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Tumor Necrosis Factor-α Prevents Desensitization of $G\alpha_s$-Coupled Receptors by Regulating GRK2 Association with the Plasma Membrane

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ABSTRACT

We have reported previously that interleukin-1 and tumor necrosis factor (TNF)-α increase expression and function of adenosine A_{2A} receptors (A_{2A}Rs), although the increased function is disproportionate to the increment in expression. We therefore studied the effect of TNF-α on A_{2A}R function and desensitization in human monocytoid THP-1 cells. We observed that TNF-α regulates activity of A_{2A}Rs and other G protein-coupled receptors (GPCRs) by altering their ligand-mediated desensitization. Pretreatment of resting cells with the A_{2A}R agonist 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) or the pan-adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine quickly desensitized cAMP responses to CGS 21680 restimulation, but TNF-α treatment prevented A_{2A}R desensitization. As expected, A_{2A}R occupancy induced translocation of GPCR kinase-2 (GRK2) to the plasma membrane (PM). We were surprised to find that after TNF-α treatment, A_{2A}R occupancy not only failed to induce GRK2 translocation to PM but also decreased GRK2 association with PM. TNF-α altered GRK2 translocation in response to the β-adrenergic receptor agonist isoproterenol in a similar manner. Similar to GRK2, β-arrestin associated with PM after A_{2A}R stimulation in control cells but not in TNF-α-treated cells. C_2-ceramide, a downstream mediator in the sphingomyelinase (SMase)-dependent pathway, mimicked the effect of TNF-α on GRK2 translocation. Moreover, inhibitors of the SMases and an inhibitor of c-Jun NH_2-terminal kinase, also a downstream effector in the SMase pathway, reversed TNF-α-mediated effects on GRK2 translocation and A_{2A}R desensitization. These results suggest a novel form of cross-talk between TNF-α receptors and GPCRs; TNF-α enhances GPCR function by preventing agonist-induced desensitization of GPCRs by diminishing agonist-dependent recruitment of GRK2 and β-arrestin to PM by a SMase pathway-mediated mechanism.

Adenosine and its receptors are well known for their role in modulating inflammation and tissue protection as well as physiological functions of multiple mammalian organs, including brain and heart (Cronstein, 1998; Ohta and Sitkovsky, 2001). We and others have demonstrated that the inflammatory cytokines IL-1 and TNF-α increase the function and expression of adenosine receptors, especially A_{2A}Rs, in what seems to be feedback regulation of inflammation (Khoa et al., 2001, 2003; Bshesh et al., 2002; Trincavelli et al., 2002). However, in our prior studies on several human cell types, cytokine-induced changes in A_{2A}R number are probably not sufficient to account for the increase in the receptor's activities (Khoa et al., 2001, 2003), leading us to speculate that TNF-α and other inflammatory mediators may also increase receptor function by modulating the desensitization mechanisms of adenosine receptors.

After receptor activation, G protein-coupled receptors (GPCRs) rapidly lose the ability to stimulate intracellular signals in a process that is mediated by specific GPCR ki-

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ABBREVIATIONS: IL, interleukin; TNF, tumor necrosis factor; A_{2A}R, adenosine A_{2A} receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; CGS 21680, 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; JNK, c-Jun NH_2-terminal kinase; PKA, protein kinase A; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; SB 202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; PBS, phosphate-buffered saline; WCL, whole cell lysate; TBS, Tris-buffered saline; ANOVA, analysis of variance; SMase, sphingomyelinase; CHF, congestive heart failure; WB, Western blot; GW4869, *N*,*N*′-bis[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]-3,3′-*p*-phenylene-bis-acrylamide dihydrochloride; SP600125, 1,9-pyrazoloanthrone anthra(1,9-*cd*)pyrazol-6(2*H*)-one; KT 5720, 2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-(9α,10β,12α)-9,12-epoxy-1*H*-diindolo(1,2,3-*fg*:3′,2′,1′-*kl*)pyrrolo (3,4-*i*)(1,6)benzodiazocine-10-carboxylic acid, hexyl ester; Ro 32-0432, (*S*)-3-(8-((dimethylamino)methyl)-6,7,8,9-tetrahydropyrido(1,2-*a*)indol-10-yl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrol-2,5-dione; PD 098059, 2′-amino-3′-methoxyflavone.

nases (GRKs) that have the unique ability to recognize and phosphorylate their substrate only when they are in their active conformation state (Inglese et al., 1993). Of the seven GRKs cloned to date, GRK2 and the less abundant GRK3 (also known as β-adrenergic receptor kinase-1 and -2, respectively) are widely distributed throughout the body (Benovic et al., 1991). GRK2 was first recognized as a kinase responsible for phosphorylation of adrenergic receptors but was later shown to be a ubiquitous member of the GRK family, which phosphorylates and desensitizes a variety of GPCRs, including adenosine receptors (Fredholm et al., 2001). Although the desensitization of GPCRs, in general, and adenosine receptors, in particular, seems to involve multiple processes (Chern et al., 1993; Palmer et al., 1994), GRK2 has been implicated as the mediator of desensitization, at least in the short term, of adenosine receptors, because targeted changes in cellular levels and/or membrane translocation of this kinase affect desensitization and activity of adenosine receptors (Mundell et al., 1997; Mundell and Kelly, 1998; Willets et al., 1999). Although GRK2 and other GRKs are regulated by both endogenous and exogenous factors (Penela et al., 2003), there has been some evidence that expression and activity of GRKs are regulated by inflammation and inflammatory cytokines in certain cells or tissues (Lombardi et al., 1999, 2001). More recent studies by Trincavelli et al. (2004) demonstrated that TNF-α reduced agonist-dependent receptor phosphorylation and attenuated agonist-mediated desensitization of A_{2B}Rs in human astroglial cells. Precise mechanisms of TNF-α regulation of adenosine receptor desensitization and the role of this cytokine in regulation of GPCR activity in general, however, remain unknown.

In this study on human monocytoid THP-1 cells, we determined whether TNF-α treatment affects agonist-dependent desensitization of A_{2A}Rs and examined the effect of TNF-α on expression and subcellular distribution of GRK2 and β-arrestins in response to stimulation with agonists of A_{2A}Rs and β-adrenergic receptors. In addition, we examined the signaling pathways mediating TNF-α regulation of GRK2 and A_{2A}R desensitization.

Materials and Methods

Reagents. Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN). CGS 21680 and 5′-*N*-ethylcarboxamidoadenosine (NECA) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-GRK2 and anti-A_{2A}R antibodies and alkaline phosphatase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-arrestin1 antibody was obtained from BD Biosciences (San Jose, CA). C_2-ceramide (*N*-acetylsphingosine), C_2-dihydro-ceramide, sphingosine, C_6-ceramide (*N*-hexanoyl-D-sphingosine), desipramine, and GW4869 were obtained from Sigma-Aldrich. The JNK inhibitor SP 600125 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). The protein kinase A (PKA) inhibitor KT 5720, protein kinase C (PKC) inhibitor Ro 32-0432, MEK1/2 inhibitor PD 098059, and p38 MAPK inhibitor SB 202190 were purchased from Sigma Chemical.

Cell Culture. Human monocytoid THP-1 cells, obtained from American Type Culture Collection (Manassas, VA), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere consisting of 5% CO_2. They were treated with 5 ng/ml TNF-α overnight or medium alone before being subject to further treatments.

Protein Extraction and Crude Membrane Preparation. To prepare total cell lysates, cells were washed with ice-cold PBS and lysed in ice-cold lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor cocktail (Sigma-Aldrich) for 15 to 20 min. The lysates were microcentrifuged at 14,000 rpm for 10 min at 4°C, and supernatants were collected. Crude membrane preparations were obtained after sonication of cells in a modification of a previously described technique (Arslan et al., 1997). In brief, cells were washed, resuspended in ice-cold PBS, and disrupted with a tissue homogenizer (three 10-s periods) in the presence of antiprotease cocktail (Sigma-Aldrich). Whole cells, nuclei, and other large debris were cleared by centrifugation at 300*g* for 10 min at 4°C, and the particulate fraction (crude membrane fraction) of the cleared supernatant was separated by microcentrifugation at maximum speed for 30 min. The protein content was determined by standard bovine serum albumin protein assay.

Western Immunoblot. Equal amounts of protein (10–40 μg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. Nonspecific antibody binding was blocked with 3% skim milk in Tris-buffered saline (TBS). Subsequently, the blot membrane was incubated with a primary antibody for 1 to 4 h and then with an alkaline phosphatase-conjugated secondary antibody for another hour in TBS. After each antibody incubation, the blot was extensively washed (three to four times; 5 min each) with TBS containing 0.1% Tween 20. Finally, the blot was exposed to fluorescent ECF substrate (Amersham Pharmacia Biotech, Piscataway, NJ) and scanned using the Storm PhosphorImager system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The band intensity was then quantitated using ImageQuant software (GE Healthcare). To screen multiple proteins, after probing with an antibody, the blot was stripped off and reprobed for β-actin or other proteins of interest.

Immunoprecipitation. Cultured cells ($2–5 \times 10^7$ cells for each treatment) were collected and washed with ice-cold PBS. Cells were resuspended and lysed in 1 ml of lysis buffer. The whole-cell lysate (WCL) was precleared for 30 min at 4°C with 0.25 μg of appropriate control (normal) IgG, corresponding to the host species of the primary antibody, and 20 μl of protein A/G-agarose (Santa Cruz Biotechnology, Inc.). The precleared cell lysates were subsequently incubated with 2 μg of the primary antibody for 1 h before the addition of 20 μl of protein A/G-agarose beads, and the incubation was continued overnight at 4°C with constant rotation. Next, the agarose beads were pelleted by centrifugation at 2500 rpm (~1000*g*) for 5 min at 4°C. After extensive washes (four to five times) with ice-cold lysis buffer, the beads were resuspended in sample loading buffer, boiled for 4 to 5 min, and subject to SDS-polyacrylamide gel electrophoresis and Western immunoblot with antibodies of interest.

Study of Adenosine Receptor Desensitization. Cells were pretreated overnight with medium alone or TNF-α and then stimulated with either 10 μM CGS 21680 for various times (0–60 min) or with 10 μM NECA (30 min). The medium was then removed, and cells were washed and resuspended with fresh medium and stimulated with 1 μM CGS 21680 for various times. Cells were then assayed for intracellular cAMP concentrations.

cAMP Quantification. After appropriate treatment, culture supernates were discarded, and cells were washed with ice-cold PBS. Intracellular cAMP levels were determined using the Biotrak cAMP enzyme immunoassay system (Amersham Pharmacia Biotech). In brief, washed cells from each treatment were lysed in 200 μl of the lysis buffer provided with the kit. Cell debris was removed by brief centrifugation. The lysate was applied in duplicate to 96-well plates provided by the manufacturer, and all steps of the assay were carried out following the manufacturer's instructions. cAMP concentrations were normalized to the protein content measured by standard protein assay.

Data Analysis. All statistical analysis was performed using Prism software (GraphPad Software, Inc., San Diego, CA). Data are presented as mean ± S.E.M. when appropriate. One- or two-way analysis of variance (ANOVA) was used to determine overall statis-

tical significance. Differences with a P value less than 0.05 were considered significant.

Results

TNF-α Diminished Agonist-Induced A_{2A}R Desensitization. Because A_{2A}Rs are generally coupled to $G\alpha_s$ and activate adenylyl cyclase, increasing cAMP formation, we evaluated the agonist-induced desensitization of A_{2A}Rs in control and TNF-α-treated THP-1 cells by measuring cAMP accumulation in response to the selective A_{2A}R agonist CGS 21680 (1 μM) after pretreatment of the cells with either 10 μM CGS 21680 or 10 μM NECA, a pan-adenosine receptor agonist. Analysis of the time course of agonist-mediated desensitization of adenosine A_{2A} receptor-stimulated increases in intracellular cAMP demonstrated a significantly greater response of TNF-α treated cells than the control cells to agonist stimulation whether or not they underwent desensitization with agonist (Fig. 1A). More importantly, these desensitization studies revealed that most of the A_{2A}Rs in control cells were quickly desensitized after 10 μM CGS 21680 pretreatment, whereas desensitization of A_{2A}Rs in TNF-α-treated cells was not only delayed but also greatly inhibited ($P < 0.01$; TNF-α-treated versus control cells; two-way ANOVA; Fig. 1, A and B). Likewise, after 30-min NECA pretreatment (10 μM), A_{2A}Rs in control cells seemed to be desensitized, and these cells were unresponsive to CGS 21680 stimulation, whereas TNF-α-treated cells were still responsive to CGS 21680 with an increase in cAMP level up to 127 ± 7% of basal level (Fig. 1C).

Effects of TNF-α on GRK2 Expression and Subcellular Localization. It has been previously demonstrated that Gαs-coupled receptors, including A_{2A}Rs, are quickly phosphorylated by GRK2 and other GRKs and subjected to desensitization after agonist exposure. To determine the mechanism for TNF-α inhibition of A_{2A}R desensitization, we next studied the effect of TNF-α on expression and subcellular distribution of GRK2 after agonist stimulation. Neither TNF-α treatment nor CGS 21680 stimulation significantly changed the total cellular level of GRK2 (Fig. 2A; data not shown). Because there were no changes in total GRK2 levels and proximity to its receptor target is probably more important for the function of this kinase, we examined the effect of TNF-α treatment on translocation of GRK2 from cytosol to the plasma membrane after agonist stimulation. As demonstrated by immunoblot, in control (untreated) THP-1 cells the level of GRK2 in the plasma membrane quickly increased after stimulation with CGS 21680 in a time-dependent manner and reached up to 124 ± 5% of baseline within 10 to 20 min (Fig. 2B). In contrast, in cells that were pretreated overnight with TNF-α, agonist stimulation failed to induce GRK2 translocation to the plasma membrane and instead resulted in a time-dependent decrease in the membrane GRK2 level to 80 ± 3% of basal level within 10 to 20 min (Fig. 2, A and B). The difference in response to CGS 21680 between control and TNF-α-treated cells in terms of GRK2 translocation was highly significant ($P < 0.001$; TNF-α versus control; two-way ANOVA). Incubation of cells with TNF-α for shorter periods also significantly inhibited CGS 21680-induced translocation of GRK2 to the plasma membrane after short-term treatment (Fig. 2C; $P < 0.01$ for all time points versus control). The effect of TNF-α on GRK2 translocation did not require protein synthesis because coincubation of cells with cycloheximide, a protein synthesis inhibitor, did not alter the inhibitory effect of TNF-α on agonist-dependent GRK2 relocalization (Fig. 2D; $P > 0.05$; two-way ANOVA).

To determine whether the regulatory effect of TNF-α on

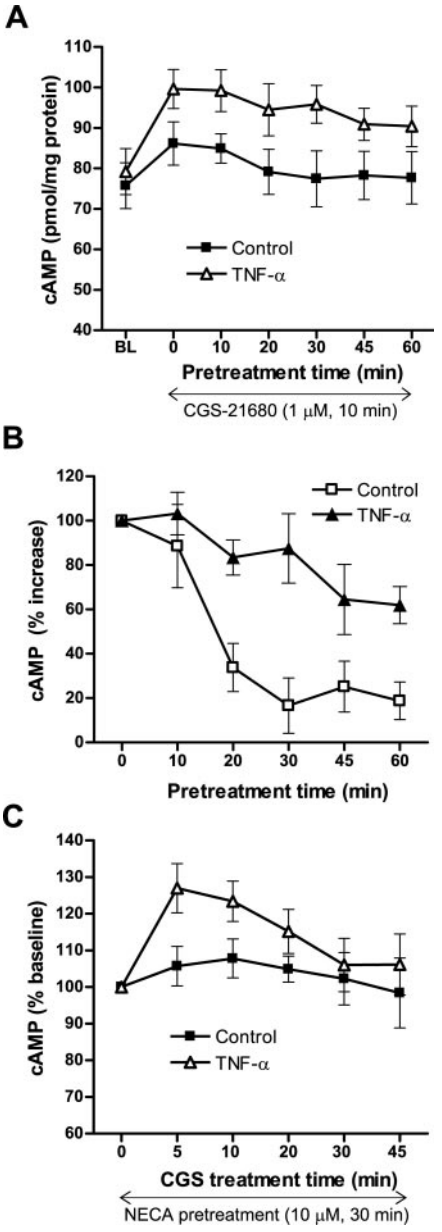

Fig. 1. TNF-α inhibits agonist-dependent desensitization of A_{2A}Rs. A, THP-1 cells were treated overnight with TNF-α (5 ng/ml) or medium alone (Control) and then stimulated with 10 μM CGS 21680 for varying intervals up to 60 min before washing and restimulation with 1 μM CGS 21680 for 10 min. Cells were collected and assayed for intracellular cAMP concentrations as described in text. Data are expressed as mean ± S.E.M. of three separate experiments. BL, basal cAMP level in control and TNF-α-treated cells without any agonist stimulation; time point 0 represents the cAMP level in cells without CGS 21680 pretreatment. B, time course of A_{2A}R desensitization. Data were deducted from A but expressed as percentage of CGS 21680-induced cAMP increase beyond the basal cAMP level. The amount of cAMP increase over the basal level at time point 0 (10.5 and 20.4 pmol/mg protein for control and TNF-α-treated cells, respectively) was considered 100%. C, after cytokine treatment, cells were prestimulated with 10 μM NECA for 30 min and then subject to stimulation with 1 μM CGS 21680 for the indicated times. Data are expressed as mean ± S.E.M. of percentage of the cAMP level in cells without CGS 21680 stimulation ($n = 3$).

agonist-induced GRK2 translocation is specific for the A_{2A}Rs or whether this regulatory mechanism is a more general phenomenon for other $G\alpha_s$-coupled receptors, we examined GRK2 relocalization in control and TNF-α-treated THP-1 cells in response to isoproterenol, a selective β-adrenergic receptor agonist. As demonstrated in Fig. 3, isoproterenol, like CGS 21680, induced translocation of GRK2 to the plasma membrane in control cells, whereas TNF-α treatment decreased the membrane-associated GRK2 level after isoproterenol stimulation ($P < 0.01$; TNF-α versus control; two-way ANOVA).

TNF-α Inhibits Agonist-Induced Receptor-GRK2 Association. It was previously reported that activated $G\alpha_s$-coupled receptors bind to membrane-bound GRKs, resulting in kinase activation and phosphorylation of these receptors (Li et al., 2003; Pao and Benovic, 2005). To determine the effect of TNF-α on interaction between A_{2A}Rs and GRK2, we immunoprecipitated A_{2A}Rs from the total cell lysates from untreated and treated cells and then probed for the presence of GRK2. As shown in Fig. 4A, without agonist stimulation little GRK2 was associated with A_{2A}Rs in either control or TNF-α-treated THP-1 cells. As expected, CGS 21680 stimulation clearly increased GRK2 association with A_{2A}Rs in control cells. However, in TNF-α-treated cells, the agonist-induced increase in A_{2A}R-GRK2 association was almost abrogated, consistent with the TNF-α-induced decrease in the membrane-associated GRK2 level during agonist stimulation (Fig. 4).

Effects of TNF-α on β-Arrestin Translocation. β-Arrestin1/2 proteins bind phosphorylated $G\alpha_s$-coupled receptors and mediate their internalization. We next determined whether TNF-α-induced changes in GRK2 translocation after agonist stimulation lead to similar changes in membrane recruitment of β-arrestin protein. As demonstrated by Western immunoblot analysis of crude membrane and cytosolic fractions of THP-1 cells, β-arrestin1 was mostly detected in the cytosol (Fig. 5A). Nonetheless, in control cells CGS 21680 stimulation quickly and significantly increased β-arrestin1 levels in the plasma membrane, up to 121 ± 5% of the basal level within 5 to 10 min ($P < 0.01$), whereas the membrane β-arrestin1 level in TNF-α-treated cells was not significantly increased after CGS 21680 stimulation (Fig. 5). The difference in agonist-induced changes in membrane-bound β-arrestin levels between control and TNF-α-treated cells was significant ($P < 0.05$; TNF-α versus control; two-way ANOVA). Neither TNF-α nor CGS 21680 induced any detectable changes in the cytosolic β-arrestin1 levels (Fig. 5A; data not shown). These results indicate that TNF-α treatment, consistent with its effect on GRK2 translocation, suppresses β-arrestin recruitment to the plasma membrane during stimulation of $G\alpha_s$-coupled receptors.

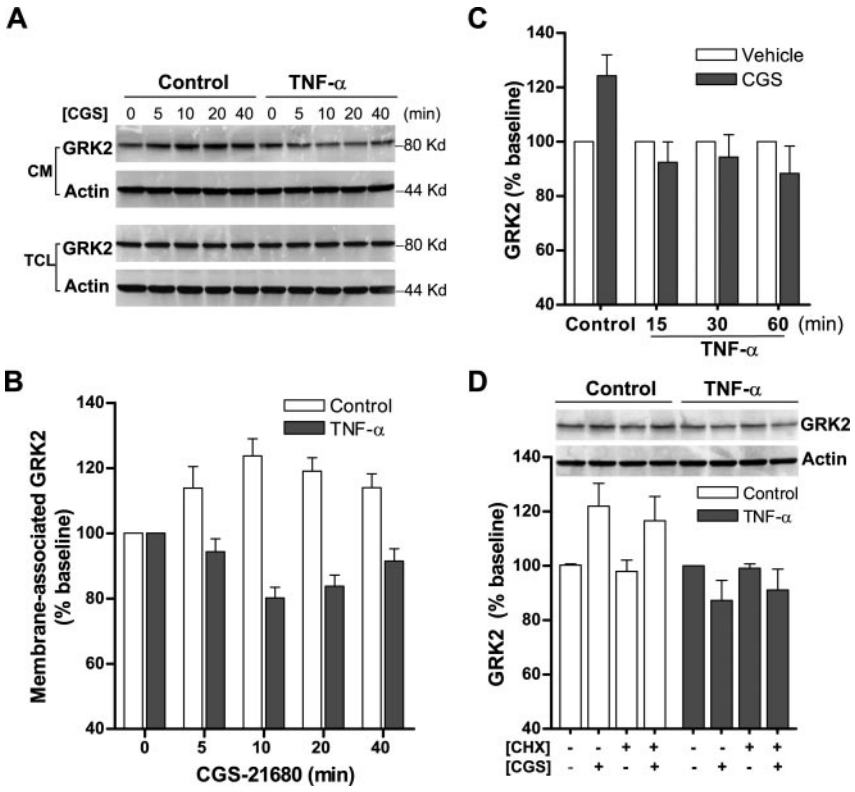

Fig. 2. Effects of TNF-α on expression and translocation of GRK2 after CGS 21680 stimulation. A and B, THP-1 cells were treated overnight with 5 ng/ml TNF-α or medium alone (Control) and then stimulated with 1 μM CGS 21680 (CGS) for the indicated times. Crude membrane preparations (CM) and total cell lysates (TCL) were prepared, and Western immunoblot was carried out as described in text. A, representative images of Western immunoblot for GRK2 and β-actin. B, densitometric analysis of immunoblotted GRK2 after normalization to β-actin levels. Note that the overall change in membrane GRK2 levels from basal level after CGS 21680 stimulation is statistically significant in both control ($P < 0.05$) and TNF-α-treated cells ($P < 0.05$). C, THP-1 cells were treated with 5 ng/ml TNF-α for 15, 30, or 60 min before CGS 21680 stimulation (1 μM; 10 min). Membrane-associated GRK2 levels, detected by Western blots, were densitometrically quantitated and normalized to β-actin levels. D, Western immunoblot and quantification of membrane-associated GRK2 and β-actin levels in THP-1 cells treated with TNF-α in the presence or absence of cycloheximide (CHX; 3 μM) before CGS 21680 stimulation (1 μM; 10 min). For B, C, and D, each bar represents mean percentages ± S.E.M. of basal membrane-associated GRK2 levels in control or TNF-α-treated cells, as appropriate, from nine (B; $n = 9$) and three (C and D; $n = 3$) independent experiments.

Involvement of Sphingomyelinase-Dependent Pathway in Mediating Effects of TNF-α on GRK2 Translocation and A_{2A}R Desensitization. Because the effect of TNF-α on agonist-dependent GRK2 translocation does not depend on new protein synthesis (Fig. 2D), we examined the role of other signaling pathways for TNF-α in the prevention of desensitization of A_{2A}Rs. TNF-α has previously been shown to signal for cellular activation in monocytes and macrophage-like cells (Wiegmann et al., 1992; Dbaibo et al., 1993; MacKichan and DeFranco, 1999; Mallampalli et al., 1999) via activation of sphingomyelinases (SMases), which generate lipid-derived signaling elements such as C_2-ceramide. Pretreatment with 20 μM C_2-ceramide decreased GRK2 levels in the plasma membrane after CGS 21680 stimulation to 84 ± 5% of basal level (Fig. 6, A and B), mimicking the effect of TNF-α. Treatment of the cells with C_2-dihydroceramide, an inactive analog of C_2-ceramide, did not change the effect of CGS 21680 on GRK2 translocation (Fig. 6A; data not shown). Of other ceramide isoforms tested, 50 μM sphingosine was toxic to the cells and 10 μM C_6-ceramide did not clearly alter the agonist-induced membrane translocation of GRK2 (data not shown).

To further understand the involvement of SMase pathways in TNF-α signaling, we examined the effects of selective inhibitors of acid and neutral SMases on the capacity of TNF-α treatment to modulate TNF-α-mediated inhibition of GRK2 translocation to the plasma membrane. Cotreatment of THP-1 cells with TNF-α and either desipramine, an inhibitor of acid SMase, or GW4869, an inhibitor of neutral SMase, partly but significantly reversed the TNF-α effect on CGS 21680-dependent GRK2 translocation ($P < 0.05$ for both inhibitors; two-way ANOVA), whereas combination of both in-

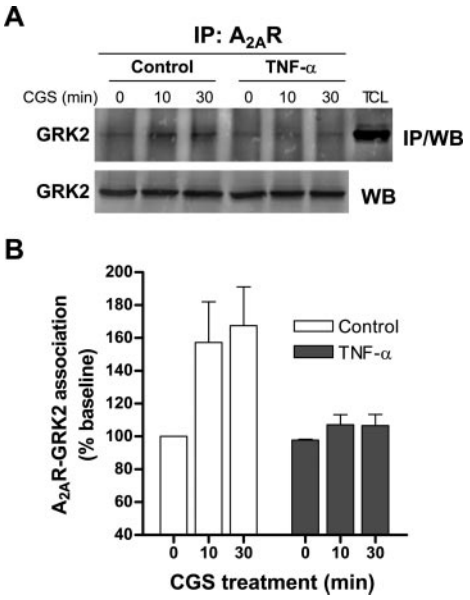

Fig. 4. TNF-α reduces agonist-dependent association of GRK2 with A_{2A}R. THP-1 cells were pretreated overnight with 5 ng/ml TNF-α or medium alone (Control) and then stimulated with 1 μM CGS 21680 (CGS) for the indicated times. Whole cell lysates were prepared and immunoprecipitated (IP) with a polyclonal A_{2A}R antibody. The precipitated proteins were immunoblotted (WB) for GRK2 as described in text. A, representative image of co-immunoprecipitation/WB for A_{2A}R-GRK2 association (top gel panel). Total cell lysates (TCL) (last lane) were used in WB as a positive control for GRK2 identity. Western immunoblot for total GRK2 expression (bottom gel panel) in whole cell lysates was also carried out to verify loading inputs for immunoprecipitation. B, level of GRK2 associated with A_{2A}Rs was densitometrically quantified and expressed as percentage (mean ± S.E.M.) of the basal GRK2 level in control cells from three independent experiments ($n = 3$).

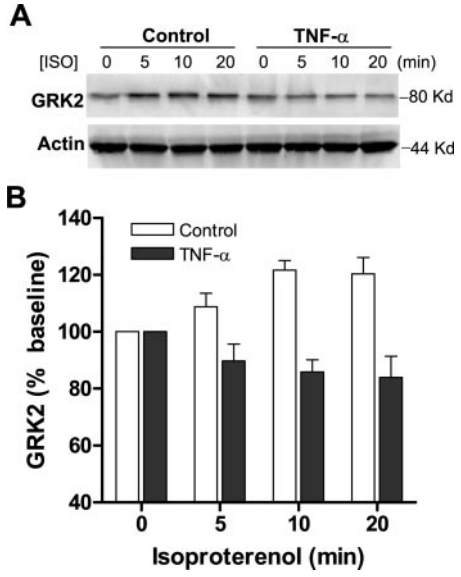

Fig. 3. Effects of TNF-α on GRK2 translocation after isoproterenol stimulation. THP-1 cells were treated overnight with 5 ng/ml TNF-α or medium alone (Control) and then stimulated with isoproterenol (ISO; 10 μM) for the indicated times. Crude membrane preparations were obtained and subject to Western immunoblot for GRK2 and β-actin as described in text. A, representative images of Western immunoblot of membrane-associated GRK2 and β-actin. B, densitometric analysis of changes in membrane-associated GRK2 levels in response to isoproterenol stimulation after normalization to β-actin. Data are expressed as percentage of either GRK2 levels in resting cells or TNF-α-treated cells, as appropriate, and represent the mean ± S.E.M. of three independent experiments.

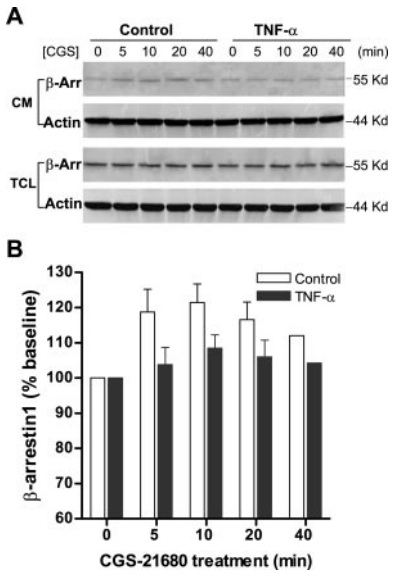

Fig. 5. TNF-α diminishes agonist-dependent recruitment of β-arrestin to the plasma membrane. THP-1 cells were treated overnight with 5 ng/ml TNF-α and then stimulated with 1 μM CGS 21680 for the indicated times. Crude membrane and cytosolic fractions were prepared and immunoblotted for β-arrestin1. A, representative images of Western blots for β-arresin1 (β-Arr) and β-actin expression in crude (CM) membrane preparations and the cytosol (TCL). B, densitometric analysis of membrane-associated β-arrestin1 levels are expressed as mean percentage ± S.E.M. of basal levels in cells without CGS 21680 stimulation from three experiments ($n = 3$). Note that CGS 21680 stimulation significantly increased membrane-associated β-arrestin1 in control ($P < 0.05$) but not in TNF-α-treated cells ($P > 0.05$).

hibitors almost completely reversed the TNF-α effect ($P < 0.01$ versus no inhibitor; two-way ANOVA; Fig. 6C). These inhibitors did not interfere with agonist-mediated GRK2 translocation in control cells (Fig. 6C). To determine whether the reversal of TNF-α inhibition of GRK2 translocation by SMAase inhibitors affects GRK2-mediated receptor desensitization, we studied the effect of desipramine and GW4869 on $A_{2A}R$ desensitization along with TNF-α treatment. TNF-α greatly inhibited $A_{2A}R$ desensitization after 30-min CGS 21680 pretreatment, as shown previously (Fig. 1), and this inhibitory effect of TNF-α was almost completely abrogated in the presence of SMase inhibitors ($P < 0.01$ versus no inhibitor; two-way ANOVA; Fig. 6D).

Effects of Protein Kinase Inhibitors on GRK2 Translocation. It was previously reported that GRK2 activity and membrane targeting are also regulated by phosphorylation of GRK2 by other protein kinases, including PKA, PKC, and MAPKs (Penela et al., 2003). To determine whether any of these kinases are involved in mediating the TNF-α effect on GRK2 translocation, we studied the effect of specific inhibitors of these kinases on the phenomenon. We found that inhibition of PKC by Ro 32-0432 moderately inhibited and PKA inhibitor KT 5720 slightly affected the capacity of CGS 21680 to stimulate GRK2 association with the plasma membrane in control cells ($P < 0.05$ for Ro 32-0432 and $P > 0.05$ for KT 5720 versus no inhibitor; two-way ANOVA; Fig. 7, A and B). None of the inhibitors, including those of p38 MAPK and MEK1/2, diminished the effect of TNF-α on CGS 21680-induced GRK2 translocation (Fig. 7, A–D).

TNF-α and ceramides are known to activate JNK signaling via activating its upstream kinases (Sathyanarayana et al., 2002; Shen et al., 2003), and other downstream protein kinases mediate effects of adenosine and other GPCRs. Treatment of cells with the selective JNK inhibitor SP 600125 (25 μM) minimally enhanced CGS 21680-induced GRK2 translocation in control cells. More interestingly, in TNF-α-treated cells JNK inhibition significantly and greatly reversed the TNF-α effect on CGS 21680-dependent GRK2 translocation (Fig. 7E; $P < 0.05$ for SP 600125 versus no inhibitor; two-way ANOVA). Moreover, the effect of SP 600125 on TNF-α inhibition of GRK2 translocation correlated with the reversal of TNF-α inhibition of $A_{2A}R$ desensitization of the cAMP response by this inhibitor (Fig. 7F).

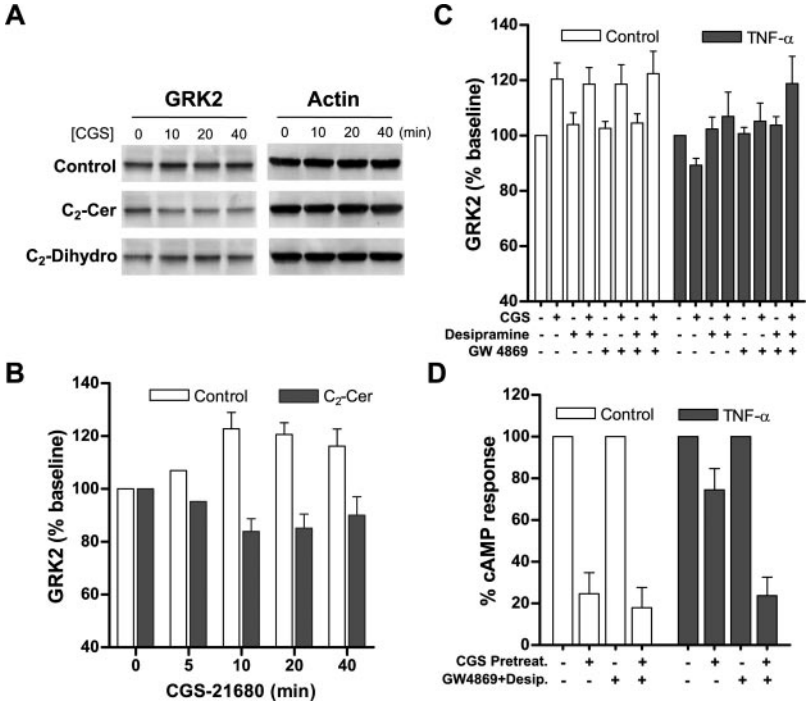

Fig. 6. Involvement of sphingomyelinase pathway in TNF-α-mediated regulation of agonist-dependent translocation of GRK2. A and B, THP-1 cells were treated with C_2-ceramide (C_2-cer; 20 μM), C_2-dihydro-ceramide (C_2-dihydro; 20 μM), or vehicle alone (Control) for 4 h and then stimulated with 1 μM CGS 21680 (CGS) for the indicated times. Crude membrane proteins were extracted and immunoblotted for GRK2 and β-actin. A, representative images of Western immunoblots for membrane-associated GRK2 and β-actin from control or ceramide-treated cells. B, densitometric analysis of CGS 21680-induced changes in membrane-associated GRK2 levels in control and C_2-ceramide-treated cells, normalized to β-actin. Data are expressed as the percentage ± S.E.M. of the basal levels observed in the absence of CGS 21680 ($n = 5$, except at time point 5 min, for which data were only available from a single experiment). Note that membrane-associated GRK2 levels were significantly increased in control cells ($P = 0.036$) and decreased in C_2-ceramide-treated cells ($P = 0.039$) after CGS 21680 treatment; $P < 0.01$ for C2-ceramide versus control, two-way ANOVA. C, THP-1 cells were treated overnight with TNF-α or medium alone (Control) in the presence or absence of the acid SMase inhibitor desipramine (10 μM) or the neutral SMase inhibitor GW4869 (20 μM) or both. Cells were then stimulated with 1 μM CGS 21680 for 10 min. The membrane-associated GRK2 level was examined by Western immunoblotting and densitometrically quantified. Data are expressed as percentage of the basal GRK2 level, and bars represent the mean ± S.E.M. of results from four experiments. D, effects of SMase inhibitors on TNF-α inhibition of $A_{2A}R$ desensitization. THP-1 cells were treated overnight with TNF-α or medium alone (Control) in the presence or absence of both desipramine (10 μM) and GW4869 (20 μM) (GW4869+Desip.). Cells were then pretreated with 10 μM CGS 21680 (CGS Pretreat.) for 30 min and washed before restimulation with 1 μM CGS 21680 for 10 min. cAMP levels were measured as described in text. Data shown are from three experiments. Bars represent percentage of cAMP response to CGS 21680 stimulation in cells without agonist pretreatment. The cAMP increase over the basal level after agonist stimulation of nonpretreated cells is defined as 100%.

Discussion

In previous studies, we and others have observed that TNF-α induces a 20 to 30% increase in $A_{2A}R$ expression. Although one would expect a shift in the dose-response curve for this receptor, we observed that there was, in fact, a threshold effect. Thus, concentrations of ligand that should stimulate maximal receptor function do not stimulate any detectable response unless the cells have been pretreated with TNF-α. To better understand this apparent shift in the threshold for $A_{2A}R$ function, we carried out the current study. We found that TNF-α inhibits agonist-dependent desensitization of $A_{2A}Rs$ in human monocytoid THP-1 cells, an observation that further elucidates the TNF-α-stimulated change in receptor function. These findings provide a more solid explanation for the enhanced function and activity of $A_{2A}Rs$ observed after TNF-α and IL-1 treatment in our previous studies (Khoa et al., 2001, 2003). Together, these data suggest that TNF-α and probably some other inflammatory cytokines promote $A_{2A}R$ signaling and function through multiple effects, not only enhancing the receptor number but also regulating mediators of receptor signaling and desensitization. Our demonstration that TNF-α inhibits $A_{2A}R$ desensitization is also consistent with the previous report by Trincavelli et al. (2004) that TNF-α inhibits desensitization of $A_{2B}Rs$ and enhances the functions of these receptors in human astroglial cells. Moreover, the current study provides further mechanistic insight into TNF-α-mediated inhibition of receptor desensitization. Our results demonstrate that the cytokine promotes the agonist-dependent dissociation of GRK2, a kinase primarily responsible for phosphorylation and desensitization of GPCRs, from the plasma membrane and diminishes recruitment of β-arrestin to the plasma membrane after stimulation with the agonists of $A_{2A}Rs$ and β-adrenergic receptors, leading to diminished phosphorylation and desensitization of these receptors. Findings from our studies and others that $A_{2A}Rs$, $A_{2B}Rs$, and β-adrenergic receptors respond similarly to TNF-α treatment suggest that this is a common regulatory mechanism for other $G\alpha_s$-coupled receptors.

Previous studies have demonstrated that the activity of GRKs decreases in lymphocytes of rheumatoid arthritis patients and that the proinflammatory cytokines interferon-γ and IL-6 down-regulate cellular expression and activity of GRK2 and GRK6 and increase sensitivity to β_2-adrenergic receptor activation (Lombardi et al., 1999). Lombardi et al. (2001) have also reported that inflammation induces a tissue-specific down-regulation of GRKs in the immune system of animals with adjuvant arthritis. Our results indicate that TNF-α decreases the activity of GRK2 as the cytokine was shown to inhibit $A_{2A}R$ desensitization and to enhance receptor function, although this effect does not seem to result from decreased expression of GRK2 but rather from diminished

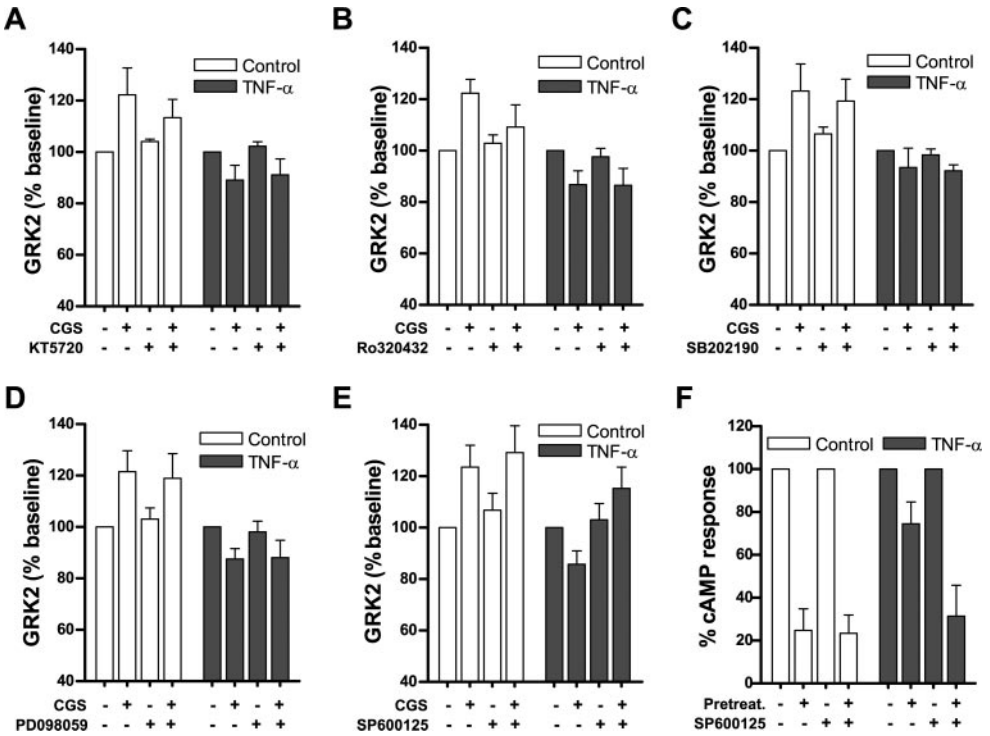

Fig. 7. JNK inhibitor reverses TNF-α effects on GRK2 translocation and $A_{2A}R$ desensitization. A to E, THP-1 cells were pretreated overnight with 5 ng/ml TNF-α or medium alone (Control) and stimulated with CGS 21680 (CGS; 1 μM; 10 min) in the presence or absence of PKA inhibitor (KT 5720; 1 μM; A), PKC inhibitor (Ro 32-0432; 2 μM; B), p38 MAPK inhibitor (SB 202190; 20 μM; C), MEK1/2 inhibitor (PD 098059; 50 μM; D), or JNK inhibitor (SP 600125; 25 μM; E). All inhibitors were added to the culture 30 min before agonist stimulation. The level of GRK2 in the crude membrane preparations was examined by Western immunoblotting and densitometrically quantified. Data are expressed as mean ± S.E.M. of percentage of the basal GRK2 level from five (SP 600125), four (KT 5720), and three (Ro 32-0432, PD 098059, and SB 202190) experiments. F, desensitization assay. THP-1 cells were treated overnight with TNF-α or medium alone (Control). Cells were then pretreated with 10 μM CGS 21680 (Pretreat.) for 30 min in the presence or absence of 25 μM SP 600125 and washed before restimulation with 1 μM CGS 21680 for 10 min. SP 600125 was added to the cells 30 min before agonist pretreatment. cAMP levels were measured as described in text. Bars represent percentage of cAMP response to CGS 21680 of cells without agonist pretreatment for nonpretreated and pretreated cells. The cAMP increase in response to CGS 21680 over the basal level in nonpretreated cells is defined as 100%. Data shown are from three experiments.

translocation of GRK2 to the plasma membrane. In sum, our data and the previously published results indicate that decreased association of GRK2 and β-arrestin with GPCRs in TNF-α-treated cells is responsible for decreased agonist-induced receptor desensitization and increased activity of corresponding GPCRs.

It is interesting that TNF-α treatment not only prevents agonist-dependent membrane translocation of GRK2 but also seems to promote agonist-dependent dissociation of GRK2 from the membrane. Unlike most other members of the GRK family, which are generally associated with the plasma membrane (Stoffel et al., 1994; Pronin et al., 1998), GRK2 resides predominantly in the cytosol and translocates to the plasma membrane via a carboxy-terminal βγ binding domain or by directly binding to phospholipids upon receptor ligation (Daaka et al., 1997; Carman et al., 2000). Multiple mechanisms have been shown to regulate the association of GRK2 with the plasma membrane and its activity, including regulation of GRK2 by other kinases, such as PKA, PKC, and MAPKs, during activation of GPCRs (Penela et al., 2003). Although inhibition of PKA and PKC might partly affect phosphorylation and membrane translocation of GRK2 in control cells, the inability of inhibitors of these kinases to alter the effect of TNF-α on the ligand-induced association of GRK2 with the plasma membrane suggests that other mechanisms are responsible for ligand-induced desensitization of adenosine A_{2A} receptors. Moreover, our results indicate involvement of the TNF-α-activated, sphingomyelinase-dependent pathway (signaling through the protein kinase JNK) (Dbaibo et al., 1993; Muller et al., 1998; Mallampalli et al., 1999) in the TNF-α-mediated blockade of A_{2A} receptor desensitization. It is still unclear, however, how ceramide and/or JNK, activated by TNF-α, modulate GRK2 subcellular localization during GPCR occupancy. An alternative explanation is suggested by the finding of Liu et al. (2002) that growth hormone-induced formation of ceramide is mediated by a $G_{\beta}\gamma$-dependent pathway and that ceramide formation is blocked by a mutated form of GRK2, which is a known $G_{\beta}\gamma$ scavenger (Koch et al., 1994). Thus, it is possible that TNF-α and its downstream effector ceramide might interfere with $G_{\beta}\gamma$-GRK2 binding and thus prevent GRK2 association with the plasma membrane during GPCR stimulation. Nonetheless, ceramide-mediated mechanisms might not be solely responsible for the TNF-α effect on GRK2 translocation. Decreased activity and membrane availability of GRK2 in TNF-α treated cells during GPCR activation may also result from increased binding of GRK2 to other cytosolic proteins, such as Raf kinase inhibitor protein, an inhibitor that can alternate binding to either Raf-1 or GRK2 (Lorenz et al., 2003). TNF-α treatment may promote Raf kinase inhibitor protein-GRK2 binding and thereby retain GRK2 in the cytosol during GPCR ligation, and this hypothesis needs to be investigated.

TNF-α, one of the most prominent inflammatory cytokines, plays a pivotal role in orchestrating host defense and inflammatory processes in vertebrates. Although critical for host defense, TNF-α, in concert with other proinflammatory cytokines, such as IL-1 and IL-6, has been strongly implicated in the pathogenesis of inflammatory and autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease, and it is responsible for progressive tissue injury and damage in those conditions (Feldmann et al., 1998). The development of TNF-α and IL-1 inhibitors has therefore been one of the most active areas of drug development over the last decade for the treatment of inflammatory diseases (Vilcek and Feldmann, 2004). Although clinically effective, these inhibitors still pose serious side effects (Cush, 2004), some of which result from unexpected interactions between TNF-α and other receptors. Our results suggest one such interaction between TNF-α and $G\alpha_s$-linked receptors that could account for a distinct toxicity of the anti-TNF-α therapies, exacerbation of congestive heart failure (CHF). In a rat model of CHF, Wang et al. (2005) demonstrated that density and protein content of β1-adrenergic receptors were increased, whereas GRK isoforms, including GRK2, and β-arrestin1 were decreased in the membrane fraction of cells from failing hearts. Increased adrenergic tone is a compensatory mechanism in the failing heart, and interference with this compensation may exacerbate heart failure. A recent study has demonstrated increased production of TNF-α and the accompanying up-regulation of $A_{2A}R$ in peripheral blood mononuclear cells from CHF patients (Capecchi et al., 2005). Thus, we speculate that drugs targeting inflammatory cytokines, such as TNF-α or IL-1, which are beneficial in the treatment of inflammatory diseases, may undercut TNF-α-induced enhancement of β-adrenergic tone, thereby exacerbating CHF.

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