

Involvement of Protein Kinase C δ /Extracellular Signal-regulated Kinase/Poly(ADP-ribose) Polymerase-1 (PARP-1) Signaling Pathway in Histamine-induced Up-regulation of Histamine H₁ Receptor Gene Expression in HeLa Cells*

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The histamine H₁ receptor (H1R) gene is up-regulated in patients with allergic rhinitis. However, the mechanism and reason underlying this up-regulation are still unknown. Recently, we reported that the H1R expression level is strongly correlated with the severity of allergic symptoms. Therefore, understanding the mechanism of this up-regulation will help to develop new anti-allergic drugs targeted for H1R gene expression. Here we studied the molecular mechanism of H1R up-regulation in HeLa cells that express H1R endogenously in response to histamine and phorbol 12-myristate 13-acetate (PMA). In HeLa cells, histamine stimulation caused up-regulation of H1R gene expression. Rottlerin, a PKC δ -selective inhibitor, inhibited up-regulation of H1R gene expression, but Go6976, an inhibitor of Ca²⁺-dependent PKCs, did not. Histamine or PMA stimulation resulted in PKC δ phosphorylation at Tyr³¹¹ and Thr⁵⁰⁵. Activation of PKC δ by H₂O₂ resulted in H1R mRNA up-regulation. Overexpression of PKC δ enhanced up-regulation of H1R gene expression, and knockdown of the PKC δ gene suppressed this up-regulation. Histamine or PMA caused translocation PKC δ from the cytosol to the Golgi. U0126, an MEK inhibitor, and DPQ, a poly(ADP-ribose) polymerase-1 inhibitor, suppressed PMA-induced up-regulation of H1R gene expression. These results were confirmed by a luciferase assay using the H1R promoter. Phosphorylation of ERK and Raf-1 in response to PMA was also observed. However, real-time PCR analysis showed no inhibition of H1R mRNA up-regulation by a Raf-1 inhibitor. These results suggest the involvement of the PKC δ /ERK/poly(ADP-ribose) polymerase-1 signaling pathway in histamine- or PMA-induced up-regulation of H1R gene expression in HeLa cells.

Histamine is a major chemical mediator of allergic reactions, and its action is mainly mediated by the histamine H₁ receptor

(H1R),² which is one of the four histamine receptor isoforms. H1R, which is a G protein-coupled receptor, is coupled to the G_q protein. The stimulation of H1R activates inositol phospholipid hydrolysis and intracellular Ca²⁺ mobilization. Typically, the activation of G protein-coupled receptors by their agonists induces down-regulation of the receptors. However, to date, a few reports have demonstrated up-regulation of G protein-coupled receptors by their agonists (1–4). Previously, we found that histamine stimulation up-regulated H1R at both the mRNA and protein level via activation of H1R in HeLa cells that express H1R endogenously (5). Up-regulation of H1R has been observed in patients with allergic rhinitis (6, 7). The strength of H1R signaling depends on the H1R expression level (8). Our recent studies have demonstrated that the level of H1R gene expression is strongly correlated with the severity of allergic symptoms in model rats and patients with pollinosis (9, 10), and compounds that suppress H1R gene up-regulation alleviate allergic symptoms (11–16). These findings suggest that the H1R gene is an allergy-sensitive gene, *i.e.* its expression level affects the severity of symptoms. Hence, understanding the molecular mechanism of H1R up-regulation may be useful for developing new anti-allergic drugs that target H1R gene expression. However, the mechanism of H1R up-regulation in response to histamine is unknown.

Previously, we demonstrated that PKC-dependent signaling is involved in up-regulation of H1R gene expression in HeLa cells (5). PKC consists of at least 11 isoforms, and based on their structures and cofactor requirements, the PKC isoforms are divided into three subgroups; that is, conventional PKC (α , β , and γ), novel PKC (δ , ϵ , η , and θ), and atypical PKC (ζ and ι/λ) (17). It has been reported that PKC α , β , δ , ϵ , and ζ isoforms are expressed in HeLa cells (18–20).

Coupling of H1R signaling with PKC α has been reported in H1R-overexpressing CHO cells (21) and native human epidermal keratinocytes (22). In these cells, however, no up-regulation of H1R gene expression was observed. In addition, NF- κ B (23), the cAMP response element-binding protein (CREB) (24), the nuclear factor of activated T-cell (25), and the serum-responsive element (26) are activated by H1R signaling. Thus,

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² The abbreviations used are: H1R, histamine H₁ receptor; PARP-1, poly (ADP-ribose) polymerase-1; PMA, phorbol 12-myristate 13-acetate; RACK, receptors for activated C kinase; Ds-Red-PKC δ , DsRed-tagged PKC δ .

H1R signaling is rather complicated, and the PKC isoform responsible for this H1R up-regulation in HeLa cells remains unknown.

Here we investigated the molecular mechanism of H1R up-regulation in HeLa cells in response to histamine and PMA. Our results indicate that the PKC δ /ERK/PARP-1 signaling pathway is involved in histamine or PMA-induced up-regulation of H1R gene expression in HeLa cells.

EXPERIMENTAL PROCEDURES

Chemicals—PolyFect transfection reagent was from Qiagen (Tokyo, Japan). pDsRed-monomer-C1, pAcGFP-N3, and pAcGFP-Golgi were from Clontech (Palo Alto, CA). The pGEM-T-Easy vector, pRL-MPK vector, and Dual-luciferase Reporter Assay System were from Promega (Madison, WI). The predeveloped TaqMan Assay Reagent of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Applied Biosystems (Foster City, CA). Minimal essential medium- α was from Invitrogen. RNAiso Plus was purchased from Takara Bio Inc. (Kyoto, Japan). Rottlerin and Ro-31-8220 were purchased from Calbiochem. The BCA protein assay kit was from Sigma. All other chemicals were of analytical grade.

Real-time Quantitative RT-PCR—HeLa cells were cultured at 37 °C under a humidified 5% CO₂, 95% air atmosphere in minimal essential medium- α containing 8% fetal calf serum and 1% antibiotic-antimycotic (Invitrogen). HeLa cells cultured to 70% confluency in 6-well dishes were serum-starved for 24 h and treated with reagents 1 h before histamine or PMA stimulation. After a 3-h treatment with histamine or PMA, the cells were harvested with 700 μ l of RNAiso Plus, mixed with 140 μ l of chloroform, and centrifuged at 15,000 rpm for 15 min at 4 °C. For the H₂O₂ experiment, cells were harvested with 700 μ l of RNAiso Plus at established time intervals after the addition of 100 μ M H₂O₂. The aqueous phase was collected, and RNA was precipitated by the addition of isopropyl alcohol. After centrifugation at 15,000 rpm for 15 min at 4 °C, the resulting RNA pellet was washed with ice-cold 70% ethanol. Total RNA was resolved in 10 μ l of diethylpyrocarbonate-treated water, and 5 μ g of each RNA sample was used for the reverse transcription reaction. RNA samples were reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). TaqMan primers and the probe were designed using Primer Express (Applied Biosystems). Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). The sequences of the primers and TaqMan probe were as follows: forward primer for H1R, 5'-CAGAGGATCAGATGTTAGGTGATAGC-3'; reverse primer for H1R, 5'-AGCGGAGCCTCTTCCAAGTAA-3'; TaqMan probe, FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA. To standardize the starting material, the human GAPDH gene (Applied Biosystems) was used, and data were expressed as the ratio of H1R mRNA to GAPDH mRNA. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. Identities of the PCR products were verified by sequencing using a genetic analysis system (Beckman CEQ 8000; Beckman Coulter, Fullerton, CA).

Promoter Assay—The human H1R reporter plasmid (pH1R) was constructed as previously reported (5). In brief, a 2.1-kbp

5'-upstream fragment (−2029 to +64; +1 indicates the putative transcription initiation site (27) of the human H1R gene) was amplified by PCR using a forward primer (5'-GCTAGCCGAATGTGGGAAGATCAGTAGTAG-3') and a reverse primer (5'-AGATCTGAAGGTCTTCTCCATGATGGCCTTC-3'), and the fragment was subcloned into the NheI-HindIII site of the promoter-less reporter plasmid pGL3-Basic vector (Promega; designated as pH1R). HeLa cells cultured in 12-well culture plates were cotransfected with pH1R and the internal control plasmid pRL-MPK (Promega) in a ratio of 100:1 using the PolyFect transfection reagent (Qiagen) according to the manufacturer's instructions. After 5 h the medium was replaced with 1 ml of serum-free medium, and the cells were starved for 24 h. The cells were then stimulated with 10 μ M histamine for 3 h in the same medium. Where appropriate, inhibitors were added 1 h before histamine stimulation. After stimulation, the cells were washed twice with 500 μ l of ice-cold phosphate-buffered saline (PBS) and lysed with 100 μ l of passive lysis buffer (Promega). The lysate was frozen for at least 3 h at −85 °C and then thawed at room temperature. Luciferase activity was determined using the Dual-luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Luminescence was measured using the Infinite M200 microplate reader (Tecan Japan, Kanagawa, Japan).

Construction of Expression Vector for PKC δ —Human PKC δ cDNA was PCR-amplified using a forward primer, 5'-GCAGGTACCACCATGGCGCCGTTTC-3', and a reverse primer, 5'-CCAGGATCCTCAATCTTCCAGGAG-3'. The fragment was cloned into the pGEM-T-Easy vector, and the nucleotide sequence was confirmed. The PKC δ cDNA was then cloned into the expression vector pDsRed-monomer C1 or pAcGFP-N3 at the KpnI and BamHI sites. The stop codon of the PKC δ cDNA was deleted before cloning into the pAcGFP-N3 plasmid. To overexpress DsRed-tagged PKC δ , HeLa cells were transfected with the expression vector using the PolyFect transfection reagent (Qiagen). After 24 h the medium was replaced with serum-free medium, and the cells were starved for 24 h. The cells were then stimulated with 100 μ M histamine for 3 h in the same medium.

Small Interfering RNA (siRNA) Transfection—HeLa cells were reverse-transfected with 7 nM PKC δ -specific siRNA (Silencer® Select siRNA, Applied Biosystems) or 21 nM control siRNA (Silencer® negative control #1 siRNA, Applied Biosystems) using siPORT NeoFX Transfection Agent according to the manufacturer's instructions (Applied Biosystems). Twenty-four hours after transfection, the cells were starved for 24 h and then treated with or without 100 μ M histamine. The H1R mRNA expression level was determined by real-time RT-PCR as described above. The PKC δ mRNA expression level was determined by RT-PCR using the primers used for the cloning of PKC δ cDNA.

Immunoblot Analysis—For the immunoblot analysis, 10 μ g of each protein sample was separated on a 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was briefly rinsed in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and then incubated for 1 h at room temperature in TBS-T containing 5% skim milk (Difco) or 3% BSA (for detecting phosphoproteins; Sigma). The mem-

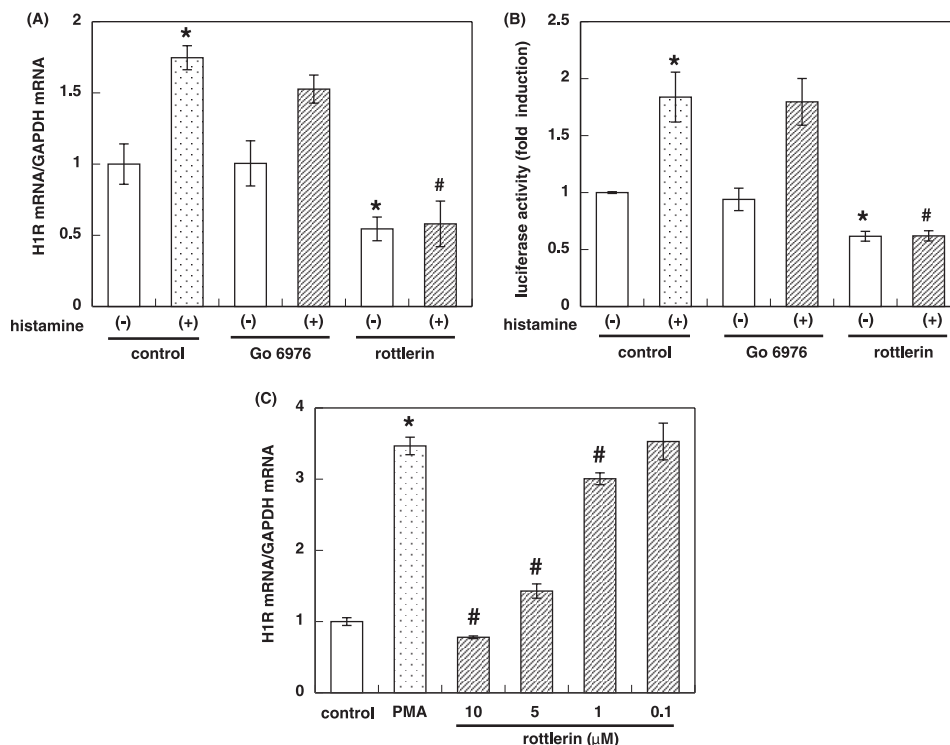


FIGURE 1. Involvement of PKC δ in histamine- or PMA-induced up-regulation of H1R mRNA expression. A, HeLa cells were serum-starved for 24 h and then treated with Go6976 (1 μ M) or rottlerin (5 μ M) 1 h before stimulation with 10 μ M histamine. After a 3-h treatment with histamine, total RNA was isolated, and the H1R mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the means \pm S.E. ($n = 4$). *, $p < 0.05$ versus control, histamine (-); #, $p < 0.05$ versus control, histamine (+). B, HeLa cells were cotransfected with pH1R and pRL-MPK vectors, as described under "Experimental Procedures," and then treated with Go6976 (1 μ M) or rottlerin (5 μ M) 1 h before stimulation with 10 μ M histamine. After 3 h of histamine stimulation, luciferase activity was determined using the Dual-luciferase Reporter Assay System. Data are presented as the means \pm S.E. ($n = 4$). *, $p < 0.05$ versus control, histamine (-); #, $p < 0.05$ versus control, histamine (+). C, HeLa cells were serum-starved for 24 h and then treated with rottlerin (0.1–10 μ M) 1 h before stimulation with 50 nM PMA. After a 3-h treatment with PMA, total RNA was isolated, and the H1R mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as means \pm S.E. ($n = 4$). *, $p < 0.05$ versus control; #, $p < 0.05$ versus PMA.

brane was then incubated with a primary antibody (PKC δ (C-20), sc-937, 1:1000; ERK (K-23), sc-94, 1:1000; phospho-ERK (E-4), sc-7383, 1:1000, (Santa Cruz Biotechnology); phospho-PKC δ (Tyr³¹¹), #2055S, 1:500; phospho-PKC δ (Thr⁵⁰⁵), #9374S, 1:1000; phospho-c-Raf (Ser³³⁸) (56A6), #9427, 1:1000; β -actin, #4697, 1:2000 (Cell Signaling)) overnight at 4 $^{\circ}$ C. Goat anti-rabbit IgG (H+L)-HRP conjugate (#170-6515, 1:10,000, Bio-Rad) or Immun-Star goat anti-mouse-HRP conjugate (#170-5047, 1:10,000, Bio-Rad) was used as the secondary antibody, and proteins were visualized with an Immobilon Western Chemiluminescent HRP substrate (Millipore).

Subcellular Localization of PKC δ —To determine the subcellular localization of PKC δ , HeLa cells were plated onto 35-mm glass-bottomed dishes (Asahi Techno Glass, Chiba, Japan). The expression plasmid encoding the DsRed-tagged PKC δ (DsRed-PKC δ) and pAcGFP-Golgi (for labeling of the Golgi) was cotransfected into the attached HeLa cells using the PolyFect transfection reagent (Qiagen). After 5 h, the medium was replaced with 1 ml of serum-free medium, and the cells were starved for 24 h. The cells were then stimulated with 100 μ M histamine or PMA for the indicated time in the same medium. The cells were treated with the PKC δ inhibitor rottlerin (10 μ M) for 1 h before histamine stimulation. After stimulation, the cells were washed once with PBS and fixed with ice-cold methanol, and the PBS was then replaced. The subcellular localization of the Ds-Red-PKC δ was determined using a confocal laser microscope (LSM510; Carl Zeiss, Oberkochen, Germany).

Statistical Analysis—The results are shown as the mean \pm S.E. Statistical analyses were performed with unpaired t tests or analysis of variance with Dunnett's test using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Identification of PKC Isoforms Involved in Histamine-induced Up-regulation of H1R Gene Expression—Our previous data indicated the involvement of PKC in histamine-induced up-regulation of H1R gene expression (5). To date, 11 PKC isoforms have been identified, and PKC α , β , δ , ϵ , and ζ isoforms are expressed in HeLa cells (18–20). Thus, we investigated which PKC isoform was involved in H1R gene up-regulation. Rottlerin, an selective inhibitor for the PKC δ isoform, suppressed histamine-induced increase in the H1R mRNA expression level in HeLa cells (Fig. 1A). Go6976, an inhibitor of the Ca²⁺-dependent PKC isoforms, did not show any significant inhibitory effects (Fig. 1A). Similar results were obtained with the H1R promoter assay (Fig. 1B). We reported that Ro-31-8220, a non-isoform selective PKC inhibitor, completely inhibited histamine-induced up-regulation of H1R gene expression (5). These data suggest that PKC δ , ϵ , and ζ are the candidates responsible for this up-regulation. The IC₅₀ for rottlerin of PKC δ has been reported to be 3–6 μ M (28). On the other hand, those of PKC ϵ and - ζ isoforms were about 100 μ M (28). The dose of rottlerin used in this study was 0.1–10 μ M, so these two

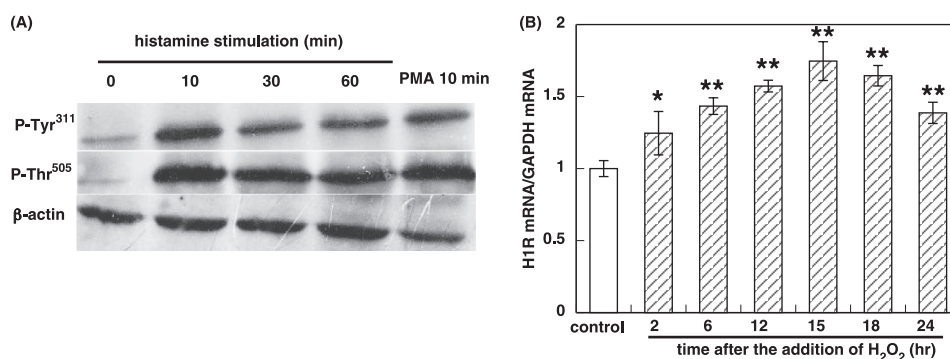


FIGURE 2. **Activation of PKC δ causes up-regulation of H1R mRNA expression.** A, HeLa cells were serum-starved for 24 h and then treated with 10 μ M histamine. After stimulation with histamine, total cell lysates were isolated at the given time intervals and subjected to immunoblot analysis. For PMA, the cell lysate was isolated 10 min after PMA (50 nM) stimulation. B, at the given time intervals after the addition of 100 μ M H₂O₂, cells were harvested with 700 μ l of RNAiso Plus, and total RNA was isolated. The H1R mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the means \pm S.E. (n = 4). *, p < 0.05 versus control; **, p < 0.01 versus control.

isoforms are not inhibited by rottlerin. This excludes the possibility that PKC ϵ and - ζ isoform were responsible for this up-regulation. These data suggest the PKC δ isoform is involved in histamine-induced up-regulation of H1R gene expression. Rottlerin also suppressed PMA-induced H1R mRNA elevation in a dose-dependent manner (IC_{50} = 5.2 μ M; Fig. 1C). These findings suggest that a common pathway downstream of PKC δ is involved in both histamine- and PMA-induced up-regulation of H1R gene expression.

Effect of PKC δ Activation on Histamine/PMA-induced Up-regulation of H1R Gene Expression—Phosphorylation and dephosphorylation of PKCs regulate their activity, stability, and function. PKC δ is phosphorylated at Tyr³¹¹ upon activation, and phosphorylation at Thr⁵⁰⁵ regulates the PKC δ substrate specificity (29). We investigated the effect of histamine or PMA stimulation on the phosphorylation of these residues. After stimulation with 100 μ M histamine, Tyr³¹¹ was phosphorylated in a maximum of 10 min (Fig. 2A). Thr⁵⁰⁵ was also phosphorylated by histamine stimulation. In addition, phosphorylation of both Tyr³¹¹ and Thr⁵⁰⁵ residues was observed after PMA stimulation. Because H₂O₂ is a well known PKC δ activator (30, 31), we investigated the effect of H₂O₂ on up-regulation of H1R gene expression. Previously we demonstrated that histamine induced a transient and time-dependent increase of H1R mRNA, and the mRNA level reached a maximum at 9 h after histamine stimulation and afterward declined gradually, returning near to the basal level after 24 h of treatment (5). Therefore, we checked the H1R mRNA level up to 24 h after H₂O₂ treatment in this study. After treatment with H₂O₂, the H1R mRNA expression level was increased in a time-dependent manner in a maximum of 15 h (Fig. 2B). A decrease in the H1R mRNA level 15 h after H₂O₂ treatment could be attributed to disappearance of H₂O₂. These data suggest that histamine/PMA activated PKC δ and that PKC δ activation caused H1R mRNA elevation.

Effect of the PKC δ Expression Level on Histamine-induced Up-regulation of H1R Gene Expression—To investigate the effect of PKC δ overexpression, we expressed Ds-Red-PKC δ (fusion protein) in HeLa cells and examined histamine-induced up-regulation of H1R gene expression. In the control (wild type) HeLa cells, histamine stimulation did not affect the expression level of endogenous PKC δ protein (M_r = 75 kDa).

This indicates that histamine stimulation does not induce the expression of PKC δ protein. On the other hand, HeLa cells transfected with the plasmid harboring Ds-Red-PKC δ cDNA expressed Ds-Red-PKC δ protein with an 110 kDa in addition to the endogenous PKC δ . This causes an increase in the total amount of PKC δ protein compares with the control cells as shown in Fig. 3A. Fig. 3A also shows that histamine stimulation does not affect expression level of either endogenous PKC δ or Ds-Red-PKC δ . Without histamine stimulation, there was no significant difference in the H1R mRNA level between the control and the Ds-Red-PKC δ -transfected cells (Fig. 3B). However, significant H1R mRNA elevation induced by histamine was observed in the Ds-Red-PKC δ -transfected cells compared with the control cells (Fig. 3B). Next we examined the effect of knockdown of PKC δ expression using a specific PKC δ siRNA. In PKC δ knockdown cells, PKC δ mRNA expression was confirmed by RT-PCR analysis, and the amount of PKC δ mRNA was decreased to \sim 48% that in control cells (Fig. 3C). This should cause a decrease in the amount of PKC δ protein expressed in the cells. No significant difference in the H1R mRNA expression was observed in both the control and siRNA-transfected cells without histamine stimulation. In PKC δ knockdown cells, H1R mRNA elevation induced by histamine stimulation was less (1.3 ± 0.3 -fold increase versus control) than that in control cells (1.8 ± 0.2 -fold increase versus control; Fig. 3D). These data suggest that the PKC δ expression level affects the H1R mRNA expression level induced by histamine stimulation.

Translocation of PKC δ by Histamine/PMA Stimulation—PKCs translocate to various organelles, including the plasma membrane, nucleus, endoplasmic reticulum, mitochondria, and Golgi in response to various stimuli, and phosphorylate isoform-specific substrates, leading to isoform- and/or stimulus-specific cellular responses. Thus, it is very important to know where PKC δ is translocated by histamine stimulation for better understanding of the signaling pathway involved in histamine-induced H1R gene expression. Therefore, we expressed Ds-Red-PKC δ in HeLa cells and investigated the translocation of this fusion protein in response to histamine. Histamine stimulation promoted PKC δ translocation from the cytosol to the Golgi (Fig. 4). PMA also promoted PKC δ translocation to the Golgi. Pretreatment with the PKC δ -specific inhibitor rottlerin

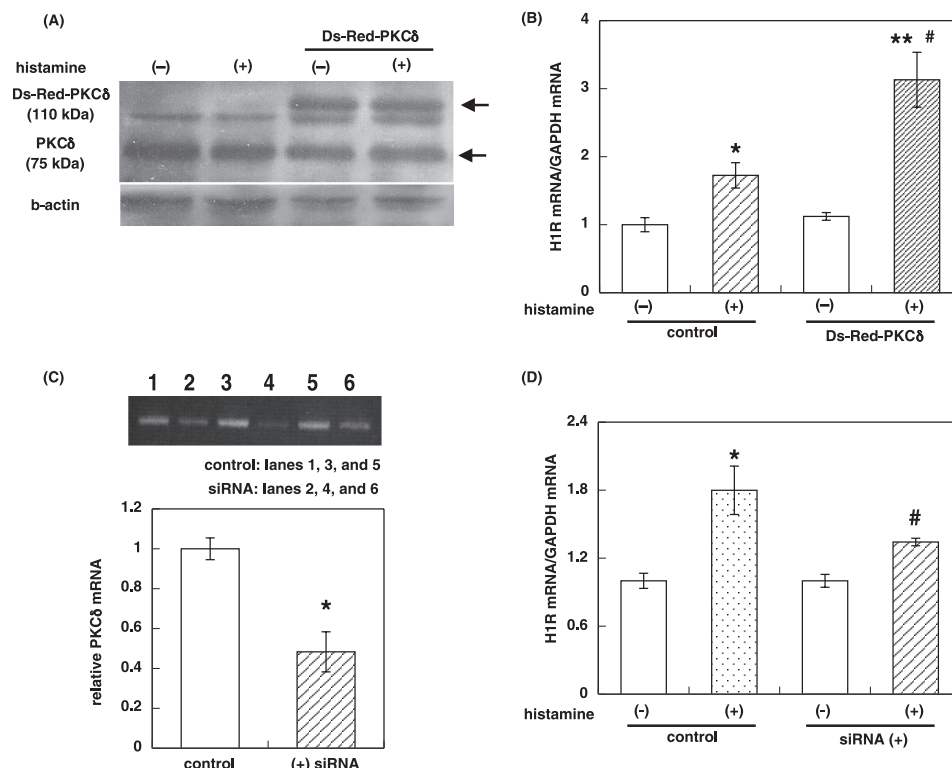


FIGURE 3. Effect of the PKC δ expression level on histamine-induced up-regulation of H1R mRNA expression. A and B, the expression plasmids encoding Ds-Red-PKC δ were transfected into the attached HeLa cells. After 24 h of transfection, HeLa cells were serum-starved for 24 h and then treated with 100 μ M histamine for 3 h. In A, total cell lysates were isolated, and the expression levels of Ds-Red-PKC δ and endogenous PKC δ were analyzed by immunoblot analysis. In B, the cells were harvested with 700 μ l of RNAiso Plus, and total RNA was isolated. The H1R mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the means \pm S.E. (n = 4). *, p < 0.05 versus control, histamine (-); **, p < 0.05 versus control, Ds-Red-PKC δ (+); #, p < 0.05 versus control, histamine (+). C and D, HeLa cells were reverse-transfected with 7 nM PKC δ -specific siRNA or 21 nM control siRNA. Twenty-four hours after transfection, the cells were starved for 24 h followed by treatment with histamine (100 μ M). In C, the PKC δ mRNA levels were determined by RT-PCR using the primers used for the cloning of PKC δ cDNA (top). The values are presented as the means \pm S.E. (n = 3). *, p < 0.05 versus control. In D, the H1R mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the means \pm S.E. (n = 4). *, p < 0.05 versus control, histamine (-); #, p < 0.05 versus control, histamine (+).

inhibited the histamine-induced PKC δ translocation to the Golgi. Similar PKC δ translocation was observed when PKC δ -AcGFP was expressed in HeLa cells (data not shown). These data suggest that PKC δ translocates to the Golgi in response to histamine/PMA stimulation.

Identification of ERK and PARP-1 as Downstream Signal Effectors of PKC δ in PMA-induced Up-regulation of H1R Gene Expression—Activation of the ERK signaling cascade, which consists of Raf-1 (c-Raf), MEK, and ERK, by PKC δ has been reported (24, 32). Thus, we investigated the effect of PKC δ activation by PMA on the ERK signaling cascade in PMA-induced up-regulation of H1R gene expression. U0126, an MEK inhibitor, suppressed PMA-induced H1R mRNA elevation in a dose-dependent manner (Fig. 5A). Similar results were obtained with the H1R promoter assay (Fig. 5B). Immunoblot analysis showed that histamine or PMA stimulation resulted in transient phosphorylation of ERK (Fig. 5C), and pretreatment with rottlerin completely inhibited this phosphorylation (Fig. 5D). PKC δ -induced phosphorylation of Raf-1 was also observed (Fig. 6A). However, real-time RT-PCR analysis showed no inhibition of PMA-induced H1R mRNA elevation by the Raf-1 kinase inhibitor I (Fig. 6B), suggesting that Raf-1 is not related to the signaling pathway. During promoter analyses of the H1R gene, we identified Ets-1, AP-1, PARP-1, Ku86, and Ku70 as promoter-

binding proteins.³ Therefore, we next investigated the effect of the PARP-1 inhibitor DPQ (33) on PMA-induced up-regulation of H1R gene expression. DPQ suppressed PMA-induced up-regulation of H1R gene expression in a dose-dependent manner (Fig. 7A). Similar results were obtained with the H1R promoter assay (Fig. 7B). These data indicate that MEK, ERK, and PARP-1, but not Raf-1, are the signal effectors downstream of PKC δ .

DISCUSSION

In the present study we studied the mechanism involved in up-regulation of H1R gene expression in response to histamine or PMA stimulation in HeLa cells that express H1R endogenously. We propose the signaling pathway involved in histamine- or PMA-induced up-regulation of H1R gene expression in HeLa cells (Fig. 8). In general, the activation of receptors by their agonists induces down-regulation of the receptor. However, our previous study demonstrated that histamine or PMA stimulation caused up-regulation of H1R gene expression via activation of H1R in HeLa cells (5). This finding led to a working hypothesis of the existence of a detrimental circuit between

³ H. Mizuguchi, T. Terao, N. Sakamoto, Y. Yamawaki, Y. Yoshimura, Y. Kitamura, N. Takeda, and H. Fukui, submitted for publication.

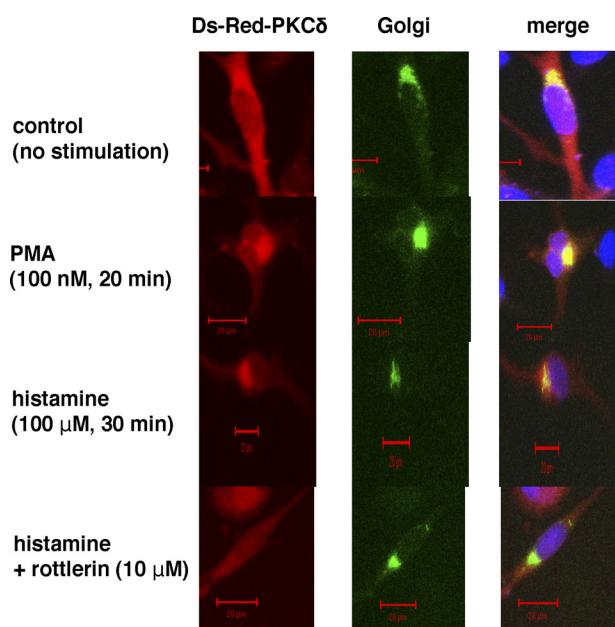


FIGURE 4. Translocation of PKC δ by histamine or PMA stimulation. The expression plasmid encoding Ds-Red-PKC δ and pAcGFP-Golgi (for labeling of the Golgi) were cotransfected into HeLa cells attached onto 35-mm glass-bottomed dishes as described in Fig. 3. The cells were serum-starved for 24 h and then stimulated with 100 μ M histamine or 100 nM PMA for the indicated times in the same medium. The cells were treated with the PKC δ inhibitor rottlerin (10 μ M) for 1 h before histamine stimulation. The subcellular localization of Ds-Red-PKC δ was determined using a confocal laser microscope. Bars = 20 μ m.

histamine and H1R in which histamine-induced up-regulation of H1R gene expression results in an increase in the amount of H1R protein, and this makes the cells more sensitive to histamine and may actually exacerbate the allergic symptoms. It was reported that expression of H1R mRNA was increased in epithelial, endothelial, and neural cells on the nasal mucosa in the patients with occupational rhinitis (34, 35). Up-regulation of H1R was also observed in patients with allergic rhinitis (6, 7). Terada *et al.* (36) reported that H1R mRNA was increased in human nasal epithelial cells after stimulation with diesel exhaust particles that are known to cause chronic airway diseases. They also showed that histamine stimulation induced production of granulocyte macrophage-colony stimulating factor and interleukin-8 (IL-8) in human nasal epithelial cells. In addition, Matsubara *et al.* (37) reported the involvement of H1R-PKC-ERK signaling pathway in eliciting granulocyte macrophage-colony stimulating factor and IL-8 production from bronchial epithelial cells stimulated by histamine. Involvement of PKC δ was also reported by Masaki *et al.* (38). These findings suggest that epithelial cells are one of the candidates responsible for histamine-induced up-regulation of H1R gene expression. It is known that HeLa cells are the cell line derived from cervical cancer cells arising in epithelial cells. Therefore, we consider that HeLa cells can be a model to analyze molecular mechanism of histamine-induced up-regulation of H1R gene expression, although HeLa cells are not representative for typical target cells involved in allergic reactions.

Bakker *et al.* (23) reported that H1R activates NF- κ B in an agonist-dependent manner in H1R-transfected COS-7 cells. Hill and co-workers (21) demonstrated that H1R stimulation

causes activation of the *c-fos* promoter via the PKC α /MEK/MAP kinase signaling pathway in CHO-H1 cells expressing H1R. However, because these cells do not naturally express H1R, these authors used cells that exogenously overexpress H1R. On the other hand, Matsubara *et al.* (22) reported that histamine activates Ca²⁺-dependent PKC isoforms that play crucial roles in the activation of Raf/MEK/ERK and IKK/I κ B/NF- κ B cascades, leading to up-regulation of cytokine expression in native human epidermal keratinocytes. However, close examination of their data revealed that up-regulation of H1R gene expression in response to histamine was not observed in these cells. Thus, it is unlikely that the signaling pathway reported in keratinocytes is involved in histamine-induced up-regulation of H1R gene expression in HeLa cells or patients with allergic rhinitis.

Our data indicate that up-regulation of H1R gene expression is PKC δ -dependent. PKC δ overexpression or knockdown experiments showed that no significant difference in the H1R mRNA expression was observed in both the control and Ds-Red-PKC δ or PKC δ -specific siRNA-transfected cells without histamine stimulation. As enough PKC δ was expressed in the wild type HeLa cells, a part of the PKC δ protein expressed in the cells might be involved in the basal transcription of H1R gene. However, with histamine stimulation, the expression level of H1R mRNA was significantly increased (in Ds-Red-PKC δ -transfected cells) or decreased (in siRNA-transfected cells) compared with that in the control cells. In this case histamine stimulation induces up-regulation of H1R gene expression, and more PKC δ should be required for this enhanced histamine signaling. Therefore, the expression level of H1R mRNA is increased (in Ds-Red-PKC δ -transfected cells) or decreased (in siRNA-transfected cells) in proportion to the amount of PKC δ available. These data indicate that the PKC δ expression level affects the H1R mRNA expression level induced by histamine stimulation, and in this context, PKC δ is the rate-limiting enzyme in this signaling. The data that rottlerin, PKC δ -selective inhibitor completely inhibits histamine or PMA-induced up-regulation of H1R gene expression in HeLa cells (Fig. 1) support this hypothesis. Recently we demonstrated that many compounds including epigallocatechin-3-O-gallate, which we identified as a compound that suppresses up-regulation of H1R gene expression, suppress PKC δ phosphorylation,⁴ which also supports this idea. Translocation analysis using Ds-Red-PKC δ (PKC δ tagged with Ds-Red at its N terminus) demonstrated that histamine/PMA stimulation induced translocation of PKC δ from the cytosol to the Golgi (Fig. 4). Yoshida *et al.* (39) reported that PKC δ is proteolytically cleaved by caspase-3 in response to apoptotic stimuli, resulting in a 40-kDa catalytically active fragment. However, PKC δ -AcGFP (PKC δ tagged with AcGFP at its C terminus) also exhibited similar translocation from the cytosol to the Golgi in response to histamine/PMA (data not shown), indicating that there was no proteolytic cleavage of PKC δ in response to histamine or PMA in HeLa cells. A similar finding was reported for the apoptosis of LNCaP cells treated with phorbol esters, for

⁴ H. Mizuguchi and H. Fukui, unpublished data.

Signaling Pathway of Histamine-induced H1R Up-regulation

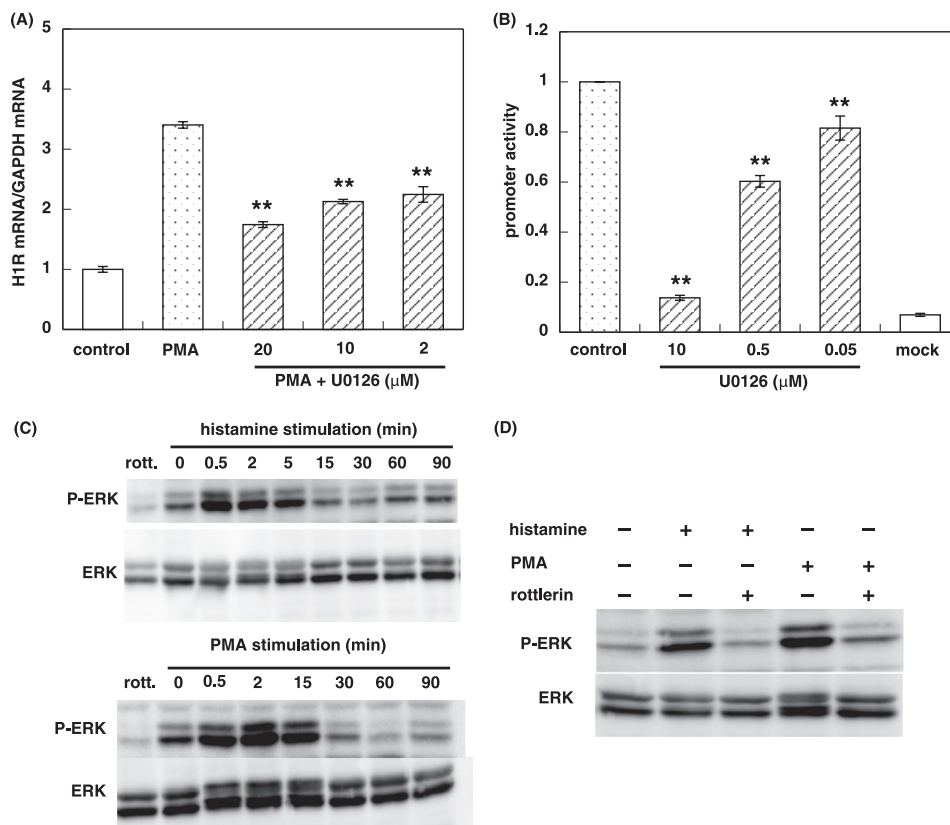


FIGURE 5. ERK is a signal effector downstream of PKC δ . A and B, HeLa cells were serum-starved for 24 h and treated with U0126 (2–20 μ M) 1 h before stimulation with 50 nM PMA for 3 h. In A, total RNA was isolated, and the H1R mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the means \pm S.E. ($n = 4$). **, $p < 0.01$ versus PMA. In B, HeLa cells were cotransfected with pH1R and pRL-MPK vectors, as described in Fig. 1B, and treated with U0126 (0.05–10 μ M) as described above. After stimulation, luciferase activity was determined using the Dual-luciferase Reporter Assay System. Data are presented as the means \pm S.E. ($n = 4$). **, $p < 0.01$ versus PMA. C, time course of histamine- or PMA-induced phosphorylation of ERK is shown. HeLa cells were stimulated with histamine (100 μ M, top) or PMA (100 nM, bottom), and at then given time intervals, total cell lysates were isolated and subjected to immunoblot analysis. Because H1R has constitutive activity, cells were also treated with rottlerin (rott) before histamine/PMA stimulation to determine the real basal level of ERK phosphorylation. In D, the cells were treated with rottlerin (10 μ M) for 1 h before stimulation with histamine (for 30 s) or PMA (for 2 min).

CHO cells treated with hydrogen peroxide, and for the apoptosis of HaCaT cells in response to UV radiation (28, 40, 41). PKC δ translocates to nearly all organelles, including the nucleus, mitochondria, Golgi, endoplasmic reticulum, and plasma membrane in response to various stimuli (42). Translocation of PKC δ to specific organelles leads to different cellular effects because of the phosphorylation of specific substrates and to the association of PKC δ with distinct proteins present in these locations. Thus, the target organelle of PKC δ is probably an important factor in the caspase-driven PKC δ degradation. Translocation of PKC δ to the Golgi may prevent PKC δ cleavage by caspase-3, although the mechanism involved in the activation of caspase-3 by PKC δ is unclear. In agreement with this assumption, there are reports indicating that ceramide stimulation induces translocation of PKC δ from the cytosol to the Golgi, whereas no significant degradation of PKC δ was observed in HeLa cells treated with ceramide (20, 43). It was also reported that a constitutively active PKC δ mutant that was targeted to the cytosol and mitochondria underwent cleavage by caspase, whereas no cleavage was observed in the PKC δ mutant that was targeted to the nucleus and endoplasmic reticulum (44).

Receptors for activated C kinase (RACKs) are a family of membrane-associated PKC-anchoring proteins that act as

molecular scaffolds to localize individual PKCs to distinct organelles in close proximity with their allosteric activators and unique intracellular substrates, and PKC-RACK interactions are essential for isoform-specific cellular responses. Kheifets *et al.* (45) reported that annexin V interacts with PKC δ upon PMA stimulation and translocates PKC δ from the cytosol to the membrane where PKC δ binds to the PKC δ -specific anchoring RACK. However, to date no studies have reported a PKC δ -specific RACK. Because PKC δ activated by H1R stimulation translocates to the Golgi and results in activation of the MEK/ERK signaling cascade, a PKC δ -specific RACK should be localized to the Golgi. Sef (similar expression to *fgf* genes) is a transmembrane protein that is localized on the Golgi. Sef binds to activated forms of MEK, inhibits the dissociation of the MEK-ERK complex, blocks nuclear translocation of activated ERK, and inhibits activation of the transcription factor Elk-1 (46). Sef was originally reported to act as a molecular switch for ERK signaling by specifically blocking ERK nuclear translocation without inhibiting its activity in the cytoplasm. However, the opposite role of Sef has also been reported wherein Sef plays a positive role in the EGFR-mediated MAPK signaling pathway (47). Because the binding of MEK-ERK complexes to Sef does not inhibit the activity of either kinase (46), ERK can phosphorylate its downstream signal protein. Activation of PKC δ by his-

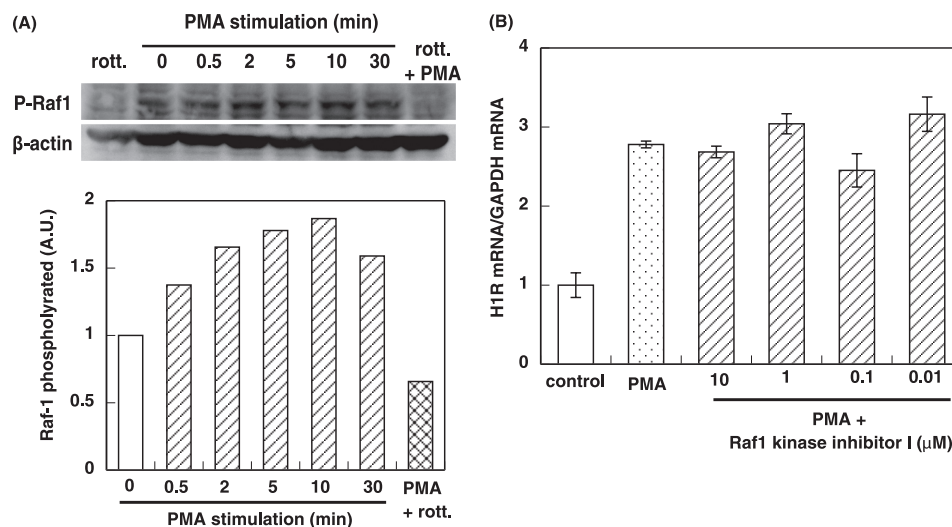


FIGURE 6. Raf-1 is not involved in PMA-induced up-regulation of H1R mRNA expression. A, shown is the time course of the PMA-induced phosphorylation of Raf-1. HeLa cells were stimulated with PMA (100 nM), and at the given time intervals total cell lysates were isolated and subjected to immunoblot analysis (top). The relative expression levels of Raf-1 normalized to that of β -actin are shown in the bottom. The cells were treated with rottlerin (rott, 10 μ M) for 1 h before stimulation with PMA for 2 min. Because H1R has constitutive activity, the cells were also treated with rottlerin before PMA stimulation to determine the real basal level of Raf-1 phosphorylation. B, HeLa cells were serum-starved for 24 h and then treated with Raf-1 kinase inhibitor I (0.01–10 μ M) 1 h before stimulation with 50 nM PMA for 3 h. Then total RNA was isolated, and the H1R mRNA levels were determined by real-time quantitative RT-PCR.

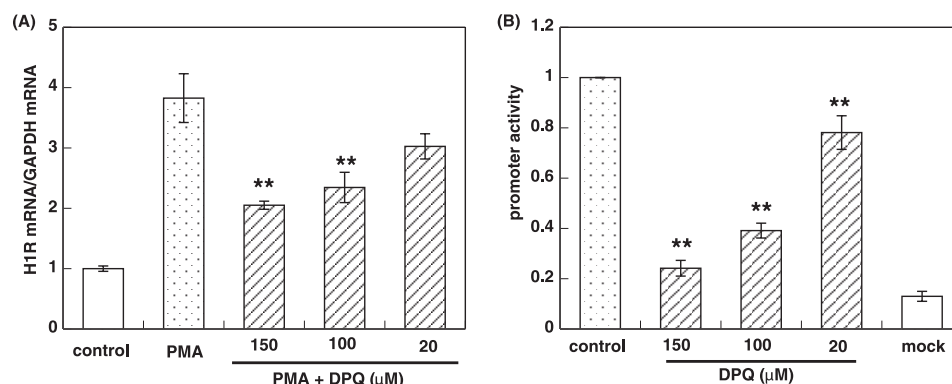


FIGURE 7. Effect of PARP-1 inhibitor on PMA-induced up-regulation of H1R mRNA expression. HeLa cells were serum-starved for 24 h and then treated with DPQ (20–150 μ M) 1 h before stimulation with 50 nM PMA for 3 h. In A, total RNA was isolated, and the H1R mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the means \pm S.E. ($n = 4$). **, $p < 0.01$ versus PMA. In B, HeLa cells were cotransfected with pH1R and pRL-MPK vectors, as described in Fig. 1B, and treated with DPQ (20–150 μ M) as described above. After stimulation, luciferase activity was determined using the Dual-luciferase Reporter Assay System. Data are presented as the means \pm S.E. ($n = 4$). **, $p < 0.01$ versus PMA.

tamine or PMA caused transient phosphorylation of ERK (Fig. 5C). Our recent studies indicate that Src, Raf-1, or ELK-1 is not involved in histamine/PMA-induced up-regulation of H1R in HeLa cells (Fig. 6).⁴ Thus, Sef (or a Sef-like scaffold protein that binds MEK-ERK) may function as a RACK for PKC δ in response to histamine, and PKC δ may phosphorylate MEK-ERK on Sef followed by activation of PARP-1 and downstream transcription factors that are involved in H1R gene transcription. We noted that ERK was phosphorylated without histamine/PMA stimulation. It is likely that this phosphorylation is due to the constitutive activity of H1R (23). The shutdown of H1R signaling by the PKC δ inhibitor rottlerin resulted in a decrease of the constitutive activity, thereby inhibiting ERK phosphorylation in both nonstimulated and histamine/PMA-stimulated cells (Fig. 5). Similar histamine-independent activation of H1R mRNA was observed, and rottlerin resulted in a further decrease in the H1R mRNA level compared with the basal level (Fig. 1A).

PARP-1 is a nuclear chromatin-associated protein that catalyzes posttranslational modification of proteins by poly(ADP-ribosylation), which affects protein-protein and protein-DNA interactions (48). PARP-1 is considered to play a role in the repair of single-stranded DNA breaks. However, recent works suggest that PARP-1 functions as a transcriptional regulator or co-regulator by direct interaction with transcription factors including YY1, NF- κ B, TFIIF, Oct-1, B-MYB, AP-2, and the HTLV Tax-1 protein (49–56). So far, more than 30 nuclear substrates of PARP-1 have been identified (48); however, poly(ADP-ribosylation) of Ku86 by PARP-1 has not been reported yet. It was reported that PARP-1 is activated by a variety of extracellular signals that activate the Raf/MEK/ERK phosphorylation cascade, and poly(ADP-ribosylated) PARP-1 amplifies ERK signals that target core histone acetylation and gene transcription (58, 59). Furthermore, PARP-1 directly interacts with NF- κ B and activates NF- κ B-dependent gene transcription (57). Therefore, it could be possible that acetylation or poly(ADP-

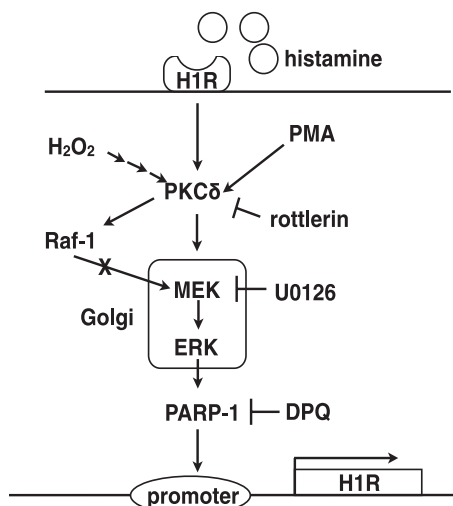


FIGURE 8. Schematic presentation of the signaling pathway involved in histamine-induced up-regulation of H1R mRNA expression in HeLa cells. Binding of histamine to H1R activates PKC δ and translocates PKC δ from the cytosol to the Golgi where PKC δ phosphorylates MEK and ERK. Phosphorylated ERK then activates PARP-1. Poly(ADP)-ribosylation of an unknown protein (or PARP-1 itself) by PARP-1 causes activation of a transcription factor, resulting in up-regulation of H1R gene transcription.

ribosyl)ation of histones induces decondensation of chromatin structure and enhances access of transcription factors to the H1R promoter, although we have not identified the target (substrate) of PARP-1 in the H1R up-regulation. PARP-1 could activate transcription factors involved in the H1R transcription regulation.

PMA-induced up-regulation of H1R gene expression was inhibited by DPQ (Fig. 7), and this up-regulation was also inhibited by another PARP-1 inhibitor, 1,5-isoquinolinediol (data not shown), suggesting that enzymatic activity of PARP-1 is required for H1R gene up-regulation in HeLa cells. Hao *et al.* (24) reported histamine-induced up-regulation of Egr-1 expression in human aortic endothelial cells via the H1R-mediated PKC δ -dependent ERK activation pathway. Their pathway is similar to our pathway reported here, but the downstream components of the ERK pathway are different. In their report, ERK activated cAMP response element-binding protein (CREB). On the other hand, the activation of PARP-1 is observed in the pathway involved in histamine-induced up-regulation of H1R gene expression.

In conclusion, we have demonstrated that the PKC δ /MEK/ERK/PARP-1 pathway is involved in histamine/PMA-induced up-regulation of H1R gene expression in HeLa cells. The H1R expression level is highly correlated to the severity of allergic symptoms, and compounds that suppress H1R gene expression alleviate allergic symptoms (12–16). Therefore, drugs targeting the proteins that compose the signaling pathway involved in histamine-induced up-regulation of H1R gene expression could be good therapeutics for allergic diseases. Our results provide information about target proteins for anti-allergic drugs that suppress H1R up-regulation. Also, our recent findings that many compounds isolated from natural resources that suppress up-regulation of H1R gene expression suppressed PKC δ phosphorylation⁴ suggest that PKC δ could be one of the most promising target proteins for anti-allergic drugs.

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