

Activation of human mast cells through the platelet-activating factor receptor

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Background: In human subjects platelet-activating factor (PAF) concentrations are markedly increased in the plasma after anaphylactic reactions, and these correlate strongly with the severity of the response. The mechanism for the systemic spread of mast cell (MC) activation in anaphylaxis is often assumed to relate to the hematogenous spread of allergen, but this is implausible, and amplification mechanisms need to be considered.

Objective: We have investigated the ability of PAF to induce human MC degranulation using skin, lung, and peripheral blood (PB)-derived cultured MCs and the signaling pathways activated in PB-derived MCs in response to PAF.

Methods: The expression of PAF receptor was investigated by means of RT-PCR and Western blot analysis. Cell-signaling pathways in PB-derived MCs in response to PAF were investigated by analyzing the effect of various inhibitors and the silencing of phospholipase C (PLC) mRNA on PAF-mediated histamine release.

Results: We show for the first time that PAF induces histamine release from human lung MCs and PB-derived MCs but not skin MCs. Activation of PAF receptor-coupled G_{αi} leads to degranulation through PLCγ1 and PLCβ2 activation in human

MCs. PAF-induced degranulation was rapid, being maximal at 5 seconds, and was partially dependent on extracellular Ca²⁺. **Conclusion:** Our findings provide a mechanism whereby PAF mediates an amplification loop for MC activation in the generation of anaphylaxis. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Anaphylaxis, histamine, human lung mast cells, human peripheral blood-derived mast cells, human skin mast cells, platelet-activating factor receptor

Mast cells (MCs) play a key role in IgE-dependent allergic diseases, such as asthma, rhinoconjunctivitis, and anaphylaxis, through the release of a variety of vasoactive and bronchospastic autacoid mediators and functionally diverse proteases, chemokines, and cytokines.^{1,2} Anaphylaxis is a systemic life-threatening event characterized by local swelling, laryngeal edema, acute bronchospasm, urticaria, gut edema and dysmotility, and cardiovascular collapse. Respiratory embarrassment and cardiovascular collapse are the life-threatening events. Sometimes seemingly minute quantities of allergen can induce severe and fatal systemic reactions. MC activation is a central component of this anaphylactic response, a view supported by the finding of increased concentrations of MC-derived β-tryptase in the peripheral circulation.^{3,4} The mechanism for the rapid systemic spread of MC activation in patients with anaphylaxis is often assumed to relate to the hematogenous spread of allergen, but this is perhaps implausible after oral exposure, and amplification mechanisms need to be considered (eg, through neurological reflexes).

Platelet-activating factor (PAF) has recently been implicated in the pathogenesis of anaphylaxis.⁵⁻⁸ Not only does it make a major contribution to the anaphylactic response in murine models,⁵⁻⁷ but also in human subjects PAF concentrations are markedly increased in the plasma after anaphylactic reactions, and these correlate strongly with the severity of the response.⁸ Furthermore, the chief metabolizing enzyme for PAF, PAF acetylhydrolase, demonstrates reduced expression in patients with anaphylaxis.⁸ There is evidence that PAF can activate human MCs through indirect mechanisms in both the skin and the lung. In the skin neural reflexes are required for the activation of MCs after administration of PAF,⁹ and isolated human skin MCs do not degranulate in response to PAF directly.¹⁰ In the lung one study reported that PAF induced the release of arachidonic acid metabolites from human lung fragments but not purified MCs, although cells from only 2 donors were studied.¹¹ Whether PAF induces human lung MC degranulation, arachidonic acid metabolism, or cytokine production is therefore not known.

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Abbreviations used

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
 GPCR: G protein–coupled receptor
 IMDM: Iscove modified Dulbecco medium
 MC: Mast cell
MRGX2: Mas-related G protein–coupled receptor family member X2
 PAF: Platelet-activating factor
 PAF-R: Platelet-activating factor receptor
 PAI-1: Plasminogen activator inhibitor 1
 PB: Peripheral blood
 PG: Prostaglandin
 PKC: Protein kinase C
 PLC: Phospholipase C
 PTX: Pertussis toxin
 shRNA: Short hairpin RNA

In this study we have investigated the ability of PAF to activate MCs using primary cells isolated from the skin and lung and cells generated *in vitro* from adult peripheral blood (PB)–derived progenitors. We show for the first time that PAF induces histamine release from lung MCs and PB-derived MCs but not skin MCs and that this signals through PAF receptor (PAF-R)–coupled $G_{\alpha i}$ and activation of phospholipase C (PLC) $\gamma 1$ and PLC $\beta 2$. Thus we provide a biologically plausible mechanism that links increased PAF concentrations in human anaphylaxis to an amplified airway MC response.

METHODS**Ethical considerations**

The study was approved by the Ethics Committee of Nihon University, Dokkyo Medical University Koshigaya Hospital, and the Leicestershire Research Ethics Committee, and all subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association.

Isolation, purification, and culture of human lung and skin MCs

Macroscopically normal human lung or skin tissue was obtained during surgery. Lung and skin cells were dispersed from chopped lung specimens and foreskin specimens by means of an enzymatic procedure and purified by using magnetic bead affinity selection with the anti-CD117 mAb (clone AC126; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), respectively, as described previously.¹² The purity of human MCs, as assessed with metachromatic staining, was more than 97%. In some experiments the dispersed lung or skin cells were cultured in serum-free methylcellulose medium, as previously described.¹³ After 8 weeks of culture, methylcellulose was dissolved in PBS. The purity of human MCs, as assessed with metachromatic staining, was greater than 98%.

Cell activation, mediator releases, and cytokine production

MCs were suspended in HBSS supplemented with 1% FCS and 1 mmol/L CaCl_2 for histamine and prostaglandin (PG) D_2 experiments. For cytokine experiments, MCs were cultured in Iscove modified Dulbecco medium (IMDM) containing stem cell factor (PeproTech EC Ltd, London, England) at 100 ng/mL and IL-6 (PeproTech EC Ltd) at 50 ng/mL with 2% FCS. MCs were challenged by means of incubation with PAF (Sigma-Aldrich, St Louis, Mo) or calcium ionophore A23187 (10^{-6} mol/L, Sigma-Aldrich) at 37°C for 30 minutes for measurement of histamine release and PGD₂ synthesis and for 16 hours for measurement of cytokine production. For aggregation of FcεRI, MCs were sensitized by means of incubation at 37°C for 16 hours with 1 μg/mL recombinant human myeloma IgE (Calbiochem, Gibbstown, NJ). After washing, the cells were challenged with rabbit anti-human IgE antibody

(Dako, Glostrup, Denmark) at 37°C for the time indicated. After incubation, cell-free culture supernatants and cell pellets were harvested to measure mediator levels. To assess the effect of various inhibitors of signal molecules on PAF (10^{-7} mol/L)– or IgE/anti-IgE (30 μg/mL)–induced histamine release, MCs were preincubated with the PAF-R antagonists CV-6209 (BIOMOL International LP, Plymouth Meeting, Pa) and BN52021 (BIOMOL International LP), the PLC inhibitor U73122 (Sigma-Aldrich), the Ca^{2+} –dependent protein kinase C (PKC) inhibitor Go6976 (Calbiochem), or the intracellular Ca^{2+} chelating agent BAPTA/AM (10 μmol/L, Calbiochem) for 30 minutes or with pertussis toxin (PTX; Calbiochem) for 2 hours before addition of PAF or anti-IgE.

Statistical analysis

Data are expressed as means $\pm$ SEMs. Data were submitted to statistical analysis by means of 1-way ANOVA and the Tukey–Kramer test for multiple comparisons ($P < .05$).

See the Methods section of this article's Online Repository at www.jacionline.org for additional information about the experimental protocols.

RESULTS**Comparison of G protein–coupled receptor expression profile between lung MCs and skin MCs: *In silico* study**

MCs express various G protein–coupled receptors (GPCRs), and because the function of human MCs is modulated by various GPCR ligands,^{14,15} the GPCRs expressed on MCs might play an important role in allergic diseases and anaphylaxis. The GPCR ligands, such as anaphylatoxins C3a and/or C5a,¹⁶ leukotrienes,¹⁷ sphingosine-1-phosphate,¹⁸ and PAF,⁸ have been reported to be mediators of anaphylaxis, acting as signaling components within the MC and as circulating mediators. Our initial microarray analysis of GPCR expression in human lung and skin MCs revealed that 68 GPCR genes were expressed in lung MCs, skin MCs, or both. Both lung and skin MCs expressed 54 GPCRs, including *C3aR1*, *C5aR1*, *CYSLTR1*,^{19,20} and *EDG1* (endothelial differentiation gene 1; sphingosine-1-phosphate receptor 1).²¹ Six GPCRs were skin MC specific, including *GPCR5B*, *EBI2* (EBV-induced gene 2), and *MRGX2* (Mas-related GPCR family member X2). Eight GPCRs were lung MC specific, including *CRHR1* and *PAFR*. Table I shows lung and skin MC–specific GPCRs. Because PAF and MCs are implicated in the pathophysiology of anaphylaxis, we investigated further the expression and function of PAF-R on human MCs. The levels of PAF-R transcripts in human lung MCs, skin MCs, and PB-derived MCs were $12.7\% \pm 2.59\%$ ($n = 4$), 2.83% ($n = 2$), and $15.6\% \pm 2.67\%$ ($n = 3$) of levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA, respectively (Table I).

Effect of PAF on histamine release from human MCs

In keeping with the expression of *PAFR* mRNA, lung MCs and PB-derived MCs released histamine in response to PAF in a concentration-dependent manner. However, skin MCs did not (Fig 1, A–C). The releasability of histamine from human lung MCs in response to PAF was variable and donor dependent (see Fig E1 in this article's Online Repository at www.jacionline.org). Therefore we used PB-derived MCs to examine further mediator production and the signaling pathway in response to PAF. The amount of PGD₂ released by PB-derived MCs in response to PAF was comparable with that released by IgE-sensitized MCs, which were stimulated with anti-IgE (Fig 1, D), whereas

TABLE I. Comparison of GPCR expression profiles between lung and skin MCs

Accession no.	GPCRs	Known/orphan	Lung MCs			Skin MCs		PB-MCs			Lung/skin
			Mean	SEM		Mean		Mean	SEM		
Lung MC-specific GPCRs											
gb:AL031588	<i>CELSR1</i>	o	4.40	1.40	P	0.55	A	1.50	0.10	P	7.98
gb:AL567376	<i>GPR39</i>	o	6.00	4.02	P	1.03	A	0.20	0.10	A	5.85
gb:M80436.1	<i>PAFR</i>	k	12.7	2.59	P	2.83	A	15.6	2.67	P	4.46
gb:NIM_017705.1	<i>PAQR5</i>	k	2.35	0.35	P	1.0	A	2.61	0.20	P	2.35
gb:NIM_004382.1	<i>CRHR1</i>	k	0.51	0.05	P	0.46	M	0.76	0.18	A	1.13
gb:AI492234	<i>GPR92</i>	k	1.07	0.19	P	1.05	A	1.44	0.20	A	1.03
gb:NIM_005288.1	<i>GPR12</i>	o	0.63	0.07	P	1.05	A	0.84	0.10	A	0.60
gb:AF348078.1	<i>GPR91</i>	k	0.39	0.07	P	0.76	A	6.50	4.12	P	0.52
Skin MC-specific GPCRs											
gb:M81778.1	<i>5-HT2C</i>	k	0.11	0.02	A	0.25	P	0.22	0.08	A	0.44
gb:AC002550	<i>GPRC5B</i>	o	1.04	0.11	A	2.33	P	0.43	0.19	A	0.44
gb:NIM_000316.1	<i>PTHR1</i>	k	0.55	0.06	A	3.37	P	0.53	0.06	A	0.16
gb:NIM_003991.1	<i>ETB</i>	k	1.24	0.37	A	12.0	P	2.60	0.13	P	0.10
gb:NIM_004951.1	<i>EBI2</i>	o	0.43	0.10	A	4.50	P	0.58	0.13	A	0.09
gb:NIM_054030.2	<i>MGRX2</i>	k	0.15	0.02	A	9.22	P	0.83	0.60	A	0.01

Data were expressed as means $\pm$ SEMs (lung MCs, $n = 4$; PB-derived MCs, $n = 3$) or means (skin MCs, $n = 2$) of mRNA expression (percentage GAPDH control). Lung/skin means a ratio of mRNA expression (percentage GAPDH control) of lung MCs to that of skin MCs.

A, Absent call; M, marginal call; P, present call; k, ligands are known; o, orphan receptor.

the amount of IL-8 and plasminogen activator inhibitor 1 (PAI-1) produced by PB-derived MCs in response to PAF was much lower than that produced by MCs after aggregation of Fc ϵ RI (Fig 1, E and F). To clarify the specific cytokine expression profile in MCs through PAF-R, we used a DNA array to compare cytokine expression levels between PAF-stimulated and anti-IgE-activated PB-derived MCs at 1 and 4 hours (see Fig E2 this article's Online Repository at www.jacionline.org). We found the cytokines induced in PAF-activated MCs were included in those induced by IgE/anti-IgE-activated MCs. Therefore there were no specific PAF-induced cytokines/chemokines. PAF upregulated mRNA for *CCL1*, *CCL3*, *CCL4*, *CSF1*, *CXCL3*, *EREG*, *IL8*, *INHBA*, and *SERPINE1* (PAI-1). Compared with Fc ϵ RI-mediated activation, PAF caused transient upregulation of mRNA for cytokines. The mRNAs for cytokines were upregulated in MCs by PAF at 1 hour but had waned at 4 hours, except for *CCL1* and *CCL3*, whereas they remained upregulated at 4 hours by IgE-mediated activation (see Fig E2). We next confirmed expression of mRNA for *PAFR* (Fig 2, A) and its protein in PB-derived MCs using RT-PCR and Western blotting (Fig 2, B), respectively. We confirmed that the sequence of *PAFR* expressed on human MCs was identical to that previously reported (data not shown).²² We also confirmed the expression of *PAFR* in human bronchial MCs in bronchial biopsy specimens by using immunohistochemistry (Fig 2, C). *PAFR* was preferentially expressed in tryptase-positive chymase-negative MCs (Fig 2, C and D).

Ca²⁺ mobilization by PAF through PAF-R in PB-derived MCs

We then assessed the ability of PAF to signal through the PAF-R. Treatment of MCs loaded with the Ca²⁺-sensitive dye Indo-1AM (Invitrogen, Carlsbad, Calif) with PAF resulted in a rapid increase in intracellular Ca²⁺ levels. This effect of PAF was concentration dependent (Fig 3, A). PAF also induced an intracellular Ca²⁺ flux in PB mononuclear cells (see Fig E3, A, in this article's Online Repository at www.jacionline.org). However, PAF did not induce a Ca²⁺ response in Jurkat cells (see Fig 3, A), which had no expression of PAF-R (Fig 2). As a positive control,

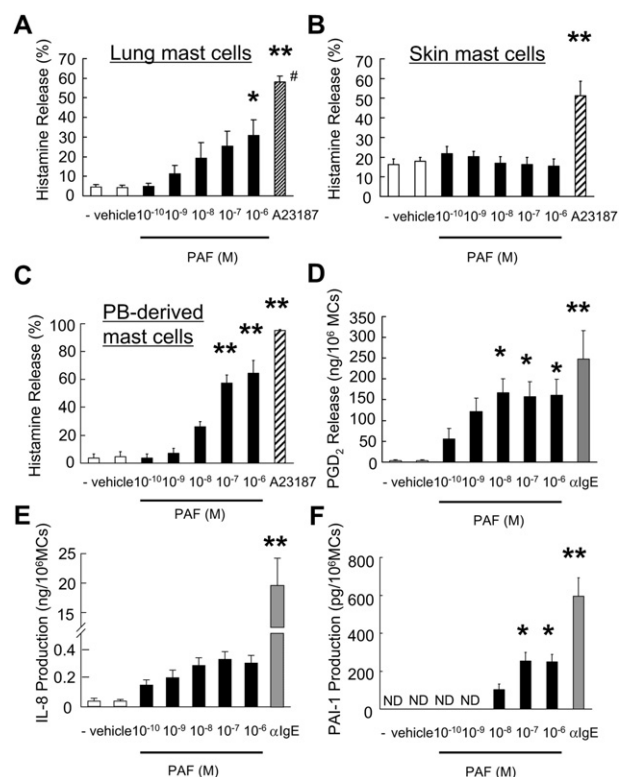


FIG 1. Mediators released by MCs in response to PAF. Histamine release by lung MCs (A, $n = 7$), skin MCs (B, $n = 3$), and PB-derived MCs (C, $n = 3$) in response to PAF and calcium ionophore A23187 (10^{-6} mol/L) are shown. PGD₂ (D, $n = 5$), IL-8 (E, $n = 3$), and PAI-1 (F, $n = 3$) production by PB-derived MCs in response to PAF and IgE/anti-IgE (α -IgE, 0.3 μ g/mL) are also shown. Data shown are means $\pm$ SEMs. * $P < .05$ and ** $P < .01$ compared with vehicle (0.1% chloroform). -, Buffer alone. # $n = 3$. ND, Not detected.

treatment of MCs (Fig 3, B) and Jurkat cells (data not shown) with calcium ionophore A23187 also triggered an immediate increase in intracellular Ca²⁺ levels. Treatment of MCs with the PAF-R antagonists CV-6209 (Fig 3, B) and BN52021 (see Fig E3, B)

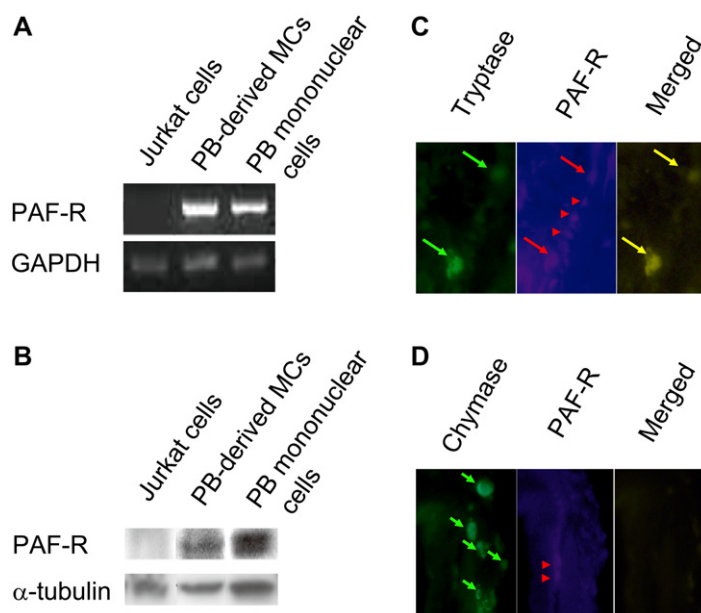


FIG 2. Expression of PAF-R in PB-derived MCs. **A**, *PAFR* mRNA expression was analyzed by means of RT-PCR. **B**, PAF-R protein expression was analyzed by means of Western blotting. Jurkat cells and PB mononuclear cells were used as negative and positive controls for PAF-R expression, respectively. The data are representative of similar results obtained from 3 independent experiments. **C** and **D**, Dual staining of bronchial MCs with anti-PAF-R antibodies (red arrows and red arrowheads indicate tryptase-positive PAF-R-positive cells and tryptase or chymase-negative PAF-R-positive cells, respectively; middle) and anti-tryptase mAb (**C**, green arrows, left) or anti-chymase mAb (**D**, green arrows, left). Yellow color indicates merged cells (Fig 2, C, right). The data are representative of similar results obtained from 6 independent experiments with MCs from different donors.

inhibited PAF-induced intracellular Ca^{2+} flux in a concentration-dependent manner but not A23187-induced Ca^{2+} flux. These findings indicate that PAF can signal through the PAF-R on MCs.

Effect of PAF on FcεRI-mediated histamine release and PGD_2 production by MCs

IgE-sensitized PB-derived MCs were simultaneously challenged with both PAF and anti-IgE to determine whether PAF synergizes with FcεRI-mediated histamine release and PGD_2 production. As shown in Fig E4, A (available in this article's Online Repository at www.jacionline.org), PAF at concentrations of 10^{-9} and 10^{-8} mol/L showed an additive effect on FcεRI-mediated histamine release, whereas PAF at higher concentrations than 10^{-8} mol/L showed a less than additive effect on FcεRI-mediated histamine release. PAF at concentrations of 10^{-10} and 10^{-6} mol/L showed a less than additive effect on FcεRI-mediated PGD_2 synthesis (see Fig E4, B), and even if suboptimal concentrations of PAF (10^{-9} mol/L) and the range of suboptimal concentrations of anti-IgE (0.03, 0.1, 0.3, and 1 $\mu\text{g}/\text{mL}$) were used in the experiment, simultaneous addition of PAF and anti-IgE to MCs showed a less than additive effect on PGD_2 synthesis (data not shown).

Time course of PAF-induced histamine release by MCs and effect of pretreatments of MCs with CV-6209 and PTX on PAF-induced histamine release

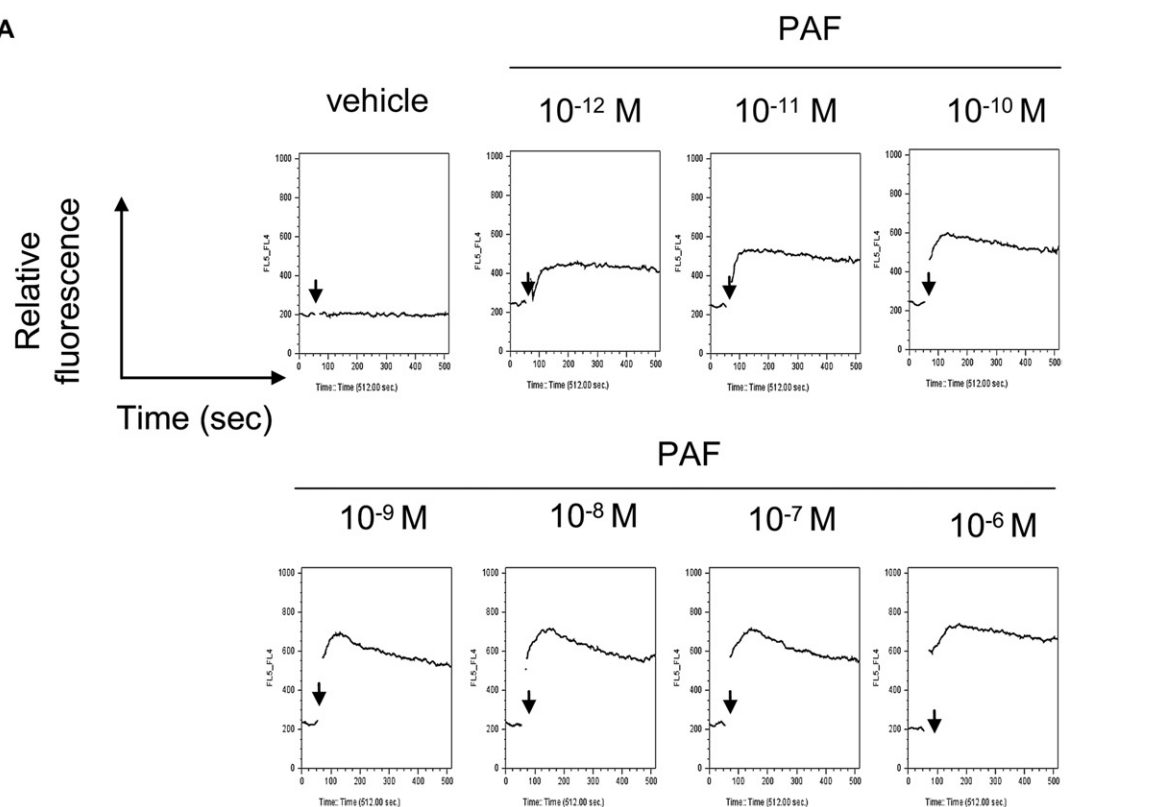
To clarify the PAF-R-mediated signaling pathways, we compared the kinetics of histamine release by MCs in response to PAF and IgE/anti-IgE and the effect of CV-6209 and PTX on PAF- or

IgE/anti-IgE-induced histamine release. PAF induced rapid histamine release by MCs, reaching a maximum at 5 seconds (Fig 4, A). Compared with the kinetics of histamine release by PAF, FcεRI-mediated histamine release was relatively slow and reached a peak at 40 seconds. Treatment of MCs with CV-6209 and PTX significantly inhibited histamine release by PAF in a concentration-dependent manner, but they did not inhibit IgE/anti-IgE-dependent release (Fig 4, B and C). Therefore PAF induces degranulation through activation of a PTX-sensitive $\text{G}_{\alpha i}$ protein associated with the PAF-R.

Activation of PLC in PB-derived MCs by PAF

To investigate the involvement of activation of PLC, a downstream signaling molecule in PAF-R-mediated signaling,^{23,24} we examined the effect of a PLC inhibitor, U73122, on PAF- and IgE/anti-IgE-induced histamine release by MCs. U73122 significantly inhibited PAF- and IgE/anti-IgE-induced histamine release by MCs in a concentration-dependent manner (Fig 5, A). Among the various isoforms of PLCβ and PLCγ, we observed that PLCβ2, PLCγ1, and PLCγ2 were dominantly expressed in PB-derived MCs, whereas the expression of PLCβ1 and PLCβ3 was not observed (Fig 5, B). PLCγ requires phosphorylation of tyrosine residues for activation, and both PLCβ and PLCγ isozymes require membrane localization for biological function.²⁵ Translocation of PLCβ2 to the membrane after activation was examined to investigate activation of PLCβ2 in MCs by IgE/anti-IgE and PAF. Both PAF and IgE/anti-IgE induced the translocation of PLCβ2 to the membrane (Fig 5, C). Densitometric analyses revealed that IgE/anti-IgE stimulation induced stronger phosphorylation of PLCγ1 than did PAF. PAF and IgE/anti-IgE

A



B

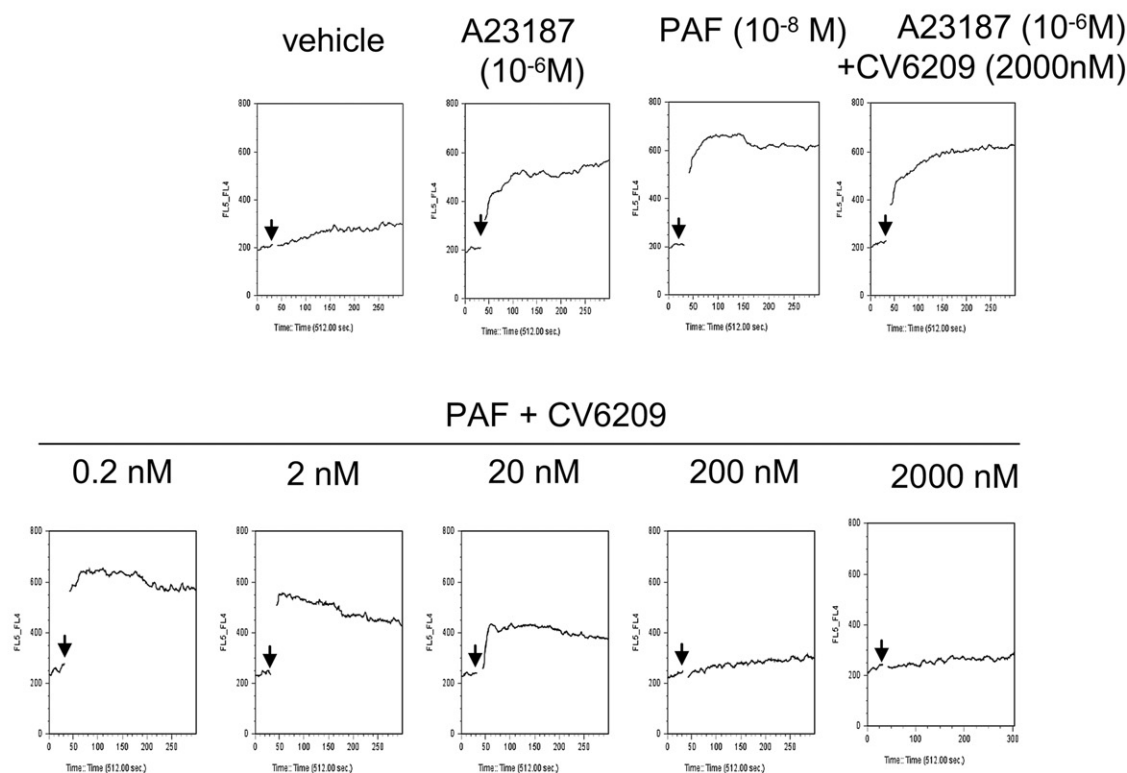


FIG 3. Ca^{2+} mobilization through PAF-R in PB-derived MCs in response to PAF. **A**, Ca^{2+} mobilization in MCs in response to PAF. **B**, Effect of the PAF-R antagonist CV-6209 on PAF- or calcium ionophore A23187-induced Ca^{2+} mobilization. The y-axis and x-axis show intracellular free calcium levels and the incubation time after stimulation, respectively. Arrows indicate the time point of addition of PAF or calcium ionophore A23187. The data are representative of similar results obtained from 3 independent experiments. The vehicle used was 0.1% chloroform.

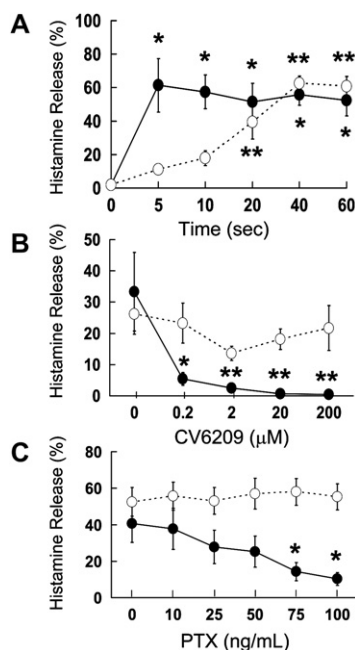


FIG 4. PAF-induced histamine release from PB-derived MCs is rapid and inhibited by pretreatments with CV-6209 and PTX. **A**, Kinetics of histamine release induced by PAF (10^{-7} mol/L, solid circles) or IgE/anti-IgE (30 μg/mL, open circles; $n = 3$). **B** and **C**, Effects of CV-6209 ($n = 6$) and PTX ($n = 7$) on PAF- or IgE/anti-IgE-induced histamine release. Data shown are means $\pm$ SEMs. * $P < .05$ and ** $P < 0.01$ compared with 0 seconds (Fig 4, A). * $P < .05$ and ** $P < .01$ compared with nontreatment (Fig 4, B and C).

stimulation did not have a significant effect on phosphorylation of PLC γ 2 up to the 40-second time point (Fig 5, D). Therefore PAF and IgE/anti-IgE appeared to regulate activation of both PLC γ 1 and PLC β 2, but the intensities of activation of PLC by PAF and IgE/anti-IgE were different.

Effect of lentiviral short hairpin RNA silencing of PLC γ 1 and PLC β 2 on Fc ϵ RI- and PAF-R-mediated degranulation

mRNA for PLC γ 1 and PLC β 2 were knocked down by using the lentiviral short hairpin RNA (shRNA) silencing technique to clarify the roles of PLC γ 1 and PLC β 2 on PAF- and IgE/anti-IgE-induced histamine release. These mRNA were selectively knocked down (Fig 6, A). Reduction of PLC γ 1 and PLC β 2 levels in MCs significantly inhibited both IgE/anti-IgE- and PAF-induced histamine release (Fig 6, B). In particular, reduction of PLC β 2 completely inhibited it.

Effects of BAPTA/AM and Go6976 on histamine release from MCs in response to PAF or IgE/anti-IgE

Because PLC activation leads to intracellular Ca^{2+} level increase and PKC activation,²³ we investigated whether these intracellular events are necessary for degranulation. Exposure of cells to BAPTA/AM almost completely inhibited PAF- or IgE/anti-IgE-induced degranulation (Fig 7, A). Removal of extracellular Ca^{2+} from the solution of PB-derived MCs completely inhibited IgE/anti-IgE-induced degranulation, although only partially, but significantly inhibited PAF-mediated degranulation. The augmented activation of PLC and increase in intracellular Ca^{2+} levels would likely extend to enhanced activation of conventional PKCs

(α , β I, β II, and γ). Both IgE/anti-IgE- and PAF-induced degranulation were effectively blocked by preincubation with Go6976, an inhibitor of Ca^{2+} -dependent isozymes of PKC (Fig 7, B).

DISCUSSION

In this study we have demonstrated for the first time that human lung MCs and PB-derived MCs release histamine in response to PAF through the PAF-R. Activation of the PAF-R-coupled PTX-sensitive G protein $G_{\alpha i}$ in PB-derived MCs leads to degranulation through activation of both PLC γ 1 and PLC β 2, which are common features of both Fc ϵ RI- and PAF-R-mediated MC activation. This has important implications for our understanding of the mechanisms of acute allergic responses, in particular anaphylaxis.

PAF is a phospholipid (1-o-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine) that is released by a variety of human cells, including platelets and MCs.²⁶ It has been reported that mean serum PAF levels were 1.5×10^{-9} mol/L (805 pg/mL) in patients with anaphylaxis and were correlated with the severity of anaphylaxis.⁸ Mean serum PAF levels in patients with grade 3 anaphylaxis were more than 10 times greater than the levels in control patients (approximately 2.5×10^{-9} mol/L). However, these concentrations are likely to be an underestimate because of rapid degradation²⁷ and lower than those achieved locally within tissue. Nevertheless, 10^{-9} mol/L PAF induced 20% to 30% of total histamine release from some lung MC preparations (see Fig E1). Therefore the PAF concentrations required for human lung MC activation correspond to physiological concentrations measured during an anaphylactic response.

MCs are known to play a central role in human anaphylaxis, as supported by the observation that there are increased concentrations of MC-specific β -tryptase in the sera of anaphylactic patients.^{3,4} It is often assumed that the systemic MC response after local exposure occurs as a result of the dissemination of allergen through the bloodstream. Although this is possible, the rapid nature of events after oral exposure suggests that other factors might be important. Increased concentrations of PAF appear to be important for human anaphylaxis, although its biological role is not known. Our finding that PAF can directly induce human MC degranulation and arachidonic acid metabolism and can potentiate IgE-dependent mediator release provides a mechanism whereby PAF directly amplifies the effects of allergen exposure. The source of PAF in human anaphylaxis is uncertain but in part might come from MCs.²⁸ Thus we suggest that PAF generated locally by MCs and perhaps other cells in response to MC activation at the point of allergen contact leads to an exaggerated MC response at more distal sites, such as the airway. We therefore propose a PAF amplification loop as a key factor leading to airway MC degranulation on exposure to oral allergens.

We did not find any effect of PAF on human skin MCs, which is in keeping with a lack of PAF-R expression in these cells. However, PAF can induce MC degranulation through the stimulation of neural reflexes,⁹ and because skin MCs can be activated by neuropeptides,²⁹ PAF might still be relevant to skin MCs. Whether cardiac and gut MCs respond to PAF requires further investigation.

Because every GPCR downstream signaling pathway can have its own unique pharmacology in a particular cell depending on the pathway stimulated by the unique ligand receptor conformation or complex involved,³⁰ we investigated the signaling pathway of PAF-R on human MCs. Previous reports have demonstrated that the PAF-R interacts with multiple G proteins, including $G_{\alpha i}$,

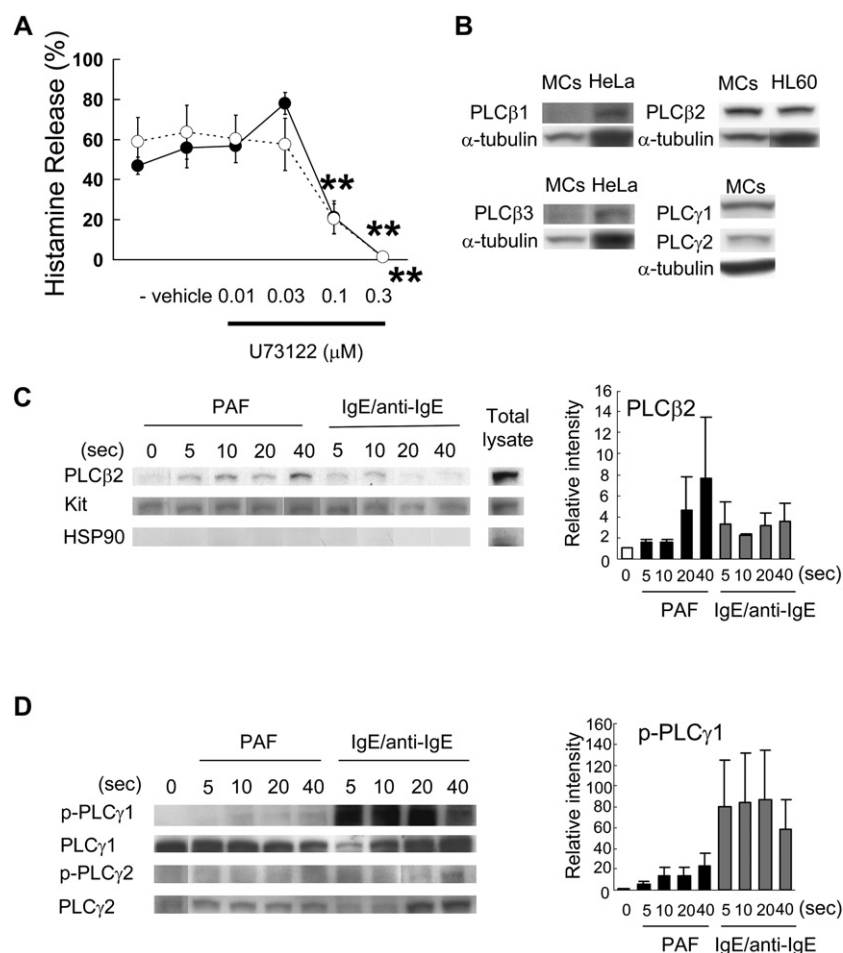


FIG 5. Activation of PLC γ 1 and PLC β 2 in PB-derived MCs by PAF. **A**, Effect of U73122 on PAF-induced (solid circles) or IgE/anti-IgE-induced (open circles) histamine release ($n = 4$). **B**, Expression of PLC β and PLC γ in MCs. HeLa cells and HL-60 cells were used as positive controls. (PLC β 1, PLC β 2, and PLC β 3, $n = 3$; PLC γ 1 and PLC γ 2, $n = 6$). **C**, Membrane translocation of PLC β 2 in response to PAF or IgE/anti-IgE. Anti-Kit was used to verify the membrane protein loading, and anti-heat shock protein (HSP) 90 was used to verify the absence of cytosolic proteins. **D**, Phosphorylation of PLC γ 1 ($n = 5$) and PLC γ 2 ($n = 4$) in response to PAF or IgE/anti-IgE. Data shown are means $\pm$ SEMs. * $P < .05$ and ** $P < .01$ compared with vehicle (0.1% dimethyl sulfoxide). The data (Fig 5, C and D) were generated by scanning the blots and then normalizing to the response at 0 seconds ($n = 3$). –, Buffer alone; p -PLC γ , anti-phospho-PLC γ .

$G_{\alpha o}$, and $G_{\alpha q/11}$, leading to simultaneous stimulation of distinct signaling pathways.²⁴ Histamine release was blocked by approximately 80% by using PTX at 100 ng/mL in PB-derived MCs, suggesting a dominant role for $G_{\alpha i}$. Further studies will be required to investigate interactions of the $G_{\alpha q}$ protein with PAF-R and involvement of $G_{\alpha q}$ for PAF-R-mediated degranulation in human MCs. Nilsson et al³¹ demonstrated that PAF induced a transient increase in intracellular Ca^{2+} concentrations and induced significant migratory responses in the human leukemia MC line HMC-1. Both PAF-induced calcium mobilization and migration of HMC-1 cells were shown to require activation of PTX-sensitive G protein. Although we have not investigated the migratory response of primary MCs to PAF, there is a possibility that PAF induces migration and degranulation of MCs simultaneously, which might result in augmented inflammation.

Several reports have characterized the ability of PAF to activate tyrosine kinases with subsequent activation of PLC γ 1, an isozyme of phospholipase that is activated by tyrosine phosphorylation in

B cells, platelets, and PAF-R-transfected cell lines.²⁴ In PAF-R-transfected RBL-2H3 cells, PAF did not activate PLC γ ,³² whereas in human umbilical vein endothelial cells PAF activated PLC β 3 through its $G_{\alpha q}$ -coupled receptor.³³ In human MCs IgE-dependent activation induces activation of both PLC γ 1³⁴ and PLC β 2, although not PLC β 3.¹⁵ We found that PAF-R activation also leads to degranulation through activation of both of PLC γ 1 and PLC β 2. Therefore the isozymes of phospholipase that are activated in response to PAF are dependent on the cell type, and PAF-R- and Fc ϵ RI-mediated histamine release in human MCs share a common pathway using activation of both PLC γ 1 and PLC β 2. The main difference in the cell-signaling pathway between PAF-R- and Fc ϵ RI-mediated histamine release is therefore upstream of PLC. This is supported by the observation that PAF-mediated degranulation was rapid (maximal at 5 seconds), and it did not completely require extracellular Ca^{2+} , which is required for sustained activation of the tyrosine kinase syk by IgE-dependent MC activation.³⁵

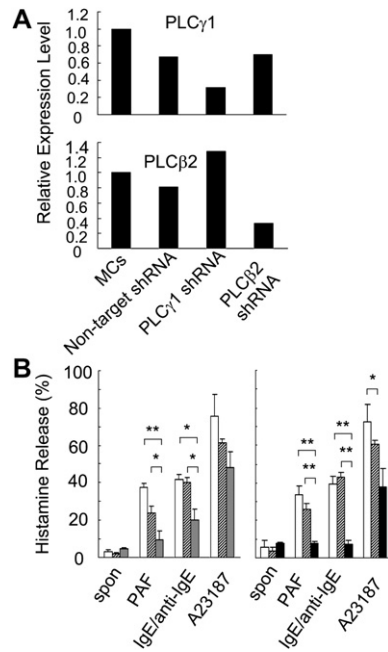


FIG 6. Lentiviral shRNA silencing of PLC γ 1 and PLC β 2 reduces Fc ϵ RI- and PAF-R-mediated degranulation. **A**, Assessment for loss of PLC γ 1 and PLC β 2 mRNA expression in nontransduced PB-derived MCs, cells transduced with nontargeted shRNA, or lentiviral shRNA constructs for PLC γ 1 and PLC β 2. **B**, Degranulation from nontransduced MCs (open bars), MCs transduced with nontargeted shRNA (hatched bars), or lentiviral shRNA constructs for PLC γ 1 (gray bars) and PLC β 2 (solid bars), which were stimulated with PAF (10^{-7} mol/L), IgE/anti-IgE (30 μ g/mL), or calcium ionophore A23187. Data shown are means $\pm$ SEMs of 4 independent experiments. * P < .05 and ** P < .01.

We found that the cytokines induced by PAF-activated MCs were also induced by IgE/anti-IgE-activated MCs (see Fig E2), but PAF-induced cytokine mRNA upregulation was only transient. We measured IL-8 and PAI-1 levels in the supernatant of activated MCs after stimulation with PAF or IgE/anti-IgE. The amounts of IL-8 and PAI-1 produced by PAF-activated MCs were much smaller than those produced by IgE/anti-IgE-activated MCs (Fig 1, E and F). The IL-8 production by PAF-activated MCs was significantly inhibited by CV-6209, but IL-8 production by IgE/anti-IgE-activated MCs was not, indicating the IL-8 production by PAF-activated MCs was mediated through PAF-R (data not shown). These data indicate that PAF-induced activation of MCs predominantly affects the early phase of allergic responses, which are the key feature of anaphylaxis.

Two common MC phenotypes recognized in human subjects are identified by their protease content. One population contains only tryptase (MC_T), whereas a second contains tryptase, chymase, carboxypeptidase A3, and cathepsin G (MC_{TC}).³⁶ The MC_T phenotype is typically found at mucosal surfaces, such as the nasal and bronchial epithelium in patients with rhinitis and asthma, respectively, and the bronchial lamina propria in states of both health and disease.^{36,37} In contrast, the MC_{TC} phenotype favors connective tissues, such as normal skin,³⁶ the airway smooth muscle bundles in patients with asthma,³⁸ and atherosclerotic lesions.³⁹ Our histochemical analyses of human lung specimens showed that PAF-R was preferentially expressed in the MC_T phenotype (Fig 2, C and D). This study also shows the marked heterogeneity of human MCs, as demonstrated by the difference in

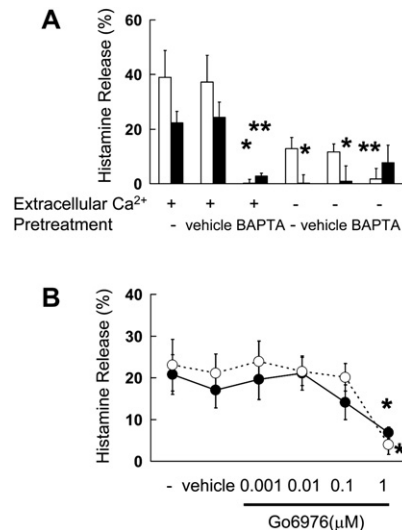


FIG 7. Effects of BAPTA/AM and Go6976 on PAF- or IgE/anti-IgE-induced histamine release. **A**, Effect of BAPTA/AM on PAF-induced (open bars) or IgE/anti-IgE-induced (solid bars) histamine release in the presence or absence of extracellular Ca²⁺ (n = 6). **B**, Effect of Go6976 on PAF-induced (solid circles) or IgE/anti-IgE-induced (open circles) histamine release (n = 10). Data shown are means $\pm$ SEMs. * P < .05 and ** P < .01 compared with vehicle (0.03% dimethyl sulfoxide) in the presence of extracellular Ca²⁺ (Fig 7, A). * P < .05 compared with vehicle (0.1% dimethyl sulfoxide; Fig 7, B).

GPCR expression in the lung and skin. Our microarray analysis of comparison of GPCR expression between human lung and skin MCs revealed specific expression of *PAFR* and *MRGX2* transcripts in lung and skin MCs, respectively. The expression of *MRGX2* in the cord blood-derived cultured MCs has been reported.⁴⁰ The ligands for *MRGX2* are basic neuropeptides, such as substance P, vasoactive intestinal peptide, and cortistatin. Taken together with previous evidence,^{8-10,31} our findings support the presence of 2 pathways for a PAF amplification loop in the generation of anaphylaxis: one provided by the direct activation of airway MCs contributing to bronchospasm and laryngeal edema and perhaps MCs in other organs, such as the heart, and the second mediated through neural reflexes in organs such as the skin.

In summary, PAF activates human lung and PB-derived MCs directly and potentiates IgE-dependent human MC activation. The effects are predominantly evident with respect to degranulation and arachidonic acid metabolism rather than cytokine mRNA expression or IL-8 release. This study therefore provides a mechanism that links the increased PAF levels found in patients with anaphylaxis to the pathophysiology of the disease. This strengthens the case for targeting PAF in the treatment of anaphylaxis.

We thank Ms Noriko Hashimoto and Ms Hiromi Wakita of the National Research Institute for Child Health and Development for her excellent technical assistance. We thank Dr Yoshihiro Ono, Kosaku Clinic, and the respiratory surgical staffs of Nihon University Hospital for supplying human specimens and for their willing and capable assistance.

Clinical implications: Blocking of PAF-R on human lung MCs might offer a novel approach to the prevention and treatment of anaphylaxis.

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METHODS

Generation of adult PB progenitor-derived MCs

Lineage-negative (CD4⁺, CD8⁺, CD11b⁺, CD14⁺, CD16⁺, and CD19⁺) mononuclear cells were selected from the PB mononuclear cells and cultured in serum-free Iscove methylcellulose medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) and IMDM containing recombinant human stem cell factor (PeproTech EC Ltd) at 200 ng/mL, recombinant human IL-6 (PeproTech EC) at 50 ng/mL, and recombinant human IL-3 (Intergen, Inc, Purchase, NY) at 1 ng/mL, as previously described.^{E1} On day 42 of culture, methylcellulose was dissolved in PBS, and the cells were resuspended and cultured in IMDM containing stem cell factor at 100 ng/mL and IL-6 at 50 ng/mL with 2% FCS. The MCs used in the experiments were 12 to 16 weeks old. High MC purity (>99%) was obtained by using Kit-positive magnetic selection with anti-CD117 mAb (clone AC126, Miltenyi Biotec GmbH). MC numbers and purity were determined by counting after metachromatic staining with toluidine blue or by means of tryptase staining (clone AA1, Dako).

Isolation of human PB mononuclear cells

Human PB mononuclear cells were isolated by means of centrifugation of whole blood on a Ficoll-Isopaque density gradient (Nycomed, Oslo, Norway).

Cell lines

Jurkat, HeLa, and HL60 cells were purchased from American Type Culture Collection (Manassas, Va). Jurkat and HeLa cells were cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% FCS and 1% penicillin-streptomycin, and HL60 cells were cultured in IMDM supplemented with 10% FCS and 1% penicillin-streptomycin.

Isolation of RNA and RT-PCR

Isolation of total RNA and RT-PCR were performed as previously described.^{E2} Amplification was performed for 30 cycles. Each cycle included denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57°C for *PAFR* and at 64°C for *GAPDH*, and extension for 1 minute at 72°C. After the final cycle, the samples were incubated for 10 minutes at 72°C. The sequences of the primers for *PAFR* were 5'-CGGACATGCTCTCTTGATCA-3' for the 5' primer and 5'-GTCTAAGACACAGTTGGTGCTA-3' for the 3' primer. The sequences of the primers for *GAPDH* were 5'-TGATGACA TCAAGAAGGTGGTGAAG-3' for the 5' primer and 5'-TCCTTGAGGCC ATGTGGGCCAT-3' for the 3' primer.

GeneChip expression analysis

Human genome-wide gene expression was assessed by using the Human Genome U133 Plus 2 probe array (GeneChip; Affymetrix, Santa Clara, Calif), which contains an oligonucleotide probe set for approximately 66,996 full-length genes and expressed sequence tags (ESTs), according to the manufacturer's protocol (Expression Analysis Technical Manual and as in previous reports).^{E2} The data obtained were analyzed with Genespring software version GX 7.3 (Silicon Genetics, San Carlos, Calif). The average difference values for all genes on a given chip were divided by the median of all measurements on that chip to normalize the staining intensity variations among chips. Data were considered significant when (1) expression changed by at least 4-fold (activation program) and (2) increased gene expression included at least 1 "present absolute call" (Affymetrix algorithm). Based on those normalized values, the genes were classified as upregulated or downregulated.

Histamine release assays

The degree of MC degranulation was monitored by measuring the histamine released into the extracellular fluid after activation with either an enzyme immunoassay kit (Immunotech A; Beckman Coulter Co, Marseille, France) or a radioenzymatic assay, as described previously.^{E3} The net percentage of histamine release was calculated from the ratio of each sample, with spontaneous release subtracted to total histamine.

PGD₂ assay

The PGD₂ MOX EIA kit (Cayman Chemical, Ann Arbor, Mich) was used to measure the amounts of PGD₂ in the supernatants.

ELISA for IL-8 and PAI-1

Human IL-8 and PAI-1 were measured with ELISA kits. The ELISA kits for IL-8 and PAI-1 were purchased from R&D Systems, Inc (Minneapolis, Minn), and from AssayPro (Brooklyn, NY). The assay sensitivities of human IL-8 and PAI-1 were 31.2 and 78 pg/mL, respectively.

Measurement of intracellular calcium

Cells were suspended in HBSS supplemented with 1% FCS and 1 mmol/L CaCl₂. Indo-1AM (3.6 μg/mL, Invitrogen) containing F127 (Sigma-Aldrich) and FCS was added, and the cells were incubated at 37°C for 30 minutes in the dark. After washing, the cells were resuspended in HBSS supplemented with 1% FCS and 1 mmol/L CaCl₂ and stimulated with PAF or calcium ionophore A23187. In some experiments MCs were preincubated with CV-6209 or BN52021 at 37°C for 15 minutes before stimulation. The fluorescence intensity of intracellular Indo-1AM was monitored and analyzed with an LSR system (BD, Franklin, NJ).

Immunoblotting

Cells were lysed in SDS sample buffer containing protease inhibitor cocktail (Roche, Indianapolis, Ind) and phosphatase inhibitor cocktail set I (Calbiochem). The whole-cell lysates were boiled for 5 minutes. In some experiments membrane fractions were prepared as previously described.^{E4} Briefly, the lysates were centrifuged to remove intact cells and nuclei. The recovered supernatants were centrifuged at 20,000g for 30 minutes at 4°C. Proteins in the pellet fractions were resuspended in SDS sample buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail set I and put on ice for 30 minutes. The samples were then boiled for 5 minutes and centrifuged at 15,000g for 15 minutes at 4°C. The recovered supernatants were saved as membrane fractions. Proteins were separated by means of electrophoresis on 4-20% or 8% Tris-Glycine Gel (Invitrogen). The separated proteins were then transferred onto polyvinylidene difluoride membranes (GE Healthcare, Uppsala, Sweden), blocked with TBS containing 5% nonfat dry milk and 0.05% Tween-20 for 1 hour at room temperature, and then probed with antibodies against PAF-R (Alexis Biochemicals, San Diego, Calif); PLCβ1 and PLCβ2 (Santa Cruz Biotechnology, Santa Cruz, Calif); PLCβ3, PLCγ1, PLCγ2, and phospho-PLCγ2 (Cell Signaling Technology, Danvers, Mass); and phospho-PLCγ1 (Invitrogen) overnight at 4°C. After washing and incubation with the horseradish peroxidase-linked secondary antibody (GE Healthcare), the blotted membranes were visualized with ECL plus Western Blotting Detection Reagents (GE Healthcare). Membranes were stripped by means of incubation in Re-Blot plus Strong Antibody Stripping Solution (Chemicon, Temecula, Calif) for 15 minutes at room temperature. After washing, membranes were blocked with Block Ace (Dainipon, Inc, Osaka, Japan) and reprobed with antibodies against α-tubulin (Sigma-Aldrich), Kit (R&D systems, Inc), heat shock protein 90 (BD Transduction Laboratories, San Diego, Calif), PLCγ1, and PLCγ2. The heat shock protein 90 and α-tubulin were used as positive controls of intracellular and protein expression, respectively. Bound proteins were quantified with ImageJ 1.39u software.

Immunohistochemical analysis

The study was conducted with the approval of the Ethics Committee of Dokkyo Medical University School of Medicine, and written informed consent for participation was obtained from each of the subjects in accordance with the Helsinki Declaration of the World Medical Association. Bronchial tissue samples were collected from the subcarinal region between the right lower lobe and middle lobe bronchi (origin of the right B6 bronchus) in the asthmatic subjects by using a pair of standard forceps during fiberoptic bronchoscopy, as previously described.^{E5} Each biopsy specimen was immediately placed in OCT medium, snap-frozen in liquid nitrogen, and stored at -80°C until cryostat sectioning. The cells were dual stained with fluorescein

isothiocyanate–conjugated anti-tryptase mAb (clone AA1, Dako) or fluorescein isothiocyanate–conjugated anti-chymase mAb (clone CC1; MBL, Tokyo, Japan) and rabbit anti-human PAF-R antibody (Cayman Chemical).^{E6} PAF-R⁺ cells were visualized by means of incubation with Alexa Fluor 647–conjugated anti-rabbit IgG (Invitrogen, Tokyo, Japan).

Lentivirus shRNA vector construction and gene transduction

The lentiviral expression plasmid with the sense and antisense oligonucleotide sequence for construction of 2 PLC γ 1 (GenBank accession no. NM_002660) and 2 PLC β 2 (GenBank accession no. NM_004573) shRNAs were purchased from Sigma-Aldrich. Packaging vector (MISSION lentiviral packaging mix, Sigma-Aldrich), the lentiviral expression vector (pLKO<-puro) with PLC shRNA (MISSION shRNA plasmid DNA) or MISSION nontarget shRNA control vector (Sigma-Aldrich), or lentiviral expression vector encoding enhanced green fluorescent protein (EGFP) were cotransfected into 293FT packaging cells with TransIT (Takara Bio, Inc, Shiga, Japan). After 6 hours, the culture supernatants were centrifuged at 3,000 rpm for 10 minutes and filtered with a 0.45- μ m filter. The filtered supernatants were centrifuged at 9,000 rpm overnight at 4°C, and the released virus was kept on ice for 1 hour. This step concentrated the supernatants from 220 mL to 1 mL. Transduction of human MCs (1×10^4) was conducted by

resuspending the cells in 100 μ L of virus containing IMDM. Two days after infection, the medium was changed to virus-free IMDM containing stem cell factor at 100 ng/mL and IL-6 at 50 ng/mL with 2% FCS, and antibiotic selection (1 mg/mL puromycin) was performed for 2 days.

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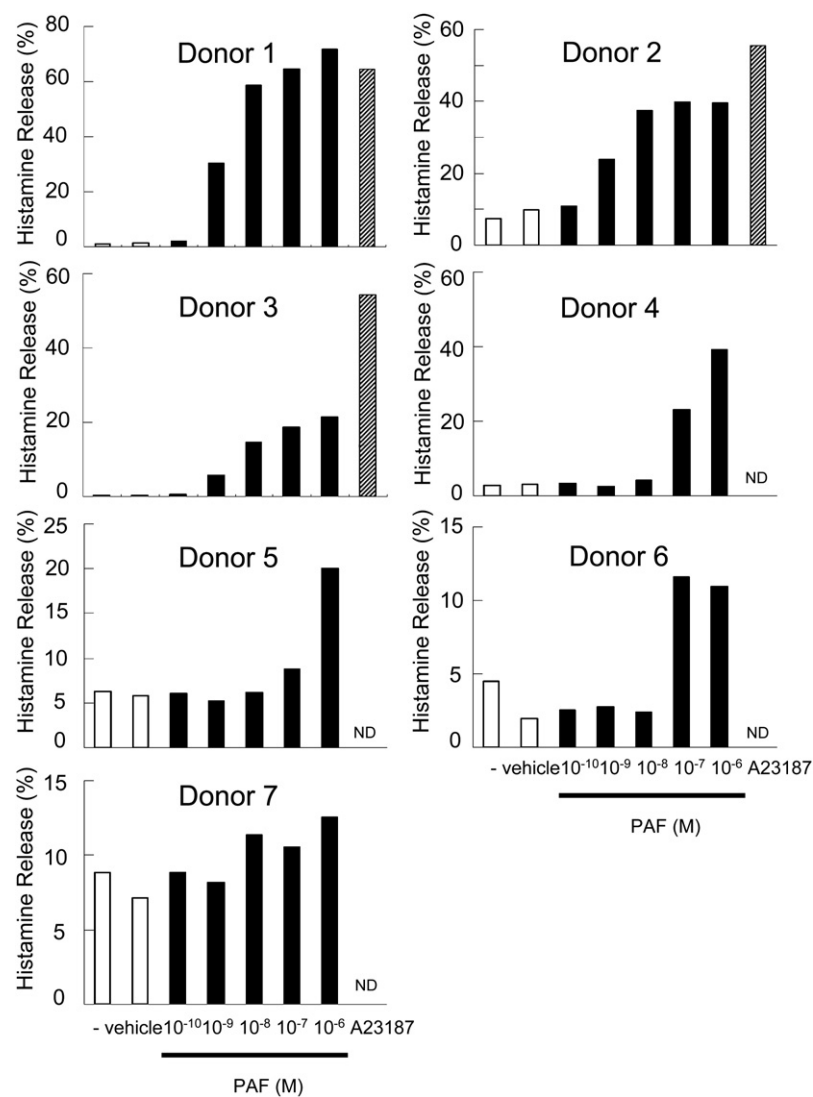


FIG E1. Variable response of histamine release from human lung MCs in response to PAF (n = 7). ND, Not determined.

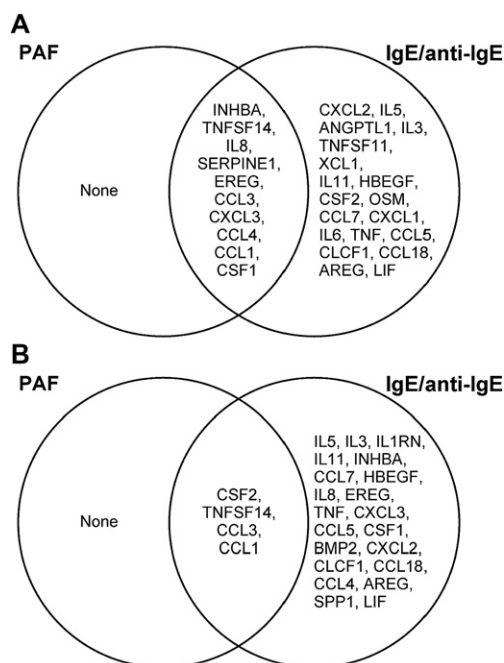


FIG E2. PAF-R- or FcεRI-mediated cytokine gene expression profiles in human PB-derived MCs. Human MCs were activated with PAF (10^{-7} mol/L) or IgE/anti-IgE (3 μ g/mL) for 1 hour (**A**) and 4 hours (**B**). Each experiment was performed with 3 independent donors, and the data are the average of 3 separate analyses. Overlapping sets of PAF- and IgE/anti-IgE-induced cytokine genes are shown. Stimulus-induced cytokine genes were selected if the ratio of the relative expression level between those with and without the stimulus was greater than 4.0.

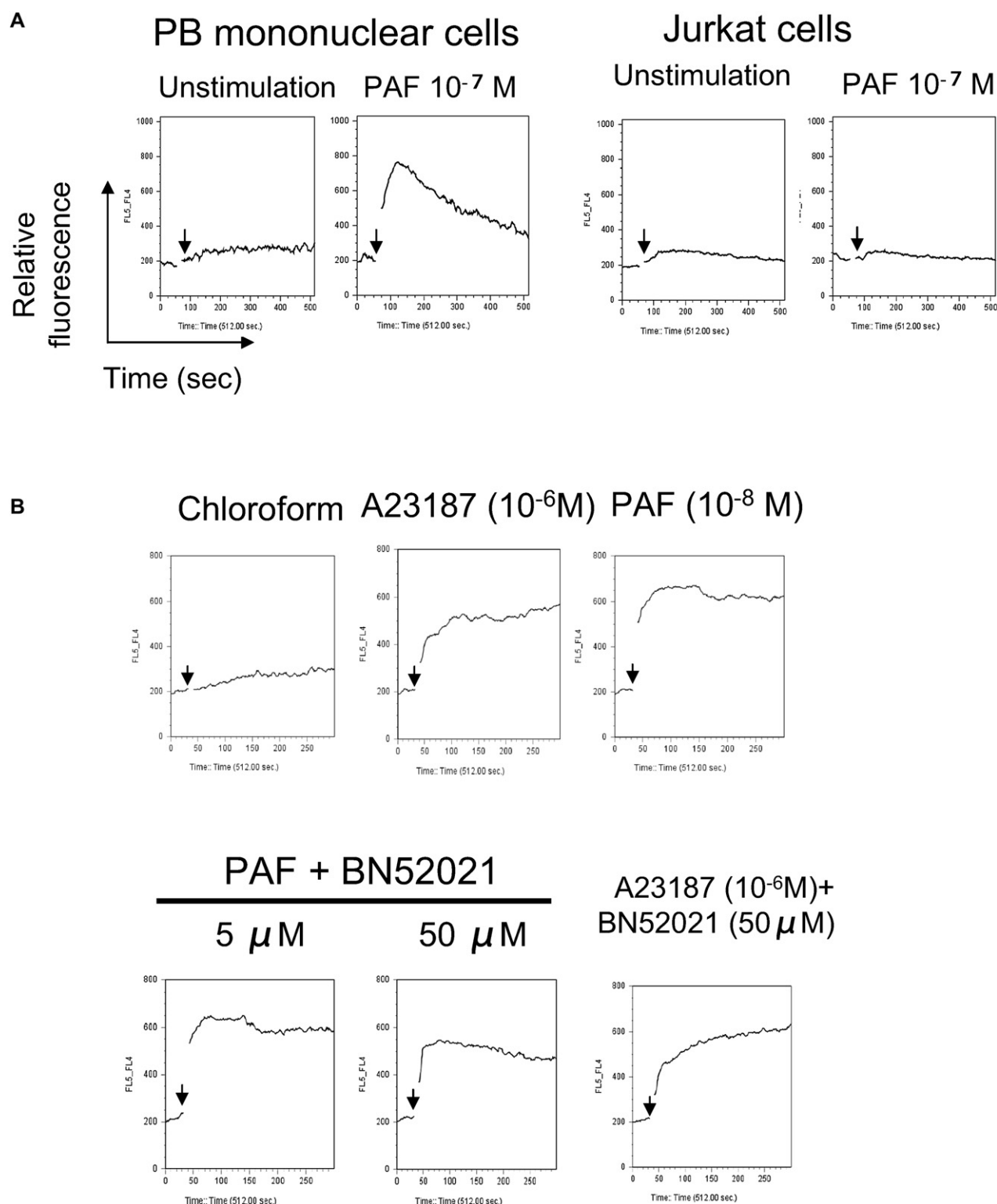


FIG E3. Ca^{2+} mobilization through PAF-R in PB-derived MCs in response to PAF. **A**, Ca^{2+} mobilization in Jurkat cells ($n = 3$) and PB mononuclear cells ($n = 3$) in response to PAF. **B**, Effect of the PAF-R antagonist BN52021 on PAF- or calcium ionophore A23187-induced Ca^{2+} mobilization ($n = 3$). Intracellular free calcium levels (y -axis) were monitored with the LSR system. The x -axis shows the incubation time after stimulation. Arrows indicate the time point of addition of PAF or calcium ionophore A23187. The data are representative of similar results obtained from 3 independent experiments with MCs from different donors.

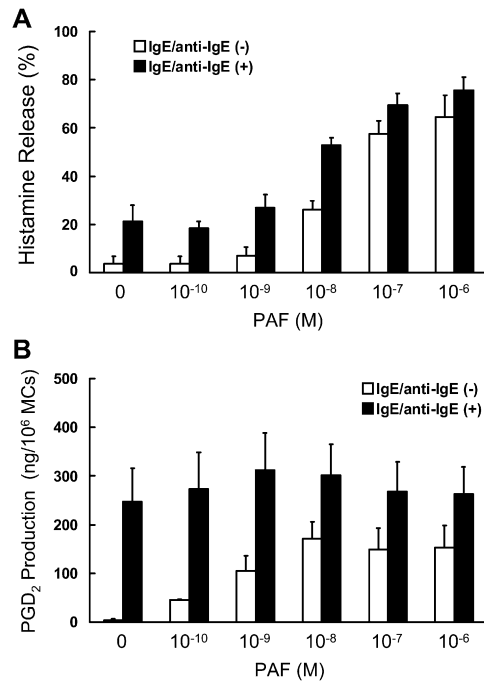


FIG E4. The additive effect of PAF on IgE-dependent histamine release and PGD₂ production by MCs. MCs were sensitized with IgE for 16 hours and then washed and challenged with PAF (10⁻¹⁰ to 10⁻⁶ mol/L) with (*IgE/anti-IgE* [+], solid bars) or without (*IgE/anti-IgE* [-], open bars; 0.3 μg/mL) anti-IgE for 30 minutes for histamine release (**A**, n = 3) and PGD₂ production (**B**, n = 4). Data shown are means ± SEMs.