but also induces a sensitization of the stimulatory pathway of AC, which may have physiological and potential therapeutic implications.

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ABBREVIATIONS: AR, adenosine receptor; CHO, Chinese hamster ovary; AB-MECA, N⁶-(4-aminobenzyl)-5'-N-methylcarboxamidoadenosine; IB-MECA, N⁶-(3-iodobenzyl)-5'-N-methylcarboxamidoadenosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; CREB, cAMP-responsive element-binding protein; NECA, 5'-N-ethylcarboxamidoadenosine; AC, adenylyl cyclase; PTX, pertussis toxin; PKC, protein kinase C; SDS, sodium dodecyl sulfate; ATF-1, activating transcription factor-1.

Experimental Procedures

Materials. Radiochemicals were obtained from DuPont-New England Nuclear (Boston, MA). AB-MECA and IB-MECA were generously donated by Dr. Kenneth A. Jacobson (National Institutes of Health, Bethesda, MD). ¹²⁵I-AB-MECA was synthesized and purified as described previously (10). PTX and RO201724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] were from Life Technologies (Grand Island, NY). Antibodies specific for CREB and Ser133-phosphorylated CREB were from New England Biolabs (Beverly, MA). Sources of other materials have been described previously (9–11).

Receptor cDNA constructs and expression. The human A₃ receptor cDNA was constructed by splicing together two partial cDNAs obtained from Dr. Sean Munro (Cambridge University, Cambridge, UK) to form a functional ORF. The open reading frame was subcloned into the *Bam*HI site of the internal ribosome entry site element containing expression vector pCIN1 (12). The stable transfected cell line 93.1.1 was generated in the following manner: 50 μg DNA (pCIN1/hA3) was linearized in 50 μl with *Ssp*I and sterilized by adding 50 μl of phenol/chloroform/isoamyl-alcohol (25:24:1) to the 50-μl DNA sample in a phase-lock tube. Contents were mixed, and the tube was spun for 30 sec. Chloroform/isoamyl-alcohol [50 μl (24:1)] was added to the upper aqueous phase, and the tube was respun. The upper aqueous phase containing the 50 μg of DNA was removed aseptically. The CSH host line 53.1.12 was maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) with 15 mM HEPES (no. 42400; Life Technologies), 10% (v/v) fetal calf serum, and 500 μg/ml Hygromycin and passaged when 50% confluent. The cells were resuspended at 1 × 10⁷/ml in the Dulbecco's modified Eagle's medium/Ham's F-12 medium. Then, 450 μl of cells plus 50 μl of sterile linearized DNA were electroporated with a single pulse in a Gene Pulsar I apparatus (Biorad, Melville, NY) using a 0.4-cm cuvette at 960 μF and 280 V. The time constant was 20 msec. The cells were diluted immediately into 30 ml of media and cultured at 37°. Media was changed every 3 days until day 12, on which the cells were dilution cloned. A single clone was chosen empirically from the 12 tested on the basis of (a) a 4-fold response to 10^{−5} M forskolin (water-soluble derivative, no. 344273; Calbiochem, San Diego, CA) in terms of secreted placental alkaline phosphatase production and (b) a dose-responsive reduction in the latter by NECA. Cells were maintained in Ham's F-12 media supplemented with 10% (w/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin as described previously (9–11).

Antibodies and immunoblotting. Antibodies versus specific G protein subunits were generously donated by Dr. Tom Gettys (Medical University of South Carolina, Charleston, SC). The generation and specificities of these antisera have been described previously (13, 14). The primary antibodies used in this study were 982 (1:8,000 dilution of serum) for detection of G_{iα-2}, 977 (1:8,000 dilution of serum) for detection of G_{iα-3}, 951 (1:16,000 dilution of serum) for detection of G_{sα}, 457 (1:1,000 dilution of protein A affinity-purified IgG) for detection of G_{qα} and G_{11α}, and 987 (1:8,000 dilution of serum) for detection of β subunits. Membranes were prepared from confluent T-75 flasks as we described previously and aliquoted for storage at −80° (9). After quantification of protein content according to the method of Bradford (15), equivalent amounts of membrane protein (typically 15 μg) were resolved by discontinuous SDS-PAGE on 10% (w/v) polyacrylamide resolving gels using a BioRad (Hercules, CA) Mini Protean 2 system. After transfer to a nitrocellulose membrane, nonspecific protein binding sites were blocked by a 60-min room temperature incubation in blocking buffer [5% (w/v) skim milk powder in phosphate-buffered saline containing 0.2% (v/v) Triton X-100 and 0.02% (w/v) thimerosal]. Nitrocellulose membranes were then incubated with the appropriate dilution of primary antibody in fresh blocking buffer overnight at 4°. After removal of anti-serum and extensive washing with three changes of blocking buffer, membranes were incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG in a high-detergent skim

milk solution. The series of washes described above was then repeated and followed by two additional washes in phosphate-buffered saline before visualization of reactive proteins by an enhanced chemiluminescence protocol. Quantification of immunoblots was by densitometric scanning as we described previously (9).

To assess CREB phosphorylation and expression, cell monolayers in six-well dishes were treated as described in the figure legends. Reactions were stopped by placing the dishes on ice and washing each well twice with phosphate-buffered saline. Cells were solubilized by scraping into 0.25 ml of a detergent buffer [1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM sodium HEPES, pH 7.5, 5 mM EDTA, 0.15 M sodium chloride, 10 mM sodium fluoride, 10 mM sodium phosphate, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A] and incubation at 4° for 1 hr. After microcentrifugation for 10 min, soluble fractions were assayed for protein content, and equivalent amounts (typically 30 μg) were subjected to SDS-PAGE and immunoblotting with anti-CREB and anti-phospho-CREB antibodies, both used at 1:500 dilution, as described above.

Radioligand binding and AC assays. Radioligand binding and AC assays were performed and analyzed exactly as we described previously, except that for AC assays, 20 μM RO201724 was used as the phosphodiesterase inhibitor instead of papaverine (10, 11).

[³H]Leucine incorporation. Triplicate wells of transfected CHO cells in six-well dishes were incubated for 6 hr in regular media supplemented with 1 μCi/well of [³H]leucine with or without 30 μg/ml cycloheximide. Incubations were terminated by placing the cells on ice, washing with ice-cold phosphate-buffered saline, and solubilizing the cell monolayers in detergent buffer as described above. An equal volume of 72% trichloroacetic acid was added to solubilized extracts, and the resulting precipitates were collected by microcentrifugation. Pellets were solubilized in 1 M sodium hydroxide, and [³H]leucine incorporation was determined by liquid scintillation counting.

Results

Functional desensitization of the human A₃AR. The cell line used for these experiments bound the A₃AR agonist radioligand ¹²⁵I-AB-MECA with high affinity ($K_d = 1.24 \pm 0.41$ nM; four experiments) and exhibited B_{max} values of 0.38–0.68 pmol/mg of protein (four experiments). Radioligand binding experiments demonstrated that exposure of transfected cells to 10 μM concentration of the AR agonist NECA for 20 hr resulted in a $73 \pm 7\%$ reduction in B_{max} versus vehicle-treated controls ($p < 0.05$; four experiments) without significantly affecting the K_d values (1.24 ± 0.41 nM for control and 1.42 ± 0.13 nM for agonist-treated cells; four experiments) (Fig. 1A). This was associated with a functional desensitization of A₃AR signaling as determined by analysis of IB-MECA-mediated inhibition of forskolin-stimulated AC activity in isolated membranes, with maximal inhibition falling from $57 \pm 8\%$ to $14 \pm 10\%$ after a 20-hr agonist treatment ($p < 0.05$ versus vehicle-treated controls; three experiments) and the IC₅₀ value for IB-MECA increasing from 19 ± 4 to 95 ± 60 nM ($p < 0.05$ versus vehicle-treated controls; three experiments) (Fig. 1B). Therefore, under conditions in which agonist treatment results in a profound reduction in agonist radioligand binding at the A₃AR, the signaling capacity of the A₃AR undergoes functional desensitization.

Effects of agonist treatment on G protein levels. We demonstrated previously that prolonged agonist exposure of the rat A₃AR expressed in CHO cells results in the down-regulation of specific G protein subunits (9). To determine whether chronic exposure of the human A₃AR to agonist

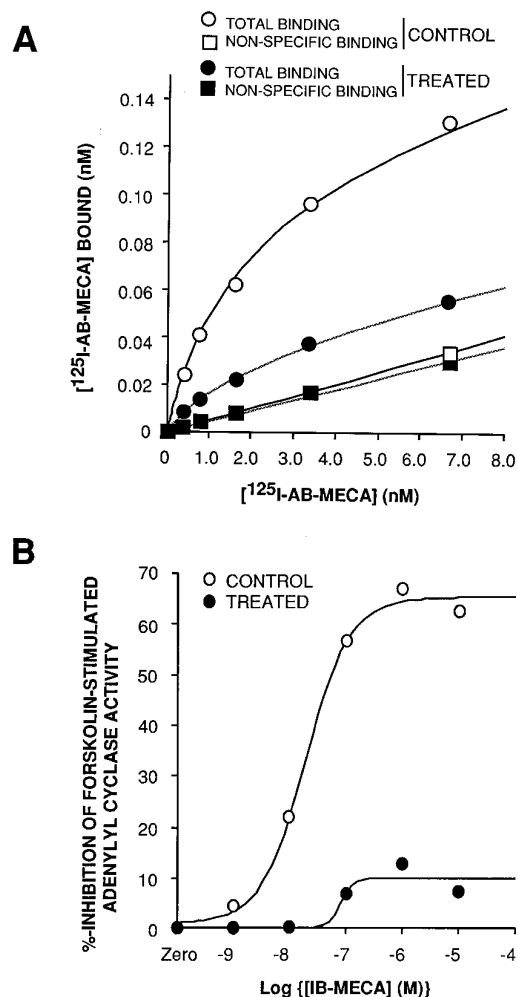


Fig. 1. Effects of chronic exposure to NECA on A_3AR function. **A**, Transfected CHO cells were incubated in the absence (*CONTROL*) or presence (*TREATED*) of $10\ \mu M$ NECA for 20 hr at 37° . After extensive washing of cell monolayers, membranes were prepared for saturation radioligand binding experiments with increasing concentrations of ^{125}I -AB-MECA and analysis as described in Experimental Procedures. Nonspecific binding was assessed by the inclusion of $50\ \mu M$ NECA. *Inset*, Scatchard transformations of the specific binding data from this experiment. This is one of four experiments that produced similar results. **B**, Membranes from transfected CHO cells treated as described in **A** were assayed for AC activity in the presence of $5\ \mu M$ forskolin and increasing concentrations of IB-MECA as described in the Experimental Procedures. This is one of three experiments that produced similar results.

could mediate similar effects, membranes from transfected cells were analyzed by comparative immunoblotting after treatment with or without $10\ \mu M$ NECA for 20 hr (i.e., conditions that produced a desensitization of human A_3AR function) (Fig. 1). Immunoblotting with antisera specific for $G_{i\alpha-2}$, $G_{i\alpha-3}$, and β subunits common to multiple G proteins demonstrated that each of these proteins were down-regulated by agonist treatment (Fig. 2, A–C, and Table 1). Moreover, the altered expression levels of each of these proteins did not reflect a nonspecific global change in the total pool of cellular G protein subunits in that levels of $G_{s\alpha}$ and $G_{q+11\alpha}$ subunits were unaffected (Fig. 2, D and E, and Table 1).

Effects of A_3AR agonist treatment on AC stimulation. Under conditions that produced A_3AR desensitization, a ~ 2 -fold increase was observed in the stimulation of AC activity obtained in the presence of GTP with or without forskolin (Table 2). This effect required the presence of a functional A_3AR because a 20-hr exposure of nontransfected CHO cells to $10\ \mu M$ NECA resulted in a $-6 \pm 17\%$ change [$p > 0.05$ (NS); three experiments] in GTP-stimulated AC activity in subsequently isolated membranes. The changes in AC stimulation were not reflected in an increased activity of AC catalytic units in these membranes because when assays were performed in the presence of forskolin and manganese chloride to uncouple the catalytic units from G protein regu-

lation (16), no difference in activity was found between membranes from control cells and those from agonist-treated cells (Table 2). This initially suggested that the down-regulation of G_i proteins induced on agonist exposure removed a low-level tonic inhibitory effect of G_i on AC activity in these cells. Moreover, if this were the case, it would be expected that this effect could be mimicked by treatment of transfected cells with PTX, which would inactivate G_i by catalyzing its ADP-ribosylation as opposed to the down-regulation induced on chronic agonist exposure. Exposure of transfected cells with sufficient PTX to abolish IB-MECA-mediated inhibition of forskolin-stimulated AC activity failed to result in a significant increase in GTP-stimulated AC activity (Fig. 3). However, PTX treatment completely attenuated the ability of NECA to sensitize AC activity (Fig. 3). Therefore, the ability of chronic A_3AR activation to sensitize AC stimulation is not a result of G_i down-regulation and requires a functional A_3AR/G_i signaling system to be manifested.

Time course experiments revealed that the agonist-induced increase in GTP-stimulated AC activity was half-maximal at 2–3 hr, maximal by 6 hr, and sustained for ≥ 20 hr after agonist addition (Fig. 4A). During this time period, no transient elevation in the level of membrane-associated $G_{s\alpha}$ was detectable (Fig. 4A), thereby ruling out any such increase as a potential mechanism for this effect. Nevertheless,

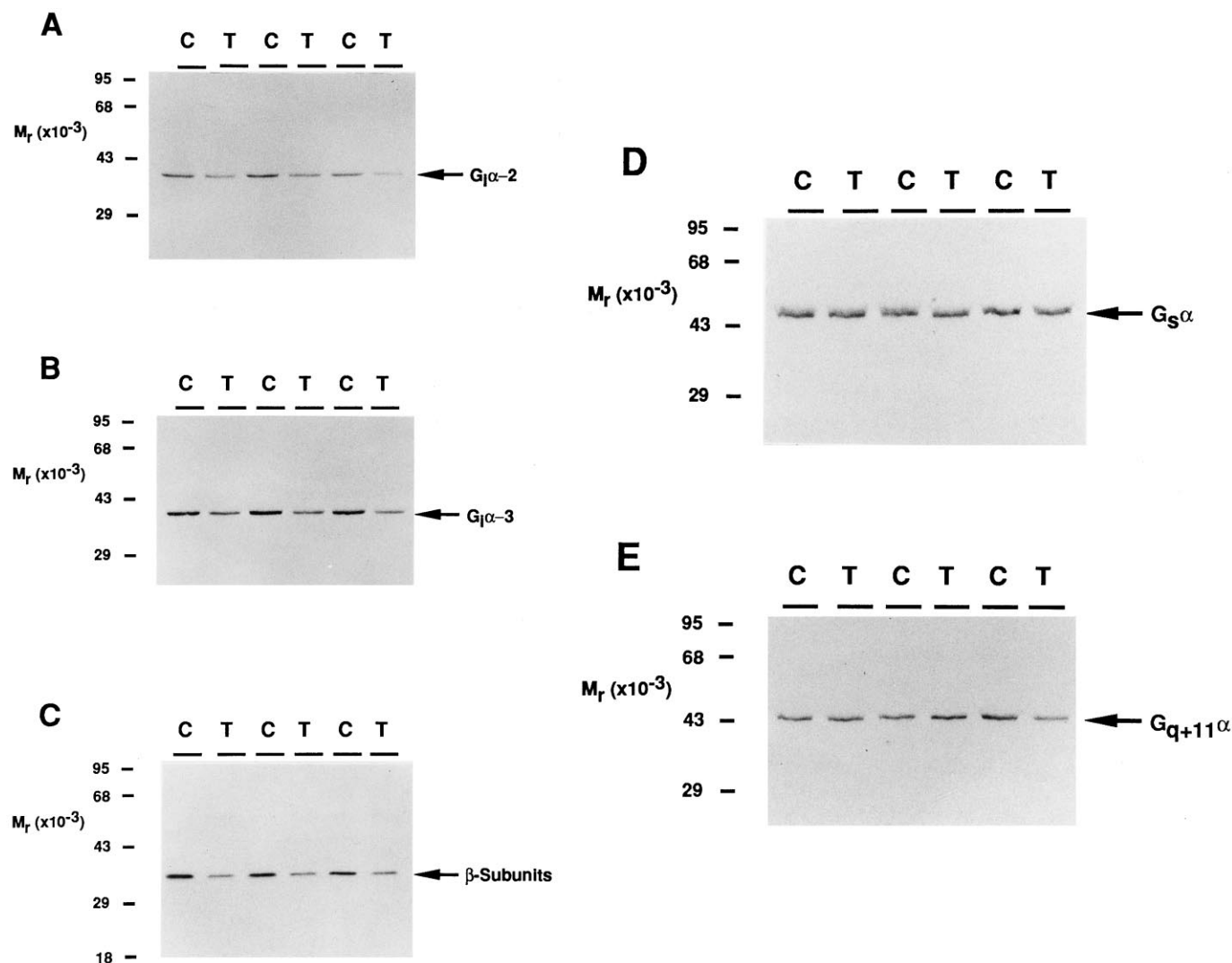


Fig. 2. Effects of chronic exposure to NECA on G protein expression. Aliquots (15 μ g) of membranes prepared from transfected CHO cells incubated in the absence (C) or presence (T) of 10 μ M NECA for 20 hr at 37° were subjected to SDS-PAGE and immunoblotting with antisera specific for G_{1α-2} (A), G_{1α-3} (B), β subunits (C), G_{Sα} (D), and G_{q+11α} subunits (E) as described in Experimental Procedures. Data pooled from three separate comparisons for each G protein subunit are given in Table 1.

TABLE 1

Agonist regulation of G protein expression in CHO cells expressing the human A₃AR

CHO cells expressing the human A₃AR were treated with or without 10 μ M NECA for 20 hr at 37° before membrane preparation and comparative immunoblotting as described in Experimental Procedures. Under these conditions, G_{qα} and G_{11α} comigrate, and therefore the signal observed on immunoblots represents a composite signal for these proteins. Data are presented as mean \pm standard error for three experiments with the signal in untreated controls set at 100%.

G protein subunit	Expression
	% of control
G _{1α-2}	53 \pm 22 ^a
G _{1α-3}	58 \pm 9 ^a
G _{Sα}	123 \pm 21
G _{qα} + G _{11α}	121 \pm 34
β Subunits	48 \pm 7 ^a

^a Significant difference ($p < 0.05$) versus control signal.

TABLE 2

Effects of chronic agonist exposure on AC activity in membranes from CHO cells expressing the human A₃AR

CHO cells expressing the human A₃AR were treated in the absence (Control) or presence (Treated) of 10 μ M NECA for 20 hr at 37° before membrane preparation and assay of AC activity in the presence of the indicated compounds as described in Experimental Procedures.

Condition	AC activity	
	Vehicle treated	NECA treated
	pmol/min/mg	
5 μ M GTP	1.30 \pm 0.15 (5)	2.85 \pm 0.52 (5) ^a
5 μ M Forskolin + 5 μ M GTP	16.00 \pm 1.55 (4)	29.02 \pm 4.25 (4) ^a
5 μ M Forskolin + 5 mM MnCl ₂	85.25 \pm 2.70 (4)	84.61 \pm 3.00 (4)
Inhibition by 1 μ M IB-MECA (%)	52 \pm 7 (5)	17 \pm 7 (5) ^a

^a Significant ($p < 0.05$) difference from the activity obtained in membranes from vehicle-treated cells for the number of experiments given in parentheses.

because agonist exposure times of several hours were necessary to observe elevated AC activation, it was possible that increased synthesis of other unidentified proteins may have been responsible. However, incubation of transfected cells

with 30 μ g/ml cycloheximide failed to alter the ability of chronic NECA exposure to elevate GTP-stimulated activity in subsequently isolated membranes (Fig. 4B). This did not reflect an inability of this concentration of cycloheximide to

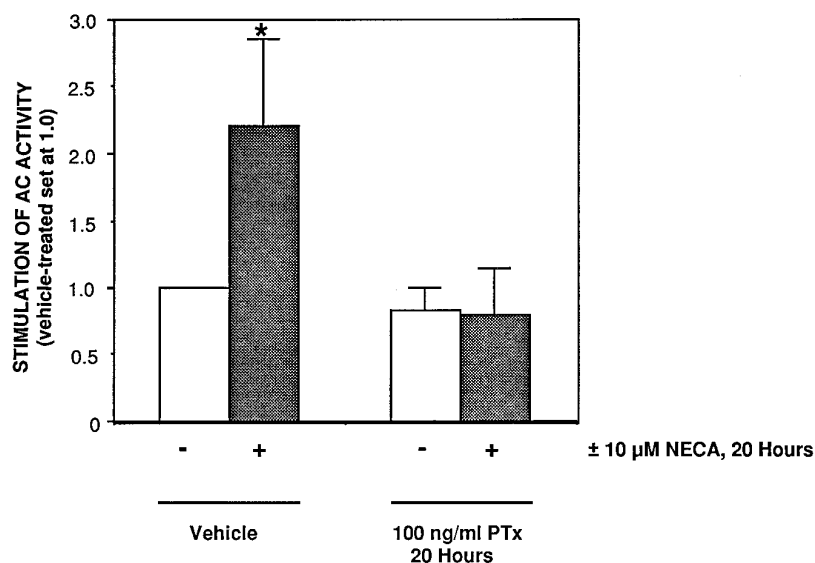


Fig. 3. Effect of PTX treatment on agonist-induced AC sensitization. Transfected CHO cells were incubated in the presence or absence of 10 μ M NECA and 100 ng/ml PTX for 20 hr at 37° as indicated. Membranes were then prepared for assay of AC activity in the presence of 5 μ M GTP as described in Experimental Procedures. Data are pooled from three experiments. The 5 μ M GTP-stimulated activity in membranes from cells treated without either NECA or PTX was 2.00 ± 0.26 pmol/min/mg. The percent inhibition of 5 μ M forskolin-stimulated AC activity elicited by 1 μ M IB-MECA for each treatment condition was $34 \pm 4\%$ (Vehicle), $9 \pm 3\%$ (NECA), $0 \pm 1\%$ (PTx + vehicle), and $1 \pm 1\%$ (PTx + NECA). *, Significant difference ($p < 0.05$) from the specific activity achieved in membranes from cells treated without agonist or PTX.

inhibit protein synthesis in these cells because in two separate [3 H]leucine labeling experiments, 30 μ g/ml cycloheximide inhibited [3 H]leucine incorporation into trichloroacetic acid-precipitated material from transfected CHO cells by 95% and 96%. Other experiments have also demonstrated an inability of the protein kinase C inhibitor GF109203X (bisindolylmaleimide I) to block AC sensitization at a concentration of 2.5 μ M, which is sufficient to abolish phorbol ester-stimulated extracellular signal-regulated kinase activity in CHO cells (data not shown). Therefore, the ability of NECA to elevate AC activity does not reflect a PKC-mediated activation of specific AC isoforms.

Effects of agonist treatment on stimulation of CREB phosphorylation. Given that the increase in GTP-stimulated AC activity induced by chronic A_3 AR activation was only 1.5–2-fold over control, it was important to determine whether this effect had consequences for long term adaptation in intact cells. This was determined by assessing the effect of agonist pretreatment on the subsequent ability of a submaximal concentration of forskolin to stimulate phosphorylation of CREB, which is regulated by cAMP in part through a well-characterized phosphorylation of Ser133 that can be detected immunologically (17). In vehicle-treated controls, 1 μ M forskolin stimulated CREB phosphorylation by 3.5 ± 1.7 -fold over basal after a 30-min incubation (Fig. 5A). In cells pretreated with 10 μ M NECA for 20 hr, 1 μ M forskolin produced levels of CREB phosphorylation $\sim 220 \pm 40\%$ greater than that seen in vehicle-treated cells ($p < 0.05$; three experiments) without changing the basal level of phosphorylation (Fig. 5A). A similar pattern of elevated forskolin-stimulated phosphorylation was observed for ATF-1, which cross-reacts with this antibody preparation (Fig. 5A). The changes in CREB phosphorylation were not the result of a parallel change in the levels of CREB expression, as determined by immunoblotting the same extracts with an antibody that recognizes CREB regardless of its phosphorylation status (Fig. 5B). In contrast, similar pretreatment of non-transfected cells failed to produce augmentation in the subsequent ability of forskolin to increase CREB or ATF-1 phosphorylation (Fig. 5A).

Discussion

On the binding of an agonist ligand to a G protein-coupled receptor, multiple cellular mechanisms may be invoked to control both the signal emanating directly from the receptor and the responsiveness of receptors that regulate other signaling pathways (18). We demonstrated that exposure to agonist of cells expressing the human A_3 AR affects both the inhibitory and stimulatory arms of AC regulation. Consistent with our previous report on the desensitization of the rat A_3 AR (9), prolonged agonist exposure resulted in a profound functional desensitization that was associated with a reduction in the number of high affinity agonist binding sites detectable by radioligand binding. The loss of high affinity binding sites is indicative of a reduction in the number of signaling-competent receptor/G protein complexes and may be due to any of several reasons. Because the human A_3 AR is capable of inhibiting AC activity in a PTX-sensitive manner, which is indicative of an ability to couple productively with G_i proteins, the large reductions in the levels of membrane-associated $G_{i\alpha-2}$ and $G_{i\alpha-3}$ may be partly responsible for the loss in high affinity binding observed on agonist exposure. On the basis of observations made with other G protein-coupled receptors, it was also possible that the A_3 AR protein was down-regulated in response to agonist. In the absence of either a high affinity antagonist radioligand or an antibody of sufficiently high sensitivity to assess A_3 AR expression, we cannot examine this possibility.

A previously unappreciated consequence of chronic A_3 AR activation was the time-dependent onset of AC sensitization. Specifically, agonist treatment enhanced the stimulation of AC activity elicited by GTP in the absence or presence of forskolin but not in the presence of forskolin and manganese chloride. This phenomenon was not a reflection of a diminution in G_i function due to down-regulation of $G_{i\alpha}$ subunits because abolition of G_i function with PTX failed to mimic the effect of chronic agonist exposure, thereby implicating the stimulatory pathway as the locus for the altered regulation of AC. We also observed that the ability of prolonged agonist treatment to sensitize AC stimulation is not restricted to the

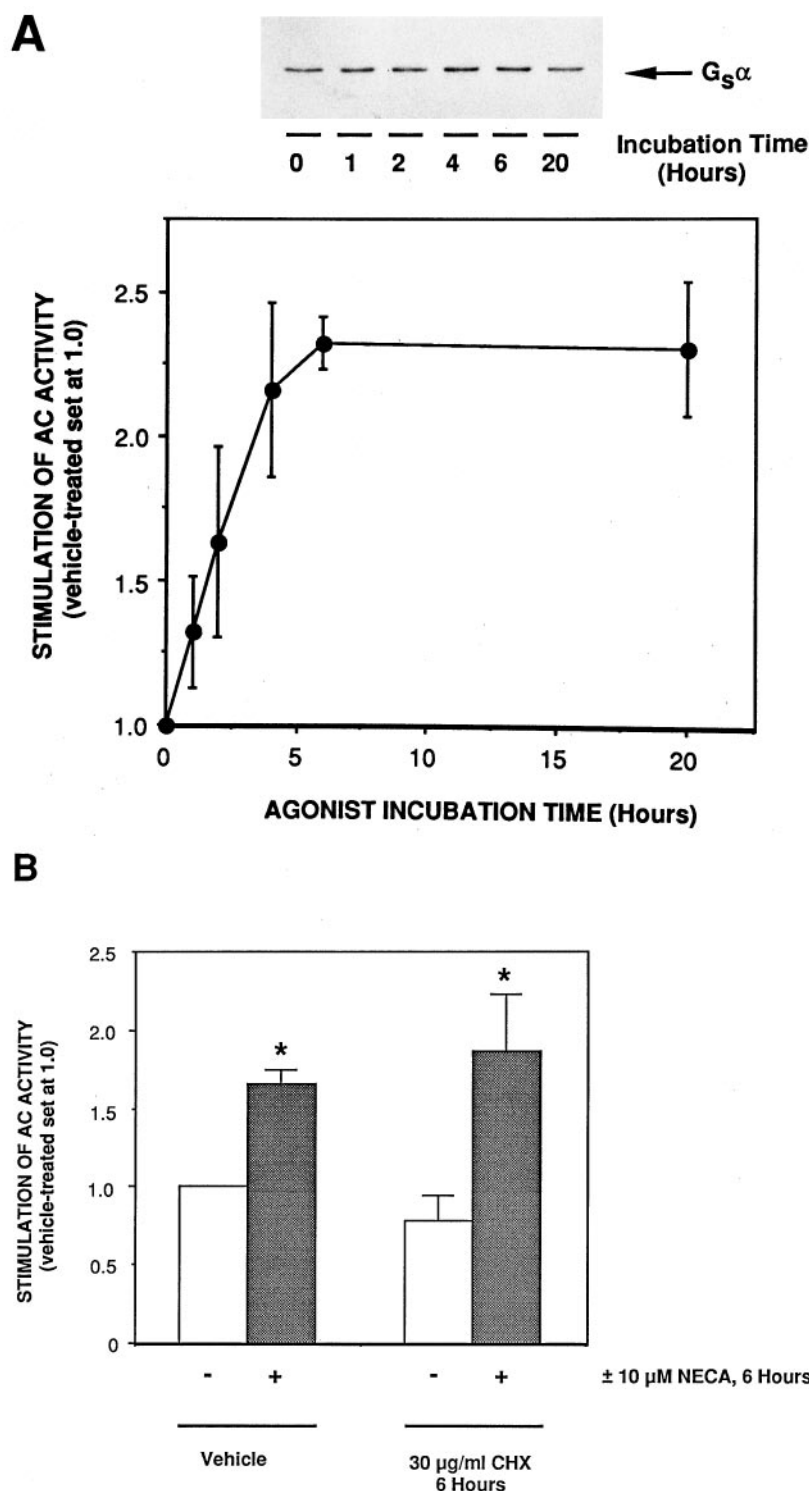


Fig. 4. Time course of agonist-induced AC sensitization. A, Transfected CHO cells were treated with 10 μM NECA at 37° for the indicated times before membranes preparation and assay of AC activity in the presence of 5 μM GTP as described in Experimental Procedures. Data are pooled from three experiments. *Inset*, immunoblot for G_sα subunits in 15-μg aliquots of membranes prepared from transfected CHO cells incubated at 37° with 10 μM NECA for the indicated times. B, Transfected CHO cells were incubated in the presence or absence of 10 μM NECA and 30 μg/ml cycloheximide (CHX) for 6 hr at 37° as indicated. Membranes were then prepared for assay of AC activity in the presence of 5 μM GTP as described in Experimental Procedures. Data are pooled from three experiments. The 5 μM GTP-stimulated activity in membranes from cells treated without either NECA or CHX was 2.28 ± 0.26 pmol/min/mg. The percent inhibition of 5 μM forskolin-stimulated AC activity elicited by 1 μM IB-MECA for each treatment condition was $54 \pm 3\%$ (Vehicle), $29 \pm 7\%$ (NECA), $56 \pm 4\%$ (CHX + Vehicle), and $30 \pm 3\%$ (CHX + NECA). *, Significant difference ($p < 0.05$) from the specific activity achieved in membranes from cells treated without agonist or CHX.

human A₃AR; similar effects on AC regulation are involved through chronic agonist exposure of the rat A₃AR.²

Forskolin binds to and activates all nine of the AC isoforms cloned to date, although there is some evidence for isoform-specific variations in the levels of activation that can be achieved (19–21). However, the interaction is greatly enhanced in the presence of G_s, a property used by several

investigators to quantify G_s/AC coupling in intact cells and isolated membranes (20, 22, 23). Therefore, the AC activity achieved in the presence of forskolin and GTP reflects the stimulation of G_s-coupled AC proteins. In contrast, the addition of manganese to the assay uncouples AC from G protein regulation; therefore, the activity observed with forskolin and MnCl₂ reflects the activity of AC catalytic units independent of G protein function (16). Hence, the lack of any enhancement of AC stimulation observed with forskolin in the

² T. M. Palmer, unpublished observations.

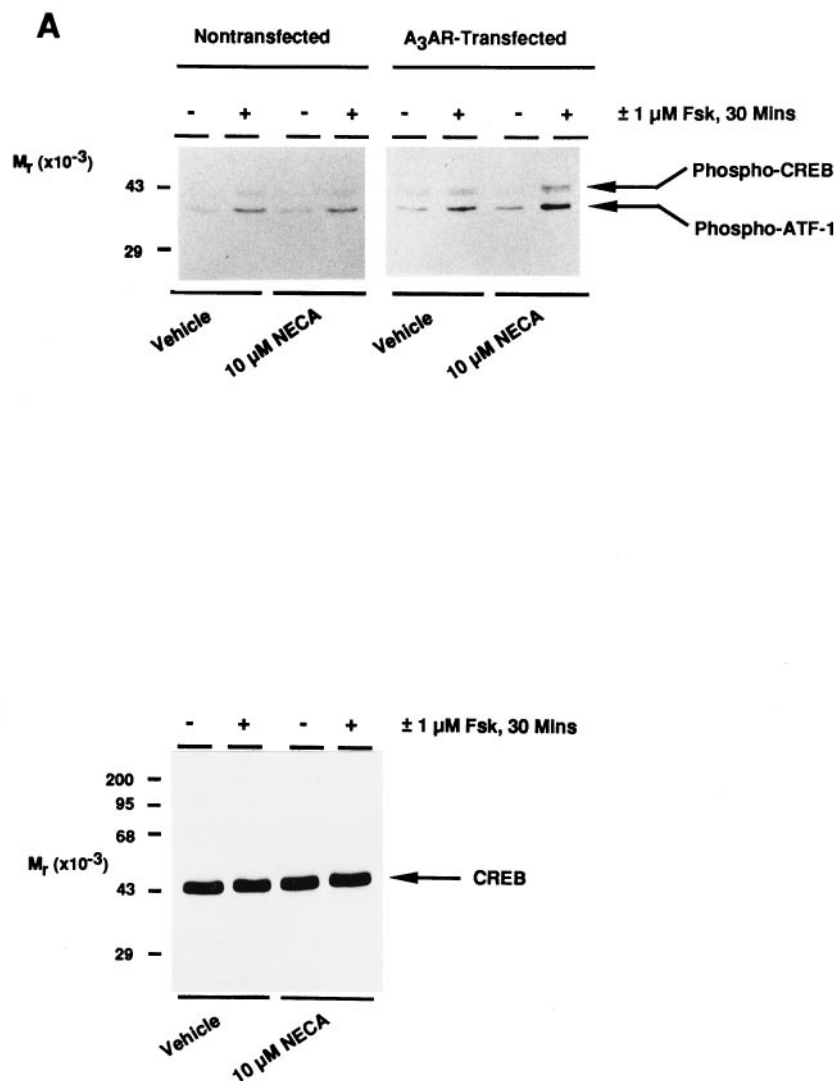


Fig. 5. Effect of chronic agonist exposure and subsequent withdrawal on CREB phosphorylation. **A**, Nontransfected and transfected CHO cells were incubated for 20 hr at 37° in the absence (*Vehicle*) or presence of 10 μM NECA before washing of cell monolayers with prewarmed phosphate-buffered saline and a subsequent 30-min incubation at 37° in the presence or absence of 1 μM forskolin (*Fsk*) as indicated. Detergent extracts were then prepared for immunoblotting with antibodies versus phosphorylated CREB as described in Experimental Procedures. Quantitative data from three experiments are presented in Results. **B**, Transfected CHO cells were incubated without (*Vehicle*) or with 10 μM NECA for 20 hr at 37° before agonist washout and a further 30-min stimulation with 1 μM forskolin (*Fsk*) as indicated. Detergent extracts were then prepared for immunoblotting with antibodies versus CREB as described in Experimental Procedures. This is one of three experiments that produced similar results.

presence of MnCl₂ suggests the functioning of the G_s/AC complex was specifically enhanced by agonist pretreatment. An increase in the levels of membrane-associated G_{sα} subunits was not responsible because over the time course in which sensitization of AC was observed, no significant change in the expression of membrane-associated G_{sα} subunits could be detected. Moreover, several studies addressing the consequences of overexpression of G_{sα} on AC regulation found that even high degrees of overexpression of this protein have little effect on the maximal AC stimulation that can be achieved (24, 25). Therefore, agonist treatment must either increase the ability of G_s to stimulate AC and/or increase the ability of AC catalytic units to respond to activated G_s. Although the mechanism for this phenomenon remains obscure, it does not seem to involve *de novo* protein synthesis, because a maximally effective concentration of cycloheximide was without effect, or a slow-onset activation of specific AC isoforms by PKC-mediated phosphorylation, because maximally effective concentrations of a selective PKC inhibitor failed to block the sensitizing effect of agonist pretreatment.

The ability of chronic treatment with inhibitory agonists to sensitize the stimulatory pathway of AC has been described for several G protein-coupled receptors, including dopamine

D₂ and muscarinic acetylcholine m2 receptor subtypes as well as the rat adipocyte A₁ adenosine receptor (26–28). However, most research has been focused on the ability of morphine, acting at the μ-opioid receptor, to sensitize AC activity both in cultured cells (29–32) and in specific regions of the brain because the sensitization phenomenon is thought to play a crucial role in the behavioral changes associated with opiate withdrawal in humans (33). The sensitivity to PTX, insensitivity to cycloheximide, and time course of onset of sensitization we described here are essentially the same properties as the morphine-induced sensitization of AC induced by a recombinant μ-opioid receptor expressed in CHO cells (30). Further experiments by the same group demonstrated that although AC I, V, VI, and VIII can be sensitized in response to prolonged μ-opioid receptor activation, AC II, III, IV, and VII are not sensitized under the same experimental conditions (31, 32). Because we have shown that the specific activity of G protein-uncoupled AC catalytic units is unaffected by conditions in which AC sensitization is observed in response to A₃AR activation, it may be that AC isoform specificity for this phenomenon reflects a sensitivity to an unknown regulatory factor capable of modulating G_s/AC interactions.

In contrast to the similarities with the μ -opioid receptor system, our data appear distinct from that of Thomas and Hoffman (27) because half-maximal sensitization by agonist occupation of the muscarinic m2 receptor in their transiently transfected human embryonic kidney 293 cell system occurred ~5 min after agonist exposure rather than the 2–3 hr we and Vogel *et al.* (30) observed in CHO cells (30). Therefore, it seems likely that the phenomenon we observed in CHO cells represents one of several potential AC sensitization mechanisms that may exist, whose contribution to the overall effect may vary in a cell type-specific manner. However, an involvement of G protein $\beta\gamma$ subunits in mediating AC sensitization in both of these systems has been proposed, although in each case this effect on AC seems to be indirect (i.e., not the result of a direct interaction between $\beta\gamma$ subunits and the classic $\beta\gamma$ -stimulated AC isoforms, AC II and IV) because ACV, which is not activated directly by $\beta\gamma$ subunits, can still be sensitized in a manner blocked by overexpression of $\beta\gamma$ scavenger proteins (27, 31). The relevance of these findings to the results presented here remains to be determined.

Because the A₃AR-induced sensitization of GTP-stimulated AC activity in isolated membranes was relatively modest (1.5–2.5-fold), it was important to determine whether this phenomenon had any effect on cAMP-regulated events in an intact cell. The phosphorylation of CREB was chosen as a measure of significance at the intact cell level because long term adaptive changes in response to extracellular stimuli typically result in altered patterns of gene expression, which requires mobilization of appropriate transcription factors. Moreover, CREB is expressed constitutively and is predominantly regulated by a well-characterized cAMP-dependent protein kinase-catalyzed phosphorylation event that is readily detectable (17). The observations that chronic A₃AR activation resulted in increased forskolin-stimulated phosphorylation of CREB (and ATF-1) on agonist removal and that this phenomenon is not observed in nontransfected cells suggest strongly that the A₃AR-induced sensitization of AC detectable in isolated membranes reflects the induction of an important adaptive process that may have consequences for cellular regulation of gene transcription.

Finally, the sensitization of AC reported here may provide a molecular basis for the observation that for several adenosine receptor-mediated events, acute agonist exposures produce opposite effects to those of chronic agonist treatments. This “effect inversion” phenomenon has been observed with several AR-mediated physiological processes, including neuroprotection from ischemia, which may involve the A₃AR (3, 34). Specifically, acute preischemic activation of gerbil A₃ARs with the A₃AR agonist IB-MECA increases postischemic cerebral damage and mortality, whereas chronic treatment with IB-MECA reduces these parameters after an ischemic insult (2). The ability of chronic A₃AR activation to sensitize signal transduction pathways diametrically opposed to those induced after acute exposure could explain this phenomenon. Interestingly, a recent study has shown that enhanced survival of dentate granule cells after hypoxic-ischemic injury in rats is associated with elevated levels of phosphorylated CREB compared with CA1 pyramidal cells, which readily undergo programmed cell death after an ischemic insult (35). Any potential relationship between these observations made

in intact animal models and the A₃AR-mediated sensitization of AC we report here remains to be elucidated.

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