G16-mediated Activation of Nuclear Factor κB by the Adenosine A1 Receptor Involves c-Src, Protein Kinase C, and ERK Signaling*

Received for publication, September 7, 2004, and in revised form, October 8, 2004 Published, JBC Papers in Press, October 12, 2004, DOI 10.1074/jbc.M410196200

Andrew M. F. Liu and Yung H. Wong‡

From the Department of Biochemistry, Molecular Neuroscience Center, and Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

The G16-linked adenosine A1 receptor has been shown to mediate anti-inflammatory actions, possibly via modulation of the transcription factor nuclear factor-κB (NFκB). Here we demonstrate that an adenosine A1 agonist, N6-cyclohexyladenosine (CHA), activated IKKα/β phosphorylation through PTX-insensitive G proteins in human lymphoblastoma Reh cells. To delineate the mechanism of action, different PTX-insensitive G proteins were expressed in human embryonic kidney 293 cells. Only Gαt5 supported the CHA-induced IKK phosphorylation and NFκB-driven luciferase activity in time-dependent, dose-dependent, and PTX-insensitive manners. Gβγ subunits also modulated IKK/NFκB, as indicated by the stimulatory actions of Gβγ2 and the abrogation of CHA-induced response by transducin. The participation of phospholipase Cβ, protein kinase C, and calmodulin-dependent kinase II in CHA-induced IKK/NFκB activation were demonstrated by employing specific inhibitors and dominant-negative mutants. Inhibition of c-Src and numerous intermediates along the extracellular signal-regulated (ERK) kinase cascade including Ras, Raf-1 kinase, and MEK1/2 abolished the CHA-induced IKK/NFκB activation. Although c-Jun N-terminal kinase and p38 MAPK were also activated by CHA, they were not required for the IKK/NFκB regulation. Similar results were obtained using Reh cells. These data suggest that the G16-mediated activation of IKK/NFκB by CHA required a complex signaling network composed of multiple intermediates.

Nuclear factor-κB (NFκB) is a ubiquitous heterodimeric transcription factor, which plays important roles in the regulation of numerous inducible genes involved in modulating inflammation, cell survival, and differentiation (1). In the resting state, the NFκB heterodimer is anchored and retained in the cytosol by inhibitor-κBα (IκBα). The NFκB transcription factor can be stimulated by various environmental factors including ultraviolet rays, as well as cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α. These extracellular signals activate a key regulatory step in the pathway, the IκB kinase (IKK) complex, comprising the catalytic subunits (IKKa and IKKβ) and a linker subunit (IKKγ/NEMO). This kinase complex, in turn, phosphorylates IκBα at Ser32 and Ser36 and signals for its ubiquitin-related degradation (2). The released NFκB is then translocated into the nucleus and promotes NFκB-dependent transcription (3).

As one of the largest superfamilies of cell surface detectors, the heptahelical G protein-coupled receptors (GPCRs) are known to regulate numerous cellular processes, ranging from photon detection to cell differentiation. The GPCRs transduce signals through the coupling of one or more heterotrimeric G proteins to generate diverse cellular responses. Increasing evidence indicates that GPCRs can actively control gene transcription and expression in different cell types (4). Practically all GPCRs are capable of activating the mitogen-activated protein kinase (MAPK) pathways (5), thereby regulating numerous cellular responses including apoptosis (6) and inflammation (7). A variety of GPCRs have now been shown to regulate inflammation and cell survival processes by controlling the activation of NFκB. They include receptors for bradykinin (8), formyl peptide (fMLP) (9), lysophosphatidic acid (10), interleukin B (11), and dopamine (12, 13). This list of NFκB-regulating GPCRs is far from complete and is rapidly expanding.

The nucleoside adenosine controls a variety of physiological responses and the adenosine receptors can be considered as novel therapeutic targets of various diseases (14). In particular, the G16-linked adenosine A1 receptor (A1R) has been shown to play critical roles in regulating apoptotic and inflammatory activities. The apoptotic effects induced by ethanol (15) and hydrogen peroxide (16) are attenuated by the activation of A1R, and the severity of multiple sclerosis is reduced by the administration of A1R-specific agonist in female 129/Sv mice (17).

Immune responses, for instance the adherence of neutrophil to endothelium (18) and chemotaxis in human dendritic cells (19), are also regulated by A1R. However, the precise mechanisms by which A1R modulates these cellular events remain elusive. Given the central role of NFκB in mediating inflammatory and immune responses, it is reasonable to predict that A1R can regulate the activity of NFκB. Signals arising from A1R can be channeled via PTX-sensitive G16 (20) and PTX-insensitive G16 (21, 22) proteins, and both pathways can potentially lead to the activation of NFκB. The fMLP receptor can employ both Gi (23) and G16-dependent (24) mechanisms to activate NFκB, but the ability of A1R to activate NFκB has not been reported. In view of the hematopoietic-specific expression of G16 (25), it will be particularly interesting to determine its ability to link.
Activation of NFκB by Adenosine A1 Receptor

AIR activation to changes in NFκB activity. In the present study, we examined the ability of AIR to stimulate the phosphorylation of IKKα/β and the up-regulation of the transcriptional activity of NFκB-dependent luciferase reporter in human lymphoblastoma Reh cells that endogenously express Go1α (26) as well as in human embryonic kidney (HEK) 293 cells transiently expressing Go1α. The possible involvement of various signaling intermediates including PKC, c-Src, and MAPKs was also determined.

EXPERIMENTAL PROCEDURES

Materials—All chemicals except for PTX were purchased from Sigma-Aldrich or CalBiochem (San Diego, CA). PTX was from List Laboratories (Campbell, CA). Cell culture reagents, including Lipofectamine PLUS and Lipofectamine (Gibco-BRL, Life Technologies, Gaithersburg, MD), were from Invitrogen. The origin of cDNAs for receptors and G proteins were as described previously (22, 27). The cDNAs of wild-type cLeuB and doubly mutated cLeu-βA were gifts from Dr. Alan Israel (Institut Pasteur, France) whereas Akt and its dominant-negative mutant cDNAs were obtained from Dr. Zhenguo Wu (Hong Kong University of Science and Technology, Hong Kong). The cDNAs for wild type and dominant-negative mutants of iPKos and iKKβ were from Dr. Richard D. Ye (University of Illinois). The luciferase reporter gene was obtained from Clontech Laboratories (Palo Alto, CA), and the luciferin substrate kit was a product of Roche Diagnostics (Mannheim, Germany). Various antisera were products of Cell Signaling Technology (Beverly, MA) and Amersham Biosciences (Piscataway, NJ). Specific anti-Go antibodies were purchased from CalBiochem (San Diego, CA), PerkinElmer Life Sciences (Boston, MA), and Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Transfection—Human embryonic kidney HEK 293 cells (CRL-1573, American Type Culture Collection) were maintained at 37 °C in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO2. To establish stably transfected HEK 293 cells carrying pNFκB-luc, HEK 293 cells were grown to confluence in a 100-mm dish. Cells were washed with fresh MEM medium 3 h before transfection, and 9 μg of pNFκB-luc luciferase reporter gene (Clontech) and 1 μg of pCDNA3 (as a selection marker) were introduced using the calcium phosphate method. One day after transfection, cells were subjected to selection with 500 μg/ml G418. HEK 293 cells stably expressing NFκB luciferase reporter gene (HEK 293-NFκB) were maintained in growth medium containing 250 μg/ml G418. For luciferase assay, 1 day prior to transfection, HEK 293-NFκB cells were seeded at a density of 15,000 cells/well into white 96-well microplates designed for luminescent work (Nunc). Cells were transfected with cDNAs encoding various receptors (12.5 ng) and G proteins (37.5 ng) using 0.2 μl of PLUS and Lipofectamine reagents (Invitrogen) in 50 μl of Opti-MEM per well. In the case where other signaling molecules were investigated, 250 ng of receptor, 30 ng of G protein, and 10 ng of the signaling molecule cDNAs were transfected per well instead. After 3 h, 25 μl of Opti-MEM containing 30% fetal bovine serum were added to the well. For immunoblotting analysis, HEK 293 cells were seeded at 500,000 cells/well into 6-well microplates 1 day prior to transfection, and the cells were transfected with 200 ng of receptor and 400 ng of G protein cDNAs per well using Lipofectamine 2000 reagent (Invitrogen). To investigate other signaling molecules, an extra 200 ng of DNA encoding the gene of interest was also transfected.

Human lymphoblastoma cell line Reh (CRL-8286, American Type Culture Collection) was cultured at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO2. For immunoblotting analysis, Reh cells, serum-starved overnight, were seeded at 1 × 105 cells/ml into a 1.5-ml Eppendorf tube for drug treatment.

Luciferase Reporter Assay—Transfectants were grown in culture medium for 30 h and then maintained in serum-free medium in the presence or absence of 10 μM N6-cyclohexyladenosine (CHA) for 16 h. Where indicated, cells were pretreated with PTX (100 ng/ml, 4 h) and/or various kinase inhibitors (30 min) before the CHA challenge. Subsequently, the growth medium was removed and replaced by 25 μl of lysis buffer provided in the Luciferase Reporter Gene Assay kit (Roche Applied Science). The 96-well microplate was shaken on ice for 30 min. Luciferase activity was determined using a microplate luminesimeter LB986 (EG&G Berthold, Germany). Injector M connected to lysis buffer and injector P connected to the luciferin substrate were set to inject 25 μl of each component into each well. A 1.6-s delay time followed by a 2-s measuring time period was assigned to injector M whereas injector P was measured for 10 s after the luciferin was introduced into the well. Results were collected by WinGlow version 1.24 and expressed as relative luminescent units. Statistical calculation was performed using KyPlot version 2.0.

Immunoblotting Analysis—30 h after transfection, HEK 293 cells were serum-starved overnight in the presence or absence of 100 ng/ml PTX. The cells were then challenged by 10 μM CHA for 10 min followed by cell lysis in 150 μl of lysis buffer containing 50 μM Tris-HCl (pH 7.5), 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, 40 mM NaF, 1 mM dipotassiumiodide, 200 μM Na3VO4, 4 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin. When kinase inhibitors were examined, the transfectants were pretreated with different kinase inhibitors for 30 min in serum-free medium. The cell lysates were shaken for 15 min, and the supernatants were collected by centrifugation at 16,000 × g for 2 min. Clarified lysates (40 μg) were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad). For Reh cells, treated cells were harvested by centrifugation for 2 min at 16,000 × g. The supernatant was removed and replaced with 150 μl of lysis buffer. Phospho-IKKα/βSer177/183/IKKβSer181, IKKα, IKKβ, phospho-ERKThr202/Tyr204, ERK, phospho-JNKThr183/Tyr185, JNK, phospho-p38 MAPKThr180/Tyr182, p38 MAPK, phospho-c-Src(Tyr416), and c-Src were detected by specific primary antisera and horseradish peroxidase-conjugated secondary antisera. The immunoblots were visualized by chemiluminescence with the ECL kit (Amersham Biosciences). Images detected on x-ray films were quantified by densitometric scanning using ImageJ software.

Statistical Analysis—Data were expressed as mean ± S.E. of at least three independent sets of experiments. The probability of the observed difference being a coincidence was evaluated by analysis of variance and paired Student’s t test using KyPlot software. Differences at values of p < 0.05 were considered significant.

RESULTS

Activation of A1R Induces IKKα/β Phosphorylation in Human Lymphoblastoid Reh Cell through PTX-insensitive G Proteins—It has been shown to induce adhesion in neurophils (18) and chemotaxis (19) in plasmacytoid dendritic cells. These inflammatory events are likely to be NFκB-dependent. However, no report has yet documented the functionality of AIR in regulating NFκB activity. We began our study by examining the ability of AIR to activate the NFκB signaling pathway in a human lymphoblastoid leukemic Reh cell line. Agonist-induced phosphorylation of IKKα/β was determined using a phospho-IKKα/β-specific antiserum. As shown in Fig. 1A, the AIR-specific agonist CHA significantly stimulated the phosphorylation of IKKα/β in a time-dependent manner in Reh cells. Maximum stimulation was observed with a 15-min CHA treatment, and the response was sustained up to 60 min. The levels of IKKα/β phosphorylation increased with increasing concentrations of CHA (Fig. 1B, solid squares). Phosphorylation of IKKα/β in Reh cells became detectable at 100 nm CHA and reached a maximum of over 3-fold at 10 μM with an EC50 of ~100 nm.

AIR has previously been demonstrated to interact with both Go1α (20) and Go1qα (21, 22). Having a hematopoietic lineage, the Reh cells are known to express Go1qα (26). To test whether the CHA-induced IKKα/β stimulation was Gi-dependent, Reh cells were pretreated with PTX (100 ng/ml, 16 h) to ADP-ribosylate the G proteins. The CHA-induced phosphorylation of IKKα/β in Reh cells was resistant to PTX treatment (Fig. 1B, open squares), indicating the lack of involvement of PTX-sensitive G proteins in this pathway. Collectively, these data suggested that activation of IKKα/β by AIR occurred in a time- and dose-dependent manner but did not require PTX-sensitive G proteins.

Activation of IKK/NFκB by AIR Is Mediated via PTX-insensitive Gα1. To identify the specific PTX-insensitive G proteins that were responsible for the AIR-mediated IKKα/β phosphorylation, we employed an HEK 293-NFκB cell stably expressing the NFκB-dependent luciferase reporter gene for heterolo-
Activation of NFκB by Adenosine A1 Receptor

A

FIG. 1. Time- and dose-dependent activation of IKKα/β by CHA in Reh cells. A, Reh cells were stimulated with 10 nM CHA for different periods of time. B, Reh cells were treated with different concentrations of CHA in the absence (closed squares) or presence (opened squares) of PTX pretreatment. Cells were stimulated by 10 nM CHA for 15 min. Cell lysates were collected and analyzed by immunoblotting for total IKKα and IKKα/β phosphorylation. The relative intensities of CHA-induced IKKα/β phosphorylation are expressed as a percentage of the basal level (set as 100%). Data shown are the mean ± S.E. and immunoblots shown are representatives of three individual sets of experiments.

phosphorylation by up to 2-fold in HEK 293 cells co-expressing A1R with Gα16 (Fig. 2A). The CHA-induced phosphorylation of IKKα/β was accompanied by a 4-fold increase in NFκB-dependent luciferase activity (Fig. 2B) in these transfectants. Indeed, a previous study has demonstrated that the constitutively active Gα16 mutant (Gα16QL) can activate NFκB in HeLa cells (24). Gα16QL was co-expressed with A1R to confirm the ability of Gα16 to regulate NFκB in HEK 293 cells. The constitutive activity of Gα16QL resulted in agonist-independent stimulation of IKKα/β phosphorylation (Fig. 2D) and NFκB-driven luciferase activity (Fig. 2B). The magnitudes of the Gα16QL-induced responses were similar to those obtained with CHA in Gα16-expressing cells (compare Fig. 2, A and B).

The ability of Gα16 to link A1R activation to the IKK/NFκB pathway is in good agreement with the results obtained with Reh cells (Fig. 1). Since Gα16 appeared to mediate the CHA-induced IKKα/β phosphorylation and NFκB-dependent luciferase expression, we went on to characterize this pathway in detail. Transfectants co-expressing A1R and Gα16 were serum-starved in the absence or presence of PTX prior to stimulation with increasing concentrations of CHA. The NFκB-driven luciferase activity was dose-dependently stimulated by CHA with an EC50 of around 100 nM (Fig. 2E), similar to that obtained with Reh cells. The CHA-induced response was essentially unaffected by PTX throughout the agonist dose range tested. In the absence of Gα16, CHA was completely unable to stimulate the NFκB-dependent luciferase activity (Fig. 2E, pcDNA1), confirming that endogenous G proteins could not support A1R-mediated activation of NFκB even at an agonist concentration of 100 μM. The Gα16 dependence and PTX insensitivity of the CHA-induced NFκB activation were further confirmed by Western blot analyses (Fig. 2F). The phosphorylation level of IKKα/β increased with increasing concentrations of CHA, reaching a peak at around 10 μM. The CHA-induced phosphorylation of IKKα/β was also unaffected by PTX (Fig. 2F).

CHA-induced NFκB Activation Is Inhibited by DPCPX and Mediated through IKK and IκBα—To determine the specificity of the CHA-induced response, PTX-treated HEK 293-NFκB cells co-expressing A1R and Gα16 were incubated with 100 nM NFκB activation inhibitor (APQ; 6-amino-4-(4-phenoxyphenyl)ethylamino)quinazoline) for 30 min before the CHA challenge (Fig. 3A). APQ significantly attenuated the CHA-induced NFκB-dependent luciferase activity as compared with the vehicle control. Furthermore, blockade by a selective A1R antagonist DPCPX (1 μM) confirmed the specific involvement of A1R in mediating the IKKα/β phosphorylation (Fig. 3B) and NFκB activation (Fig. 3A). Next, we asked if dominant negative mutants of IKKα and IKKβ can attenuate the CHA-induced activation of NFκB. Introduction of IKKα, IKKβ, or both into HEK 293-NFκB cells did not affect the agonist-induced NFκB activation (Fig. 3C). In contrast, the dominant negative mutants of IKKα and IKKβ partially attenuated the CHA-mediated luciferase transcription by ~25 and 45%, respectively. When both dominant negative mutants were co-expressed in the cells, the CHA-induced NFκB activation was suppressed by 70% (Fig. 3C). Similarly, overexpression of the NFκB inhibitor protein, IκBα, significantly reduced the CHA-induced NFκB-dependent luciferase activity by ~30% as compared with the vector control (Fig. 3D). A doubly mutated IκBα protein (IκBαAA) in which the phosphorylation sites have been removed was then employed. IκBαAA is resistant to the induction of degradability by IKKα/β and thus can inhibit NFκB activation. By introducing IκBαAA into HEK 293-NFκB cells in conjunction with A1R and Gα16, CHA treatment was indeed significantly suppressed as compared with the vector control (Fig. 3D). The suppressive effect of this non-degradable IκBαAA protein was slightly but
significantly stronger than the wild-type degradable IkB protein.

**Involvement of the PLCγ/PKC/CaMKII Cascade in CHA-mediated IKKa/β Phosphorylation and NFkB Activation**—We have previously established that activation of G16-coupled A1R leads to the stimulation of PLCγ and Ca2+ mobilization (22, 27), and the subsequent activation of PKC and CaMKII is required for Gα16-mediated stimulation of STAT3 (29). Thus, we examined if the PLCγ/PKC/CaMKII pathway is similarly required for Gα16-mediated stimulation of NFkB. First, we employed specific inhibitors against these signaling molecules to block the CHA-induced IKKa/β phosphorylation and NFkB-dependent luciferase activity in PTX-treated HEK 293-NFkB cells co-expressing A1R and Gα16. U73122, a specific PLCγ inhibitor, at 10 μM inhibited the CHA-induced NFkB-dependent luciferase expression by over 30%, whereas its inactive analogue, U73343, was totally ineffective (Fig. 4A). Similar observations were obtained with regard to CHA-induced IKKa/β phosphorylation (Fig. 4B). Likewise, pretreating the transfecants with 100 nM calphostin C (CalC; selective PKC inhibitor) significantly attenuated the CHA-induced NFkB-dependent transcription of luciferase by around 40% (Fig. 4A) and the phosphorylation of IKKa/β were also reduced (Fig. 4C). The possible involvement of CaMKII was examined with its selective inhibitor, KN92. At 10 μM, KN92 significantly attenuated the CHA-induced responses (Fig. 4, A and D). The inactive analogue of this CaMKII inhibitor, KN92, was incapable of inhibiting the CHA-induced responses.

Next, we investigated the effects of different isoforms of PKC on A1R-mediated activation of NFkB. PKCα and PKCε were selected for the study as representatives of calcium-sensitive and -insensitive PKC isoforms, respectively. The wild-type and kinase-deficient mutant (KR) of these PKC isoforms were transfected into the cells along with A1R and Gα16. Expressions of wild-type PKCα and PKCε enhanced the CHA-induced NFkB-dependent luciferase activity by 25 and 30%, respectively, as compared with the vector control (Fig. 4E). Correspondingly, these wild-type PKC proteins enhanced the CHA-induced phosphorylation of IKKa/β (Fig. 4F). In contrast, the KR mutants of PKCα and PKCε significantly inhibited the A1R-mediated luciferase expressions by over 50% compared with the vector control (Fig. 4E) and comparable results were obtained by monitoring the phosphorylation of IKKa/β (Fig. 4F). Collectively, these results demonstrate the participation of
the PLCβ/PKC/CaMKII pathway in G16-mediated activation of IKK/NF-κB.

**Gβγ Plays a Role in A1R-mediated IKKα/β Phosphorylation and NF-κB Activation**—Because a number of studies have implicated the involvement of Gβγ in GPCR-mediated regulation of NF-κB (8, 13), we asked if Gβγ also plays a role in G16-mediated activation of the IKK/NF-κB pathway. To test this hypothesis, transducin (Gαt) was co-expressed in conjunction with A1R and Gαt and potent interference from Gε-associated Gβγ was eliminated by PTX treatment. Acting as a Gβγ scavenger, co-expression of Gαt significantly reduced CHA-induced luciferase activity (Fig. 5A). As shown in Fig. 5B, the co-expression of Gαt attenuated the CHA-induced IKKα/β phosphorylation. To confirm the participation of Gβγ subunits in G16-mediated NF-κB activation, we overexpressed Gβ2 and Gγ12, along with A1R and Gαt. Co-expression of Gβ2 γ12 stimulated the CHA-induced NF-κB-dependent luciferase activity by over 80% (Fig. 5A). The stimulatory signal was primarily carried by Gβ2 because its overexpression, but not that of Gε2, enhanced (by ~45%) the CHA-induced NF-κB activation. In agreement with the luciferase reporter data, the enhancement of NF-κB activation was accompanied by increased phosphorylation of IKKα/β (Fig. 5C). These results suggest that Gβγ subunits, such as Gβ2 γ12, released from activated Gαt, are indeed involved in CHA-induced activation of IKKα/β and NF-κB.

**Ras but Not Rac1 Mediates the Activation of IKKα/β Phosphorylation and NF-κB**—A number of studies have illustrated that small GTPases are involved in the NF-κB activation (11, 30). To define the role of the small GTPases in the activation of NF-κB mediated by A1R, we transiently transfected the wild-type (WT) and constitutively active (CA) mutants of Ras and Rac1 into HEK 293-NF-κB and evaluated the NF-κB-dependent transcription of luciferase (Fig. 6A). Overexpression of wild-type Ras and Rac1 proteins did not alter the basal NF-κB-induced luciferase production. However, the constitutively active mutant of Ras (RasCA) was capable of stimulating the NF-κB-dependent transcription by ~3-fold whereas the constitutively active mutant of Rac1 (RacCA) was ineffective (Fig. 6A). To further investigate the involvement of these small GTPases in the A1R-stimulated IKK phosphorylation, we then introduced the dominant negative mutants of Rac1 (RacDN) and Ras (RasDN) into the cells (Fig. 6B). Indeed, overexpression of RasDN abrogated the CHA-stimulated IKKα/β phosphorylation whereas RacDN failed to intervene. These data suggest that the activation of IKK/NF-κB cascade requires only Ras, but not Rac1.

**ERK Is Important in the G16-mediated Regulation of IKK/NF-κB by A1R**—Activation of NF-κB activity by Gαi-coupled receptor through ERK has been described previously (13). Because the constitutively active GαiQL can activate all three MAPKs in HEK 293 cells (29), we asked if the coupling of A1R to GαiQL can similarly stimulate the MAPKs. HEK 293 cells were transfected with A1R and GαiQL, treated with PTX, and then stimulated by CHA (10 μM) for 10 min. Immunodetection with phosphospecific antisera revealed that all three MAPKs were activated upon CHA treatment (data not shown).
Fig. 5. NFκB activation by CHA treatment is inhibited by expression of transducin (Goα) and stimulated by the overexpression of GB1γt- A, HEK 293-NFκB cells were transfected with Goα (10 ng/well) or GB1γt and/or GB2γt (5 ng/well each) in conjunction with A1R (10 ng/well) and Goαγtα (30 ng/well). Transfectants were pretreated with PTX and followed by 10 μM CHA for 16 h. B and C, HEK 293 cells were transfected with Goα (200 ng/well) or GB2γt and GB2γt (100 ng/well) together with A1R (200 ng/well) and Goαγtα (400 ng/well). Subsequent to PTX treatment, the transfectants were treated with 10 μM CHA for 10 min. Cell lysates were analyzed as described in the legend to Fig. 2 for luciferase activity assay and immunodetection. Data shown are the mean ± S.E. from three individual experiments. Representative results from at least three sets of experiments are shown. *, CHA-induced response was significantly enhanced over the vector control; paired Student’s t test, p ≤ 0.05.

Fig. 6. Ras participates in the CHA-induced activation of IKK/ NFκB. A, HEK 293-NFκB cells were transfected with wild-type or constitutive active (CA) mutants of Rac1 or Ras small GTPases (30 ng/well) for 30 h. Cells were serum-starved for 16 h before luciferase detection. B, HEK 293 cells were transfected with Rac, dominant-negative mutant of Rac (RacDN), Ras or RasDN (200 ng/well) along with A1R (200 ng/well) and Goαγt (400 ng/well). The transfectants were pretreated with PTX followed by 10 min of CHA induction. Cell lysates were analyzed as described in Fig. 2 for luciferase activity assay and immunodetection. Data shown are the mean ± S.E. from three individual experiments. Representative results from at least three sets of experiments are shown. *, CHA-induced response was significantly enhanced over the vector control; paired Student’s t test, p ≤ 0.05.

Next, to investigate the involvement of MAPKs in the activation of IKK/NFκB signaling cascade, a panel of MAPK inhibitors was applied to HEK 293-NFκB cells co-expressing the A1R and Goαγt. Raf-1 kinase inhibitor (10 μM) and the selective MEK1/2 inhibitors (PD98059 and U0126; each at 10 μM) inhibited the CHA-induced NFκB-dependent luciferase activity by 30–50% (Fig. 7A). In contrast, the inactive analogue of U0126 (U0124; 10 μM) failed to attenuate the luciferase activity. The CHA-induced IKKα/β phosphorylation were similarly affected by these inhibitors (Fig. 7B). U0126, Raf-1 kinase inhibitor, and PD98059, but not U0124, were able to inhibit CHA-induced ERK phosphorylation (Fig. 7B). The effect of JNK on IKK/ NFκB signaling was examined using SP600125, a selective JNK inhibitor. The application of SP600125 (10 μM) neither affected the NFκB-induced luciferase expression (Fig. 7A) nor the phosphorylation of IKKα/β (Fig. 7C). Finally, for p38 MAPK, two p38 MAPK inhibitors, SB202190 and SB203580 at 10 μM were also unable to inhibit the CHA-induced luciferase transcription (Fig. 7A) and IKKα/β phosphorylation (Fig. 7D). These findings clearly demonstrate the participation of ERK, but not JNK or p38 MAPK, in CHA-mediated activation of IKK/NFκB by A1R.

c-Src Participates in the Goαγt-mediated activation of IKK/NFκB Activation—In the activation of NFκB by the Gt-coupled dopamine D2 receptor, the participation of the c-Src kinase is clearly evident (13). Thus, we sought to investigate whether c-Src is also involved in Goαγt-mediated activation of IKK/NFκB. We first examined if c-Src can be activated by A1R. As shown in Fig. 8A, 10 μM CHA stimulated the phosphorylation of c-Src at Tyr416 in HEK 293 cells co-expressing A1R and Goαγt. A selective c-Src kinase inhibitor (PP2) was then used to confirm its involvement in CHA-induced IKK/NFκB activation. PP2 at 10 μM for 30 min significantly attenuated the CHA-induced NFκB-dependent luciferase activity, whereas the inactive PP3 had no inhibitory effect under identical conditions (Fig. 8B). Similar results were obtained on the ability of PP2 to inhibit CHA-induced phosphorylation of IKKα/β (Fig. 8C). Additionally, the effect of overexpressing c-Src on CHA-induced activation of IKK/NFκB was examined. Co-expression of wild-type c-Src with A1R and Goαγt did not affect the ability of CHA to stimulate NFκB-dependent luciferase expression (Fig. 8D). When the dominant negative mutant of c-Src was co-expressed, the CHA-induced luciferase activity was attenuated by over 40% as compared with the level generated by the wild-type c-Src (Fig. 8D). In agreement with the reporter gene assays, overexpres-
FIG. 8. c-Src is involved in the Gαq-mediated activation of NFκB by CHA. HEK 293 (A and C) and H9293-NFκB (B) cells were transiently transfected with AIR and Gtq16. D, H9293-NFκB cells were transfected with c-Src, c-SrcDN, or pcDNA1 (5 ng/well) in conjunction with AIR (15 ng/well) and Gtq16 (30 ng/well). E, H9293 cells were transiently transfected with c-Src, c-SrcDN, or pcDNA1 (200 ng/well) along with AIR (200 ng/well) and Gtq16 (400 ng/well). After to PTX treatment, the transfectants were stimulated with 10 μM CHA for 10 min. c-Src phosphorylation was detected by anti-phospho-c-Src(Y416). Equivalent protein loading was monitored by anti-c-Src antibody in A, B, and C. Transfectants were pretreated with 25 μM PP2 (a Src inhibitor) or its negative control PP3 for 30 min before CHA treatment. Cell lysates were analyzed as described in the legend to Fig. 2 for luciferase activity assay and immunoblotting detection. Data represent the mean ± S.E. from three independent experiments. The immunoblots shown were representatives of three sets of experiments. #, CHA-induced response was significantly inhibited as compared with the controls; paired Student’s t test, p < 0.05.

Numerous signaling components have been implicated in G protein-dependent activation of NFκB but there is no clear indication as to which pathway is predominant. The signaling specificity becomes even more complicated for those GPCRs that employ multiple G proteins for signal transduction. The present study provides evidence to support a role of Gq in AIR-mediated activation of NFκB in human lymphocytic Reh and H9293 cells.

Although AIR is functionally coupled to Gq, proteins in H9293 cells (20), it cannot utilize endogenous Gq pathways to activate NFκB in both cell types (Figs. 1 and 2). In contrast, co-expression of Gα16 allows AIR to efficiently stimulate the IKK/NFκB pathway (Fig. 2). The collective use of the AIR selective antagonist DPCPX, IκBα and its non-degradable mutant, IκKαβ and their dominant-negative mutants, as well as the NFκB activation inhibitor APQ confirmed the specificity of the pathway. The PTX insensitivity of CHA-induced NFκB activation in Reh cells signifies the involvement of Gq or G12 family members. As Gα16 is present in the Reh cells (26) and is known to interact with AIR (21, 22), while neither Gαq nor Gα12 has been reported to associate with AIR, the activation signal for NFκB is presumably transmitted via Gαq. Mechanistically, both cell types appear to employ a complicated network of intermediates for signal propagation. They include an effector (PLCβ), a small GTPase (Ras), a non-receptor tyrosine kinase (c-Src), serine kinases (Raf-1, PKC, and CaMKII), and also a MAPK (ERK). These signaling molecules can be divided into two major cascades: PLCβ/PKC/CaMKII and Ras/Raf-1/MEK/ERK (Fig. 10).

Regulation of NFκB by Gq proteins has previously been suggested to depend on both Gα and Gβγ subunits. Gαq stimulates NFκB via the PLCβ/PKC/CaMKII pathway (10) whereas Gβγ propagates the signal through phosphatidylinositol-3 kinase

FIG. 9. Characterization of CHA-induced phosphorylation of IKKαβ in Reh cells. A–C, PTX-treated Reh cells were treated for 30 min with 1% MeSO (vehicle), U73122, U73343, KN62, KN92, Raf-1 kinase inhibitor, U0126, U0124, SB202190, SB203580 (10 μM each), calphostin C (Cal C; 100 nM), SP600125 (30 μM), PP2 (25 μM), or PP3 (25 μM) as indicated. Cells were then stimulated by 10 μM CHA for 15 min, and cell lysates were analyzed as described in the legend to Fig. 2 for immunoblotting detection. Immunoblots shown are representatives of three independent sets of experiments.

DISCUSSION

Adenosine has recently been shown to possess anti-inflammatory actions in rodents (34) and it inhibits cytokine production in mature plasmacytoid dendritic cells (19). Given that NFκB is an important transcription factor in regulating inflammatory and immune responses, adenosine receptors may employ G protein-dependent pathways to modulate the NFκB activity. Indeed, a number of GPCRs have been shown to activate NFκB via Gq or G12-dependent pathways (8–13, 23, 24).
Activation of NFκB by Adenosine A1 Receptor

FIG. 10. A mechanistic model of Gα16-mediated activation of IKK/NFκB by A1R. CHA-bound A1R activates Gα16 and releases Gβγ. The activated Gα16 stimulates the PLCβ cascade. Through both Gα and Gβγ, Ras/Raf-1/MEK1/2/ERK cascade becomes activated and c-Src is activated indirectly. These activations lead to the phosphorylation of IKKα/β and signal IκBα degradation. The released NFκB is translocated to the nucleus and promotes transcription of the luciferase reporter. Solid-lined arrows illustrate findings based on previous studies and the putative interactions are indicated with dash-lined arrows. The experimental evidence supporting individual pathways and the interactions between their intermediates are described in the text.

cascade (8). Both arms of the signal appear to operate in the G16-mediated activation of NFκB. Inhibition of CHA-induced IKK/NFκB activities by U73122, calphostin C, and KN62 provides evidence that PLCβ and its downstream effectors mediate the NFκB activation. Both Ca2+-dependent and -independent PKCs have been shown to regulate NFκB. The Ca2+-sensitive PKCα is capable of activating the IKK complex in T-lymphocytes (32) while the Ca2+-independent PKCe is implicated in the modulation of IKK activity based on the use of PKCe-deficient mice (33). Indeed, our data support a role of PKCα and PKCe in G16-mediated activation of IKK/NFκB by CHA (Fig. 4). However, other PKC isoforms may also be involved since the G16-coupled A1R is known to regulate c-fos through PKCβ (34). The deployment of PLCβ and PKCα in G16-mediated activation of NFκB by A1R resembles that of the formyl peptide receptor (24). Elevation of intracellular Ca2+ level can also alter the activity of NFκB through the actions of CaMKs. In particular, CaMKII is known to mediate phorbol ester-induced activation of IKK (35). The fact that KN62, but not KN92, suppresses the CHA-induced IKK phosphorylation and NFκB-dependent luciferase activity (Fig. 4) implicates the involvement of CaMKII. Thus, the classical PLCβ/PKC/CaMKII cascade appears to play an important role in the regulation of IKK/NFκB by the G16-coupled A1R.

Despite the fact that Gα16 can propagate stimulatory signals to NFκB, the released Gβγ also takes part in the regulation. Attenuation by the co-expression of Gαi (a Gβγ scavenger) and potentiation by the overexpression of Gβ1γ2 confirm the participation of Gβγ in G16-mediated activation of NFκB by A1R. Signals arising from Gα and Gβγ subunits are often integrated at downstream loci (36, 37). One locus for signal integration is the small GTPase Rac (38). Recently, the linkage of Gα16 to Ras is provided by a novel adaptor protein named tetrastricopeptide repeat 1 (39) while Gβγ has long been shown to activate Ras in HEK 293 cells (38). Ras is known to initiate the Raf-1/MEK/ERK signaling cascade and ERK has previously been demonstrated to activate the IKK complex through direct interaction (40). Thus, it is not surprisingly that activation of IKK/NFκB by the G16-coupled A1R is attenuated in the presence of Raf-1 and MEK1/2 inhibitors (Fig. 7). Moreover, the G16-mediated IKK phosphorylation by A1R is effectively attenuated in the presence of RasDN, whereas RasCA induces NFκB-driven luciferase expression (Fig. 6). The ability of ERK to exert a stimulatory effect on IKK/NFκB is not shared by the other two MAPKs. Although both JNK (41) and p38 MAPK (42) can activate NFκB, neither is required for G16-mediated stimulation of IKK/NFκB by A1R (Fig. 7). The lack of involvement of JNK and p38 MAPK is further supported by the inability of Rac1DN to suppress the G16-mediated activation of IKK/NFκB (Fig. 6). Stimulation of A1R by CHA can, nevertheless, lead to the activation and phosphorylation of JNK and p38 MAPK, and such activities can be effectively abolished by specific inhibitors of the two kinases. The requirement of Ras/Raf-1/MEK/ERK pathway, but not JNK or p38 MAPK, for G16-mediated activation of IKK/NFκB is highly reminiscent of the regulation of STAT3 by Gα16 (29).

Another site for possible signal integration is c-Src, a non-receptor tyrosine kinase, which becomes phosphorylated upon activation of A1R (Fig. 8A). The combined use of selective inhibitors and dominant negative mutants of c-Src (Fig. 8, B–E) clearly demonstrates the involvement of c-Src in G16-mediated stimulation of IKK/NFκB by A1R. Although constitutively active Gα16 can activate c-Src in HEK 293 cells, the interaction is probably indirect because Gα16 does not directly associate with c-Src (29). It is interesting to note that in human epithelial cells, TNFα-induced cyclooxygenase-2 expression is mediated via c-Src/NFκB in a PKC-dependent manner (43). Similarly, suppression of the c-fos gene promoter by c-SrcDN indicates that c-Src is required for CaMKII-induced activation in cultured rat mesangial cells (44). As both PKCα and CaMKII participate in G16-mediated activation of NFκB (Fig. 4), it is conceivable that they can bridge the gap between Gα16 and c-Src. Additionally, c-Src can be activated by Gβγ (45) even though no direct binding between them can be established (46). Indeed, activation of NFκB by the dopamine D2 receptor in HeLa cells is mediated via c-Src in a Gβγ-dependent manner (13). Direct interaction between c-Src and the IKK complex (47) leads to the phosphorylation of IKK (43) and, subsequently, IκBα (48). These findings provide a pathway connecting the agonist-stimulated GPCR and G proteins to IKK/NFκB.

In considering the signal routing from Gα16 to NFκB, it is important to note that signal diversification as well as convergence may occur at multiple loci (Fig. 10). For instance, c-Src is known to play regulatory roles in Ras (45) and ERK (49) sig-
naling, and each of these regulatory intermediates has been shown to modulate the NFκB activation through the upstream IKK complex. The present study has revealed an intricate signaling network for G16-coupled receptors to regulate IKK/NFκB pathway. Some of the signaling intermediates such as PLCβ and PKCα have previously been shown to mediate the activation of NFκB by constitutively active Gα16 (24). Other molecular players like Gβγ and c-Src are known to be required for G1-mediated stimulation of NFκB (13). Yet we are far from fully appreciating all the intricacies of the complex signaling network. Because dysregulation of NFκB activity has been implicated in the pathogenesis of a variety of human diseases, G16-coupled receptors may represent attractive targets for therapeutic intervention. This is especially applicable to inflammatory and immune diseases as Gα16 is primarily expressed in hematopoietic cells.

Acknowledgments—We thank the following individuals for kindly providing the various cDNAs: Drs. Alain Israel, Richard Ye, Zhenguo Wu, and Shengcai Lin. We thank Dr. David New for helpful discussions and valuable comments.

REFERENCES

1. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 6, 225–260
2. Maniatis, T. (1997) Science 278, 818–819
3. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
4. Gutkind, J. S. (1998) Oncogene 17, 1331–1342
5. Crespo, P., and Gutkind, J. S. (2004) Methods Mol. Biol. 250, 203–210
6. Berestetskaya, Y. V., Faure, M. P., Ichijo, H., and Vojno-Yasenetskaia, T. A. (1998) J. Biol. Chem. 273, 27416–27423
7. Druey, K. M. (2000) Expert Opin. Ther. Targets. 4, 475–484
8. Xie, P., Browning, D. D., Hay, N., Mackman, N., and Ye, R. D. (2000) J. Biol. Chem. 275, 24907–24914
9. Browning, D. D., Fan, X., and Manning, D. R. (1999) Mol. Pharmacol. 55, 857–864
10. Shahrestanifar, M., Fan, X., and Manning, D. R. (1999) J. Biol. Chem. 274, 3828–3833
11. Mansell, A., Khelef, N., Cossart, P., and O’Neill, L. A. (2001) J. Biol. Chem. 276, 36008–36013
12. Chen, J. S., Lee, J. W., Ho, M. K., and Wong, Y. H. (2000) Mol. Pharmacol. 57, 700–708
13. Lowes, V. L., Ip, N. Y., and Wong, Y. H. (2002) Neurosignals 11, 5–19
14. Ito, A., Satoh, T., Kazuo, Y., and Itoh, H. (1995) FEBS Lett. 368, 163–187
15. Marty, C., Browning, D. D., and Ye, R. D. (2003) Mol. Cell. Biol. 23, 3847–3858
16. Zhao, Q., and Lee, F. S. (1999) J. Biol. Chem. 274, 8355–8358
17. Tuyt, L. M., Dokter, W. H., Birkenkamp, K., Koopmans, S. B., Lummen, C., Kruijer, W., and Vellenga, E. (1999) J. Immunol. 162, 4983–4992
18. Vanden Berghe, W., Plaisance, S., Boone, E., De Bosscher, K., Schmitz, M. L., Fiers, W., and Haegeaman, G. (1998) J. Biol. Chem. 273, 3285–3290
19. Huang, W. C., Chen, J. J., and Chen, C. C. (2003) J. Biol. Chem. 278, 9944–9952
20. Wang, Y., Mishra, R., and Simonson, M. S. (2003) J. Am. Soc. Nephrol. 14, 995–1006
21. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 19443–19450
22. Huang, W. C., Chen, J. J., Inoue, H., and Chen, C. C. (2003) J. Immunol. 170, 4767–4775
23. Fan, C., Li, Q., Ross, D., and Engelhardt, J. F. (2003) J. Biol. Chem. 278, 2072–2080
24. Kraus, S., Renard, O., Naor, Z., and Seger, R. (2003) J. Biol. Chem. 278, 32618–32630