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Rocaglamide Derivatives Are Immunosuppressive Phytochemicals That Target NF-AT Activity in T Cells

Peter Proksch,* Marco Giaisi,† Monika K. Treiber,† Katalin Palfi,† Anette Merling,† Herbert Spring,‡ Peter H. Krammer,† and Min Li-Weber††

Aglaia (family Meliaceae) plants are used in traditional medicine (e.g., in Vietnam) for the treatment of inflammatory skin diseases and allergic inflammatory disorders such as asthma. Inflammatory diseases arise from inappropriate activation of the immune system, leading to abnormal expression of genes encoding inflammatory cytokines and tissue-destructive enzymes. The active compounds isolated from these plants are derivatives of rocaglamide. In this study we show that rocaglamides are potent immunosuppressive phytochemicals that suppress IFN-γ, TNF-α, IL-2, and IL-4 production in peripheral blood T cells at nanomolar concentrations. We demonstrate that rocaglamides inhibit cytokine gene expression at the transcriptional level. At the doses that inhibit cytokine production, they selectively block NF-AT activity without impairing NF-κB and AP-1. We also show that inhibition of NF-AT activation by rocaglamide is mediated by strong activation of JNK and p38 kinases. This article must therefore be hereby marked with 18 U.S.C. Section 1734 solely to indicate this fact.

In recent years, natural bioactive products have attracted considerable attention as a new source of medicinal and agrochemical compounds. In several countries of southeast Asia, crude extracts from leaves and flowers of different Aglaia (family Meliaceae) plants are used in traditional medicine (e.g., in Vietnam) for the treatment of inflammatory skin diseases and allergic inflammatory disorders such as asthma. The active compounds isolated from these plants are derivatives of rocaglamide. Rocaglamide and its naturally occurring congeners are tetrahydrobenzofurans that occur exclusively in members of the genus Aglaia (1). In the past, these natural products have attracted attention due to their strong insecticidal activity (2). More recently, certain rocaglamide derivatives have also been found to have an inhibitory effect on the activity of the proinflammatory transcription factor NF-κB (3). Inflammatory diseases arise from inappropriate activation of the immune system, leading to abnormal expression of genes encoding inflammatory cytokines and tissue-destructive enzymes (4). Many inflammatory genes are regulated at the transcriptional level by proinflammatory transcription factors, such as NF-κB and AP-1 (5, 6).

During the immune response, Th cells produce various cytokines required for an efficient suppression of infections. Th1 cytokines IFN-γ and TNF-α promote cell-mediated immunity, and Th2 cytokines IL-4, IL-5, IL-6, IL-10, and IL-13 promote humoral (Ab) immunity (7). However, uncontrolled expression of these cytokines is dangerous and causes inflammatory diseases. Dysregulation of IFN-γ and TNF-α production may contribute to the pathogenesis of many chronic inflammatory diseases, including rheumatoid arthritis, diabetes, and hepatitis (4, 8). Overexpression of IL-4 leads to atopic disorders, including allergen-induced asthma, rhinoconjunctivitis, and anaphylaxis (9, 10). The expressions of IL-4, IFN-γ, and TNF-α are regulated by a number of inducible transcription factors, including NF-κB, AP-1 (Fos/Jun), and NF-AT (11–16). In resting T cells, NF-κB is sequestered into an inactive state by the cytoplasmic inhibitor of NF-κB (IκB). T cell activation through TCR leads to the rapid activation of the IκB kinases (IKKs) via protein kinase C and results in phosphorylation, ubiquitylation, and subsequent degradation of IκB proteins, which allows nuclear translocation of NF-κB (6). In contrast, NF-AT family proteins are calcium- and calcineurin-regulated transcription factors. In resting T cells, NF-AT proteins are phosphorylated and reside in the cytoplasm. T cell activation leads to the activation of the Ca2+-dependent phosphatase calcineurin, resulting in rapid dephosphorylation of NF-AT and its translocation to the nucleus (17). T cell activation also induces the MAPKs, including ERKs, JNKs, and p38 that promote the synthesis, phosphorylation, and activation of AP-1 transcription factors (18). MAPKs have also been implicated in phosphorylation and thereby prevention of the nuclear localization of NF-AT (19–21). In addition, the NF-AT proteins are frequently found to act synergistically with AP-1 on composite promoter/enhancer elements that contain adjacent NF-AT and AP-1 binding sites (14).

In this study we investigated the effects of rocaglamides on the expression of several cytokine genes in peripheral blood T cells. We show that rocaglamides are potent immunosuppressive phytochemicals that suppress IFN-γ, TNF-α, IL-2, and IL-4 production in peripheral blood T cells at nanomolar concentrations. We also show that rocaglamides, at the doses that inhibit cytokine production, selectively inhibit the activity of NF-AT without impairing NF-κB and AP-1 activities. Our study suggests that rocaglamide derivatives may serve as a new source of NF-AT-specific inhibitors for the treatment of certain inflammatory diseases.


Received for publication September 24, 2004. Accepted for publication March 22, 2005.

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2 Abbreviations used in this paper: IKK, IκB kinase; CsA, cyclosporin A; LSM, laser scan microscopy.
Materials and Methods

Cells and cell culture

Jurkat T leukemia cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS, 50 μg/ml gentamicin (Invitrogen Life Technologies), 6 mM HEPES (Invitrogen Life Technologies; 1 M solution), and 2 mM L-glutamine (Invitrogen Life Technologies; 200 mM solution) at 37°C in 5% CO₂.

Rocaglamide derivatives

The rocaglamide derivatives used in this study (Fig. 1) had been isolated previously from various Aglaia species, as reported in the literature (2, 22). The structures of the compounds were unequivocally elucidated based on their nuclear magnetic resonance and mass spectra as described previously.

Purification of T lymphocytes and apoptosis measurement

Human PBMC were prepared by Ficoll-Paque (Pharmacia Biotech) density centrifugation. Adherent cells were removed by adherence to plastic culture vessels for 1 h. T cells were isolated from the PBMC by resetting with 2-amino-ethylisothyo-uronium-bromide-treated SRBC (23). Apoptotic cell death was assessed by propidium iodide uptake and was analyzed by FACS (23).

Determination of IL-4, IL-2, TNF-α, and IFN-γ proteins

Freshly isolated peripheral blood T cells (1 × 10⁶/ml) were stimulated with plate-bound anti-CD3 (OKT3; 10 μg/ml) and anti-CD28 (5 μg/ml) Abs for 24 h. Supernatants were tested for the presence of IL-4, IL-2, TNF-α, and IFN-γ using an ELISA specific for human IL-4, IL-2, TNF-α, and IFN-γ proteins (BD Pharmingen) according to the manufacturer’s instruction.

Flow cytometric analysis of CD69 surface expression

Cells were washed twice with PBS and stained with fluorescent-tagged Ab to CD69 (mAb, FITC; BD Biosciences) on ice in the dark. After 30 min of staining, cells were washed twice with PBS and analyzed by FACScan (BD Biosciences).

Preparation of nuclear proteins, total cell lysates, and immunoblotting

T cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM PMSF, 25 mM sodium fluoride, 1% SDS, 1 mM DTT, 0.2 mM Na₂VO₄, and 20 μl/ml protease inhibitors) for 30 min. Nuclear proteins were isolated as described previously (24). The widely used p38 kinase and JNK inhibitors, SB 203580 and SP 600125, were purchased from Alexis Biochemicals. Equal amounts of proteins were separated on 10% SDS-PAGE gels and transferred onto a Hybond-ECL nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 5% milk powder in PBS/0.1% Tween 20 for 1 h, washed with the same solution, and incubated with Abs at 37°C for 1 h or overnight at 4°C. The blots were washed with PBS/ Tween 20 and developed with HRP-coupled Abs, followed by enhanced Chemiluminescence Reagent Plus (PerkinElmer). The following Abs were used: Abs against NF-κB p65 (A; sc-109) and IκBα (C21; sc-371; Santa Cruz Biotechnology), anti-c-Jun mAb (BD Pharmingen), anti-phospho-IκBα (Ser32) and anti-phospho-ERK Abs (Cell Signaling); anti-active p38 Ab (Promega), and anti-NF-ATc1 mAb (7A6; Alexis Biochemicals). For stripping, membranes were incubated for 30 min at 56°C in a buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM 2-ME.

EMSA

EMSA were performed essentially as described previously (25). The synthetic oligonucleotides used for EMSA were the IL-4 NF-AT binding site (TAACGAAAATTTCCAATGTA) and the Eo NF-Y binding site (CACCTTTTAACCAATC).

RNA isolation and RT-TCR

Total cellular RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed with oligo(dT). PCR amplification was performed for 30–35 cycles with specific primers (Stratagene) for human IL-2 (457-bp PCR product), human IL-4 (456-bp PCR product), human IFN-γ (501-bp PCR product), and human IFN-γ (501-bp PCR product), and human IFN-γ (501-bp PCR product), and human IFN-γ (501-bp PCR product), and human IFN-γ (501-bp PCR product), and
human β-actin (661-bp PCR product) as previously described (24). The PCR products were then subjected to agarose gel electrophoresis.

**Quantitative real-time PCR**

The principle of TaqMan quantitative real-time PCR has previously been described in detail (26). The sequences for primers of IL-2, IL-4, IFN-γ, β-actin, and fluorescent-labeled probes used in these studies were described previously (25). The primers and probe of IFN-γ are: forward, 5'-GGAGAAGGGTGACCGACTCA-3'; reverse, 5'-TGCCCAGACTCGGCAAG-3'; and probe, 5'-CGCTGAGATCAATCGGCCCGACTA-3'. PCR was performed in a 12.5-μl reaction mixture (PCR kit; Eurogentech) that contained 0.08 μg of reverse-transcribed cDNA and the proper amounts of primers and probe. For each sample, three PCRs were performed. The resulting relative increase in reporter fluorescent dye emission was monitored by the TaqMan system (GeneAmp 5700 sequence detection system and software; PerkinElmer). The level of cytokine mRNA, relative to β-actin, was calculated using the formula: relative mRNA expression = $2^{-\Delta\Delta Ct}$, where Ct is the threshold cycle value.

**Confocal laser scan microscopy (LSM)**

Jurkat T cells stimulated with PMA/ionomycin for 30 min in the presence or the absence of rocaglamides were fixed with Cytofix/Cytoperm (BD Pharmingen) and washed with Perm/Washing solution (BD Pharmingen) three times at 4°C. The cells were incubated with anti-NF-ATc1 for 30 min, washed three times with Perm/Washing solution as before, and then stained with anti-IgG Cy3 Abs for 30 min. The cells were again washed.
with Perm/Washing solution three times and subjected to LSM. Slides were coated with poly-L-lysine to increase adhesion and prevent cell motility during microscopy. Confocal LSM was performed using a Zeiss LSM 510 UV microscope operating with an argon ion laser (488 nm) and a helium-neon laser (543 nm; Zeiss).

**Plasmid constructs and transient transfections**

Luciferase reporter construct containing human IL-2 (\(\text{H11002}^{300}\)) promoter, and multiple IL-4 NF-AT (P1) luciferase constructs were generated previously (24, 27). Multiple copies of the AP-1 binding site from SV40 enhancer (CGGTTGCTGACTAATTG), the NF-\(\beta\)-B consensus sequence (GGAAATTCCCC), and the human IL-2 NF-AT (\(\text{H11002}^{282}\)) element (GAAAGGAGGAAAACTGTTTCATA CAGAAGGC) were constructed by ligation of the DNA sequence into the multiple cloning site of the pTATA-Luc vector. The luciferase reporter construct containing the human IFN-\(\beta\) (\(\text{H11002}^{854}\)) promoter was constructed by ligation of the IFN-\(\beta\) promoter sequence generated by PCR into the luciferase reporter vector. All constructs were confirmed by sequencing analysis. The pLuc-Bax promoter reporter plasmid was provided by M. L. Schmitz (University of Bern, Bern, Switzerland).

Jurkat T cells were transfected by electroporation as previously described (24). After overnight recovery, the cells were divided and treated with different doses of rocaglamides, for 1 h and then stimulated with PMA/ionomycin for 2 h. Total RNA was prepared and analyzed for cytokine mRNA expression levels by RT-PCR. β-Actin mRNA expression levels were analyzed as controls. Data are representative of two separate experiments.

**Determination of intracellular calcium**

Cells were pretreated with different doses of rocaglamide or 10 μM BAPTA/AM (Molecular Probes) for 10 min, then loaded with 1 μM fluo-4 (Molecular Probes) at 37°C in the dark for 30 min, washed three times with PBS, and resuspended in PBS. The loaded cells were measured by flow cytometry in a FACScan (BD Biosciences) after adding 1 μM ionomycin (Calbiochem). Calcium influx was assayed for 300–512 s in fluorescence-1.

**Calcineurin phosphatase assay**

Calcineurin phosphatase activity was measured using a calcineurin assay kit (Calbiochem). Each reaction (total, 50 μl) contained 50 mM Tris (pH 7.5), 100 mM NaCl, 6 mM MgCl2, 0.5 mM CaCl2, 0.5 mM DTT, 0.025% Nonidet P-40, 0.25 μM calmodulin, and 40 U calcineurin. The reaction was initiated at 30°C by adding the phosphopeptide substrate to a final concentration of 0.15 mM. In the case of calcineurin inhibition, 100–200 nM rocaglamides or 400 ng/ml cyclosporin A (CSA) plus 200 ng/ml cyclophilin A (BIOMOL) was included in the reaction mixture.

**Results**

**Rocaglamides inhibit cytokine production in peripheral blood T cells**

Three rocaglamide derivatives (Roc-1, -2, and -3) were tested for their ability to inhibit the expression of cytokine genes in T cells. These compounds differed mainly in the substitution patterns on the aromatic rings (Fig. 1) and were prepared to at least 98% purity, as determined by HPLC (22). After overnight recovery, the cells were divided and treated with different doses of rocaglamides, then additionally cultured in the absence or the presence of PMA (10 ng/ml) and ionomycin (Calbiochem). Calcium influx was assayed for 300–512 s in fluorescence-1.
IL-2, and IL-4 were determined 24 h after stimulation by ELISA. Stimulation of T cells via TCR resulted in high levels of cytokine production. However, in the presence of rocaglamides, the production of IFN-γ, TNF-α, IL-2, and IL-4 was suppressed in a dose-dependent manner. In contrast, the compound Ad1, a putative biogenetic precursor of rocaglamides (representing one-half of the full rocaglamide skeleton), which was isolated from Aglaia species by the same method, had no effect on the production of the cytokines tested (Fig. 2A). At a concentration of 50 nM, rocaglamides completely blocked T cell activation-induced expression of IL-4 and IFN-γ and led to a 60–85% reduction in IL-2 and TNF-α production. In comparison, the expression levels of the T cell surface protein CD69 were only slightly reduced in the presence of rocaglamides (Fig. 2B). At this dose, rocaglamide was not toxic to T cells. These data indicate that rocaglamides may suppress T cell activation and down-regulate cytokine gene expression.

Rocaglamides suppress cytokine mRNA expression in T cells

We next analyzed the effect of rocaglamides on cytokine expression in peripheral blood T cells at the mRNA level by quantitative real-time PCR. Consistent with the results obtained by ELISA, mRNA analysis showed that at a concentration of 50 nM, rocaglamides significantly suppressed IFN-γ, TNF-α, IL-2, and IL-4 mRNA expression in peripheral blood T cells (Fig. 3A). We also investigated the molecular mechanisms by which rocaglamides suppress the production of cytokines using the human leukemic T cell line Jurkat as a model system. Jurkat T cells express mRNAs of IFN-γ, TNF-α, IL-2, and IL-4 and are often used in studies of various cytokine genes. Cytokine mRNA expression levels in Jurkat T cells were analyzed by RT-PCR and real-time PCR. Similar to the results obtained with the peripheral blood T cells, rocaglamides suppressed mRNA expression of all four cytokine genes tested in Jurkat T cells in a dose-dependent fashion, analyzed by RT-PCR and real-time PCR (Fig. 3, B and C).

To further investigate whether the suppression of mRNA expression by rocaglamides occurred at the transcriptional level, luciferase reporter plasmids containing promoters of the human IFN-γ, IL-2, and IL-4 genes were subjected to transient transfection studies. Transfected Jurkat T cells were stimulated by PMA and ionomycin in the presence or the absence of various doses of rocaglamides. In agreement with the expression levels of the endogenous mRNAs, the promoter activities of the three cytokine genes tested were down-regulated by rocaglamides, but not by Ad1, in a dose-dependent manner (Fig. 4). In the control experiment, the activity of a promoter reporter construct containing a constitutive noncytokine gene, bax, was not influenced by rocaglamides. These experiments demonstrate that rocaglamide derivatives may directly inhibit cytokine gene expression at the transcriptional level.

Rocaglamides inhibit nuclear expression of NF-AT and c-Jun

Many inflammatory cytokine genes are transcriptionally activated by the inducible, ubiquitous transcription factors NF-AT, AP-1, and NF-κB (11–16). Therefore, we first examined the effects of the rocaglamide derivatives on nuclear expression levels of NF-AT, the AP-1 subunit c-Jun, and the NF-κB subunit p65 in activated T cells. Nuclear proteins were prepared from Jurkat T cells that had been stimulated by PMA/ionomycin for 2 h in the presence or the absence of various doses of rocaglamides. Immunoblotting analysis of nuclear levels of these transcription factors showed a dose-dependent reduction of the T cell activation-induced nuclear expression of NF-AT (Fig. 5A). To examine the nuclear expression levels of
NF-AT, the nuclear extracts were subjected to EMSA. In agreement with the immunoblotting results, EMSA showed that rocaglamides-mediated reduction of nuclear fractions of NF-AT correlated with reduced DNA binding activity (Fig. 5B). Rocaglamides also down-regulated the nuclear expression of c-Jun (Fig. 5C). Cyclosporin A is a well-known immunosuppressive reagent that targets NF-AT activity. In comparison, CsA strongly inhibits NF-AT, but only moderately influences c-Jun nuclear expression (Fig. 5C). Previously, certain rocaglamide derivatives, e.g., Roc-1 (previously named Roc-4), were shown to inhibit NF-κB activity induced by TNF-α or PMA (3). At the doses used in our experiments, no reduction in the nuclear level of p65 was seen. Instead, the nuclear level of p65 was slightly elevated in Roc-1-treated cells (Fig. 5C). As controls, rocaglamides did not influence the protein levels of tubulin or the constitutively expressed transcription factor YY-1. These data indicate that rocaglamides may differentially impair NF-AT and c-Jun expression in activated T cells.

We also investigated the nuclear expression of NF-AT in vivo by confocal LSM. To date, three calcium-regulated NF-AT, NF-ATc1, NF-ATc2, and NF-ATc3, have been identified as expressed in immune cells. Among them, NF-ATc1 is not only translocated into the nucleus upon T cell stimulation, but is also inducibly expressed by T cells (28). As shown in Fig. 6, a dramatic increase in nuclear expression of NF-ATc1 was seen after stimulation of Jurkat T cells with PMA/ionomycin. In the presence of CsA or rocaglamides, the nuclear expression levels of NF-ATc1 were significantly reduced. As a control, treatment of Jurkat T cells with Ad1 did not prevent the PMA/ ionomycin-induced increase in NF-ATc1 expression (Fig. 6). Thus, rocaglamides can inhibit NF-AT expression in T cells.

**Rocaglamides inhibit NF-AT, but not c-Jun and NF-κB, activity**

Characteristically, NF-AT family proteins act synergistically with AP-1 (Fos/Jun) proteins to activate genes containing composite NF-AT and AP-1 binding sites (5). Many cytokine genes, including IL-2, IL-4, and IFN-γ, contain NF-AT/AP-1 composite sites in their promoter/enhancer regions (28). To investigate whether inhibition of NF-AT and c-Jun expression by rocaglamides would lead to suppression of their transcriptional activities, we performed transfection studies with luciferase reporter constructs containing multiple copies of the NF-AT-binding elements derived from the IL-4 and IL-2 promoters and the consensus binding sequences for AP-1 and NF-κB, respectively. As expected, in the presence of rocaglamides, but not Ad1, transcriptional activities mediated by the IL-4 or the IL-2 NF-AT elements were down-regulated in a dose-dependent manner (Fig. 7A). Consistent with the immunoblotting data, rocaglamides had no inhibitory effect on NF-κB activity at concentrations up to 100 nM. By contrast, a subtle increase in NF-κB activity was seen in cells treated with Roc-1 (Fig. 7A). Moderate inhibition (~20%) of NF-κB-dependent transcription was seen in cells treated with Roc-2 and Roc-3 at doses >150 nM. At doses >200 nM, all three rocaglamide derivatives inhibited NF-κB activity to a variable degree (data not shown). Unexpectedly, AP-1-mediated transcriptional activity was substantially enhanced at concentrations of rocaglamides that suppressed NF-AT activity (Fig. 7A). We noticed that rocaglamide treatment induced phosphorylation of c-Jun (Fig. 5B, indicated by arrows). Because c-Jun activity can be enhanced by phosphorylation (18), we assumed that rocaglamides may increase AP-1-dependent transcription via phosphorylation of c-Jun. Indeed, treatment of Jurkat T cells with rocaglamides alone led to a modest increase in AP-1-dependent gene expression (Fig. 7B). To investigate whether rocaglamides can cooperate with the known NF-AT inhibitor CsA to suppress NF-AT-mediated transcription, suboptimal doses of rocaglamides and CsA were used in a transfection study with the luciferase-NF-AT reporter construct. As shown in Fig. 7C, NF-AT activity was additionally down-regulated by a combination of rocaglamide and CsA. In contrast, Ad1 did not down-regulate any further the CsA-mediated suppression of NF-AT activity.

**Rocaglamides inhibit NF-AT via activation of the MAPKs JNK and p38**

The NF-AT family of proteins is activated by the calcium/calmodulin-dependent phosphatase calcineurin that phosphorylates and promotes nuclear translocation of NF-AT (14, 17). Therefore, we first investigated whether rocaglamides interfere with Ca2+ signaling during T cell activation. Jurkat T cells were loaded with the Ca2+ indicator fluo-4, then stimulated with ionomycin in the presence or the absence of rocaglamides. The experiment showed that rocaglamides do not interfere with the rate of intracellular Ca2+ mobilization. As a control, the Ca2+ chelator BAPTA/AM completely blocked ionomycin-induced intracellular Ca2+ influx (Fig. 8A). To further investigate whether rocaglamides directly inhibit calcineurin activity, we performed an in vitro phosphatase assay in the presence or the absence of rocaglamides. The experiment showed no reduction in calcineurin activity in the presence of rocaglamides. In contrast, CsA strongly inhibited calcineurin phosphatase activity (Fig. 8B). These experiments demonstrate that rocaglamides do not directly inhibit calcineurin activity.
The above experiments showed that at doses ranging from 25 to 100 nM rocaglamides selectively inhibited NF-AT function without impairing the activities of AP-1 and NF-κB. The NF-AT family of proteins is activated by the calcium/calmodulin-dependent phosphatase calcineurin that dephosphorylates and promotes nuclear translocation of NF-AT (14, 17). Glycogen synthase kinase-3 is the constitutive NF-AT kinase that induces rephosphorylation and, therefore, inactivation of NF-AT (29, 30). Inactivation of NF-AT may also be regulated by the cellular MAPKs JNK and p38 that phosphorylate NF-AT and promote NF-AT nuclear export (19–21). To investigate the molecular mechanisms of rocaglamide-mediated suppression of NF-AT activity, we examined the effects of rocaglamides on activities of MAPKs. We found that treatment with rocaglamides alone induced p38 phosphorylation in Jurkat T cells. At a concentration of 25 nM, rocaglamides induced phosphorylation of p38 similar to that observed by PMA and ionomycin (Fig. 9A). In PMA/ionomycin-stimulated Jurkat T cells, rocaglamides further enhanced T cell activation-induced phosphorylation of p38 (Fig. 9A). Rocaglamides also synergized with PMA/ionomycin to induce the expression of phosphor-JNK (Fig. 9B). Subtle differences in their ability to induce JNK phosphorylation were observed among the three rocaglamide derivatives. Roc-1 and -3 were stronger inducers of phosphor-JNK than Roc-2. Consistent with the immunoblotting and transfection studies, rocaglamides did not inhibit PMA/ionomycin-induced degradation of IkB at concentrations of 25–100 nM (Fig. 9C).
To investigate whether activation of MAPKs by rocaglamides is the cause of the reduction in NF-AT activity, PMA/ionomycin-activated Jurkat T cells were treated with rocaglamides in the presence or the absence of the widely used p38 kinase and JNK inhibitors, SB 203580 and SP 600125, respectively. Immunoblotting analysis showed that rocaglamide-enhanced phosphorylation of p38 and JNK was largely inhibited in the presence of such specific inhibitors (Fig. 10, A and B). In contrast, rocaglamide did not induce phosphorylation of ERK, and the p38 and JNK inhibitors had no effect on ERK activities (Fig. 10C). Inhibition of p38 and JNK phosphorylation by the inhibitors correlated with prevention of rocaglamide-mediated reduction in NF-AT proteins in the nucleus (Fig. 10D). As control, nuclear levels of the NF-κB subunit p65 were not influenced by the dose of rocaglamide used in the same experiments. In addition, they were not significantly affected by the p38 or the JNK kinase inhibitors (Fig. 10E). Thus, inhibition of p38 and JNK activities prevents rocaglamide-mediated inhibition of NF-AT nuclear expression. These data demonstrate that rocaglamide-mediated inhibition of NF-AT activity is associated with overactivation of JNK/p38 kinases.

**Discussion**

In this study we show that rocaglamide derivatives are potent immunosuppressive phytochemicals that inhibit cytokine gene expression at the transcriptional level. A very low dose (50 nM) of rocaglamides could completely inhibit IL-4 and IFN-γ production and suppress 60–85% of IL-2 and the proinflammatory cytokine TNF-α production. This strong inhibitory effect of rocaglamides may explain the effect of the crude plant extracts in treatment of inflammatory skin disease and allergic asthma in traditional medicine, e.g., in Vietnam. We have also shown that at concentrations of 25–100 nM, rocaglamides selectively inhibit NF-AT-dependent gene expression without impairing the activities of other inducible transcription factors, such as AP-1 and NF-κB. A previous study showed that rocaglamides inhibit NF-κB activity (IC50 values of most compounds tested were in the range of 200–500 nM) (3). It was shown that 200 nM rocaglamide blocked TNF-α- and PMA-induced IkB degradation in Jurkat T cells (3). The doses used in this study are higher than those used in our studies. We showed that at doses of 25–100 nM, none of the three rocaglamides investigated (that suppress NF-AT activity) blocked p65 nuclear expression or degraded IκB after T cell stimulation. Consistent with the previous study, at higher concentrations (150 nM), an ~30% inhibition of NF-κB activity by Roc-2 and Roc-3 was observed (Fig. 7A). Our studies indicate that rocaglamides may serve as potential NF-AT inhibitors.

NF-AT is essential for activating the transcription of most T cell cytokine genes. T cells lacking NF-AT proteins have been shown to produce almost no cytokines upon stimulation (31). For the majority of cytokine genes, cooperative binding of NF-AT and AP-1 to composite NF-AT/AP-1 binding sites has been demonstrated on the promoter/enhancer regions (5, 31, 32). The consequence is that the NF-AT/AP-1 complex formed by cooperative binding is much more stable than the complex formed by any of its individual components bound independently to the composite DNA site (14, 28). The IL-2 promoter contains five NF-AT-binding elements. All five NF-AT sites are essential for the full induction of promoter activity in response to TCR stimulation. Four of the five NF-AT sites are part of composite elements able to bind AP-1 in association with NF-AT (28, 31). The IL-4 promoter contains five NF-AT/AP-1 composite binding sites (16, 32). It has
been shown that the expression of IL-2 and IL-4 mRNA is absolutely dependent on cooperation between NF-AT and AP-1 (14). Rocaglamides reduce nuclear levels of both NF-AT and c-Jun proteins. Therefore, reduction of NF-AT and its partner AP-1 proteins may account for the rocaglamide-mediated inhibition of NF-AT activity. Although rocaglamides suppress c-Jun expression and down-regulate NF-AT activity, they do not inhibit AP-1-dependent transcriptional activity stimulated by PMA/ionomycin. This may well be due to the fact that rocaglamides strongly induce phosphorylation of c-Jun by activation of JNK. Because phosphorylation of c-Jun enhances its activity (18), treatment of T cells with low doses of rocaglamides may increase AP-1-dependent transcription, as demonstrated in our study and also in a previous one (3). However, a higher dose of rocaglamides may lead to suppression of AP-1 activation due to limited amounts of c-Jun proteins.

Several rocaglamide compounds were previously shown to be potent inhibitors of NF-kB activation (3). Roc-1 (named Roc-4 in a previous study) inhibits TNF-α-induced NF-κB activation in Jurkat T cells (3). In this study we have shown that rocaglamides are potent inhibitors of NF-AT activity in T cells, and that all three rocaglamide derivatives tested inhibited NF-AT-dependent transcription at doses that did not impair AP-1- and NF-κB-dependent transcription upon T cell activation. At concentrations <100 nM, Roc-1 even substantially increased NF-κB activity. This feature suggests the potential to develop rocaglamides to control the expression of NF-AT-dependent genes.

CsA and FK-506 (tacrolimus) are NF-AT inhibitors that have been used in organ transplantation to prevent graft-vs-host disease (33). However, the toxicity of these drugs due to their ability to inhibit calcineurin in cells outside the immune system has precluded their use in other clinical situations, such as allergy, inflammation, and autoimmune disease (34, 35). Rocaglamides down-regulate NF-AT activity via a mechanism different from that of CsA and FK-506. Rocaglamides strongly activate p38 and further increase the phosphorylation of p38 and JNK in activated T cells. JNK and p38 MAPKs have been reported to oppose calcineurin activities and regulate the nuclear export of NF-AT by physical interaction with the NF-AT proteins and direct phosphorylation of the functionally important residues involved in regulating NF-AT subcellular localization (19, 20). In addition, JNK may phosphorylate and inactivate the calcineurin-targeting domain of NF-AT. For JNK1, a proposed mechanism is that phosphorylation of the SPRIEIT calcineurin-docking site of NF-ATc1 blocks the interaction of NF-ATc1 with calcineurin (21). Overexpression of JNK or p38 can block ionomycin-induced NF-AT nuclear translocation, whereas treatment of cells with MAPK inhibitors, PD98059 and SB202190, triggers NF-AT nuclear localization (20). In agreement with previous studies, we show that the widely used p38 kinase and JNK inhibitors, SB203580 and SP600125, prevent rocaglamide-induced reduction of nuclear NF-AT. Furthermore, Jnk−/− T cells were associated with increased nuclear accumulation of NF-ATc1 (36). Therefore, induction of p38 and JNK overactivation may account for rocaglamide-mediated inhibition of NF-AT activity.

Dysregulation of cytokine production or action has been recognized to contribute to the pathogenesis of many autoimmune and inflammatory diseases. The accepted model is that naive T cells become activated by Ag and produce IL-2, which, in turn, induces clonal expansion and promotes the production of other proinflammatory cytokines, such as TNF-α and IFN-γ. Administration of IL-2, IFN-γ, and TNF-α is associated with a variety of autoimmune disorders, including immune thyroiditis, rheumatoid arthritis, and other arthropathies (4). Abs against TNF-α have been developed in anti-TNF-α therapy for rheumatoid arthritis (8). We have shown that rocaglamides suppress IL-2, TNF-γ, and TNF-α production at very low concentrations (50 nM). This raises the potential of using rocaglamides in the treatment of those inflammation-associated diseases. In southeast Asia, crude extracts from various Aglaia species are also used in treatment of allergic

FIGURE 8. Rocaglamides do not interfere with intracellular calcium mobilization and have no direct inhibitory effect on calcineurin phosphatase activity. A, Jurkat T cells were loaded with the Ca2+ indicator fluo-4 and then stimulated with ionomycin in the presence or the absence of rocaglamides. As a control, the Ca2+ chelator BAPTA/AM was included in the assay. B, Calcineurin phosphatase activity was measured using a calcineurin assay kit as described in Materials and Methods.

FIGURE 9. Rocaglamides activate MAPKs p38 and JNK. A, Jurkat T cells were stimulated with PMA and ionomycin for 2 h in the absence or the presence of various concentrations of rocaglamides (added 1 h before stimulation). Total cell lysates were immunoblotted with Abs against phosphorylated p38 (p-p38) and then stripped for immunoblot to anti-tubulin. B, Total cell lysates were immunoblotted with Abs to phosphorylated JNK and then stripped for immunoblot to anti-tubulin. C, Total cell lysates were immunoblotted with Ab to IκBα and then stripped for immunoblot to antitubulin. All data are representative of two or three reproducible experiments.
asthma. IL-4 plays a central role in the development of allergic asthma by promoting production of the proinflammatory cytokines IL-5 and IL-13 (8, 16). We have shown that at a 50-nM concentration, rocaglamides may completely block IL-4 expression in peripheral blood T cells. At this concentration, rocaglamides do not impair the function of AP-1 and NF-κB and have no obvious toxicity on primary blood T cells. This may partially explain the antiasthma effect of Aglaia species. Taken together, our data suggest a potential role for rocaglamides in the development of drugs against inflammation and graft rejection after transplantation.

Acknowledgments

We thank Dr. M. L. Schmitz for providing the pluc-Bax plasmid, and Dr. D. Macasew for critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References


