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Protein Kinase Cα but not p44/42 Mitogen-Activated Protein Kinase, p38, or c-Jun NH_2-Terminal Kinase Is Required for Intercellular Adhesion Molecule-1 Expression Mediated by Interleukin-1β: Involvement of Sequential Activation of Tyrosine Kinase, Nuclear Factor-κB-Inducing Kinase, and IκB Kinase 2

CHING-CHOW CHEN, JUN-JIE CHEN, and CHIAN-YING CHOU

Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan

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ABSTRACT

IL-1β induced an increase in ICAM-1 expression in human A549 epithelial cells and immunofluorescence staining confirmed this result. Tyrosine kinase inhibitors (genistein or tyrphostin 23) or phosphatidylcholine-specific phospholipase C inhibitor (D609) attenuated IL-1β-induced ICAM-1 expression. IL-1β produced an increase in PKC activity and this effect was abolished by D609. PKC inhibitors (staurosporine, Ro 31-8220, calphostin C, or Go 6976) also inhibited IL-1β-induced response. TPA, a PKC activator, stimulated ICAM-1 expression as well, this effect being inhibited by tyrosine kinase inhibitors. Treatment of cells with IL-1β resulted in stimulation of p44/42 MAPK, p38, and JNK. However, neither the mitogen activated protein kinase kinase inhibitor PD 98059 nor the p38 inhibitor SB 203580 affected IL-1β-induced ICAM-1 expression. NF-κB DNA-protein binding and ICAM-1 promoter activity were enhanced by IL-1β and these effects were inhibited by tyrphostin 23, but not by PD 98059 or SB 203580. TPA also stimulated NF-κB DNA-protein binding and ICAM-1 promoter activity as well, these effects being inhibited by tyrosine kinase inhibitors. Dominant-negative PKCα, NIK, or IKK2, but not IKK1 mutant, inhibited IL-1β- or TPA-induced ICAM-1 promoter activity. IKK activity was stimulated by either IL-1β or TPA, and these effects were inhibited by Ro 31-8220 or tyrphostin 23. Taken together, IL-1β activates phosphatidylcholine-specific phospholipase C and induces activation of PKCα and protein tyrosine kinase, resulting in the stimulation of NIK, IKK2, and NF-κB in the ICAM-1 promoter, then initiation of ICAM-1 expression. However, activation of p44/42 MAPK, p38, and JNK is not involved.

Adhesion of circulating polymorphonuclear leukocytes to the vascular endothelium is a critical step in the inflammatory response. This event is mediated by the molecules present on the surface of endothelial cells and polymorphonuclear leukocytes (Lo et al., 1989). Intercellular adhesion molecule-1 (ICAM-1), an inducible cell surface glycoprotein that belongs to a member of the immunoglobulin superfamily, is expressed by endothelial cells (Simmons et al., 1988; Staunton et al., 1988). The regulation of ICAM-1 expression is fundamental to leukocyte trafficking. As the counter-receptor for the leukocyte β2 integrins, ICAM-1 plays a central role in a number of inflammatory and immune responses. Up-regulation of its expression on cytokine-activated vascular endothelial cells controls the targeted transmigration of leukocytes into specific area of inflammation (Dustin et al., 1988; Smith et al., 1989). Similar processes govern leukocyte adhesion to lung airway epithelial cells and may contribute to the damage to these cells seen in asthma (Montefort et al., 1992; Tossi et al., 1992; Bloemen et al., 1993).

The cytokine interleukin-1 (IL-1) is a potent contributor to inflammation and is involved in the late asthmatic response (Barnes, 1994). High levels of IL-1β, mainly secreted by macrophages, have been observed in bronchoalveolar lavage flu-

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ABBREVIATIONS: ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; IL-1RI, interleukin-1 type I receptor; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; NIK, nuclear factor-κB-inducing kinase; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; PKC, protein kinase C; PC-PLC, phosphatidylcholine-specific phospholipase C; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDTC, pyrrolidine dithiocarbamate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; TTBS, Tween-20/Tris-buffered saline; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; MEK, mitogen-activated protein kinase kinase; DAG, diacylglycerol; LPS, lipopolysaccharide; GST, glutathione *S*-transferase; MBP, myelin basic protein.

ids from asthmatic patients (Borish et al., 1992). IL-1β acts by inducing many genes, including cytokines, chemokines, proteases, adhesion molecules, and cyclooxygenases. The extent and duration of the expression of these genes are crucial in regulating the intensity of inflammatory processes (Dinarello, 1996). Two IL-1 receptors (type I and type II), expressed on many different cell types (Martin and Falk, 1997), have been cloned. Only the type I receptor (IL-1RI), heterodimerized to the IL-1 receptor accessory protein, is capable of signal transduction (Wesche et al., 1997). Many signaling systems (e.g., protein kinases A and C, phospholipase C, and G proteins) have been reported to be used in IL-1-induced responses (Dinarello, 1996). IL-1RI shares no significant homology with conserved protein kinase domains and must recruit specific cytoplasmic proteins to transmit signals. One such protein, recruited to the receptor in response to IL-1 stimulation, is the IL-1 receptor-associated protein kinase (Cao et al., 1996a), which is physically associated with a protein belonging to the TNF receptor-associated factor family and known as TRAF6 (Cao et al., 1996b). Recent advances in IL-1 signaling suggest that IL-1 activates at least four protein kinase cascades. One cascade involves the association of an IL-1 receptor-associated protein kinase and TRAF6 with the IL-1 receptor complex (Cao et al., 1996a,b), leading to activation of a NF-κB-inducing kinase (NIK), which activates the IκB kinase (IKK) complex (Malinin et al., 1997). The other IL-1-activated cascades are those activating the three best known types of mitogen-activated protein kinase (MAPK), namely, p44/42 MAPK, p38, and stress-activated protein kinase/JNK (Guan et al., 1998; Larsen et al., 1998). The intracellular signaling pathways by which IL-1β causes ICAM-1 expression are not well understood and only one report of PKC activation has been published (Ballestas and Benveniste, 1995). In the present study, we explored the intracellular signaling pathway involved in IL-1β-induced ICAM-1 expression in a human alveolar epithelial cell line A549. The results show that IL-1β can activate phosphatidylcholine-specific phospholipase C (PC-PLC), resulting in the activation of PKCα, protein tyrosine kinase, NIK, IKK2, and NF-κB in ICAM-1 promoter, followed by ICAM-1 expression. In contrast, IL-1β-induced activation of p44/42 MAPK, p38, and JNK is not involved in ICAM-1 expression in these cells.

Experimental Procedures

Materials. Mouse monoclonal anti-human ICAM-1 antibody and recombinant human IL-1β were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibodies directed against IKKβ, p42 MAPK (ERK2), and JNK1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific for the phosphorylated form of Tyr-204 p44/42 MAPK or Tyr-182 p38 or for p38 and T4 polynucleotide kinase were from New England Biolabs (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, and streptomycin were from Life Technologies (Gaithersburg, MD). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was from LC Services (Woburn, MA). Staurosporine, pyrrolidine dithiocarbamate (PDTC), tyrphostin 23, *O*-phenylenediamine dihydrochloride, and histone III-S were from Sigma (St. Louis, MO). D609, genistein, calphostin C, Go 6976, Ro 31-8220, PD 98059, and D-*erythro*-sphingosine, *N*-acetyl (C2 ceramide) were from Calbiochem (San Diego, CA). SB 203580 was a gift from SmithKline Beecham Pharmaceuticals. Poly(dI/dC) was from Amersham Pharmacia Biotech (Piscataway, NJ). Reagents for SDS-PAGE were from Bio-Rad (Richmond, CA). [γ-^{32}P]ATP (3000 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA). Horseradish peroxidase-labeled donkey anti-rabbit second antibody and the ECL detecting reagent were purchased from Amersham Pharmacia Biotech. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was from Cappel (Aurora, OH).

Plasmids. The ICAM-1 promotor constructs (pIC-339 and pIC-174) were a generous gift of Dr. P. T. van der Saag (Hubrecht Laboratory, Utrecht, the Netherlands). The dominant-negative mutant of ERK2 was from Dr. M. Cobb (South-Western Medical Center, Dallas, TX), p38 (T180A/Y182F) was from Dr. J. Han (The Scripps Research Institute, San Diego, CA), JNK (T183A/Y185F) and NIK (KK429–430AA) were from Dr. M. Karin (University of California, San Diego, CA), IKK1 (K44 M) and IKK2 (K44 M) were from Signal Pharmaceutical (San Diego, CA), and PKCα (K/R) was from A. Altman (La Jolla Institute for Allergy and Immunology, San Diego, CA). pGEX-IκBα (1–100) was from Dr. H. Nakano (Juntendo University, Tokyo, Japan).

Cell Cultures. A549 cells, an aveolar epithelial cell carcinoma, were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well plates (ICAM-1 expression), on 24-mm glass coverslips in 35-mm dishes (immunofluorescence staining for ICAM-1), in six-well plates (transfection), in 6-cm dishes (PKC activity measurement), or in 10-cm dishes (MAPKs activation, NF-κB gel shift assay and IKK activation).

Quantification of ICAM-1 Expression. The level of cell surface ICAM-1 expression was determined by an ELISA. After treatment with IL-1β at 37°C, the cells were washed twice with PBS and fixed at room temperature with 1% paraformaldehyde for 30 min. After washing with PBS, they were then blocked with 1% BSA in Tris-buffered saline containing 0.05% Tween-20 (TTBS) for 15 min before being incubated successively with anti-ICAM-1 antibody (1:100) for 1 h and horseradish peroxidase-labeled anti-mouse antibody (1:1000) for 30 min. After each incubation, the cells were washed two times with PBS. *O*-Phenylenediamine dihydrochloride substrate [0.4 mg/ml in phosphate-citrate buffer, pH 5.0; 24.3 mM citric acid; 51.4 mM $Na_2HPO_4 \cdot 12\ H_2O$; 12% H_2O_2 (v/v)] was then applied to the cells for 30 min and 3 M sulfuric acid added to stop the reaction. The absorbance was measured at 450 nm by an ELISA reader (Bio-Tek, Burlington, VA). Each assay was performed in triplicate. In pretreatment experiments, cells were incubated with the tyrosine kinase inhibitors genistein or tyrphostin 23; the PC-PLC inhibitor D609; the PKC inhibitors staurosporine, calphostin C, Ro 31-8220 or Go 6976; the MEK inhibitor PD 98059; or the p38 inhibitor SB 203580 for 30 min before addition of IL-1β.

Immunofluorescence Staining. A549 cells, grown on coverslips, were treated for 17 h with IL-1β or TPA in growth medium. The cells were then rapidly washed with PBS and fixed at room temperature for 10 min with 2% paraformaldehyde. After washing with PBS, cells were blocked for 15 min with 1% BSA in TTBS, and then incubated with anti-ICAM-1 antibody (1:100) for 1 h, washed extensively, and stained for 30 min with anti-mouse IgG-fluorescein (1:1000). After additional washes, the coverslips were mounted on glass slides using mounting medium (2% *n*-propyl gallate in 60% glycerol and 0.1 M PBS, pH 8.0). Optical sections of the immunostained cells were visualized and photographed with a Zeiss Axiovert inverted microscope equipped with photoMicroGraph digitized integration system.

PKC Activity Assay. Cells treated with IL-1β for 10 min, 1 h, or 4 h were scraped and collected, and membrane fractions were prepared and assayed for PKC activity as previously described (Chen, 1994); the assay was performed at 30°C for 5 min in 25 μl of 30 mM Tris-HCl buffer, pH 7.5, containing 6 mM magnesium acetate, 0.12 mM [γ-^{32}P]ATP, 0.4 mM $CaCl_2$, 40 μl/ml LPS, 8 μg/ml 1,2-dioleoylglycerol, 1 mg/ml histone III-S, and the enzyme preparation (2.5–5.0 μg of protein). The Ca^{2+}/phospholipid-independent activity was mea-

sured under the same conditions in the absence of Ca^{2+} and phospholipid and in the presence of 2 mM EGTA.

Preparation of Cell Extracts and Western Blot Analysis of Phosphorylated p44/42 MAPK, Phosphorylated p38, Phosphorylated JNK, p42 MAPK, p38, and JNK1, and p38 Activity Assay. After treatment with IL-1β or with PD 98059 or SB 203580 before challenge with IL-1β for 10 min, the cells were rapidly washed with PBS, and then lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 5 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM NaF, and 1 mM Na_3VO_4) as described previously (Chen et al., 1998) and the lysates were subjected to SDS-PAGE using a 7.5% running gel. The proteins were transferred to nitrocellulose paper and immunoblot analysis performed as described previously. Briefly, the membrane was incubated successively with 0.1% milk in TTBS at room temperature for 1 h, with rabbit antibodies specific for phosphorylated MAPKs or nonphosphorylated MAPKs for 1 h, and then with horseradish peroxidase-labeled anti-rabbit second antibody for 30 min. After each incubation, the membrane was washed extensively with TTBS and the immunoreactive band was detected with ECL-detecting reagents and developed with Hyperfilm-ECL.

To measure p38 activity, 30 μg of total cell lysate was incubated with 0.6 μg of anti-p38 antibody for 1 h at 4°C and the antibody-bound material collected using protein A-Sepharose CL-4B beads. The beads were washed with lysis buffer and incubated for 30 min at 30°C with 30 μl of kinase reaction mixture containing 20 mM HEPES, pH 6.4, 10 mM $MgCl_2$, 100 μM Na_3VO_4, and 50 μM [γ-^{32}P]ATP, together with 0.3 mg/ml MBP. The reaction was stopped by the addition of Laemmli buffer and subjected to SDS-PAGE, phosphorylated MBP being visualized by autoradiography.

Preparation of Nuclear Extracts and the Electrophoretic Mobility Shift Assay (EMSA). Control cells or cells pretreated with tyrphostin 23, D609, staurosporine, calphostin C, Ro 31-8220, PD 98059, or SB 203580 were treated with IL-1β for 1 h, and then nuclear extracts were prepared as described previously (Chen et al., 1998). Briefly, cells were washed with ice-cold PBS and pelleted. The cell pellet was resuspended in a hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 mM NaF, and 1 mM Na_3VO_4) and incubated for 15 min on ice, and then lysed by the addition of 0.5% NP-40 followed by vigorous vortexing for 10 s. The nuclei were pelleted and resuspended in extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM NaF, and 1 mM Na_3VO_4), and the tube vigorously shaken at 4°C for 15 min on a shaking platform. The nuclear extracts were then centrifuged and the supernatants aliquoted and stored at −80°C.

Oligonucleotides corresponding to the downstream NF-κB consensus sequences in human ICAM-1 promoter (5′-AGC**TTGGAAATTCCG**GA-3′) were synthesized, annealed, and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The nuclear extract (6–10 μg) was incubated at 30°C for 20 min with 1 ng of ^{32}P-labeled NF-κB probe (40,000–60,000 cpm) in 10 μl of binding buffer containing 1 μg of poly(dI-dc), 15 mM HEPES, pH 7.6, 80 mM NaCl, 1 mM EGTA, 1 mM DTT, and 10% glycerol as described previously (Chen et al., 1998). DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 6% polyacrylamide gel, and then the gel was vacuum dried and subjected to autoradiography using an intensifying screen at −80°C. When supershift assays were performed, polyclonal antibodies specific for p65, p50, or p52 were added to the nuclear extracts 30 min before the binding reaction, and the DNA/nuclear protein complexes were separated on a 4.5% polyacrylamide gel.

In NF-κB (p65) translocation studies, both cytosolic and nuclear extracts were used, whereas only cytosolic extracts were used in IκB-α degradation studies. The extracts were subjected to SDS-PAGE using a 10% running gel and immunoblot analysis performed as described above.

Transient Transfection and Luciferase Assay. A549 cells were grown in six-well plates. The human ICAM-1 firefly luciferase plasmids (pIC-339 or pIC-174) were transfected using Tfx-50 (Promega, Madison, WI) according to manufacturer's recommendations. Briefly, reporter DNA (0.5 μg) and β-galactosidase DNA (0.1 μg) were mixed with 2.7 μl of Tfx-50 in 1 ml of serum-free DMEM. We used the plasmid pRK containing β-galactosidase gene driven by the constitutively active simian virus 40 promoter to normalize the transfection efficiency. After a 10- to 15-min incubation at room temperature, the mixture was applied onto the cells. One hour later, 1 ml of DMEM containing 20% FCS was added and then the cells were grown in medium containing 10% FCS. The following day, cells were exposed to 1 ng/ml IL-1β or 1 μM TPA for 4.5 h, and then cell extract was prepared and luciferase (Promega Biotech System) and β-galactosidase activity were measured. The luciferase activity of each well was normalized to β-galactosidase activity. In dominant-negative mutant experiment, cells were cotransfected with reporter and β-galactosidase and either the dominant-negative ERK2 (p42 MAPK), p38, JNK, PKCα, NIK, IKK1 or IKK2 mutant, or the empty vector.

In Vitro Kinase Assays. The IKK proteins contained in the cell extracts were immunoprecipitated. Fifty micrograms of total cell extract was incubated for 1 h at 4°C with 0.5 μg of anti-IKKβ antibody and collected using protein A-Sepharose CL-4B beads (Sigma). The beads were then washed two times with lysis buffer without Triton X-100 and incubated for 30 min at 30°C in 20 μl of kinase reaction mixture containing 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM Na_3VO_4, 1 mM DTT, 1 μg of bacterially expressed GST-IκBα (1–100), and 10 μM [γ-^{32}P]ATP. The reaction mixture was stopped by the addition of Laemmli buffer and subjected to 10% SDS-PAGE, and phosphorylated-GST-IκBα (1–100) was visualized by autoradiography.

Results

IL-1β-Induced ICAM-1 Cell Surface Expression in A549 Cells. As shown by ELISA, exposure of A549 epithelial cells to 1 ng/ml IL-1β stimulated the expression of ICAM-1, but not of vascular cell adhesion molecule-1 or E-selectin (data not shown). IL-1β induced ICAM-1 expression in a concentration- and time-dependent manner. For an exposure period of 4.5 h, maximum ICAM-1 expression was obtained using 1 ng/ml IL-1β (Fig. 1A). When cells were treated with 1 ng/ml IL-1β for various times, ICAM-1 expression was significant at 3 h and maximal at 18 h, and then slightly declined after 40 h (Fig. 1B). IL-1β-induced ICAM-1 expression was further demonstrated by immunofluorescence staining. As shown in Fig. 2, no ICAM-1 expression was seen in the basal state (Fig. 2B), but was induced in the plasma membrane after IL-1β treatment (Fig. 2D). In the following ICAM-1 expression experiments, the cells were treated with 1 ng/ml IL-1β for 4.5 h to avoid the cytotoxic effect of various inhibitors seen with longer incubation times, under these conditions, both the transcriptional and translational inhibitors actinomycin D and cycloheximide inhibited the IL-1β-induced ICAM-1 expression in a dose-dependent manner (data not shown).

Inhibitory Effect of Tyrosine Kinase Inhibitors, a PC-PLC Inhibitor or PKC Inhibitors on IL-1β-Induced ICAM-1 Expression and Activation of PKC by IL-1β. To study the intracellular signaling pathway involved in IL-1β-induced ICAM-1 expression, the tyrosine kinase inhibitors genistein and tyrphostin 23 were used. When cells were pretreated for 30 min with 30 to 300 μM genistein or tyrphostin 23, IL-1β-induced ICAM-1 expression was inhibited

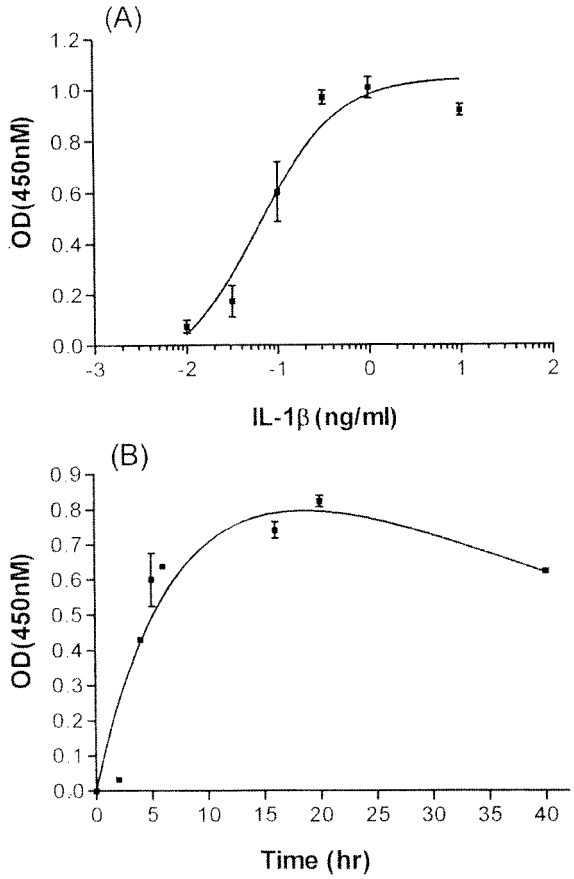

Fig. 1. Concentration- and time-dependent IL-1β-induced stimulation of ICAM-1 expression in A549 epithelial cells. Cells were incubated at 37°C with various concentrations of IL-1β for 4.5 h (A) or with 1 ng/ml IL-1β for various time intervals (B). Surface expression of ICAM-1 was measured by ELISA using anti-ICAM-1 antibody as described under *Experimental Procedures*. Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate.

in a dose-dependent manner (Fig. 3A). When cells were pretreated with 100 or 200 μM D609 (a PC-PLC inhibitor), IL-1β-induced ICAM-1 expression was inhibited by 14 or 50%, respectively, whereas 10 μM U73122 (a PI-PLC inhibitor) or 100 μM propranolol (a phosphatidate phosphohydrolase inhibitor) had no effect (Fig. 3B).

Because IL-1β-induced ICAM-1 expression was inhibited by D609, indicating the involvement of the PC-PLC pathway, which increases diacylglycerol (DAG) levels, and then activates PKC, PKC activity was assayed after treatment with 1 ng/ml IL-1β. As shown in Fig. 4A, membrane PKC activity was increased after treatment with IL-1β for 10 min and this effect was maintained for up to 4 h of treatment. This IL-1β-induced increase in PKC activity was completely inhibited by D609, but was not inhibited by genistein or tyrphostin 23 (Fig. 4B). When the specificity of genistein, tyrphostin 23, and D609 was tested by pretreating the cells with 300 μM genistein or tyrophostin 23 or 200 μM D609, TPA-induced PKC activation was not affected (data not shown), confirming that, in A549 cells, these inhibitors act on tyrosine kinase and PC-PLC, but not directly on PKC.

To determine whether activation of PKC by IL-1β was involved in the regulation of IL-1β-induced ICAM-1 expression, PKC inhibitors were used. Pretreatment of cells with staurosporine, calphostin C, Ro 31-8220, or Go 6976 inhibitedIL-1β-induced ICAM-1 expression in a dose-dependent manner (Fig. 5, A and B). Because PKC had been shown to be involved, the effect of direct TPA-mediated activation of PKC on ICAM-1 expression was examined. TPA (1 μM) also induced a time-dependent increase in ICAM-1 expression, and this effect was also inhibited by actinomycin D and cycloheximide (data not shown). When cells were pretreated with 30 to 300 μM genistein or tyrphostin 23, TPA-induced ICAM-1 expression was inhibited in a dose-dependent manner, and

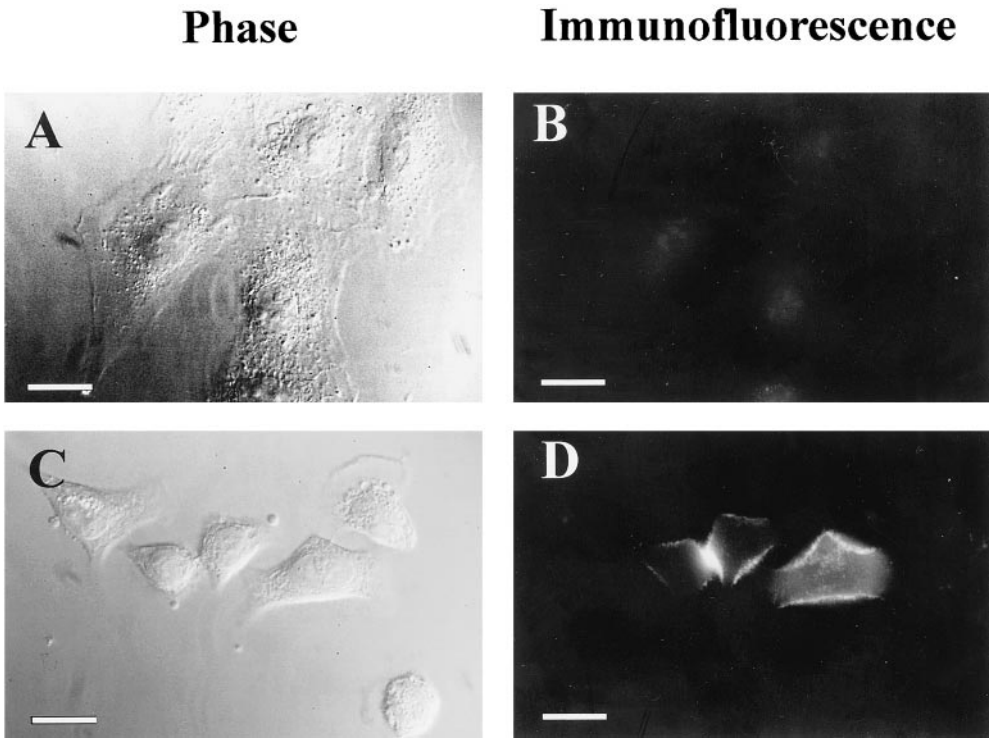

Fig. 2. ICAM-1 is located on the cell membrane. Immunofluorescent staining of A549 epithelial cells with affinity-purified ICAM-1 antibody (1:100). Cells were fixed and stained as described under *Experimental Procedures*. Control (A and B) and after 18 h treatment with 1 ng/ml IL-1β (C and D). Bar, 200 μM

100 nM staurosporine completely inhibited the TPA effect (Fig. 6).

IL-1β-Induced Activation of p44/42 MAPK, p38, and JNK, and Lack of Inhibition by PD 98059 and SB 203580 of IL-1β-Induced ICAM-1 Expression. In A549 cells, IL-1β activated p44/42 MAPK, p38, and JNK. As shown in Fig. 10, when cells were treated with 1 ng/ml IL-1β for 10, 30, or 60 min, maximal activation of these three MAPKs was seen after treatment for 10 min; sustained, or no activation of p44/42 MAPK was also seen after 30- or 60-min treatment, respectively, whereas lower or no activation of p38 and JNK was seen after 30- or 60-min treatment, respectively. The expression of p42 MAPK, p38, and JNK1 was not affected by these treatments (Fig. 7A).

To determine whether activation of p44/42 MAPK and p38 was involved in the regulation of IL-1β-induced ICAM-1 expression, an MEK inhibitor, PD 98059, and a p38 inhibitor, SB 203580, were used. As shown in Fig. 7C, neither PD 98059 (50 or 100 μM) nor SB 203580 (30 or 50 μM) inhibited IL-1β-induced ICAM-1 expression, whereas 50 μM PD 98059 completely blocked IL-1β-induced p44/42 MAPK activation without any effect on p38 and JNK activation, and 50 μM SB 203580 caused almost complete inhibition of p38 activity without affecting p44/42 MAPK and JNK activation (Fig. 7B). TPA-induced ICAM-1 expression was also not affected by 50 μM PD 98059 or SB 203580, despite the fact that TPA induced p44/42 MAPK activation (data not shown).

NF-κB Induction in the Nuclei of IL-1β-Stimulated A549 Cells, and Inhibition by D609 but not by Tyrphostin 23, Staurosporine, Calphostin C, Ro 31-8220, PD 98059, or SB 203580. The time course of NF-κB activation after treatment with IL-1β was examined. Nuclear extracts prepared from A549 cells were assayed for activated NF-κB in an EMSA. In nonstimulated A549 cells, one faint NF-κB-specific DNA-protein complex was identified. IL-1β rapidly (10 min) activated NF-κB; similar activation was seen after 1 h, whereas, after 18 h, slightly less DNA-protein complex was seen, although it was still more abundant than in resting cells (Fig. 8A). For the EMSA, cells were treated with IL-1β for 1 h. To identify the specific subunits involved in the formation of the banding pattern of the NF-κB dimer after IL-1β stimulation, supershift assays were performed in the presence of antibodies specific for the p65, p50, or p52 subunit. As shown in Fig. 8B, incubation with anti-p65 or anti-

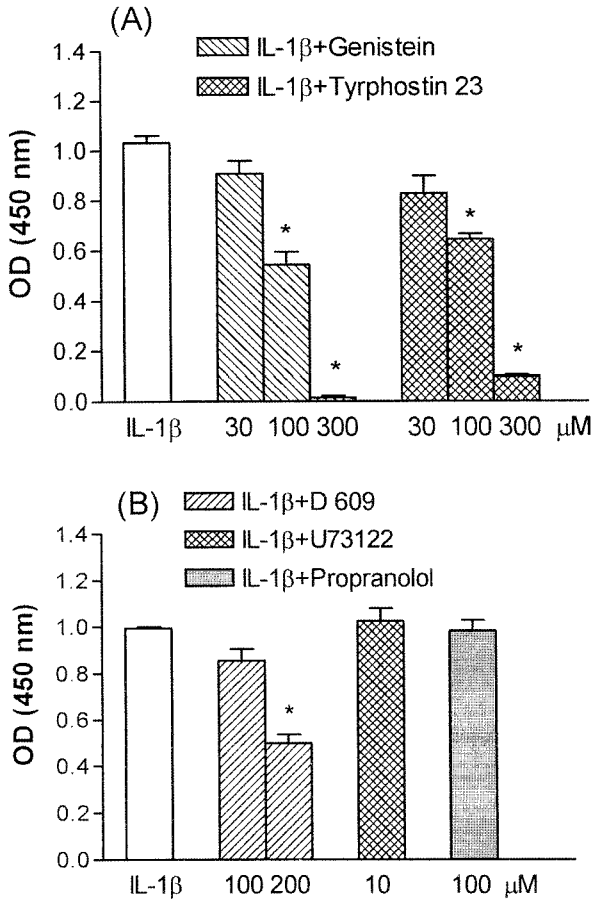

Fig. 3. Concentration-dependent inhibitory effect of genistein, tyrphostin 23, or D609 on IL-1β-induced ICAM-1 expression in A549 epithelial cells. Cells were pretreated with the indicated concentrations of genistein, tyrphostin 23 (A), D609, U73122, or propranolol (B) for 30 min before incubation with 1 ng/ml IL-1β for 4.5 h. Surface expression of ICAM-1 was measured by ELISA using anti-ICAM-1 antibody as described under *Experimental Procedures*. Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *$P < .05$ compared with IL-1β alone.

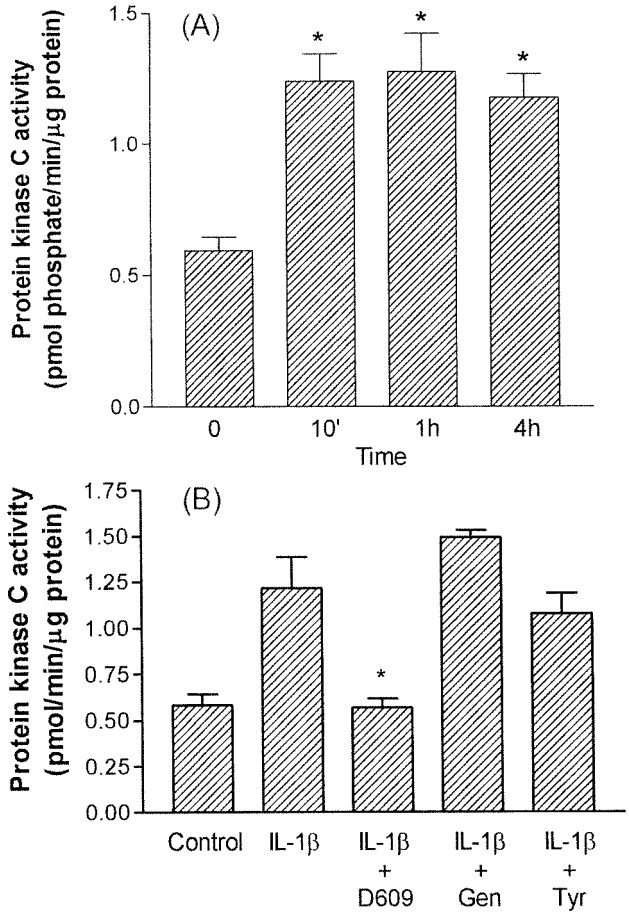

Fig. 4. PKC activity in the membrane in response to IL-1β, and the effect of D609, genistein, or tyrphostin 23 on IL-1β-stimulated PKC activity in A549 epithelial cells. Cells were incubated with 1 ng/ml IL-1β for the indicated time (A) or pretreated with 200 μM D609, or 300 μM genistein (Gen) or tyrphostin 23 (Tyr) for 30 min before incubation with IL-1β for 10 min (B), and then separated into cytosolic and membrane fractions. PKC activity in the membrane was measured as described under *Experimental Procedures*. The results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *$P < .05$ compared with the basal level (A) or IL-1β alone (B).

p50 antibodies induced a supershift (arrows a and b, respectively), but there was no shift in the presence of anti-p52 antibody. Thus, our data demonstrate the presence of the p65/p50 heterodimer of NF-κB in A549 cells. To characterize the proteins involved in NF-κB activation, the amount of p65 in cytosolic and nuclear extracts from activated cells was assayed by Western blotting. As shown in Fig. 9C, p65 was rapidly (10 min) translocated from the cytosol to the nuclear compartment in stimulated cells, and remained constant after 1 h of IL-1β treatment. Because the amount of NF-κB protein released to migrate to the nucleus is thought to be proportional to the degradation of IκB, the IκB-α protein level in the cytosol was measured. As shown in Fig. 8C, IL-1β rapidly induced complete degradation of IκB-α, but the level was restored after 1 h of IL-1β treatment.

After pretreatment of cells for 30 min with 200 μM D609, IL-1β-elicited activation of NF-κB specific DNA-protein complex formation was inhibited. However, DNA-protein complex formation was not affected by 300 μM tyrphostin, 300 nM staurosporine or calphostin C, 10 μM Ro 31-8220, and 50 μM PD 98059 or SB 203580 (data not shown). After exposure of cells to 1 μM TPA for 1 h, activation of NF-κB-specific DNA-protein complex formation was also seen and this effect was inhibited by 300 μM genistein or tyrphostin 23 (data not shown).

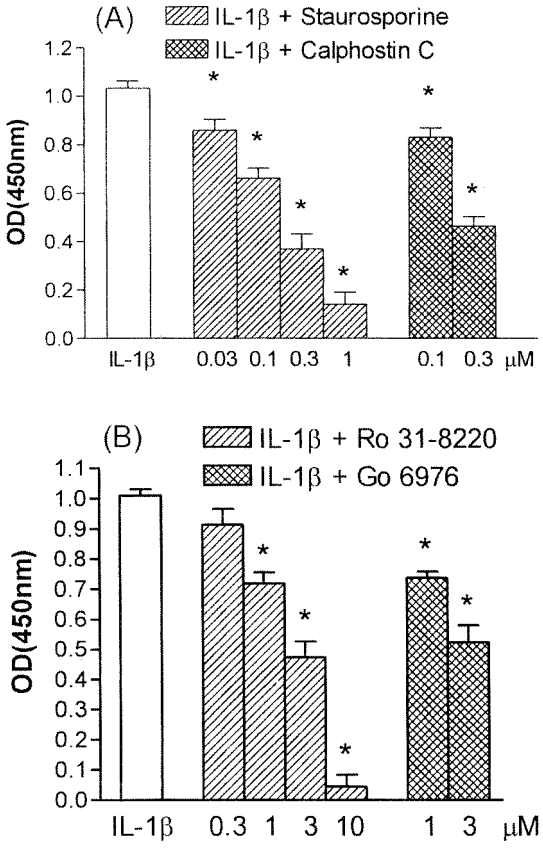

Fig. 5. Concentration-dependent inhibitory effect of PKC inhibitors on IL-1β-induced ICAM-1 expression in A549 epithelial cells. Cells were pretreated with the indicated concentrations of staurosporine or calphostin C (A) or Ro 31-8220 or Go 6976 (B) for 30 min before incubation with 1 ng/ml IL-1β for 4.5 h. Surface expression of ICAM-1 was measured by ELISA using anti-ICAM-1 antibody as described under *Experimental Procedures*. Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *$P < .05$ compared with IL-1β alone.

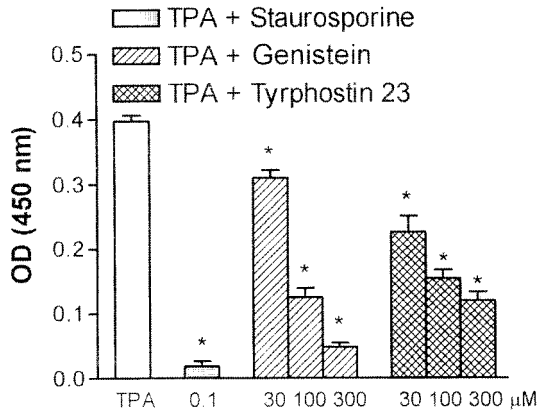

Fig. 6. Effect of staurosporine, genistein, or tyrphostin 23 on TPA-induced ICAM-1 expression in A549 epithelial cells. Cells were pretreated with 100 nM staurosporine or the indicated concentration of genistein or tyrphostin 23 for 30 min before incubation with 1 μM TPA for 4.5 h. Surface expression of ICAM-1 was measured by ELISA using anti-ICAM-1 antibody as described under *Experimental Procedures*. Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *$P < .05$ compared with TPA alone.

Induction of ICAM-1 Promoter Activity by IL-1β and the Inhibitory Effect of D609, PKC, or Tyrosine Kinase Inhibitors, or Dominant-Negative Mutant for PKCα, NIK, or IKK2, but not of PD 98059, SB 203580, or Dominant-Negative Mutant for ERK2, p38, JNK, or IKK1. To further investigate the involvement of PKC but not MAPKs signaling pathway in IL-1β-induced ICAM-1 expression, transient transfections were performed using human ICAM-1 promotor-luciferase constructs pIC-339 (−339/0) and pIC-174 (−174/0) (Van De Stolpe et al., 1994). pIC-339 construct contains the downstream NF-κB site (−188/−177) in ICAM-1 promoter, which is deleted in pIC-174 construct. Treatment with IL-1β or TPA led to an ~3.2- and ~2.2-fold increase, respectively, in ICAM-1 promoter activity when cells were transfected with pIC-339; this effect was completely blocked by PDTC. When pIC-174 construct was used, ICAM-1 promoter activity was not induced by IL-1β or TPA (Fig. 9A). These data indicate that downstream NF-κB site is responsible for mediating the effects of IL-1β or TPA in A549 cells. The IL-1β-induced ICAM-1 promoter activity using pIC-339 was inhibited by D609, PKC inhibitors (Ro 31-8220, calphostin C, or staurosporine), or tyrosine kinase inhibitors (genistein or tyrphostin 23), whereas TPA-induced activity was inhibited by tyrosine kinase inhibitors (Fig. 9B). In cotransfection experiments, the induction of ICAM-1 promoter activity by IL-1β and TPA was inhibited by the dominant-negative mutants PKCα/KR, NIK (KKAA) or IKK2 (KM), but not by the dominant-negative mutant IKK1 (KM) (Fig. 9D). In contrast, the induction of ICAM-1 promoter activity by IL-1β was not affected by PD 98059, SB 203580, or dominant-negative mutant for ERK2, p38, or JNK (Fig. 9C).

Induction of IKK Activation by IL-1β or TPA, and Inhibitory Effect of Tyrosine Kinase Inhibitor. The endogenous IKK complex was isolated by immunoprecipitation with anti-IKKβ antibody and tested for in vitro kinase activity. As shown in Fig. 10, both IL-1β and TPA induced IKK activation. The IL-1β-induced IKK activity was inhibited by Ro 31-8220 or tyrphostin 23 (Fig. 10A), that induced by TPA was inhibited by tyrphostin 23 (Fig. 10B).

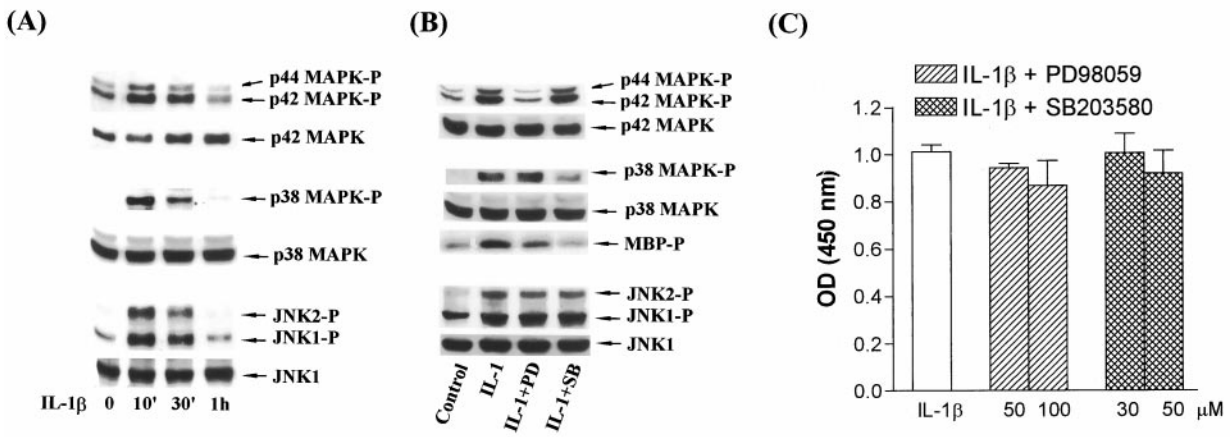

Fig. 7. Time-dependent activation of p44/42 MAPK, p38, and JNK by IL-1β and effect of PD 98059 or SB 203580 on IL-1β-induced p44/42 MAPK, p38, and JNK activation and ICAM-1 expression in A549 epithelial cells. Cells were treated with 1 ng/ml IL-1β for 10, 30, or 60 min (A), or pretreated with 50 μM PD 98059 (PD) or SB 203580 (SB) for 30 min before incubation with 1 ng/ml IL-1β for 10 min (B). Whole-cell lysates were prepared and subjected to Western blotting using antibodies specific for the phosphorylated form of p44/42 MAPK, p38, or JNK, or for p42 MAPK, p38, or JNK1, or immunoprecipitated with anti-p38 antibody, followed by autoradiography for phosphorylated MBP as described under *Experimental Procedures*. C, cells were pretreated with 50 or 100 μM PD 98059 or 30 or 50 μM SB 203580 for 30 min before incubation with 1 ng/ml IL-1β for 4.5 h. Surface expression of ICAM-1 was measured by ELISA using anti-ICAM-1 antibody as described under *Experimental Procedures*. Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate.

Discussion

The inflammatory cytokine IL-1 is a potent immunoregulatory and proinflammatory agent involved in a variety of pathological processes, such as the response to infection, activated lymphocyte products, microbial toxins, and other stimuli (Taub and Openheim, 1994). High levels of IL-1β have been observed in bronchoalveolar lavage fluids from asthmatic patients and are involved in the late asthmatic response (Borish et al., 1992; Barnes, 1994). We have shown that IL-1β induced ICAM-1 expression in A549 epithelial cells, and that this occurred in the plasma membrane as demonstrated by immunofluoresence staining. The promoter region of human ICAM-1 has been cloned and sequenced, and shown to contain putative recognition sequences for a variety of transcriptional factors, including NF-κB and activator protein-1, as well as activator protein-2, glucocorticoid receptor element, and interferon-stimulated response element (Degitz et al., 1991; Voraberger et al., 1991; Stratowa and Audette, 1995). Of these, the NF-κB appears to be essential for the enhanced ICAM-1 expression after exposure to cytokines in human umbilical vein endothelial cells and transformed embryonal kidney cell line 293 (Ledebur and Parks, 1995; Aoudjit et al., 1997; Paxton et al., 1997). NF-κB was also demonstrated to be critical in the induction of ICAM-1 expression in A549 cells because the ICAM-1 promoter activity induced by IL-1β was almost completely blocked by PDTC and deletion of downstream NF-κB site in ICAM-1 promoter (−188/−177) abolished the IL-1β-induced promoter activity (Fig. 9A). EMSA studies showed rapid activation of NF-κB in response to IL-1β stimulation (10 min), together with the parallel translocation of p65 into the nucleus. Almost complete degradation of IκB-α was also seen (Fig. 8C). It had been reported that all known NF-κB activators (IL-1, LPS, TNF, and TPA) induce IκB-α degradation (Beg et al., 1993). IL-1β treatment resulted in the rapid loss of IκB-α protein, as also seen in RAW 264.7 cells exposed to LPS (Chen and Wang, 1999) and in NCI-H292 cells exposed to TNF-α (Chen et al., 2000), and, as previously reported (Beg et al., 1993; Chen and Wang, 1999; Chen et al., 2000), this was resynthesized within 1 h (Fig. 8C). The renewed synthesis of IκB-α protein might be due to activation of the IκB-α gene by activated nuclear NF-κB because the IκB-α gene promoter contains κB

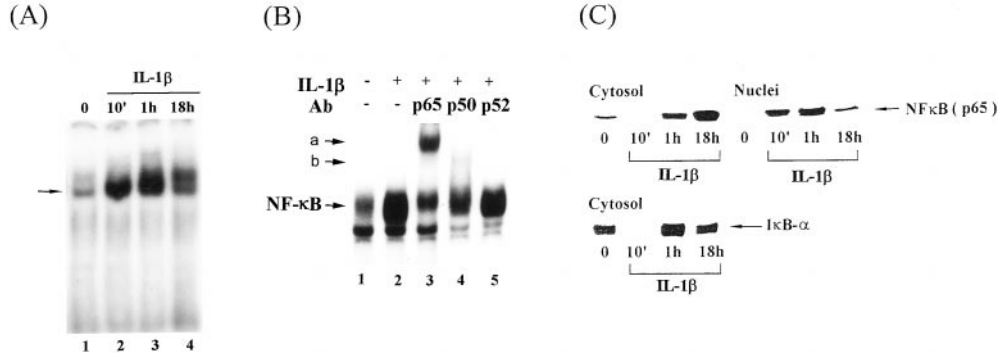

Fig. 8. Kinetics of IL-1β-induced NF-κB-specific DNA-protein complex formation, NF-κB translocation, and IκB-α degradation in A549 epithelial cells. Cell were treated with 1 ng/ml IL-1β for 10 min, 1 h, or 18 h (A), and then cytosolic and nuclear extracts were prepared. In A, NF-κB specific DNA-protein binding activity in nuclear extracts was determined by EMSA as described under *Experimental Procedures*. B, supershift assays were performed using 2 μg of the indicated antibodies as described under *Experimental Procedures*. C, cytosolic and nuclear levels of NF-κB (p65) proteins and cytosolic levels of IκB-α were immunodetected using NF-κB (p65) or IκB-α specific antibodies as described under *Experimental Procedures*.

binding sites (Brown et al., 1993). Supershift assay demonstrated the p65/p50 heterodimer of NF-κB in A549 cells (Fig. 8B). This is different from p65/p65 homodimer and p65/p50 heterodimer in human umbilical vein endothelial cells and human Hep G2 hepatoma cells (Hou et al., 1994; Ledebur and Parks, 1995), p65/p50 and p65/c-Rel in human melanoma cells (Johnke and Johnson, 1995), and p65/p65 and p65/c-Rel in human embryonal kidney cells (Aoudjit et al., 1997).

We demonstrated that four PKC inhibitors (staurosporine, calphostin C, Ro 31-8220, and Go 6976) inhibited the IL-1β-stimulated ICAM-1 expression in a dose-dependent manner, indicating that PKC activation is an obligatory event in IL-1β-mediated ICAM-1 expression in these cells. This was further confirmed by the result that the dominant-negative PKCα mutant PKCα/KR inhibited the IL-1β-induced ICAM-1 promoter activity (Fig. 9D). IL-1β caused PKC activation, this phenomenon occurring after 10-min treatment and being maintained for 4 h. PKC is activated by the physiological activator DAG, which can be generated either directly by the action of PLC or indirectly by a pathway involving the production of phosphatidic acid by PLD, followed by a dephosphorylation reaction catalyzed by phosphatidate phosphohydrolase. Normally, the PLC involved in the production of DAG is PI-PLC, but PC-PLC can also be involved (Exton, 1994; Nishizuka, 1995). The PC-PLC inhibitor D609, but neither the PI-PLC inhibitor U73122 nor the phosphatidate phosphohydrolase inhibitor propranolol, inhibited IL-1β-induced ICAM-1 expression. D609 also completely inhibited IL-1β-stimulated PKC activation (Fig. 3B). Thus, IL-1β acts through the PC-PLC, but not the PI-PLC or PC-PLD, pathway to induce PKC activation in A549 cells. Genistein or tyrphostin

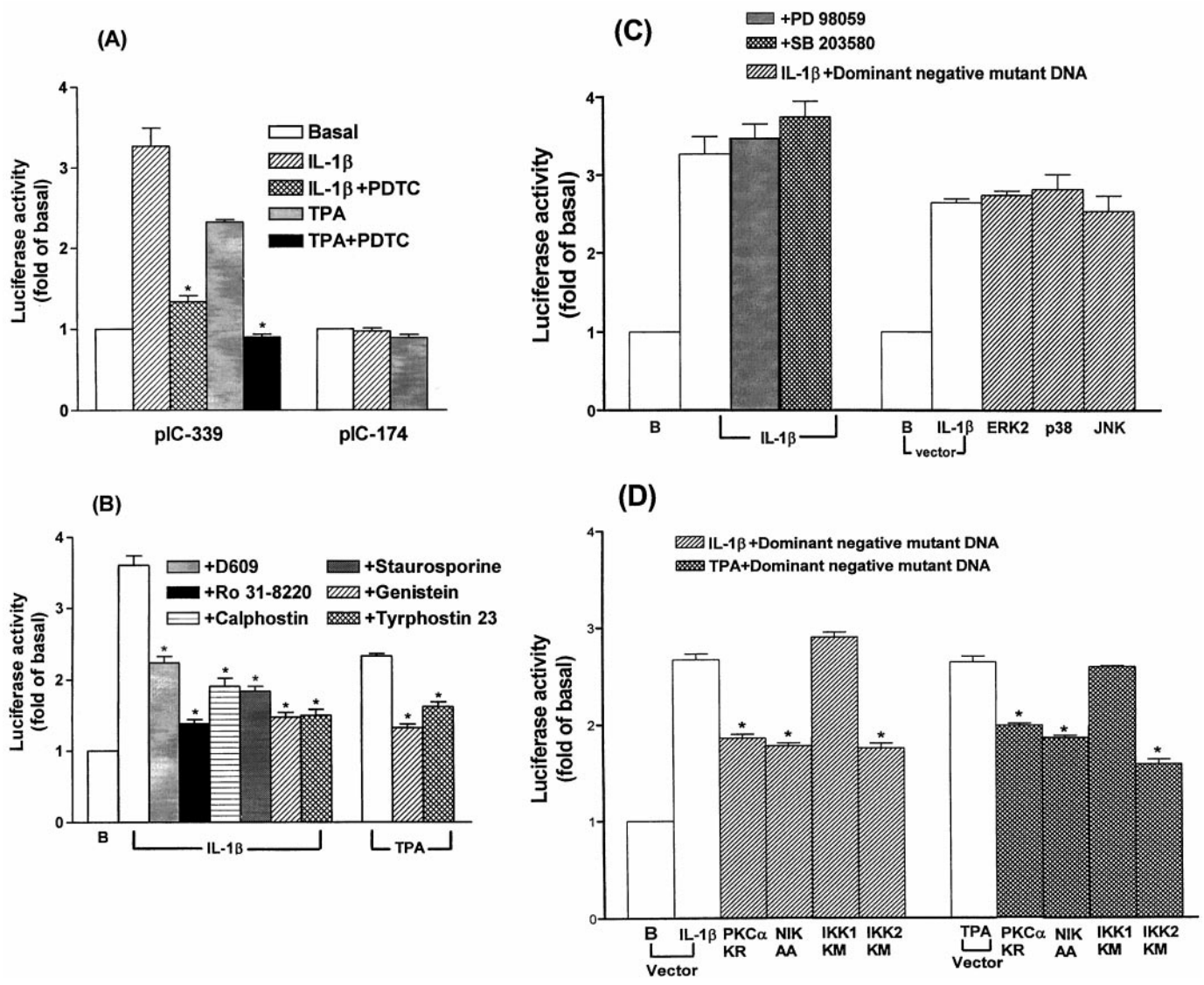

Fig. 9. NF-κB-dependent activation of ICAM-1 promoter by IL-1β and TPA and effects of various inhibitors or dominant-negative mutants. Cells were transfected with pIC-339 (A–D) or pIC-174 (A) of ICAM-1 luciferase expression vector as described under *Experimental Procedures*, and then pretreated with 100 μM PDTC (A), or 200 μM D609, 10 μM Ro 31-8220, 300 nM calphostin C or staurosporine, 100 μM genistein or tyrphostin 23 (B), or 50 μM PD 98059 or SB 203580 (C) for 30 min before incubation with 1 ng/ml IL-1β (A, B, and C) or 1 μM TPA (A and B) for 4.5 h. Dominant-negative mutant for PKCα, NIK, IKK1, or IKK2 (D), or ERK2, p38, or JNK (C) or empty vector was cotransfected with pIC-339 construct. Luciferase activity was assayed as described under *Experimental Procedures*. The results were normalized using the β-galactosidase activity and expressed as the mean ± S.E.M. $^*P < .05$ compared with the IL-1β or TPA alone.

23 did not inhibit IL-1β-induced PKC activation (Fig. 4B), indicating the lack of requirement for an initial protein tyrosine phosphorylation event in the PKC activation process. Tyrosine kinase or PKC inhibitors inhibited the ICAM-1 promoter activity (Fig. 9B), but not NF-κB DNA-protein binding induced by IL-1β (data not shown). These inhibitors are reported to attenuate IL-1β-induced NF-κB transactivation, but not NF-κB nuclear translocation and DNA binding in A549 cells (Bergmann et al., 1998). Because PKC had been shown to be involved in the IL-1β effect, direct activation of PKC by TPA was tested and found to increase ICAM-1 expression, as shown both by ELISA (Fig. 6) and immunofluorescence staining (data not shown). This TPA-induced ICAM-1 expression was inhibited in a dose-dependent manner by genistein and tyrphostin 23 (Fig. 6), as was IL-1β-induced ICAM-1 expression (Fig. 3A). TPA also stimulated NF-κB DNA-protein binding and ICAM-1 promoter activity, and these effects were inhibited by genistein or tyrphostin 23, as was IL-1β-induced activation of NF-κB-specific DNA-protein complex formation and ICAM-1 promoter activity. These results indicated that protein tyrosine kinase might act downstream of PKC to induce NF-κB activation. Further evidences demonstrated this conclusion (see below).

IL-1β activated p44/42 MAPK, p38, and JNK in the present A549 cells. We used the specific MEK inhibitor PD 98059 and the p38 inhibitor SB 203580 to study the relationship between the IL-1β-elicited activation of p44/42 MAPK and p38, and ICAM-1 expression. PD 98059 completely blocked IL-1β-elicited p44/42 MAPK activation, but had no effect on either p38 and JNK activation or IL-1β-induced ICAM-1 expression. Similarly, SB 203580 almost completely inhibited IL-1β-elicited p38 activation, but had no effect on either p44/42 MAPK or JNK activation or IL-1β-induced ICAM-1 expression. The IL-1β-induced NF-κB DNA-protein binding and ICAM-1 promoter activity were not inhibited by PD 98059 or SB 203580, either. Furthermore, dominant-negative mutant for ERK2, p38, or JNK did not affect the induction of ICAM-1 promoter activity by IL-1β. These results indicate that activation of p44/42 MAPK, p38, or JNK pathway is not involved in IL-1β-induced ICAM-1 expression. This notion was further confirmed by the fact that C2

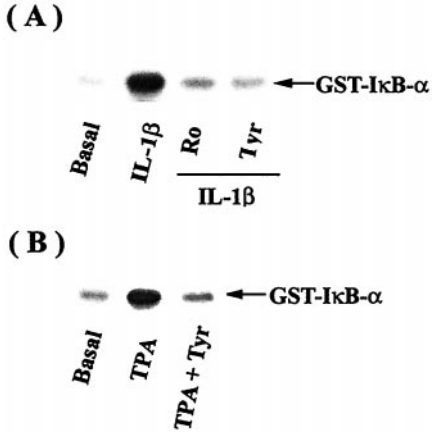

Fig. 10. IL-1β- and TPA-induced IKK activation in A549 epithelial cells. Cell were treated with 1 ng/ml IL-1β (A) or 1 μM TPA (B) for 10 min or pretreated with 10 μM Ro 31-8220 (Ro) or 100 μM tyrphostin 23 (Tyr) for 30 min before addition of IL-1β or TPA. Whole-cell lysates were immunoprecipitated with anti-IKKβ antibody, and autoradiography of phosphorylated GST-IκBα (1–100) was detected as described under *Experimental Procedures*.

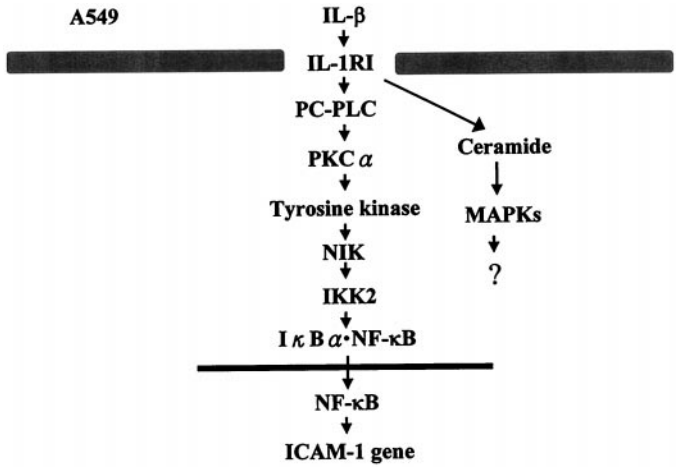

Fig. 11. Schematic representation of the signaling pathway of IL-1β-induced ICAM-1 expression in A549 epithelial cells. IL-1β binds to IL-1RI, and activates PC-PLC to induce PKCα and tyrosine kinase activation. This results in stimulation of NIK and IKK2, and NF-κB in the ICAM-1 promoter, initiating ICAM-1 expression. IL-1β also activates MAPKs via ceramide formation. However, MAPKs are not involved in ICAM-1 expression.

ceramide did not induce ICAM-1 expression or ICAM-1 promoter activity despite activating p44/42 MAPK, p38, and JNK (data not shown). The functional role of IL-1β-induced p44/42 MAPK, p38, and JNK activation in A549 cells is unknown, but might be related to cyclooxygenase-2, inducible nitric-oxide synthase, and IL-6 gene expression, as reported in other cell types (Guan et al., 1998; Larsen et al., 1998; Miyazawa et al., 1998).

In nonstimulated cells, NF-κB dimers are present as cytoplasmic latent complexes due to the binding of specific inhibitors, the IκBs, which mask their nuclear localization signal. Upon stimulation by proinflammatory cytokines, the IκBs are rapidly phosphorylated at two conserved NH_2-terminal serines, this posttranslational modification being rapidly followed by their polyubiquitination and proteasomal degradation (Thanos and Maniatis, 1995; Chen et al., 1996). This results in the unmasking of the nuclear localization signal in NF-κB dimers, which is followed by their translocation to the nucleus, binding to specific DNA sites (κB sites), and targeting gene activation. The protein kinase that phosphorylates IκBs in response to proinflammatory stimuli has been identified biochemically and molecularly (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Named IKK, it exists as a complex, termed the IKK signalsome, which is composed of at least three subunits, IKKα (IKK1), IKKβ (IKK2), and IKKγ (Zandi and Karin, 1999). IKK1 and IKK2 are very similar protein kinases that act as the catalytic subunits of the complex (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). In mammalian cells, IKK1 and IKK2 form a stable heterodimer that is tightly associated with IKKγ, a regulatory subunit (Rothwarf et al., 1998). The IKKs bind NIK (Regnier et al., 1997; Woronicz et al., 1997), a member of MAPK kinase kinase family, that interacts with TRAF6 or TRAF2, thus linking IκB degradation and NF-κB activation to the IL-1 or TNF receptor complex (Malinin et al., 1997). It has been demonstrated that NIK activates and phosphorylates IKK1 in cotransfection experiments, but is unable to phosphorylate IKK2 (Ling et

al., 1998). However, both IKK1 and IKK2 activity are reported to be regulated by NIK (Nakano et al., 1998). Our results showed that IL-1β-induced ICAM-1 promoter activity in A549 cells was inhibited by the dominant-negative mutants for NIK (KKAA) or IKK2(KM) but not by IKK1(KM). This was consistent with the findings that IKK2(KM), IKK2(AA), or IKKβ(KA) has a more pronounced effect than IKK1(KM), IKK1(AA), or IKKα(KA) in inhibiting TNF-α-induced κB-dependent transcription in HeLa and 293 cells (Mercurio et al., 1997; Woronicz et al., 1997). TPA-induced ICAM-1 promoter activity was also inhibited by the dominant-negative mutant NIK(KKAA) or IKK2(KM), but not by IKK1(KM) (Fig. 9D), indicating that NIK and IKK2 were involved in the downstream of PKC activation in ICAM-1 expression induction. However, both IKK1 and IKK2 are involved in cyclooxygenase-2 expression in NCI-H292 cells (Chen et al., 2000). IKK activity was stimulated by both IL-1β and TPA in A549 cells and inhibited by Ro 31-8220 or tyrphostin 23 (Fig. 10), indicating that tyrosine kinase activation occurs downstream of PKC in IKK activation. Similar phenomena have been observed in TNF-α-induced cyclooxygenase-2 expression in NCI-H292 cells (Chen et al., 2000) and this tyrosine kinase has been demonstrated to be Src family member, c-Src or Lyn (unpublished observations). Wild-type NIK induced ICAM-1 promoter activity in A549 cells and this effect was not affected by either Ro 31-8220 or tyrphostin 23 (data not shown), confirming that NIK was involved in the downstream of IL-1β-induced PKC and tyrosine kinase activation. PKC activating IKK2 in 293 cells, and only IKK2 being the target of PKC in T lymphocytes have also been reported (Lallena et al., 1999; Trushin et al., 1999). However, further demonstration of tyrosine kinase and NIK in the downstream of PKC to induce IKK2 activation is shown in this article.

In summary, the signaling pathway involved in IL-1β-induced ICAM-1 expression in human A549 epithelial cells has been explored. IL-1β activates PC-PLC to induce activation of PKCα and protein tyrosine kinase, resulting in the stimulation of NIK, IKK2, and NF-κB in the ICAM-1 promoter, and then initiates ICAM-1 expression. Although IL-1β also induces p44/42 MAPK, p38, and JNK activation, these MAPKs are not involved in this event. A schematic representation of the signaling pathway for the IL-1β-induced ICAM-1 expression in A549 epithelial cells is shown in Fig. 11.

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Send reprint requests to: Dr. Ching-Chow Chen, Institute of Pharmacology, College of Medicine, National Taiwan University, No. 1, Jen-Ai Road, 1st Section, Taipei 10018, Taiwan. E-mail: ccchen@ha.mc.ntu.edu.tw