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Protein Kinase C Enhances Tight Junction Barrier Function of Human Nasal Epithelial Cells in Primary Culture by Transcriptional Regulation[S]

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ABSTRACT

The epithelium of upper respiratory tissues such as human nasal mucosa forms a continuous barrier via tight junctions, which is thought to be regulated in part through a protein kinase C (PKC) signaling pathway. To investigate the mechanisms of the regulation of PKC-mediated tight junction barrier function of human nasal epithelium in detail, primary human nasal epithelial cells were treated with the PKC activator 12-*O*-tetradecanoylophorbol-13-acetate (TPA). In primary human nasal epithelial cells, treatment with TPA led not only to activation of phosphorylation of PKC, myristoylated alanine-rich C kinase substrate, and mitogen-activated protein kinase but also expression of novel PKC-δ, PKC-θ, and PKC-ε. Treatment with TPA increased transepithelial electrical resistance, with tight junction barrier function more than 4-fold that of the control, together with up-regulation of tight junction proteins, occludin, zona occludens (ZO)-1, ZO-2 and claudin-1 at the transcriptional level. Furthermore, it affected the subcellular localization of the tight junction proteins and the numbers of tight junction strands. The up-regulation of barrier function and tight junction proteins was prevented by a pan-PKC inhibitor, and the inhibitors of PKC-δ and PKC-θ but not PKC-ε. In primary human nasal epithelial cells, transcriptional factors GATA-3 and -6 were detected by reverse transcription-polymerase chain reaction. The knockdown of GATA-3 using RNA interference resulted in inhibition of up-regulation of ZO-1 and ZO-2 by treatment with TPA. These results suggest that TPA-induced PKC signaling enhances the barrier function of human nasal epithelial cells via transcriptional up-regulation of tight junction proteins, and the mechanisms may contribute to a drug delivery system.

The epithelial barrier of the upper respiratory tract, which is the first site of exposure to inhaled antigens, plays a crucial role in host defense in terms of innate immunity. The epithelium of the upper respiratory tract, such as that of the nasal mucosa, forms a continuous barrier against a wide variety of exogenous antigens (Herard et al., 1996; van Kempen et al., 2000). The epithelial barrier is regulated in large part by the apical-most intercellular junctions, referred to as tight junctions (Takano et al., 2005; Koizumi et al., 2007; Kurose et al., 2007).

Tight junctions, the most apical component of intercellular junctional complexes, separate the apical from the basolateral cell surface domains to establish cell polarity (performing the function of a fence). Tight junctions also possess a barrier function, inhibiting the flow of solutes and water through the paracellular space (Gumbiner, 1993). They form a particular netlike meshwork of fibrils created by the integral membrane proteins occludin and claudin and members

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ABBREVIATIONS: ZO, zona occludens; PKC, protein kinase C; DAG, diacylglycerol; nPKC, novel PKC; TPA, 12-*O*-tetradecanoylophorbol-13-acetate; MARCKS, myristoylated alanine-rich protein kinase C substrate; MAPK, mitogen-activated protein kinase; GF109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride; PD98059, 2′-amino-3′-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; PI3K, phosphatidylinositol 3-kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; EGFR, epidermal growth factor receptor; PD153035, 4-((3-bromophenyl)amino)-6,7-dimethoxyquinazoline; PBS, phosphate-buffered saline; siRNA, small interfering RNA; RT-PCR, reverse transcription-polymerase chain reaction; TER, transepithelial electrical resistance; FITC, fluorescein isothiocyanate.

of the Ig superfamilies junctional adhesion molecule (JAM) and Coxsackie adenovirus receptor (CAR) (Tsukita et al., 2001; Sawada et al., 2003; Schneeberger and Lynch, 2004). Several peripheral membrane proteins, ZO-1, ZO-2, ZO-3, 7H6 antigen, cingulin, symplekin, Rab3B, Ras target AF-6, and ASIP, an atypical protein kinase C-interacting protein, have been reported (Tsukita et al., 2001; Sawada et al., 2003; Schneeberger and Lynch, 2004). More recently, tricellulin was identified at tricellular contacts at which there are three epithelial cells and the tricellulin has a barrier function (Ikenouchi et al., 2005). Furthermore, ZO-1 and ZO-2 can independently determine whether and where claudins are polymerized (Umeda et al., 2006).

Protein kinase C (PKC) is a family of serine-threonine kinases known to regulate epithelial barrier function (Tsukamoto and Nigam, 1999; Andreeva et al., 2001; Seth et al., 2007). PKC has been shown to induce both assembly and disassembly of tight junctions depending on the cell type and conditions of activation (Stuart and Nigam, 1995; Andreeva et al., 2001). The activation of PKC causes an increase in permeability in the renal epithelial cell lines LLC-PK1 and Madin-Darby canine kidney (Ellis et al., 1992; Clarke et al., 2000), whereas it causes a decrease in permeability in the human colon carcinoma cell line HT29 (Sjö et al., 2003). Bryostatin enhances tight junction barrier function in T84 cells through a PKC signaling pathway (Yoo et al., 2003). PKC seems to regulate the subcellular localization, phosphorylation states, and transcription of several tight junction-associated proteins (Banan et al., 2005), although the isozyme specificity has not been clearly elucidated. At least 11 different isozymes of PKC are known. These can be subdivided in three classes according to their responsiveness to activators (Newton, 1997). The classic or conventional isozymes (α, βI, βII, and γ) are both Ca^{2+}- and diacylglycerol (DAG)-dependent. The novel (nPKC) isozymes (δ, ε, θ, η, and μ) are Ca^{2+}-independent but DAG-dependent. The atypical isozymes (ι/λ and ζ) are neither Ca^{2+}- nor DAG-dependent. In the human intestinal epithelial cell lines HT-29 and Caco-2, stimulation with TLR2 ligands leads to activation of specific PKC isoforms PKC-α and PKC-δ and enhances barrier function through translocation of ZO-1 on activation (Cario et al., 2004).

Furthermore, activation of PKC by 12-*O*-tetradecanoylophorbol-13-acetate (TPA) causes increases in transcription of occludin, ZO-1, and claudin-1 in T84 cells and melanoma cells (Weiler et al., 2005; Leotlela et al., 2007). The claudin-2 promoter is activated by CDX2, HNF-1α, and GATA-4 in a cooperative manner (Escaffit et al., 2005). Although activation of PKC exerts its effect directly at the transcriptional level, the responsible transcription factors related to PKC activation remain unknown.

We reported previously that in the epithelium of human nasal mucosa from patients with allergic rhinitis, occludin, JAM-A, ZO-1, and claudin-1, -4, -7, -8, -12, -13, and -14 were detected together with continuous tight junction strands that formed well developed networks (Takano et al., 2005). However, the detailed mechanisms of regulation of tight junctions in human nasal epithelial cells remain unclear. In this study, to investigate the mechanisms of regulation of tight junctions through a PKC signaling pathway, primary cultures of human nasal epithelial cells were treated with TPA as a PKC activator. We found that short treatment with TPA greatly enhanced epithelial barrier function together with an increase in expression of occludin, ZO-1, ZO-2, and claudin-1 at the transcriptional level. When we focused on the transcriptional factor GATA family to investigate the transcriptional mechanisms, up-regulation of ZO-1 and ZO-2 by treatment with TPA was regulated by GATA-3 via a PKC signaling pathway.

Materials and Methods

Antibodies and Inhibitors. Rabbit polyclonal anti-occludin, anti-ZO-1, anti-ZO-2, anti-claudin-1, anti-claudin-7, and mouse monoclonal anti-occludin and anti-claudin-4 antibodies were obtained from Zymed Laboratories (South San Francisco, CA). Rabbit polyclonal anti-phospho-pan-PKC, anti-phospho-MARCKS, and anti-phospho-MAPK (Thr202/Tyr204) and mouse monoclonal anti-phosphothreonine antibodies were obtained from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal anti-PKC-α, anti-PKC-γ, anti-PKC-δ, anti-PKC-θ, anti-PKC-ε, and mouse monoclonal anti-phosphoserine antibodies were obtained from BD Pharmingen (San Diego, DA). Rabbit polyclonal anti-extracellular signal-regulated kinase 1/2 antibodies were obtained from Promega (Madison, WI.). Rabbit polyclonal anti-actin was obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal anti-panPKC and mouse anti-GATA-3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

PKC inhibitor GF109203X, MAPK inhibitors PD98059 and U0126, p38 MAPK inhibitor SB203580, PI3K inhibitor LY294002, epidermal growth factor receptor (EGFR) inhibitor PD153035, PKC-δ inhibitor rottlerin, PKC-θ inhibitor myristoylated PKC-θ pseudosubstrate peptide inhibitor, and PKC-ε inhibitor PKC-ε translocation inhibitor peptide were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA).

Human Nasal Mucosa Tissues. Human nasal mucosa tissues were obtained from patients with hypertrophic rhinitis and chronic sinusitis who underwent inferior nasal turbinectomy. Informed consent was obtained from all patients, and the study was approved by the ethics committee of Sapporo Medical University, the Sapporo Hospital of the Hokkaido Railway Company, and the KKR Sapporo Medical Center Tonan Hospital.

Isolation and Cell Culture. Human nasal mucosa tissues were minced into pieces 2 to 3 mm^3 in volume and washed with phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza Walkersville, Walkersville, MD) four times. These tissue specimens were suspended in 10 ml of dispersing solution with 0.5 μg/ml DNase I (Sigma) and 0.08 mg/ml Liberase Blenzyme 3 (Roche, Basel, Switzerland) in PBS and then incubated at 37°C for 20 min. The dissociated specimens were subsequently filtrated with 300-μm mesh followed by filtration with 40-μm mesh. After centrifugation at 1000*g* for 4 min, the cells were cultured in serum-free bronchial epithelial basal medium (Lonza Walkersville) supplemented with 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 μg/ml triiodothyronine, 50 μg/ml gentamicin, 50 μg/ml amphotericin B, 0.1 ng/ml retinoic acid, and 0.5 ng/ml epidermal growth factor (Lonza Walkersville), bovine pituitary extract [1% (v/v); Pel-Freez Biologicals, Rogers, AR], 100 U/ml penicillin, and 100 μg/ml streptomycin. The isolated human nasal epithelial cells were plated with modified bronchial epithelial basal medium containing 10% fetal bovine serum (PAA Laboratories, Etobicoke, ON, Canada) on 35- or 60-mm culture dishes (Corning Life Sciences, Acton, MA), which were coated with rat tail collagen (500 μg of dried tendon/ml of 0.1% acetic acid) in a humidified, 5% CO_2/95% air incubator at 37°C (Koizumi et al., 2007; Kurose et al., 2007). The first-passaged cells using 0.05% trypsin-EDTA (Sigma) were used at day 7 after plating for the experiments.

The cells were treated with 10 or 100 nM TPA (Sigma) for 6 h. Some cells were treated with 2 μM GF109203, 10 μM PD98059, 20

μM U0126, 2 μM PD153035, 10 μM SB203580, 10 μM LY294002, 15 μM rottlerin, 5 μM myristoylated PKC-θ pseudosubstrate peptide inhibitor, or 10 μM PKC-ε translocation inhibitor peptide for 2 h before treatment with 100 nM TPA.

Transfection with siRNA. Stealth siRNA duplex oligonucleotides against human GATA-3 were synthesized by Invitrogen (Carlsbad, CA). The sequences were as follows: GATA-3 RNA interference: sense (5′-AUAUUGUGAAGCUUGUAGUAGAGCC-3′) and antisense (5′-GGCUCUACUACAAGCUUCACAAUAU-3′). The cells were passaged at subconfluence and plated 1 day before transfection. The cells were transfected with 100 pM siRNA of GATA-3 using Lipofectamine RNAiMAX (Invitrogen). At 48 h after transfection, the cells were treated with 100 nM TPA for 1 h and examined for Western blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses.

Measurement of Transepithelial Electrical Resistance. The cells were cultured to confluence on inner chambers of 12-mm Transwell inserts with 0.4-μm pore-size filters (Corning Life Sciences). TER was measured using an EVOM voltameter with an ENDOHM-12 (World Precision Instruments, Sarasota, FL) on a heating plate (Fine, Tokyo, Japan) adjusted to 37°C. The values are expressed in standard units of ohms per square centimeter and presented as the mean ± S.D. For calculation, the resistance of blank filters was subtracted from that of filters covered with cells.

Measurement of Permeability. To determine the paracellular flux, the cells were cultured on 12-mm Transwell, 0.4-μm pore-size filters, and then FITC-labeled dextran (MW: 70 kDa)-containing medium was added to the inner chamber. Samples were collected from the outer chamber at 15, 30, 60, and 120 min and were measured with a Wallac 1420 multilabeled counter (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Western Blot Analysis. The cells grown on the inner chambers were scraped in 300 μl of buffer (1 mM $NaHCO_3$ and 2 mM phenylmethylsulfonyl fluoride), collected in microcentrifuge tubes, and then sonicated for 10 s. The protein concentrations of the samples were determined using a BCA Protein Assay Reagent Kit (Pierce Chemical Co, Rockford, IL). Aliquots of 15 μg of protein/lane for each sample were separated by electrophoresis in 4/20% SDS polyacrylamide gels (Daiichi Pure Chemicals Co., Tokyo, Japan). After electrophoretic transfer to a nitrocellulose membrane (Immobilon; Millipore, Billerica, MA), the membrane was saturated for 30 min at room temperature with blocking buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Tween 20, and 4% skim milk) and incubated with anti-phospho-pan-PKC, anti-phospho-MARCKs, anti-phospho-MAPK, anti-extracellular signal-regulated kinase 1/2, anti-PKC-α, anti-PKC-γ, anti-PKC-δ, anti-PKC-θ, anti-PKC-ε, anti-occludin, anti-ZO-1, anti-ZO-2, anti-claudin-1, anti-claudin-4, anti-claudin-7, anti-GATA3, and anti-actin antibodies at room temperature for 1 h. The membrane was incubated with horseradish peroxidase-conjugated anti-rabbit or mouse IgG (Dako Denmark A/S, Copenhagen, Denmark) at room temperature for 1 h. The immunoreactive bands were detected using an ECL Western blotting system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Immunoprecipitation. The dishes were washed with PBS twice, and 300 μl of Nonidet P-40 lysis buffer (50 mM Tris-HCl, 2% Nonidet P-40, 0.25 mM sodium deoxycholate, 150 mM NaCl, 2 mM EGTA, 0.1

TABLE 1
PCR primers

Gene	Primer Sequence	Product Size
		bp
Occludin		189
Forward	TCAGGGAATATCCACCTATCACTTCAG	
Reverse	CATCAGCAGCAGCCATGTACTCTTCAC	
ZO-1		712
Forward	CGGTCCTCTGAGCCTGTAAG	
Reverse	GGATCTACATGCGACGACAA	
ZO-2		220
Forward	CGGATTCCAGACAAGGTGTT	
Reverse	CCTTCAGAGACCCAGACTGC	
Claudin-1		593
Forward	AACGCGGGGCTGCAGCTGTTC	
Reverse	GGATAGGGCCTTGGTCTTGGGT	
Claudin-4		249
Forward	AGCCTTCCAGGTCCTCAACT	
Reverse	AGCAGCGAGTCGTACACCTT	
Claudin-7		252
Forward	AGGCATAATTTTCATCGTGG	
Reverse	GAGTTGGACTTAGGGTAAGAGCG	
GATA-1		377
Forward	TCAATTCAGCAGCCTATTCC	
Reverse	TTCGAGTCTGAATACCATCC	
GATA-2		279
Forward	TGTTGTGCAAATTGTCAGACG	
Reverse	CATAGGTGCCATGTGTCCAGC	
GATA-3		251
Forward	AAGTGCATGACTCACTGGAGG	
Reverse	TAGGCTTCATGATACTGCTCC	
GATA-4		263
Forward	CTGGCCTGTCATCTCACTACG	
Reverse	GGTCCGTGCAGGAATTTGAGG	
GATA-5		290
Forward	TCGCCAGCACTGACAGCTCAG	
Reverse	TGGTCTGTTCCAGGCTGTTCC	
GATA-6		300
Forward	TTCTAACTCAGATGATTGCAGC	
Reverse	GCTGCACAAAAGCAGACACG	
Glucose-3-phosphate dehydrogenase		452
Forward	ACCACAGTCCATGCCATCAC	
Reverse	TCCACCACCCTGTTGCTGTA	

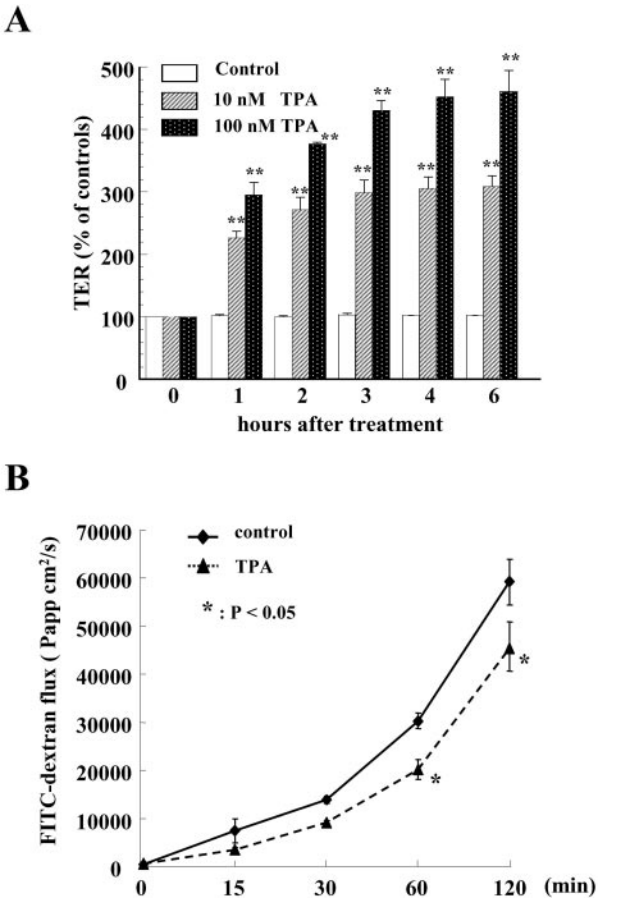

Fig. 1. Barrier function measured as TER A, and paracellular flux using FITC-dextran (70 kDa) in primary human nasal epithelial cells treated with TPA. A, the TER was markedly increased beginning from 1 to 6 h after treatment with 10 or 100 nM TPA in a dose-dependent manner. n = 6. **, $p < 0.01$ versus control. B, paracellular flux of FITC-dextran of 70 kDa was significantly decreased after treatment with 100 nM TPA compared with the control. n = 6. *, $p < 0.05$ versus control.

mM Na_3VO_4, 10 mM NaF, and 2 mM phenylmethylsulfonyl fluoride) was added to 60-mm dishes. The cells were scraped and collected in microcentrifuge tubes and then sonicated for 10 s. Cell lysates were incubated with protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden) for 1 h at 4°C and then clarified by centrifugation at 15,000*g* for 10 min. The supernatants were incubated with the polyclonal anti-occludin antibody bound to protein A-Sepharose CL-4B overnight at 4°C. After incubation, immunoprecipitates were washed extensively with the same lysis buffer and were subjected to Western blot analysis using anti-phosphoserine and anti-phosphothreonine antibodies.

RNA Isolation and RT-PCR Analysis. Total RNA was extracted and purified using TRIzol (Invitrogen). One microgram of total RNA was reverse-transcribed into cDNA using a mixture of oligo (dT) and Superscript II reverse transcriptase under the recommended conditions (Invitrogen). Synthesis of each cDNA was performed in a total volume of 20 μl for 50 min at 42°C and terminated by incubation for 15 min at 70°C. PCR containing 100 pM primer pairs and 1.0 ml of the 20-ml total RT reaction mixture was performed in 20 ml of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, and 0.5 U of *Taq* DNA polymerase (Takara, Kyoto, Japan), employing 25 or 30 cycles with cycle times of 15 s at 96°C, 30 s at 55°C, and 60 s at 72°C. Final elongation time was 7 min at 72°C. Ten microliters of the 20-μl total PCR reaction mixture was analyzed in 1% agarose gel after staining with ethidium bromide. The PCR primers used to detect occludin, ZO-1, ZO-2, claudin-1, -4, -7, GATA-1, -2, -3, -4, -5, -6, and glucose-3-phosphate dehydrogenase are indicated in Table 1.

Immunocytochemistry. The cells grown on inner chambers were fixed with ice-cold absolute acetone or a 1:1 ethanol/acetone mixture at −20°C for 10 min. The cells were stained with anti-occludin, anti-ZO-1, anti-ZO-2 and anti-claudin-1 antibodies overnight at 4°C. Alexa Fluor 488 (green)-conjugated anti-rabbit IgG and Alexa Fluor 592 (red)-conjugated anti-mouse IgG (Invitrogen) were used as secondary antibodies. The specimens were examined and photographed with an Axioskop 2 plus microscope (Carl Zeiss, Jena, Germany) and confocal laser scanning microscope (MRC 1024; Bio-Rad Laboratories, Hercules, CA). Phase-contrast photomicrographs were taken with a Zeiss Axiovert 200 inverted microscope.

Freeze-Fracture Analysis. For freeze-fracture experiments, primary cultured human nasal epithelial cells grown on 60-mm dishes were centrifuged into pellets and then immersed in 40% glycerin solution after fixation in 2.5% glutaraldehyde in 0.1 M PBS, pH 7.3. The specimens were mounted on a copper stage, frozen in liquid nitrogen, fractured at −150°C- −160°C, and replicated by platinum/carbon from an electron beam gun positioned at a 45° angle, followed by carbon applied from overhead in a JFD-7000 freeze-fracture device (JEOL, Tokyo, Japan). After the replicas were thawed, they were floated on filtered 10% sodium hypochlorite solution for 10 min in a Teflon dish. Replicas were washed in distilled water for 30 min, mounted on copper grids, and examined at 80 kV using a JEOL 1200EX transmission electron microscope. Morphometric analysis was performed on freeze-fracture replica images of tight junctions, which were printed at a final magnification of 20,000×. The mean number of tight junction strands was determined by taking numer-

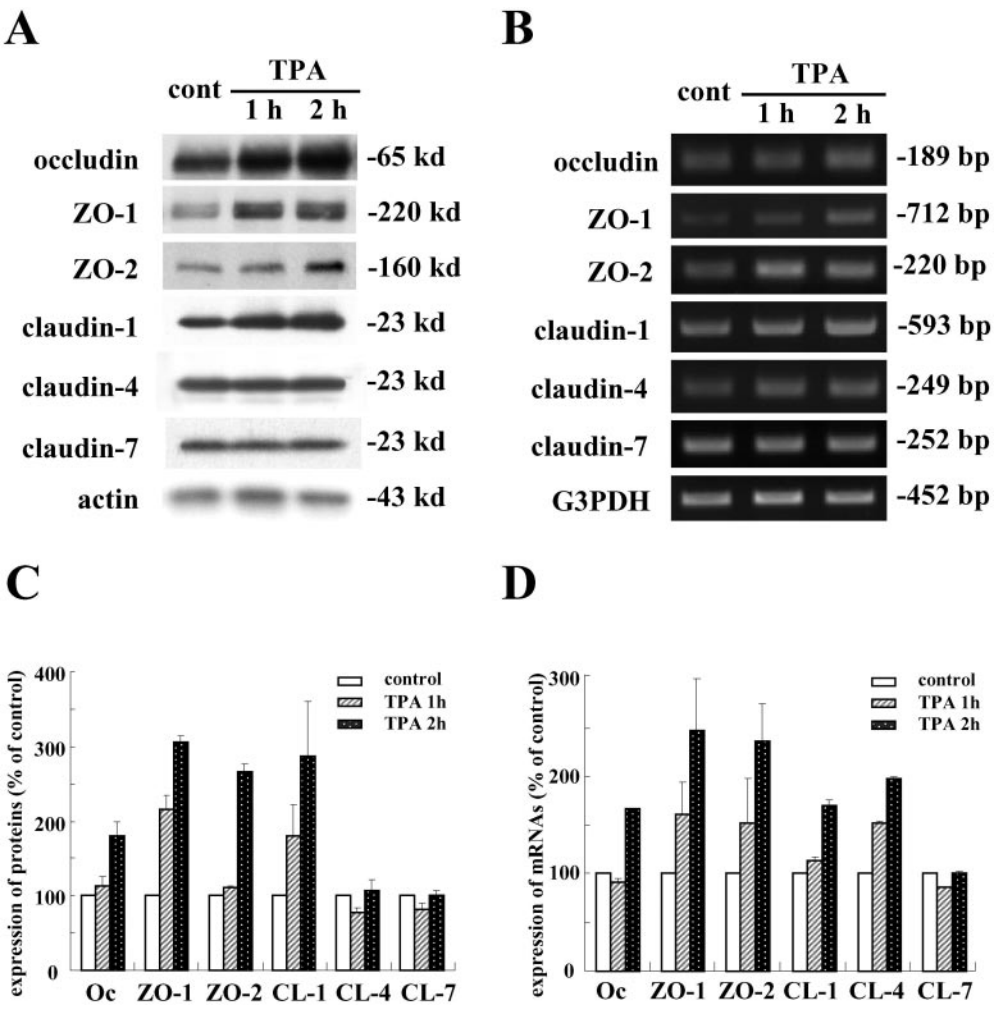

Fig. 2. Western blotting (A) and RT-PCR (B) for occludin, ZO-1, ZO-2, claudin-1, -4, and -7 in primary human nasal epithelial cells at 1 and 2 h after treatment with 100 nM TPA. A, up-regulation of proteins of occludin, ZO-1, ZO-2, and claudin-1, but not claudin-4 and -7, is observed at 1 and 2 h after treatment with 100 nM TPA. B, up-regulation of mRNAs of occludin, ZO-1, ZO-2, claudin-1 and -4, but not claudin-7, is observed at 1 and 2 h after treatment with 100 nM TPA. C and D, the corresponding expression levels are shown as bar graphs.

ous counts along a line drawn perpendicular to the junctional axis at 200-nm intervals (Stevenson et al., 1988).

Data analysis. Signals were quantified using Scion Image Beta 4.02 Win (Scion Corporation, Frederick, MD). Each set of results shown is representative of three separate experiments. Results are given as means ± S.E.M. Differences between groups were tested by the two-tailed Student's *t* test for unpaired data.

Results

TPA Enhances Tight Junction Barrier Function in Primary Cultures of Human Nasal Epithelial Cells. PKC is a family of serine-threonine kinases known to regulate epithelial barrier function (Tsukamoto and Nigam, 1999; Andreeva et al., 2001; Seth et al., 2007). To investigate effects of the PKC activator TPA on tight junction barrier function of human nasal epithelial cells, primary cultures of human nasal epithelial cells at day 7 after plating were treated with 10 or 100 nM TPA and then examined for TER and paracellular flux of FITC-labeled dextran (molecular mass, 70 kDa).

In primary human nasal epithelial cells cultured with 10% fetal bovine serum at day 7 after plating, the maximum value of TER was 200 ± 20 Ω/cm^2. The TER was markedly increased beginning from 1 h after treatment with 10 or 100 nM TPA in a dose-dependent manner (Fig. 1A). The TER values from 2 to 6 h after treatment with 100 nM TPA were more than 4-fold those of the control (Fig. 1A). The TER value at 24 h after treatment with 10 or 100 nM TPA recovered to the level of the control (data not shown).

When the paracellular flux of FITC-labeled dextran (70 kDa) was measured in the cells at 2 h after treatment with

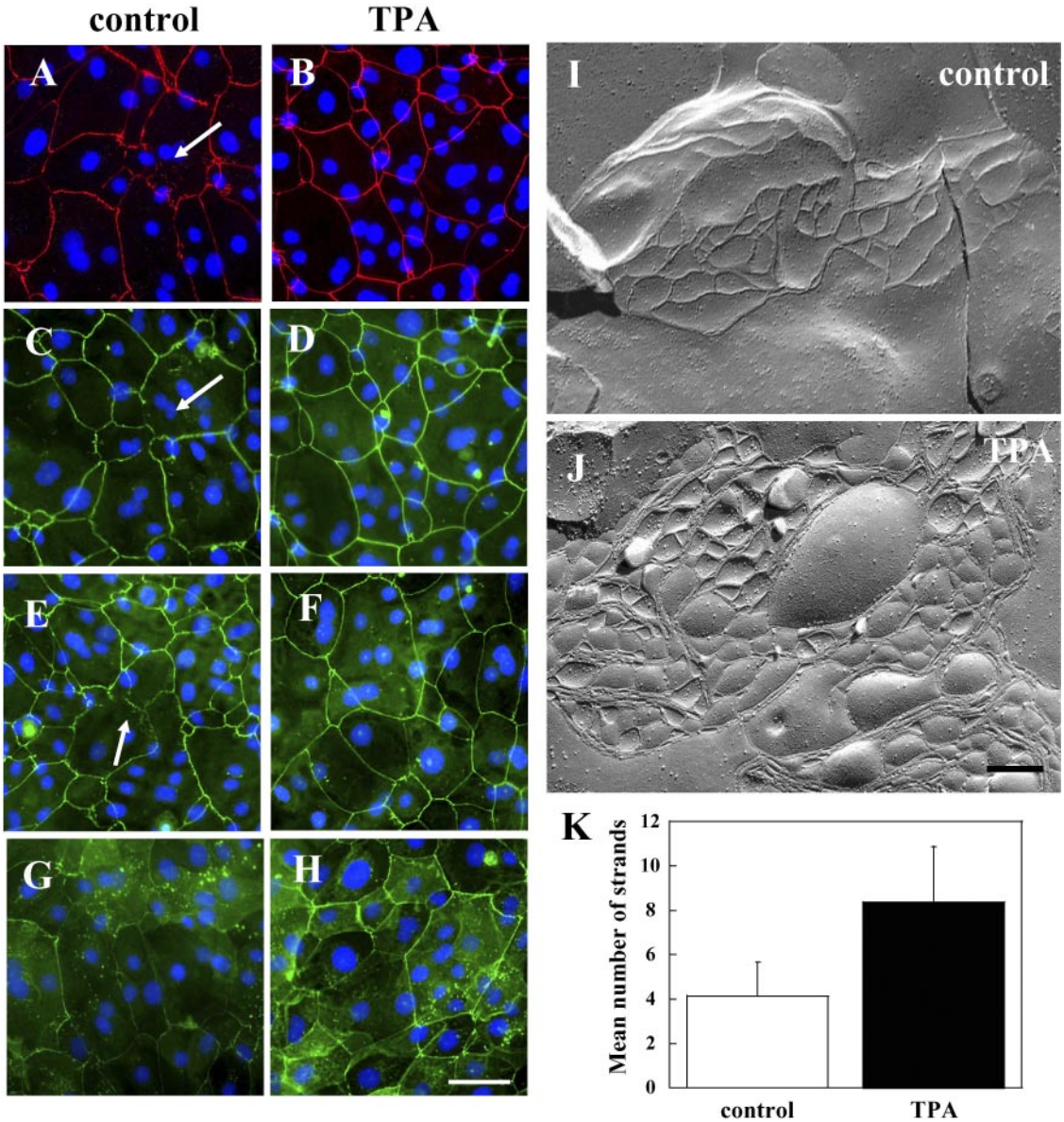

Fig. 3. Immunocytochemistry for occludin (A and B), ZO-1 (C and D), ZO-2 (E and F), and claudin-1 (G and H) in primary human nasal epithelial cells treated with (B, D, F, and H) and without (A, C, E, and G) TPA. In the cells after treatment with TPA, expression of occludin, ZO-1, ZO-2, and claudin-1 is observed as distinct continuous lines at cell borders, whereas in the control, discontinuous lines of occludin, ZO-1, and ZO-2 and weak expression of claudin-1 are observed in some cells. Bar in H, 20 μm. Freeze-fracture replicas of primary human nasal epithelial cells treated with (J) and without (I) TPA. Bar in J, 50 nm. K, morphometric analysis of tight junction strands is shown as a bar graph. In the cells after treatment with TPA, the numbers of tight junction strands are increased compared with control.

100 nM TPA, in which the TER was more than 4-fold compared with the control, the paracellular flux after treatment with TPA was significantly decreased compared with the control (Fig. 1B). The paracellular flux at 2 h after treatment with 10 nM TPA was also decreased compared with the control and similar to that of 100 nM TPA (Supplemental Fig. 1)

Increase of Tight Junction Proteins at Levels of Protein and mRNA by Treatment with TPA. We previously reported expression patterns of tight junction proteins in primary cultures of human nasal epithelial cells using this experiment (Koizumi et al., 2007; Kurose et al., 2007). In primary human nasal epithelial cells at 1 and 2 h after treatment with 100 nM TPA, in which a marked increase of TER was observed (Fig. 1A), we investigated the changes in expression of tight junction proteins occludin, ZO-1, ZO-2, claudin-1, -4, and -7 using Western blot and RT-PCR analyses. In Western blotting, up-regulation in proteins of occludin, ZO-1, ZO-2, and claudin-1, but not claudin-4 and -7, was observed at 1 and 2 h after treatment with TPA (Fig. 2, A to C). In RT-PCR, up-regulation of mRNAs of occludin, ZO-1, ZO-2, claudin-1 and -4, but not claudin-7, was observed at 1 and 2 h after treatment with 100 nM TPA (Fig. 2, B and D). No changes in expression of claudin-2, -8, -9, and -12 and JAM-A, which were detected in primary cultures of human nasal epithelial cells, were observed (data not shown).

Changes in Distribution of Tight Junction Proteins by Treatment with TPA. To investigate changes in localization of tight junction proteins in the cells at 2 h after treatment with 100 nM TPA, we performed immunocytochemistry for occludin, ZO-1, ZO-2, and claudin-1. In the control at day 5 after plating, discontinuous lines of occludin, ZO-1, and ZO-2 and weak expression of claudin-1 were observed in some cells (Fig. 3A, C, E, and G). In the cells after treatment with TPA, expression of occludin, ZO-1, ZO-2, and claudin-1 was observed as continuous wide lines at cell borders (Fig. 3, B, D, F, and H).

Changes of Tight Junction Strands Induced by Treatment with TPA. To investigate changes of tight junction strands in the cells at 2 h after treatment with 100 nM TPA, we performed freeze-fracture analysis. In the control, a network composed of several continuous tight junction strands was observed (Fig. 3I). In the cells after treatment with TPA, the mean number of tight junction strands was increased compared with the control and a well developed network of tight junction strands was observed (Fig. 3, J and K).

Changes of PKC- and MAPK-Associated Proteins Induced by Treatment with TPA. There are at least 11 different isozymes of PKC classified into three broad groups (Newton, 1997). To investigate which specific PKC isozymes affect expression and function of tight junction proteins, we performed Western blotting to examine the expression of the five PKC isoforms PKC-α, PKC-γ, PKC-δ, PKC-θ, and PKC-ε and the phosphorylation status of PKC, MARCKs, and MAPK in the cells at 1 and 2 h after treatment with 100 nM TPA. In the cells at 1 and 2 h after treatment with TPA, increases in expression of PKC-δ, PKC-θ, and PKC-ε and activation of phosphorylation of panPKC, MARCKS, and MAPK were observed (Fig. 4, B and D).

PKC Inhibitors but Not MAPK and EGFR Inhibitors Prevent Increase of Barrier Function Induced by Treatment with TPA. TPA is known to induce phosphorylation of MAPK, not only through phosphorylation of PKC but also through EGFR (Barbosa et al., 2003). TPA is involved in multiple signal transduction pathways, including c-Jun NH_2-terminal kinase and PI3K (Huang et al., 1997; Yu et al., 2006). To investigate which signaling pathways af-

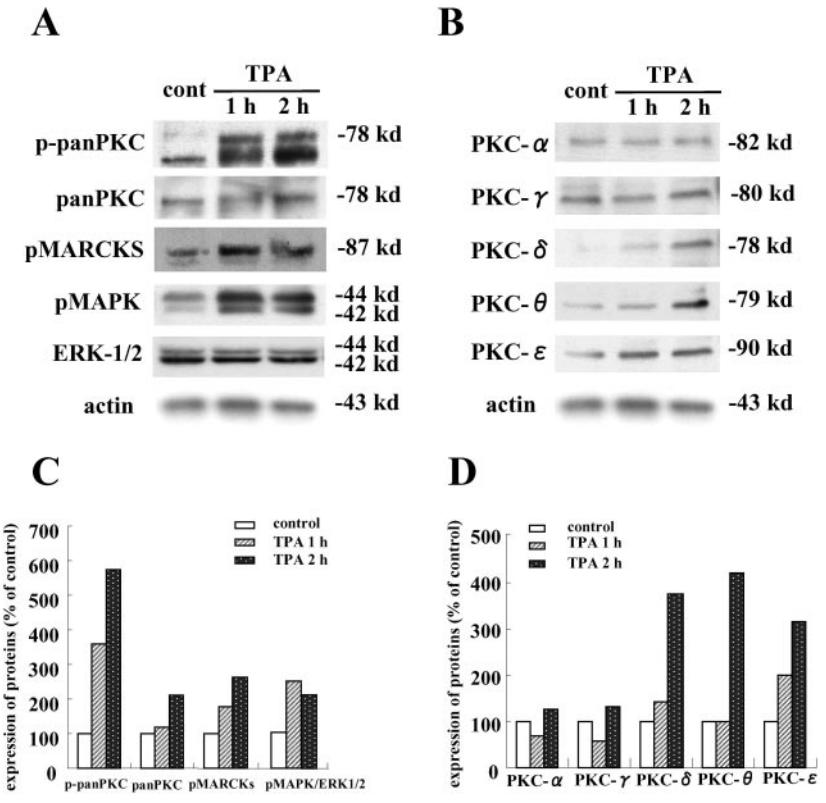

Fig. 4. A, Western blotting for phospho-pan-PKC, phospho-MARCKS, and phospho-MAPK in primary human nasal epithelial cells at 1 and 2 h after treatment with 100 nM TPA. In the cells at 1 and 2 h after treatment with TPA, activation of phosphorylation of pan-PKC, MARCKS, and MAPK is observed. B, Western blotting for PKC-α, PKC-γ, PKC-δ, PKC-θ, and PKC-ε in primary human nasal epithelial cells at 1 and 2 h after treatment with 100 nM TPA. In the cells at 1 and 2 h after treatment with TPA, increases in expression of PKC-δ, PKC-θ, and PKC-ε are observed. C and D, the corresponding expression levels are shown as bar graphs.

fected the increase of barrier function measured as TER in the cells after treatment with TPA, we used PKC inhibitor GF109203X, MAPK inhibitors PD98059 and U0126, and EGFR inhibitor PD153035. Treatment with GF109203X but not with PD98059, U0126, or PD153035 completely prevented the marked increase of TER values at 2 h after treatment with 100 nM TPA (Fig. 5A, Supplemental Fig. 1). Although the p38 MAPK inhibitor SB203580 and the PI3K inhibitor LY294002 prevented up-regulation of TER after treatment with TPA, the effect of the inhibition was weak (Fig. 5B).

PKC Inhibitor Prevents an Increase in Expression of Tight Junction Proteins by Treatment with TPA. We investigated changes in expression of tight junction proteins and PKC isozymes in the cells treated with the PKC inhibitor GF109203X before pretreatment with TPA. Treatment with GF109203X inhibited up-regulation of occludin, ZO-1, ZO-2, and claudin-1 at the levels of protein and mRNA at 2 h after treatment with 100 nM TPA. (Fig. 6, A–D). Furthermore, the increase of claudin-4 mRNA after treatment with TPA was also inhibited by treatment with GF109203X (Fig. 6, B and D). Treatment with GF109203X inhibited increases in expression of PKC-δ, PKC-θ, and PKC-ε at 2 h after treatment with 100 nM TPA (Fig. 7, A and C).

Furthermore, we investigated changes in expression of tight junction proteins in the cells treated with the PKC-δ inhibitor rottlerin, the PKC-θ inhibitor myristoylated PKC-θ pseudosubstrate peptide inhibitor, and the PKC-ε inhibitor PKC-ε translocation inhibitor peptide before pretreatment with TPA. Treatment with inhibitors of PKC-δ and PKC-θ but not PKC-ε prevented up-regulation of occludin, ZO-1, ZO-2 and claudin-1 at 2 h after treatment with 100 nM TPA. (Fig. 7, B and D).

Transcriptional Factor GATA-3 Up-Regulates Tight Junction Proteins after Treatment with TPA. Tight junction proteins are regulated by various transcription factors in a cooperative manner (Escaffit et al., 2005). Transcriptional factor GATA-3 is associated with differentiation of the luminal cells in the mammary gland (Kouros-Mehr et al., 2006; Asselin-Labat et al., 2007). When we investigated expression of GATA-1, -2, -3, -4, -5, and -6 in primary cultures of human nasal epithelial cells by RT-PCR, mRNAs of GATA-3 and -6 were detected (Fig. 8A). Up-regulation of mRNAs of GATA-3 and -6 was observed at 1 h but not 2 h after treatment with 100 nM TPA (Fig. 8A). Treatment with the PKC inhibitor GF109203X prevented up-regulation of mRNAs of GATA-3 but not GATA-6 at 1 h after treatment with 100 nM TPA (Fig. 8B).

To investigate whether GATA-3 was an essential transcriptional factor for up-regulation of tight junction proteins in the cells after treatment with TPA, GATA-3 was down-regulated by its siRNA of GATA-3 before treatment with 100 nM TPA. Transfection with siRNA of GATA-3 decreased expression of GATA-3 at the protein and mRNA levels and prevented up-regulation of proteins and mRNAs of ZO-1 and ZO-2 but not occludin and claudin-1 at 1 h after treatment with 100 nM TPA (Fig. 8, C–F).

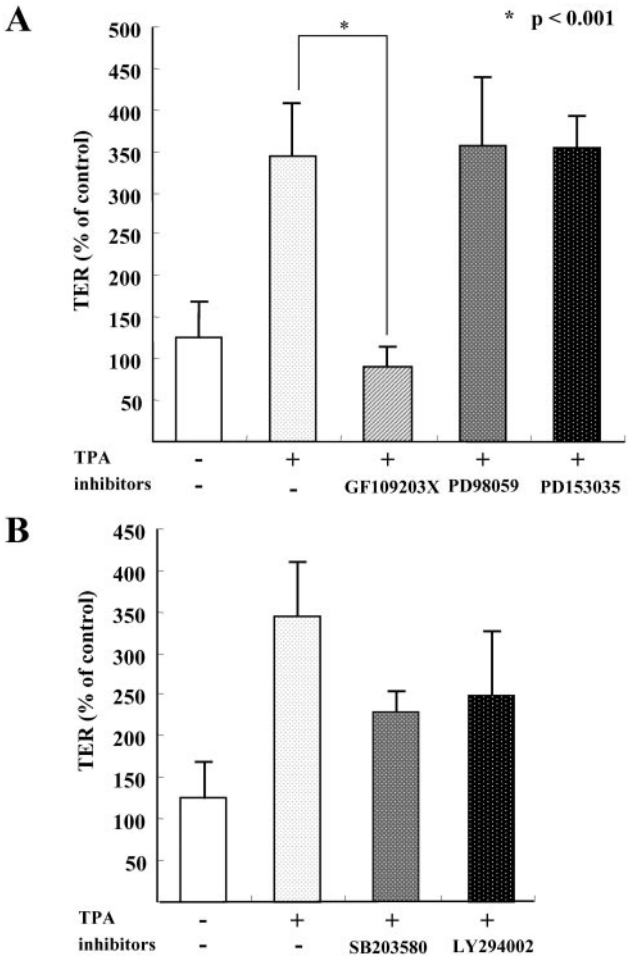

Fig. 5. Barrier function measured as TER in primary human nasal epithelial cells treated with inhibitors before treatment with TPA. A, treatment with PKC inhibitor GF109203X but not MAPK inhibitor PD98059 or EGFR inhibitor PD153035 prevented an increase of TER values at 2 h after treatment with 100 nM TPA. **, $p < 0.01$ versus control. B, p38 MAPK inhibitor SB203580 and PI3K inhibitor LY294002 slightly prevented an increase of TER values 2 h after treatment with 100 nM TPA.

Discussion

The PKC family of serine-threonine kinases is known to regulate epithelial barrier function via tight junctions (Tsukamoto et al., 1999; Andreeva et al., 2001; Seth et al., 2007). PKC has been shown to induce both assembly and disassembly of tight junctions depending on the cell type and conditions of activation (Stuart and Nigam, 1995; Andreeva et al., 2001). In this study, TPA-induced nPKC signaling greatly enhanced the barrier function of human nasal epithelial cells in primary culture together with an increase in expression of tight junction-associated proteins, occludin, ZO-1, ZO-2, and claudin-1 at the transcriptional level. The transcriptional up-regulation of ZO-1 and ZO-2 was controlled via transcriptional factor GATA-3.

PKC seems to regulate the subcellular localization, phosphorylation states, and transcription of several tight junction-associated proteins, although the PKC isozyme specificity has not been clearly elucidated (Banan et al., 2005). Conventional PKC-α participates in tight junction disassembly, whereas nPKC-ε plays a role in tight junction formation of kidney epithelial cells (Andreeva et al., 2006). In the human intestinal epithelial cell lines HT-29 and Caco-2, stimulation with TLR2 ligands leads to activation of specific PKC isoforms PKC-α and PKC-δ and enhances barrier function

through translocation of ZO-1 on activation (Cario et al., 2004). In the primary human nasal primary cells of the present study, treatment with TPA induced expression of specific nPKC-δ, -ε, and -θ and greatly enhanced tight junction barrier function together with increases in expression of occludin, ZO-1, ZO-2, and claudin-1, whereas the pan-PKC inhibitor GF109230X prevented all the changes. Furthermore, treatment with inhibitors of nPKC-δ and -θ but not -ε

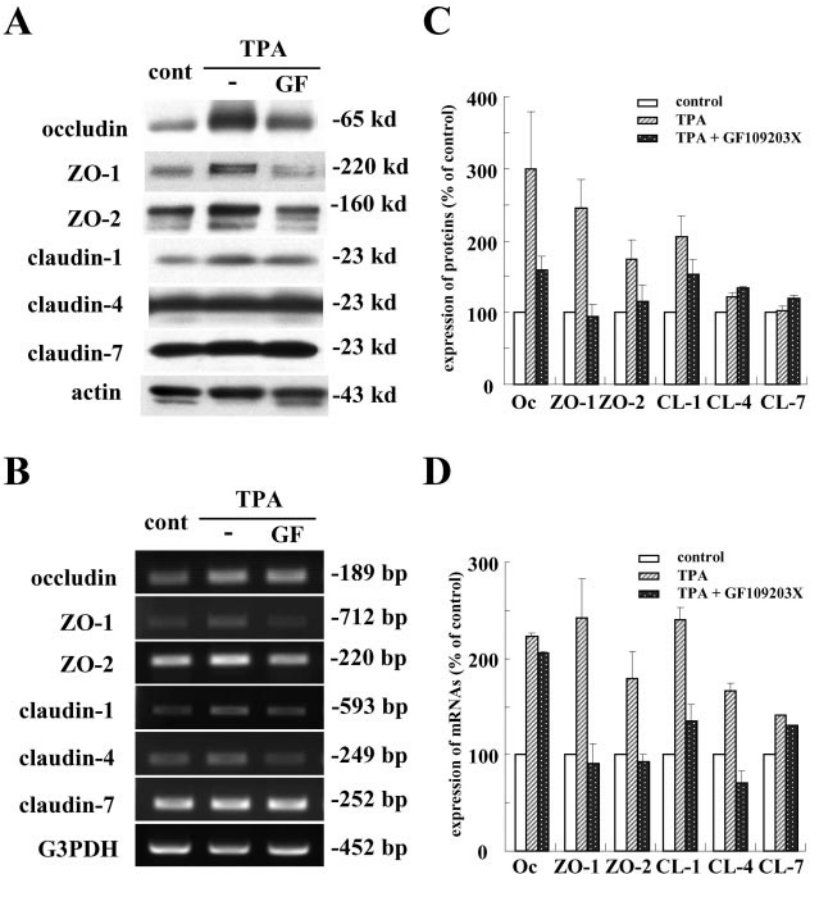

Fig. 6. A, Western blotting for occludin, ZO-1, ZO-2, and claudin-1 in primary human nasal epithelial cells treated with PKC inhibitor GF109203X before treatment with 100 nM TPA. Treatment with GF109203X inhibited up-regulation of occludin, ZO-1, ZO-2, and claudin-1 proteins at 2 h after treatment with 100 nM TPA. B, RT-PCR for occludin, ZO-1, ZO-2, and claudin-1 in primary human nasal epithelial cells treated with PKC inhibitor GF109203X before treatment with 100 nM TPA. Treatment with GF109203X inhibited up-regulation of occludin, ZO-1, ZO-2, claudin-1, and claudin-4 mRNAs 2 h after treatment with 100 nM TPA. C and D, the corresponding expression levels are shown as bar graphs.

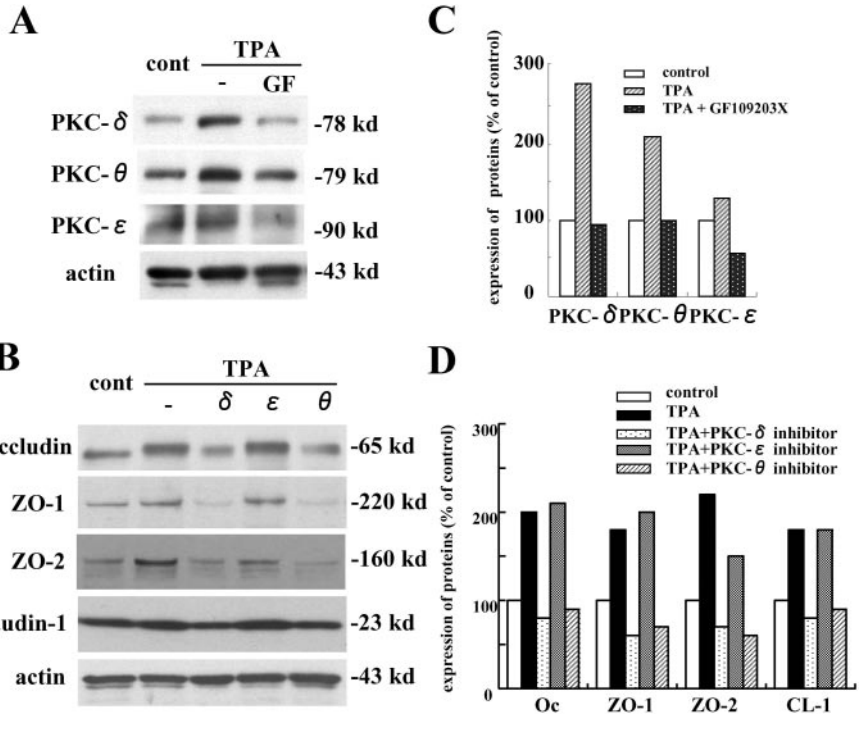

Fig. 7. A, Western blotting for PKC-δ, -θ, and -ε in primary human nasal epithelial cells treated with PKC inhibitor GF109203X before treatment with 100 nM TPA. Treatment with GF109203X inhibited increases in expression of PKC-δ, -θ, and -ε 2 h after treatment with 100 nM TPA. B, Western blotting for occludin, ZO-1, ZO-2, and claudin-1 in primary human nasal epithelial cells treated with inhibitors of PKC-δ, -θ, and -ε before treatment with 100 nM TPA. Treatment with inhibitors of PKC-δ and -θ but not -ε prevented up-regulation of occludin, ZO-1, ZO-2, and claudin-1 proteins 2 h after treatment with 100 nM TPA. C and D, the corresponding expression levels are shown as bar graphs.

prevented the up-regulation of tight junction proteins. Treatment with TPA affected the subcellular localization of the tight junction proteins together with an increase of tight junction strands. These indicate that in human nasal epithelial cells in primary culture, TPA induced nPKC-δ and -θ may enhance tight junction barrier function by assembly of tight junctions.

It is reported that activation of PKC by TPA causes increases in transcription of occludin, ZO-1, and claudin-1 in T84 cells and melanoma cells (Weiler et al., 2005; Leotlela et al., 2007). In the primary human nasal epithelial cells used in the present study, activation of PKC by TPA increased expression of occludin, ZO-1, ZO-2, and claudin-1 at the transcriptional level. Although activation of PKC exerts its effect on transcription directly, the responsible transcription factors related to PKC activation remain unknown. The claudin-2 promoter is activated by CDX2, HNF-1α, and GATA-4 in a cooperative manner (Escaffit et al., 2005). Tight junctions are regulated via direct repression of the gene expression of claudins/occludin by transcription factor Snail during epithelial to mesenchymal transition (Ikenouchi et al., 2003). In this study, to investigate the transcriptional mechanisms, we focused on the transcriptional factor GATA family. The GATA family, included in zinc finger transcription factors, has been recognized in the differentiation of the endoderm in several evolutionarily diverse organisms (Reiter et al., 2001). They form an ancient family of transcription factors that evolved into six related factors in mammals (Clarke and Berg, 1998). GATA-1, -2, and -3 are predominantly associated with the hematopoietic cell lineage, whereas GATA-4, -5, and -6 are mainly associated with development of endodermally derived organs such as the liver, lung, pancreas, and heart (Lieuw et al., 1997). More recently, it has been reported that GATA-3 is associated with differentiation of the luminal cells in the mammary gland, not only in embryos but also in adults (Kouros-Mehr et al., 2006; Asselin-Labat et al., 2007). In the primary human nasal epithelial cells of the present study, GATA-3 and -6 were detected by RT-PCR and their expres-

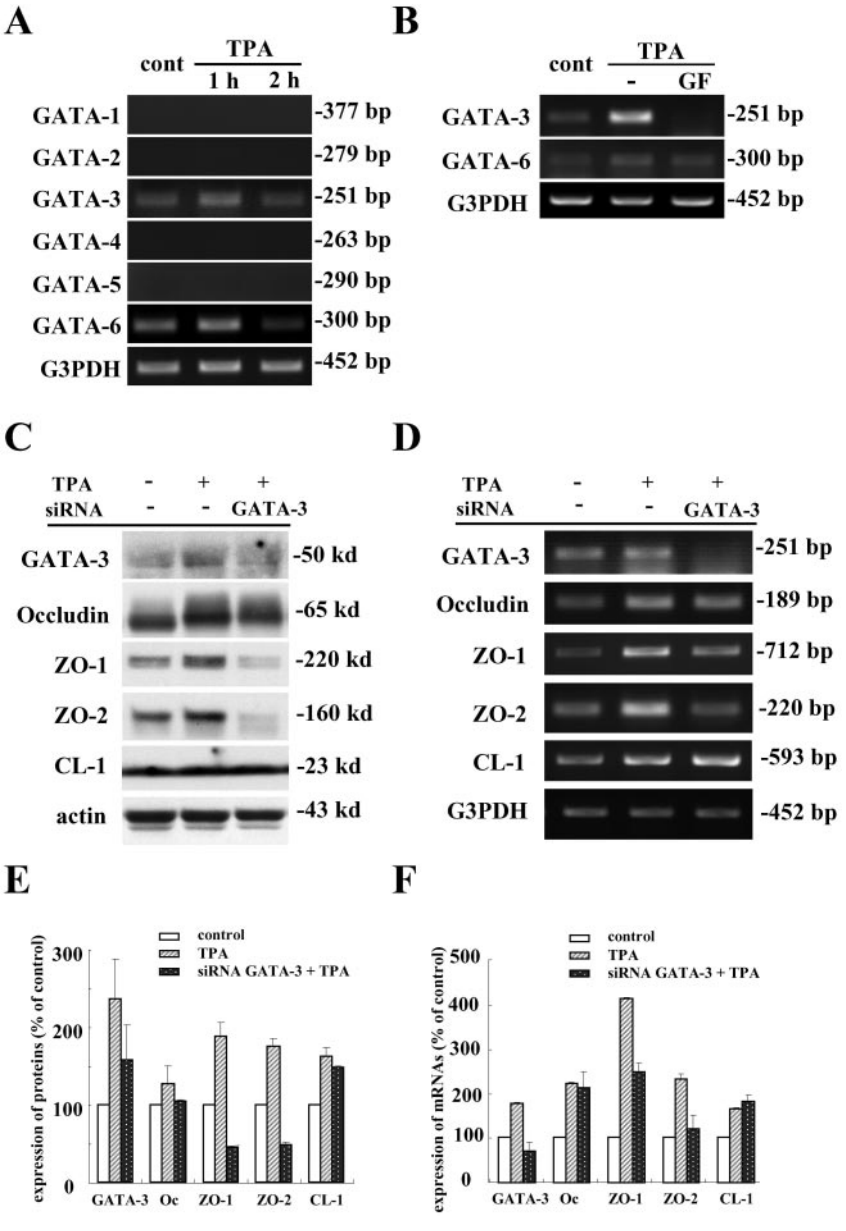

Fig. 8. A, RT-PCR for GATA-1, -2, -3, -4, -5, and -6 in primary human nasal epithelial cells at 1 and 2 h after treatment with 100 nM TPA. GATA-3 and -6 mRNAs are detected in primary human nasal epithelial cells and are increased at 1 h treatment with 100 nM TPA. B, RT-PCR for GATA-3 and -6 in primary human nasal epithelial cells treated with PKC inhibitor GF109203X before treatment with 100 nM TPA. The increase in mRNA of GATA-3 but not GATA-6 1 after treatment was inhibited by GF109203X. C, Western blotting for occludin, ZO-1, ZO-2, and claudin-1 in primary human nasal epithelial cells treated with 100 nM TPA after transfection with or without siRNA of GATA-3. D, RT-PCR for occludin, ZO-1, ZO-2, and claudin-1 in primary human nasal epithelial cells treated with 100 nM TPA after transfection with or without siRNA of GATA-3. Transfection with siRNA of GATA-3 decreased expression of GATA-3, and prevented up-regulation of ZO-1 and ZO-2 but not occludin and claudin-1 1 h after treatment with 100 nM TPA. E and F, the corresponding expression levels are shown as bar graphs.

sion was increased by treatment with TPA. The PKC inhibitor GF109230X prevented up-regulation of GATA-3 but not GATA-6. Furthermore, knockdown of GATA-3 by its RNAi inhibited up-regulation of expression of ZO-1 and ZO-2 via the PKC signaling pathway induced by treatment with TPA. These results suggest that in human nasal epithelial cells, GATA-3 is one of the transcription factors responsible for PKC activation induced by treatment with TPA and may in part regulate ZO-1 and ZO-2. ZO-1 and ZO-2 are membrane-associated guanylate kinase homologs that can bind to transmembrane tight junction proteins, occludin, claudins, and JAMs, the cytoskeleton, and signal transduction molecules (Tsukita et al., 2001; Sawada et al., 2003; Schneeberger and Lynch, 2004). Furthermore, ZO-1 and ZO-2 are closely associated with polymerization of claudins (Umeda et al., 2006). In this study, it is possible that up-regulation of the expression of ZO-1 and ZO-2 might have indirectly affected the expression and localization of occludin and claudin-1.

Although TPA is a typical PKC activator, it induces phosphorylation of MAPK not only through phosphorylation of PKC but also through that of EGFR (Barbosa et al., 2003). Furthermore, TPA is involved in multiple signal transduction pathways, including c-Jun NH_2-terminal kinase and PI3K (Huang et al., 1997; Yu et al., 2006). In primary human nasal epithelial cells, to investigate which signaling pathways mainly affect up-regulation of barrier function by treatment with TPA, we used PKC inhibitor GF109203X, MAPK inhibitor PD98059, EGFR inhibitor PD153035, p38MAPK inhibitor SB203580, and PI3K inhibitor LY294002. Treatment with the PKC inhibitor, but not inhibitors of MAPK and EGFR, completely prevented up-regulation of barrier function measured as TER values. Furthermore, inhibitors of p38 MAPK and PI3K also prevented up-regulation of barrier function by treatment with TPA, although the effect was weak. These results suggested that the increase of barrier function induced by treatment with TPA in human nasal epithelial cells was directly regulated by a PKC pathway, not via MAPK pathway, and was indirectly controlled by distinct signaling pathways, including p38 MAPK and PI3K.

TPA induces the rapid phosphorylation of occludin in Madin-Darby canine kidney cells cultured in low extracellular calcium medium with concomitant translocation of occludin to the regions of cell-cell contact (Andreeva et al., 2001). Phosphorylation of claudin-4 via PKC-ε by treatment with TPA regulates tight junction barrier function in ovarian cancer cells (D'Souza et al., 2007). PKC-θ alters barrier function through changes in phosphorylation and cellular localization of claudin-1 and -4 in Caco-2 (Banan et al., 2005). In the human nasal epithelial cells used in the present study, treatment with TPA slightly increased activities of serine and threonine phosphorylation of occludin protein (Supplemental Fig. 2). The serine and threonine phosphorylation of occludin leads to up-regulation of barrier function (Andreeva et al., 2001; Seth et al., 2007). In this study, an increase in not only expression of tight junction proteins but also serine and threonine phosphorylation of occludin might have contributed to the enhanced tight junction barrier function of human nasal epithelial cells.

PKC regulates a number of fundamental processes, such as membrane trafficking, cytoskeletal organization, ion transport, cell growth, and differentiation (Venkatachalam et al., 2004). In the human nasal epithelial cells of the present study, treatment with TPA caused phosphorylation of the endogenous PKC substrate MARCKS, which serves as an actin cross-linking protein (Hartwig et al., 1992), potentially participating in modulation of epithelial integrity. It is possible that PKC-mediated human nasal epithelial barrier enhancement results not only from up-regulation of tight junction proteins but also from potential convergence of various signaling interactions and pathways downstream of PKC, which may interact by bridging to the actin cytoskeleton.

In conclusion, the tight junction barrier function of human nasal epithelial cells is up-regulated in part by transcriptional factor GATA-3 via an nPKC signaling pathway, and the PKC-enhanced epithelial tight junction barrier may play a crucial role in innate immunity against a wide variety of exogenous antigens. The up-regulation of human nasal epithelial cells via the nPKC signaling pathway may also be caused by the stimuli such as the cigarette smoke and various dusts (Wyatt et al., 1999; Romberger et al., 2002). Furthermore, the regulation of PKC signaling may contribute to a drug delivery system via human nasal epithelium.

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