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Protein kinase C-α mediates TNF release process in RBL-2H3 mast cells

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1 To clarify the mechanism of mast cell TNF secretion, especially its release process after being produced, we utilized an antiallergic drug, azelastine (4-(*p*-chlorobenzyl)-2-(hexahydro-1-methyl-1*H*-azepin-4-yl)-1-(2*H*)- phthalazinone), which has been reported to inhibit TNF release without affecting its production in ionomycin-stimulated RBL-2H3 cells.

2 Such inhibition was associated with the suppression of an ionomycin-induced increase in membrane-associated PKC activity rather than the suppression of Ca^{2+} influx, suggesting that PKC might be involved in TNF release process.

3 To see whether conventional PKC family (cPKCs) are involved, we investigated the effects of a selective cPKC inhibitor (Gö6976) and an activator (thymeleatoxin) on TNF release by adding them 1 h after cell stimulation. By this time, TNF mRNA expression had reached its maximum. Gö6976 markedly inhibited TNF release, whereas thymeleatoxin enhanced it, showing a key role of cPKC in TNF post-transcriptional process, possibly its releasing step.

4 To determine which subtype of cPKCs could be affected by azelastine, Western blotting and live imaging by confocal microscopy were conducted to detect the translocation of endogenous cPKC (α, βI and βII) and transfected GFP-tagged cPKC, respectively. Both methods clearly demonstrated that 1 μM azelastine selectively inhibits ionomycin-triggered translocation of αPKC without acting on βI or βIIPKC.

5 In antigen-stimulated cells, such a low concentration of azelastine did not affect either αPKC translocation or TNF release, suggesting a functional link between αPKC and the TNF-releasing step.

6 These results suggest that αPKC mediates the TNF release process and azelastine inhibits TNF release by selectively interfering with the recruitment of αPKC in the pathway activated by ionomycin in RBL-2H3 cells.

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Abbreviations: ADAM-17, A disintegrin and a metalloproteinase-containing enzyme; cPKC, conventional protein kinase C; DNP–BSA, 2,4-dinitrophenyl–bovine serum albumin; FcεRI, high-affinity IgE receptor I; RBL-2H3 cells, rat basophilic leukemic-2H3 cells; TNF, tumor necrosis factor; TACE, TNF-alpha-converting enzyme

Introduction

TNF is a multifunctional cytokine and a key mediator of immune and inflammatory responses (Tracey & Cerami, 1994). Inappropriate production of TNF or sustained activation of TNF signaling has been implicated in the pathogenesis of a wide spectrum of human diseases including sepsis, cerebral malaria, diabetes, cancer, osteoporosis and allograft rejection (Aggarwal *et al*., 2002; Pfeffer, 2003; Borst, 2004). Mast cells are a potential source of inflammatory cytokines such as TNF (Robbie-Ryan & Brown, 2002) and play a key role in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases and Crohn's disease (Lee *et al*., 2002, Xu *et al*., 2004). Therefore, better understanding the mechanism underlying TNF release from mast cells should be helpful in developing future therapies for these inflammatory diseases.

The activation of mast cells *via* aggregation of their high-affinity receptor for IgE (FcεRI) leads to the release of granular mediators and arachidonic acid metabolites as well as the production of various cytokines including TNF (Paul *et al*., 1993; Galli & Wershil, 1996). The first recognized

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biochemical event of the cytoplasmic signal transduction cascade is tyrosine phosphorylation of FcεRI β- and γ-subunits by Lyn kinase. Phosphorylation of FcεRI γ-subunits allows the protein tyrosine kinase Syk to associate with the receptor, launching a cellular signal transduction cascade (Drâberovâ *et al*., 2004; Rivera, 2004). An immediate consequence is the mobilization of calcium and activation of PKC subtypes followed by degranulation and cytokine production and release (Rivera, 2004). Ca^{2+} ionophores such as ionomycin are believed to bypass these receptor-mediated events by directly conveying Ca^{2+} across the plasma membrane to activate PKC (Bennett *et al*., 1979; Hide *et al*., 1993; Hanson & Ziegler, 2001). It has been found that TNF is preformed and stored in secretory granules of mast cells and is newly synthesized following mast cell activation (Gordon & Galli, 1990; 1991). The RBL-2H3 mast cell line does not store TNF in secretory granules, but releases it by a Golgi-dependent mechanism, which unlike constitutive secretion, is highly regulated by Ca^{2+} and PKC (Baumgartner *et al*., 1994). However, the mechanism which regulates TNF secretion, especially its releasing step following TNF production, remains to be clarified.

The PKCs consist of a family of at least 10 serine/threonine kinases that participate in signal transduction events in response to hormonal, neuronal and growth factor stimuli (Nishizuka, 1992; Hofmann, 1997; Mellor & Parker, 1998). Differences in their structure and substrate requirement have permitted division of the isoforms into three groups: (1) conventional PKCs (cPKC) (α, βI, βII and γ), which are Ca^{2+} dependent and activated by both phosphatidylserine (PS) and the second messenger diacylglycerol (DAG); (2) novel PKCs (nPKC) (ε, δ and θ), which are Ca^{2+} independent and regulated by DAG and PS; and (3) atypical PKCs (aPKC) (ζ and λ), which are also Ca^{2+} independent and do not require DAG for activation, although PS regulates its activity (Newton, 1997; Kanashiro & Khalil, 1998; Liu & Heckman, 1998). cPKCs and nPKCs, which possess regulatory C1 domains , are known to be translocated from cytosol to membranes, where they may be activated by DAG or phorbol esters (Kraft *et al*., 1982). Therefore the translocation of PKC is a good marker of its activation.

Azelastine, 4-(*p*-chlorobenzyl)-2-(hexahydro-1-methyl-1*H*-azepin-4-yl)-1-(2*H*)- phthalazinone, is an antiallergic drug with high affinity for histamine H_1 receptors and is able to block histamine release from mast cells. Previously, we reported that azelastine inhibited TNF release at lower concentrations than those needed for the inhibition of degranulation. This effect of azelastine was independent of its blocking action on H_1 receptors. Inhibition of TNF release by azelastine was particularly prominent when cells were activated by ionomycin (Hide *et al*., 1997). This inhibition might occur at the release stage of TNF rather than at its synthesis through interference with PKC activation. So far, however, there is no direct evidence to indicate which PKC subtype might participate in the TNF release process.

In the present study, we have investigated the role of cPKC in TNF release by using a specific inhibitor and an activator of cPKC, added 1 h after cell activation, and to identify which cPKC subtype is involved in the release, we have examined the effects of azelastine on the translocation of cPKCs, α, βI and βII, in RBL-2H3 cells, by conventional immunoblotting analysis and live cell imaging.

Methods

Cell culture

The rat mast cell line (RBL-2H3) was obtained from Dr M.A. Beaven (National Institutes of Health, Bethesda, MD, U.S.A.) and cells were grown in RPMI-1640 supplemented with 10% FCS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin solutions (cRPMI). The cells were cultured in 12-well plates (4×10^5 cells 0.8 ml^{-1}well^{-1}) in growth media in preparation for TNF release. In the case of antigen-stimulated cells, the cultures were incubated overnight with 0.5 μg ml^{-1} of 2,4-dinitrophenyl (DNP)-specific IgE to ensure maximal occupancy of the high-affinity IgE receptor, FcεRI.

Measurement of TNF release

Following the washing step, RBL-2H3 cells were stimulated with either 10 ng ml^{-1} antigen, DNP–BSA (DNP–BSA), or 1 μM ionomycin, 1 h later 3 μM Gö6976 or 500 nM thymeleatoxin was added to the medium and the released TNF was measured before and at 1, 2, 3 and 4 h after antigen or ionomycin stimulation. Control experiments were performed without the addition of Gö6976 or thymeleatoxin for the same time course. In the study of azelastine effects on TNF release, cells were incubated with cRPMI containing the indicated concentrations of azelastine for 10 min at 37°C, and then stimulated for 3 h with 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin. For measurement of intracellular TNF content, RBL-2H3 cells were incubated with 1 μM azelastine for 10 min, and then stimulated by the same concentrations of antigen or ionomycin for 2 h. Cells lysates were prepared in 0.1% Triton X-100 in cRPMI, which had no effect on measurement by ELISA. Samples of both medium and cell lysate were stored at −80°C for TNF assay.

TNF assay

TNF was assayed in 50 μl samples of the medium using a rat TNF ELISA kit (Biosource International, Camarillo, CA, U.S.A.). The assay was performed according to the manufacturer's instructions. Standard solutions of rTNF were prepared in cRPMI to give the same composition as supernatant samples.

Isolation of total RNA and real-time quantitative RT–PCR

Control RBL-2H3 cells were stimulated directly with 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin for 1 h. Treated cells were incubated with 1 μM azelastine for 10 min, and then stimulated with the same concentrations of antigen and ionomycin for 1 h. Total mRNA was isolated from the cells using TRIzol reagent (Life Technologies, Inc., Grand Island, NY, U.S.A.) according to the manufacturer's protocol. The mRNA of TNF was measured by real-time quantitative RT–PCR (ABI Prism model 7700 sequence detection system, PE Applied Biosystems, Foster City, CA, U.S.A). RT–PCR was carried out using TaqMan® one-step RT–PCR Maser Mix reagents kit according to the manufacturer's protocol (Applied Biosystems). The sequences of the forward and reverse primers were 5′-ACAAGGCTGCCCCGACTAC-3′ and 5′-TCCTGG

TATGAAATGGCAAACC-3′, respectively. The TaqMan® fluorogenic probe was 5′-6FAM-TGCTCCTCACCCACACC GTCAGC-TAMIRA-3′.

During PCR amplification, 5′ nuclease activity of AmpliTaq Gold® DNA polymerase cleaves the TaqMan probe separating the 5′ reporter dye from the 3′ quencher dye, resulting in increased fluorescence of the reporter. The threshold cycle, C_T, which correlates inversely with the target mRNA levels, was measured as the cycle number at which reporter fluorescent emission increases above a threshold level. The TNF mRNA levels were corrected for the C_T values of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) RNA using VIC™ probe according to the manufacturer's protocol.

Western blot analysis (endogenous cPKCs translocation)

Endogenous cPKCs translocated to the membranes were detected by Western blotting. Cells were cultured in 60 mm dishes (3×10^6 cells 3 ml^{-1} dish^{-1}) and incubated overnight with 0.5 μg ml^{-1} of DNP-specific IgE at 37°C. For control dishes, following a washing step, the cells were stimulated with either 10 ng ml^{-1} antigen, DNP–BSA, or 1 μM ionomycin for 0, 1, 3 and 5 min in glucose–Pipes buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, 1 mg ml^{-1} BSA, 25 mM Pipes/NaOH, pH 7.4). For azelastine-treated dishes, the cells were pretreated with 1 μM of azelastine for 10 min at 37°C, and then stimulated with either 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin for 0, 1, 3 and 5 min in glucose–Pipes buffer. After washing in ice-cold glucose–Pipes buffer, the cells were scraped off and sonicated in extraction buffer (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 50 μg ml^{-1} PMSF and 5 μg ml^{-1} Leupeptin). The homogenate was centrifuged at 180,000 × *g* for 20 min at 4°C and the pellet was resuspended with sonication in 1 ml of extraction buffer and centrifuged at 180,000 × *g* for 20 min at 4°C. The membrane fraction was solubilized in 100 μl of extraction buffer containing 0.1% Triton X-100. Protein concentrations were determined by Bradford assay. The homogenate was diluted in SDS sample buffer and boiled at 95°C for 5 min. The membrane proteins were separated by SDS–PAGE and blotted onto PVDF membranes. Western blotting was performed according to the manufacturer's protocol and the immunoreactivity was detected by ECL detection reagents.

Recording of GFP-tagged cPKCs translocation using laser scanning microscope (LSM)

The expression plasmids bearing cDNA of αPKC-GFP, βIPKC-GFP and βIIPKC-GFP were prepared as described previously (Sakai *et al*., 1997; Shirai *et al*., 1998). Transfected, GFP-tagged cPKCs translocated to membranes were monitored by LSM. The cells were cultured in 35 mm cell culture dish (5×10^4 cells 0.1 ml^{-1} dish^{-1}) and incubated overnight in a CO_2 incubator at 37°C. Next day, following a washing step with Opti-MEM, the cells were transfected with GFP-tagged PKCs, that is, αPKC-GFP, βIPKC-GFP and βIIPKC-GFP using Fugene 6 as transfection reagent, and then incubated in a CO_2 incubator at 37°C for 4 h. For antigen-stimulated dishes, the cultures were incubated overnight with 0.5 μg ml^{-1} of DNP-specific IgE. On the third day, the cells were pretreated with or without 1 μM azelastine for 10 min, then stimulated with either 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin. The translocation of αPKC-GFP, βIPKC-GFP and βIIPKC-GFP was monitored using LSM 510 Meta (Carl Zeiss, Germany) using a ×63 oil objective lens. The transfection efficiency was less than 5% in RBL-2H3 cells. Time series of 101 confocal images were recorded for each experiment at intervals of 6 s.

Materials

Reagents were obtained from the following sources: ionomycin, Gö6976 and thymeleatoxin from Calbiochem (La Jolla, CA, U.S.A.); antigen, DNP–BSA, DNP-specific monoclonal IgE were a kind gift from Professor K. Maeyama (Ehime University, School of Medicine, Ehime, Japan); all materials for cell culture were from Life Technologies (Grand Island, NY, U.S.A.); Fugene 6 was from Roche Applied Science (Indianapolis, IN, U.S.A.); azelastine hydrochloride was supplied from Eisai Co. (Tokyo, Japan); TaqMan® one-step RT–PCR Master Mix reagents kit and TaqMan® rodent GAPDH control reagents were from PE Applied Biosystems (Foster City, CA, U.S.A.). Mouse monoclonal antibodies IgG against αPKC and rabbit polyclonal IgG against βIPKC and βIIPKC were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Mouse monoclonal IgG against βPKC was from BD Bioscience Pharmingen (San Jose, CA, U.S.A.). Anti-rabbit IgG HRP-linked antibody and anti-mouse IgG HRP-linked antibody were from Cell Signaling Technology (Beverly, MA, U.S.A.)

Statistical analysis

The results of the experiments were expressed as means ± s.e.m. from three independent experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. Probability values (P) less than 0.05 were considered to be statistically significant.

Results

Effects of the cPKC inhibitor (Gö6976) and the activator (thymeleatoxin) on TNF release induced by antigen or ionomycin in RBL-2H3 cells

It is known that TNF secretion is totally dependent on the presence of extracellular Ca^{2+} and the activation of protein kinase C (Baumgartner *et al*., 1994). We previously reported that, azelastine inhibited TNF release and PKC-mediated protein phosphorylation, without affecting the intracellular TNF protein level or Ca^{2+} signals in ionomycin-stimulated RBL-2H3 cells (Hide *et al*., 1997), suggesting a role of PKC in the TNF-release process, following its synthesis. To determine which group of PKCs is involved, we first investigated the role of cPKC in the TNF-releasing step using the selective inhibitor or activator of cPKC, Gö6976 and thymeleatoxin, respectively (Ryves *et al*., 1991; Martiny-Baron *et al*., 1993; Llosas *et al*., 1996). Since βPKC is reported to be involved in TNF gene expression (Kawakami *et al*., 2003) and we also confirmed that Gö6976 markedly suppressed TNF mRNA expression in antigen- or ionomycin-stimulated RBL-2H3 cells (data not shown), we examined the effects of Gö6976 added after 1 h of

stimulation with antigen or ionomycin, because by this time TNF mRNA expression had reached its maximum, and the produced TNF started to be released (Hide *et al*., 1997). In antigen- or ionomycin-stimulated cells, the amount of TNF released into the medium was detectable after 1 h of stimulation with 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin and progressively increased reaching a maximum after 4 h of stimulation (Figure 1a and b). Gö6976 (3 μM) added after 1 h of stimulation with either antigen or ionomycin caused complete suppression of TNF release, and this effect continued over the next 3 h of its addition (Figure 1a and b). Furthermore, selective activation of cPKC by thymeleatoxin after 1 h of stimulation markedly enhanced TNF release compared to that of control antigen or ionomycin stimulation, and this enhancing effect of thymeleatoxin also remained even 3 h after its addition (Figure 1a and b). These results strongly indicate that the releasing step of TNF is regulated by cPKC in both antigen and ionomycin stimulation. It is of note that more TNF was released by ionomycin than by antigen and the enhancement by thymeleatoxin was also more in ionomycin-stimulated cells. These results prompted us to investigate the effects of azelastine on TNF release in the context of its possible effect on cPKC activation.

Effects of azelastine on antigen- or ionomycin-induced TNF release, TNF mRNA expression and TNF production in RBL-2H3 cells

Azelastine inhibits ionomycin-induced TNF release at lower concentrations than those needed to inhibit antigen-induced TNF release. In addition, unlike in ionomycin-stimulated cells, the inhibitory effects of azelastine on TNF release and its production seem to be well-correlated in antigen-stimulated cells (Hide *et al*., 1997). To determine a concentration of azelastine that could be used to discriminate between antigen- and ionomycin-induced TNF release in RBL-2H3 cells, we examined the effects of different concentrations of azelastine on TNF release from RBL-2H3 cells stimulated by 10 ng ml^{-1} antigen or 1 μM ionomycin. Azelastine inhibited both antigen- and ionomycin-induced TNF release in a concentration-dependent manner with IC_{50} values of 21.25 ± 2.3 μM ($n=3$) and 1.07 ± 0.04 μM ($n=3$), respectively (Figure 2a). Since 1 μM azelastine inhibited ionomycin-induced but not antigen-induced TNF release; we focused on this concentration to further investigate the step at which 1 μM azelastine acts, TNF production or TNF release. Pretreatment of RBL-2H3 cells with 1 μM azelastine did not inhibit TNF mRNA expression or protein production in antigen- or ionomycin-stimulated cells compared to control only (Figure 2b), suggesting that azelastine at this concentration (1 μM) blocks the TNF release process (Figure 2a) without affecting its production. We therefore used this concentration for subsequent experiments.

Effects of azelastine on the translocation of endogenous cPKC (α, βI and βII) induced by antigen or ionomycin (Western blotting analysis)

An attempt was undertaken to identify the cPKC subtype that is involved in the TNF release process by studying further the effect of a low concentration (1 μM) of azelastine. First, we investigated the effect of azelastine on cPKC translocation induced by antigen or ionomycin by Western blotting analysis. After stimulation with 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin, all endogenous αPKC, βIPKC and βIIPKC translocated to the membranes of RBL-2H3 cells (Figure 3a and b). Treatment of the cells with 1 μM azelastine did not inhibit any antigen-induced translocation of αPKC, βIPKC or βIIPKC (Figure 3a). By contrast, in ionomycin-stimulated cells, the treatment with 1 μM azelastine inhibited selectively the translocation of αPKC to the membranes, without

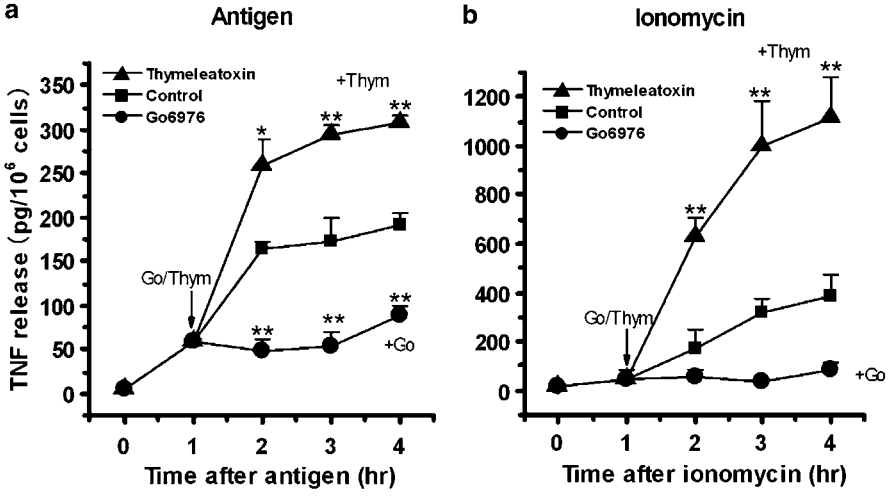

Figure 1 Effects of the cPKC inhibitor (Gö6976) and the activator (thymeleatoxin) on TNF release induced by antigen (a) or ionomycin (b) in RBL-2H3 cells. Cells were stimulated with either 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin, 1 h later 3 μM Gö6976 or 500 nM of thymeleatoxin were added to the culture. TNF released (pg 10^{-6} cells) was assessed in the medium directly before and at 1, 2, 3 and 4 h after the addition of antigen, DNP–BSA, or ionomycin. Control cells were not treated with either Gö6976 or thymeleatoxin. Values are expressed as mean ± s.e.m. of TNF release from three independent experiments with three cultures. At each time point, the difference between TNF released from Gö6976- or thymeleatoxin-treated cells compared to that of control cells was tested with one-way ANOVA followed by Dunnett's test and considered significant with $P<0.05$ (*) or $P<0.01$ (**).

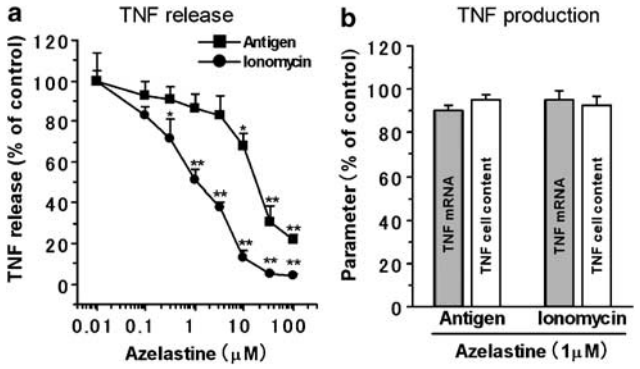

Figure 2 Effects of azelastine on antigen- or ionomycin-induced TNF release (a), and TNF mRNA expression and production (b) in RBL-2H3 cells. For TNF release, cells were incubated for 10 min with different concentrations of azelastine then challenged with either 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin, for 3 h. For TNF mRNA and production, cells were incubated for 10 min with 1 μM of azelastine then stimulated with either 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin, for 1 h (in case of TNF mRNA) and 2 h (in case of TNF production). The levels of TNF mRNA were measured by real-time RT–PCR and intracellular content of TNF was measured in cell lysates as described in Methods. Values are expressed as mean $\pm$ s.e.m. of the percentage of TNF release, mRNA expression and production relative to control (without azelastine) in three independent experiments. Values of 100% for TNF release were 241.7 $\pm$ 31.9 pg 10^{-6} cells and 390.7 $\pm$ 17.33 pg 10^{-6} cells for antigen and ionomycin, respectively. The difference between control and treated cells was tested with one-way ANOVA followed by Dunnett's test and considered significant with $P<0.05$ (*) or $P<0.01$ (**).

affecting that of βIPKC or βIIPKC (Figure 3b). The inhibition of αPKC translocation was partial after 1 min but completed within 5 min of ionomycin stimulation. This selective inhibition raises the possibility that αPKC is required for the TNF releasing step in RBL-2H3 cells.

The high concentration of azelastine (10 μM) caused a partial but significant inhibition of TNF release in antigen-stimulated RBL-2H3 cells (Figure 2a). However, this concentration of azelastine partially suppressed the translocation of not only αPKC, but also βPKC, and TNF mRNA expression to some extent in antigen-stimulated cells (data not shown). In the case of ionomycin stimulation, 10 μM azelastine also inhibited the translocation of βPKC and TNF mRNA expression (data not shown). These results provide additional evidence that only ionomycin-stimulated pathway to activate αPKC was so sensitive to such low concentration (1 μM) of azelastine, and higher concentration (10 μM) was required to interfere with the activation of αPKC and βPKC generated by antigen or ionomycin. Additionally, these data also suggest a role of βPKC in TNF mRNA expression.

Translocation of GFP-tagged cPKC (α, βI and βII) induced by antigen or ionomycin: effects of azelastine

To obtain further information about cPKC subtypes involved in TNF release from RBL-2H3 cells, we conducted real-time imaging of the translocation of PKC fused to GFP, expressed in transfected cells and examined the effects of azelastine. Confocal LSM images showed that αPKC-GFP, βIPKC-GFP and βIIPKC-GFP were all translocated to the plasma membrane of RBL-2H3 in response to 10 ng ml^{-1} antigen,

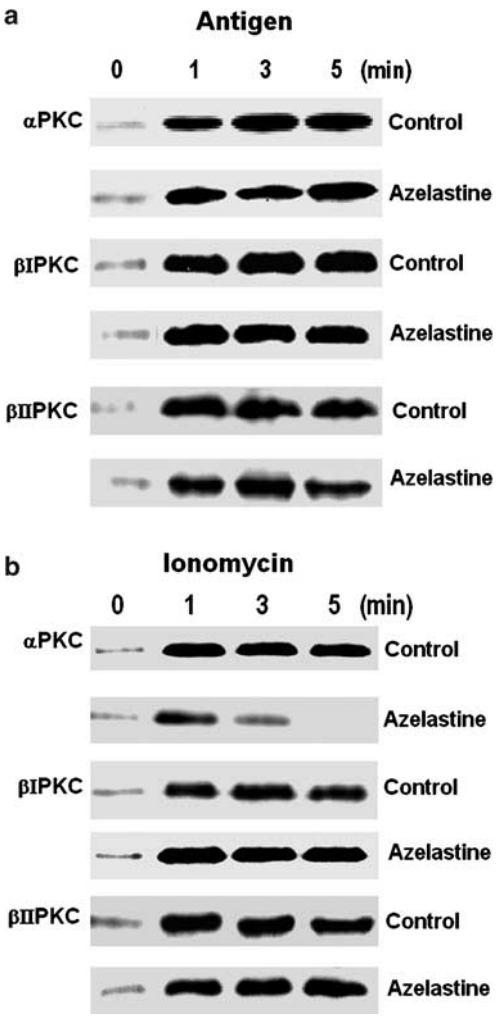

Figure 3 Effects of azelastine on the translocation of endogenous cPKC (α, βI and βII) induced by antigen, DNP–BSA (a) or ionomycin (b). Control RBL-2H3 cells were stimulated with either 10 ng ml^{-1} of antigen, DNP–BSA or 1 μM ionomycin for 0, 1, 3 and 5 min. For azelastine treated, cells were incubated with 1 μM of azelastine for 10 min before stimulation. The membrane fractions were prepared as described in Methods. Detection of each cPKC subtype in the membrane fractions was performed by SDS–PAGE and Western blotting using specific antibodies against αPKC, βIPKC or βIIPKC. Similar results were obtained from three independent experiments.

DNP–BSA or 1 μM ionomycin (Figure 4a and b). In agreement with the Western blotting data, pretreatment with 1 μM azelastine had no effect on antigen-induced translocation of transfected αPKC-GFP, βIPKC-GFP and βIIPKC-GFP (Figure 4a). Ionomycin (1 μM) also caused rapid translocation of αPKC-GFP, βIPKC-GFP and βIIPKC-GFP, from the cytosol to the membranes of transfected RBL-2H3 cells (Figure 4b), but it is notable that αPKC and βIPKC appeared to target not only the plasma membrane but also a perinuclear region, possibly the Golgi apparatus, in some ionomycin-stimulated cells. Again, pretreatment with 1 μM azelastine prevented αPKC-GFP from translocating to the plasma membrane in response to ionomycin (Figure 4b), while the translocation of βIPKC-GFP and βIIPKC-GFP was not affected (Figure 4b). Therefore, the finding that ionomycin-induced translocation of αPKC (endogenous or transfected)

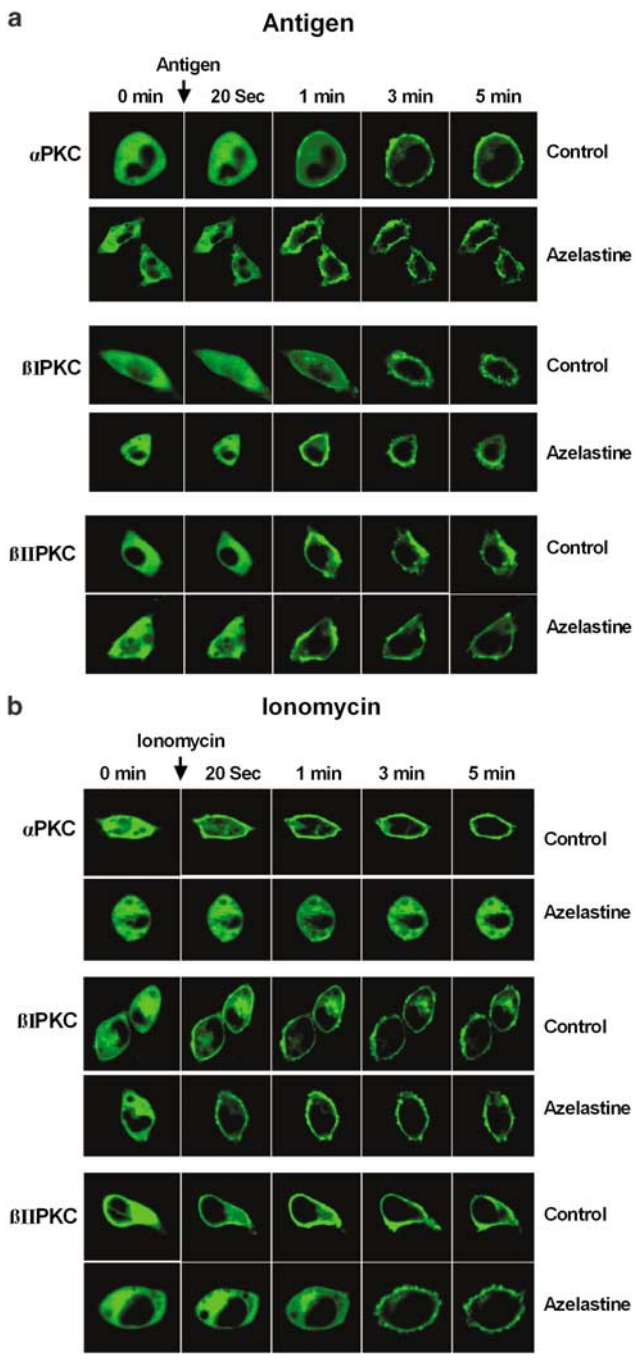

Figure 4 Effects of azelastine on the translocation of GFP-tagged cPKC (α, βI and βII) induced by antigen, DNP–BSA (a) or ionomycin (b). RBL-2H3 cells were transfected with αPKC-GFP, βIPKC-GFP and βIIPKC-GFP. Control cells were stimulated with 10 ng ml^{-1} antigen, DNP–BSA or 1 μM ionomycin for 10 min. Treated cells were incubated with 1 μM azelastine for 10 min, and then stimulated by either antigen or ionomycin for 10 min. The translocations of αPKC-GFP, βIPKC-GFP and βIIPKC-GFP, were monitored and recorded using LSM. Similar results were obtained from three independent experiments.

was inhibited by pretreatment with 1 μM azelastine shown by Western blotting, and was confirmed by the live imaging data obtained by LSM. Furthermore, this real-time morphological analysis revealed that ionomycin induced faster translocation of all cPKCs used in this study, compared with that induced by antigen. It also elicited targeting to a perinuclear region of the cells, possibly the Golgi apparatus from which pro-TNF is transported.

Discussion

In the present study, we have provided evidence that, in RBL-2H3 mast cells, the TNF-releasing step is controlled by cPKC. Furthermore, because the same low concentration of azelastine that inhibits TNF release without affecting TNF production, selectively blocked the translocation of αPKC in ionomycin-stimulated RBL-2H3 cells, we show that among cPKCs, αPKC is likely to be particularly important for the final TNF releasing step.

Upon cell activation, TNF gene transcription starts, and TNF protein is synthesized and subsequently released to the extracellular space. The TNF molecule is initially synthesized as a 26 kDa membrane-bound precursor (pro-TNF), which is transported through a Golgi-dependent pathway to the cell surface and is processed there to generate the soluble 17 kDa mature form (Mohan *et al*., 2002 & Solomon *et al*., 1999; Newton *et al*., 2001). This processing utilizes a cell-associated metalloproteinase enzyme, which has been isolated and cloned by Black *et al*. (1997) and Moss *et al*. (1997), and it has been termed TNF-alpha-converting enzyme (TACE) or a disintegrin and a metalloproteinase-containing enzyme (ADAM)-17. The TNF releasing steps including vesicular transport, exocytosis of pro-TNF and a TNF shedding process may be regulated independently of the TNF production, because sustained cell activation is required to complete TNF release (Baumgartner *et al*., 1994). Although the regulation of TNF mRNA expression and TNF release have been widely investigated, so far little information is available about the regulation of the release following protein synthesis.

A role for cPKC in TNF release was indicated in the present study by examining the effects of Gö6976, a selective cPKC inhibitor and thymeleatoxin, a selective PKC activator on TNF-releasing step from RBL-2H3 cells. Even after 1 h of stimulation with antigen, DNP–BSA or ionomycin, TNF release was completely suppressed after the addition of Gö6976, although TNF mRNA expression had reached its maximum and produced TNF was ready to be released by this time (Hide *et al*., 1997), suggesting that prolonged activation of cPKC may be required not only for TNF mRNA expression but also for TNF release. Moreover, thymeleatoxin added 1 h after stimulation with antigen or ionomycin markedly enhanced the release of TNF, supporting a key role of cPKC in the TNF-releasing step. It is of note that TNF release from cells stimulated with ionomycin was more sensitive to thymeleatoxin than release from cells stimulated with antigen.

Antiallergic drugs including azelastine are known to inhibit histamine release, mainly by interfering with Ca^{2+} influx in mast cells. Our previous report (Hide *et al*., 1997) showed that azelastine inhibited TNF release more potently than it inhibited degranulation in RBL-2H3 cells, suggesting that the drug might be effective for treating allergic inflammation as well as immediate allergic reactions. Furthermore, azelastine inhibited TNF release in response to ionomycin more than that in response to antigen. Inhibition of ionomycin-induced TNF release by azelastine at low concentration was not associated with the inhibition of Ca^{2+} influx, but was closely associated

with the inhibition of the ionomycin-induced increase in membrane-associated PKC activity (Hide *et al*., 1997). Our results show that, while a low concentration (1 μM) of azelastine inhibited TNF release, it did not affect TNF mRNA expression or protein synthesis, specifically in ionomycin-stimulated RBL-2H3 cells, suggesting that at low concentrations azelastine may specifically interfere with TNF release, possibly by inhibiting a signal other than Ca^{2+}. Like the selective cPKC inhibitor, Gö6976 (Figure 1), azelastine (1 μM) inhibited TNF release; therefore, it is conceivable that cPKCs may be a target for the inhibitory action of low concentrations of azelastine.

At least five PKC subtypes are present in the RBL-2H3 cell line (Ozawa *et al*., 1993). They include the conventional Ca^{2+}-dependent α and β (I, II) PKCs, but not γPKC, and the Ca^{2+}-independent ε, δ and ζ PKC subtypes. To identify which cPKC subtype (α, βI, βII) may be affected by azelastine and involved in TNF-releasing step from RBL-2H3 cells, we carried out not only Western blotting that shows the translocation of endogenous PKCs but also the real-time imaging of transfected cells expressing GFP-tagged cPKC, which makes it possible to monitor the spatiotemporal dynamics of PKCs translocation through visualization in living cells (Sakai *et al*., 1997). All cPKC subtypes (α, βI, βII) translocated to the plasma membrane of RBL-2H3 cells stimulated with antigen, DNP–BSA or ionomycin, although the time course and the target of translocation were not identical for each PKC subtype and stimulus. Ionomycin caused more rapid translocation of cPKC than antigen. In addition, all cPKC were mainly localized in the plasma membrane after antigen stimulation, while after ionomycin stimulation αPKC and βIPKC were observed to move to a perinuclear region, possibly the Golgi apparatus where pro-TNF is transported. Both Western blotting and live cell imaging revealed that, of the cPKCs, only αPKC translocation in response to ionomycin was potently inhibited by pretreatment with 1 μM azelastine, which acts at a terminal TNF-releasing stage. This suggests a role of αPKC in the TNF-releasing step.

The mechanism underlying the regulation of the TNF-releasing step by αPKC remains to be determined. Recent reports indicate that αPKC is implicated in phospholipase D (PLD) activation (Mukherjee *et al*., 1996; Siddiqi *et al*., 2000; Meacci *et al*., 2001; Oka *et al*., 2002). Also in RBL-2H3 mast cells, αPKC is involved in PLD activation (Powner *et al*., 2002). PLD hydrolyzes phosphatidylcholine to generate a lipid mediator, phosphatidic acid, which can be converted to DAG and lysophosphatidic acid. Phosphatidic acid and its dephosphorylated product DAG are important second messengers (Exton, 1999; Frohman *et al*., 1999; Liscovitch *et al*., 2000). Recent studies indicate that phosphatidic acid and its immediate metabolites, affect numerous cellular pathways, including ligand-mediated secretion and cytokine release (Steed & Chow, 2001). TNF release was suppressed, in macrophage cell line by using a combination of PLD and PLC inhibitors, indicating that PLD may contribute to TNF release (Loegering & Lennartz, 2004). Therefore, azelastine at a concentration of 1 μM may inhibit ionomycin-induced αPKC translocation, and consequently inhibit downstream signaling mechanisms induced by PLD and terminate TNF release from RBL-2H3.

Another possible action of αPKC might be related to the regulation of TACE activation. After cell activation, a number of cell surface proteins, including TNF, are released into the surrounding medium. Such shedding has been found to depend on a metalloproteinase enzyme (TACE) (Newton *et al*., 2001). This enzyme is expressed constitutively (Black *et al*., 1997), although it appears to require activation to become a pro-TNF processing protease (Pradines-Figurees & Raetz, 1992). Recently, it has been reported that activation of PKC dramatically increases the activity of TACE in endothelial cells (Tsou *et al*., 2001). Hurtado *et al*. (2001) have reported that TACE activity was inhibited by bisindolylmaleimide I, indicating that TACE activity is regulated by PKC. TACE is expressed in TNF-producing cells such as monocytes, neutrophils and peripheral blood T cells (Black *et al*., 1997). RBL-2H3 cells use the same enzyme for cleavage of membrane-bound pro-TNF to mature TNF (Hide *et al*., 1997). Thus, an alternative action of azelastine is to inhibit the processing mechanism of TNF on the cell surface by blocking αPKC activation.

Targeting the cytokine-specific release pathway is essential for developing strategies for preventing mast cell-mediated diseases. Our primary aim in this study was to clarify the molecular mechanism that controls the delivery of preformed TNF to the external milieu. Understanding this mechanism would be helpful in developing future therapies for diseases that may be caused by TNF, including chronic inflammation (Bruunsgaard & Pedersen, 2003), as well as the recruitment of neutrophils and eosinophils in asthma (Hart, 2001; Thomas, 2001).

In conclusion, our results suggest that cPKCs play a critical role in TNF release and that a low concentration of azelastine (1 μM) selectively inhibits ionomycin-induced TNF release without affecting its production, by interfering with PKC translocation generated by ionomycin. We also provide evidence that among cPKCs, αPKC is particularly involved in the TNF release process. This finding raises the possibility that modulation of αPKC activation may provide a novel approach towards therapeutic intervention in mast cell TNF-mediated diseases.

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