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Inhibitory effects of polymethoxy flavones isolated from *Citrus reticulate* on degranulation in rat basophilic leukemia RBL-2H3: Enhanced inhibition by their combination

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ABSTRACT

Polymethoxy flavones (PMFs) are present in fruit tissues of *Citrus* species. It has been reported that flavonoids isolated from several *Citrus* have been shown to suppress the degranulation as inferred by histamine release in rat basophilic leukemia RBL-2H3 cells. In this study, we examined the effect of PMFs (PMF-1: 6,7,4′,5′-tetramethoxy-5-monohydroxyflavone, PMF-2: 5,6,8,3′,6′-pentamethoxy flavone, PMF-3: 5,6,7,3′,4′,5′-hexamethoxy flavone) on the degranulation in RBL-2H3 cells. All the PMFs suppressed the degranulation from Ag-stimulated RBL-2H3 cells. Interestingly, PMF-combination (PMF-1 + PMF-2; PMF-1 + PMF-3) treatment enhanced the inhibition of degranulation compared with PMF-single treatment. In order to clarify the inhibitory mechanism of degranulation by PMFs, we examined the activation of intracellular signaling molecules such as Lyn, Syk, and PLCγs. All the PMFs significantly suppressed the activation of Syk and PLCγs. In Ag-mediated activation of FcεRI on mast cells, three major subfamilies of mitogen-activated protein kinases, especially ERK44/42, were activated. These PMFs reduced the level of phospho-ERKs. The intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) was elevated by FcεRI activation, and PMF treatment reduced the elevation of $[Ca^{2+}]i$ by suppressing Ca^{2+} influx. Thus, it was suggested that the suppression of Ag-stimulated degranulation by these PMFs mainly is due to the Syk/PLCγs/PKC pathway and Ca^{2+} influx.
Furthermore, to be noted in the PMF-combination treatment, inactivation of Syk was enhanced compared with PMF-single treatment. But the inhibitory effect of degranulation by PMF-combination treatment was not associated with the suppression of Ca^{2+} influx.

1. Introduction

Allergy is classified into five types (type I: anaphylactic type (e.g., anaphylactic shock), type II: antibody-mediated cytotoxic type (e.g., autoimmune hemolytic anemia), type III: immune complex type (e.g., lomerulonephritis), type IV: cellular immunity type (e.g., tuberculin reaction), and type V: stimulative type (e.g., Graves' disease)). At type I allergy, binding of antigen (Ag) to the high affinity IgE receptor (FcεRI) on the surface of mast cell and basophils induces the release of preformed intragranular mediators such as histamine, arachidonic acid metabolites, proteases, serotonin, and heparin.[1–3] Thus, mast cell is bearing the important key in the allergic reaction. In Ag-mediated degranulation process, FcεRI cross-linking on basophils and/or mast cells activates receptor-associated protein tyrosine kinases such as Lyn and Syk. The tyrosine phosphorylation of these protein tyrosine kinases induces hydrolysis of phosphatidylinositol-3,4,5-bisphosphate (PIP_2) by phospholipase C-γ1 (PLC-γ1) and/or -γ2 (PLC-γ2), resulting in the generation of inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DG). These second messengers lead to release of Ca^{2+} from internal stores and activate protein kinase C, respectively.

Recently, it has been found that several flavonoids suppressed the degranulation in human basophilic leukemia KU 812 cells[4] and that flavonoids suppress the activation of PKC and protein tyrosine kinase Syk in the degranulation process.[5] In the present

Abbreviations: AA, arachidonic acid; Ag, antigen; A23187, calcimycin, calcium ionophore; Btk, Bruton's tyrosine kinase; CRAC channels, Ca^{2+} release-activated conducting Ca^{2+}; DNP-BSA, dinitrophenylated bovine serum albumin; ERK, extracellular signal-regulated kinase; EGCG, (−)-epigallocatechin gallate; EGTA, *O,O′*-bis(2-aminoethyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; IgE, immunoglobulin E; SAPK/JNK, the stress-activated protein kinase/Jun-N-terminal kinase; LAT, linker for activation of T cells; LTs, leukotriens; Lyn, Src family protein kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; Syk, Syk/Zap-70 family protein kinase, spleen tyrosine kinase; SLP-76, SH-2-containing leukocyte-specific protein of 76 kDa.

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study, we investigated the effect of polymethoxy flavones (PMFs) isolated from *Citrus reticulate* on activation of intracellular signaling pathways leading to the degranulation in Ag-mediated rat basophilic leukemia RBL-2H3 cells. Among the PMFs tested, three PMFs such as 6,7,4′,5′-tetramethoxy-5-monohydroxyflavone: PMF-1, 5,6,8,3′,6′-pentamethoxy flavone: PMF-2, and 5,6,7,3′,4′,5′-hexamethoxy flavone: PMF-3 strongly inhibited the degranulation from RBL-2H3 cells. It was found that PMFs suppressed the activation of the Syk/PLCγs/PKC pathway and the elevation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$). Interestingly, the combination of the PMFs inhibited the degranulation more strongly than the single treatment. PMF-combinations (PMF-1 + PMF-2; PMF-1 + PMF-3) enhanced the inactivation of Syk. However, the suppression of $[Ca^{2+}]i$ elevation was not involved in the augment of degranulation inhibition (see Fig. 1).

2. Results

2.1. Inhibition of Ag-stimulated histamine release by polymethoxy flavones

To investigate the effects of the PMFs on degranulation, we measured the release of histamine from Ag-stimulated RBL-2H3 cells. Release of histamine by DNP-BSA stimulation for 10 min in Siraganian buffer was inhibited by PMFs in a dose-dependent manner (Fig. 2A). Furthermore, it was to be noted that the PMF-combination (PF-1 + 2, PF-1 + 3) suppressed the Ag-stimulated histamine release more strongly than each single treatment. A23187-induced histamine release was also inhibited by treatments with the PMFs (Fig. 2B). However, the suppression of A23187-stimulated degranulation by PMFs was much less compared with that of Ag-mediated one. The inhibitory effect of degranulation by PMF-3 was considered to be mainly due to the inhibition of Ca^{2+}-dependent degranulation process.

2.2. Inhibition of $[Ca^{2+}]i$ elevation by polymethoxy flavones

In order to investigate the mechanism underlying inhibition of degranulation by the PMFs, we examined involvement of Ca^{2+}. In Ag-stimulated FcεRI activation of mast cells, $[Ca^{2+}]i$ level was increased. PMF-1 and -2 significantly suppressed the elevation of $[Ca^{2+}]i$ in the IgE-sensitized RBL-2H3 cells. Especially, PMF-1 completely blocked $[Ca^{2+}]i$ increase (Fig. 3A). PMF-3 did not show any effect (Fig. 3B). The suppression of $[Ca^{2+}]i$ by PMF-1 was recovered or canceled by the combination treatment with PMF-2 (Fig. 3A) or PMF-3 (Fig. 3B).

2.3. Effects of polymethoxy flavones on Ag-stimulated intracellular ROS production on RBL-2H3 cells

In order to reveal the production of intracellular ROS, we measured the intracellular ROS level by using CM-H_2DCF-DA fluorescent probe. DCF oxidation was gradually increased by Ag-treatment (Fig. 4). PMFs were also found to show similar results as shown in Ag-treatment. We measured the radical-scavenging activity of PMFs by DPPH radical-scavenging method. As shown in Table 1, these PMFs did not exhibit radical-scavenging activity. Thus, the suppression of degranulation by PMFs was not associated with intracellular ROS.

2.4. Polymethoxy flavones suppress activation of Syk and PLCγ1/2 in Ag-stimulated RBL-2H3 cells

To further get insight into the mechanism underlying inhibitory effect of degranulation by PMFs, we examined the early intracellular signaling pathway. FcεRI cross-linking on mast cells activates non-receptor-associated protein tyrosine kinases such as Lyn and Syk.[6,7] Firstly, we analyzed phosphorylation of Lyn, Syk, and PLC-γs, which plays important roles for degranulation response. As shown in Figure 4A, Lyn was not affected by the PMFs, whereas phosphorylation of Syk was remarkably suppressed by the PMFs (Fig. 5A). Phosphorylation of PLCγ1/γ2 was also reduced significantly by PMFs. Moreover, the PMF-combinations (PF-1 + 2 and PF-1 + 3) suppressed phosphorylation of Syk and PLC-γs more markedly than each PMF-single treatment (Fig. 5A).

In Ag-stimulated mast cells, three major mitogen-activated protein kinases (MAPKs; ERKs, JNKs, p38) are activated. As shown in Figure 5B, phosphorylation of ERK1/2 was suppressed by the PMFs. It should be noted that the combination (PF-1 + 2, PF-1 + 3) caused greater inhibition of Ag-stimulated ERK1/2 phosphorylation. On the other hand, phosphorylation of JNK1/2 and p38 MAP kinases was not affected by the treatment with PMFs. Phospho-Akt/total-Akt ratio was slightly suppressed by the PMFs.

2.5. Polymethoxy flavones suppress arachidonic acid cascade by ERK activation of in IgE-activated RBL-2H3 cells

Cytosolic phospholipase A_2 ($cPLA_2$) is activated by the increase of $[Ca^{2+}]i$ and MAP kinase activation.[8–11] Phosphorylation of $cPLA_2$

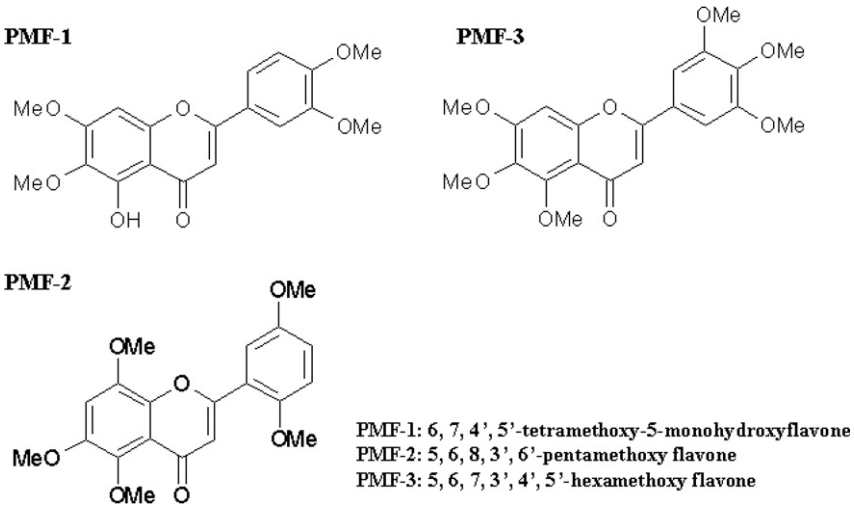

Figure 1. Chemical structures of polymethoxy flavones.

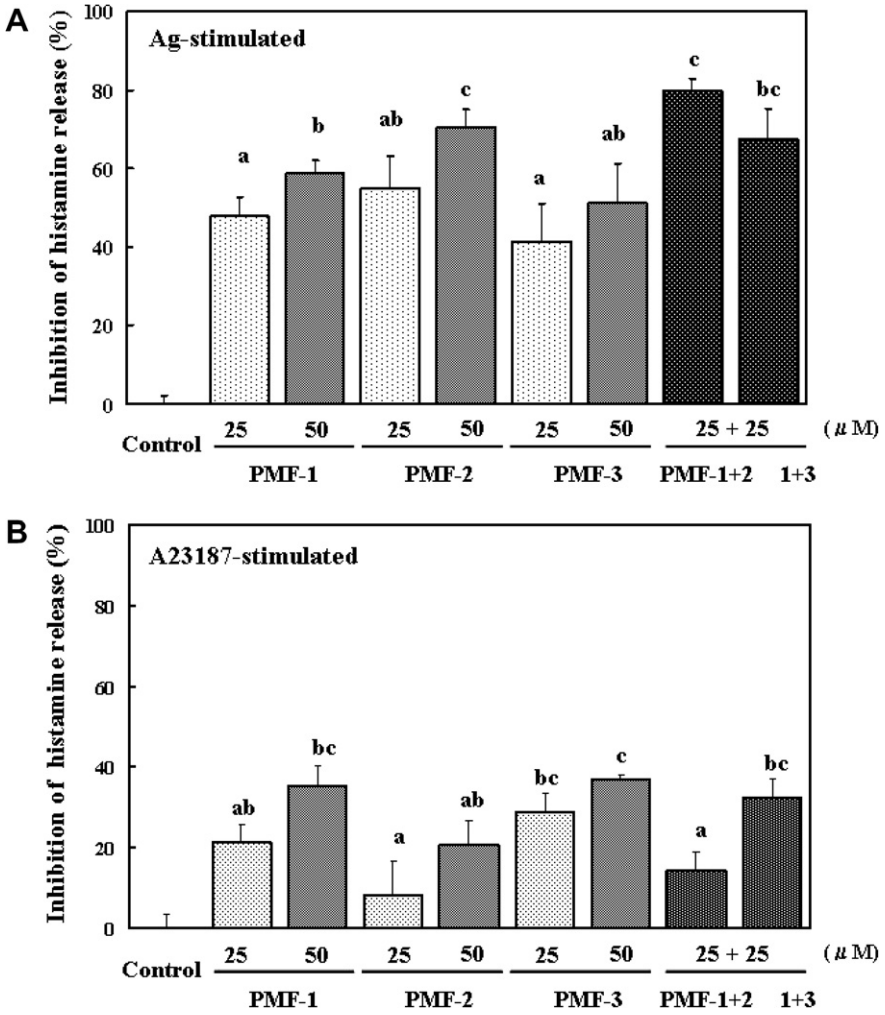

Figure 2. Inhibitory effects of the polymethoxy flavones on Ag-stimulated histamine release from rat basophilic leukemia RBL-2H3 cells. (A) IgE-sensitized RBL-2H3 cells were stimulated with DNP-BSA in the presence of each polymethoxy flavone (PMF) or the mixture of two PMFs. (B) RBL-2H3 cells were stimulated with A23187 in the presence of each polymethoxy flavone (PMF) or the mixture of two PMFs. As a marker of degranulation, we measured the release of histamine as follows: 75 mg of NaCl, 50 μl of 1 N NaOH, and 500 μl of a 3:2 (v/v) mixture of *n*-butanol and chloroform were added to 200 l of Ag-stimulated Siraganian buffer and mixed for 5 min. The organic solvent layer was recovered and mixed with 15 μl of 1 N NaOH and of 0.2% *o*-pthalaldehyde (10 μl), and kept for 5 min. The reaction was terminated by 0.5 N H_2SO_4 (15 μl), and then the fluorescence intensity was measured using a fluorocount microplate reader. Values are means ± SEM ($n = 30$) of the inhibition of histamine release. Means values with different letters are significantly different ($p < 0.05$, one-way analysis of variance followed by Fisher's multiple range test).

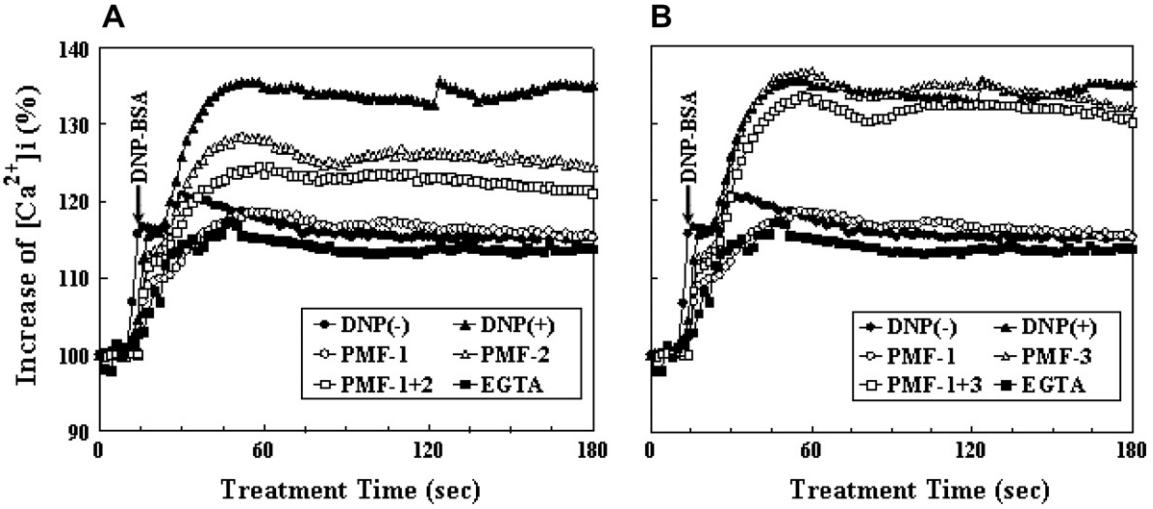

Figure 3. Suppression of the elevation of $[Ca^{2+}]i$ by PMFs in Ag-stimulated RBL-2H3 cells. Measurement of intracellular calcium Ag-stimulated RBL-2H3 cells was stimulated with either DNP-BSA in loading buffer, DNP-BSA in loading buffer including PMFs, or DNP-BSA in calcium-free loading buffer including EGTA (1 mM; selective Ca^{2+} chelating reagent) for indicated periods. Intracellular calcium was measured as described in Section 4. Each value represents the mean with SEM ($n = 32$).

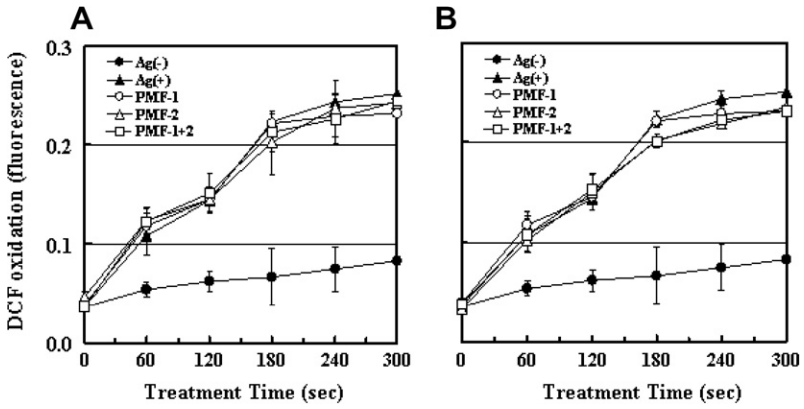

Figure 4. Effect of PMFs on the intracellular ROS production in Ag-stimulated RBL-2H3 cells. CM-H_2DCF-DA-loaded cells were incubated with PMFs (single: 50 μM, combination: 25 + 25 μM) for 30 min. CM-H_2DCF-DA-PMF treated cells were stimulated by DNP-BSA (10 μg/ml). ROS-mediated DCF oxidation was measured using a microplate fluorometer as described in Section 4.

Table 1
Anti-oxidant activity of polymethoxy flavones

	DPPH radical-scavenging activity ED_{50} (μM)
PMF-1	<100
PMF-2	<100
PMF-3	<100
EGCG	7
Vitamin C	18
Vitamin E	78

DPPH radical-scavenging activity was measured in a reaction mixture containing 0.5 mM DPPH radical solution 0.1 ml, 99% ethanol 0.8 ml, and 0.1 ml if PMF solution, The solution was rapidly mixed and the scavenging capacity was measured by monitoring the decrease in absorbance at 517 nm.

by Ag-stimulation was moderately suppressed by each treatment of PMFs and more markedly by the combination (PMF-1 + 2, PMF-1 + 3) (Fig. 5C).

3. Discussion

In this study, we have shown the inhibitory effects on degranulation by PMFs isolated from *C. reticulate* in RBL-2H3 cells. Interestingly, the inhibition of degranulation by PMFs was enhanced by the combined treatment of PMFs, as compared with their single treatment. As for the mechanism of the inhibition of degranulation, the inactivation of Syk and the suppression of Ca^{2+} influx play a major role (Fig. 6).

Recent studies reported that Syk-deficient mast cells completely abrogated the degranulation, elevation of $[Ca^{2+}]i$, and activation of the ERK and JNK MAP kinase pathways.[12,13] Akt is regulated by Lyn/Syk/Btk.[14] These observations indicated that Syk is essential for the degranulation signal transduction. Thus, inactivation of Syk by PMFs was thought to affect the Ag-mediated degranulation.

In this study, PMFs suppressed the elevation of $[Ca^{2+}]i$, and it was considered that PMFs mainly blocked the Ca^{2+} influx from extracellular medium rather than the IP_3-mediated release of Ca^{2+} from endoplasmic reticulum. Ca^{2+} influx through Ca^{2+} release-activated conducting Ca^{2+} (CRAC) channels has been reported to be activated by $cPLA_2$.[15] PMFs were shown to remarkably suppress the activation of $cPLA_2$ and ERKs in this study. Therefore, it was thought that PMFs suppress Ca^{2+} influx through the CRAC channels.

Moreover, several reports indicated that endogeneous ROS is a critical regulator in mast cell response.[16–18] The intracellular ROS production was observed immediately after Ag-stimulation and was most likely due to an NADPH oxidase.[19] However, in present study these PMFs did not suppress intracellular ROS production and exhibited radical-scavenging activity. Therefore, the intracellular ROS was thought not to be associated with the inhibition of degranulation by PMFs.

It was to be noted that the combination of PMFs enhanced the inhibition of degranulation induced by Ag-stimulation. It was suggested that enhancement of the inhibition of degranulation by the combined treatment of PMFs was mainly ascribed to inhibition of Syk/PLCγs/PKC pathway. It was, however, considered that the suppression of $[Ca^{2+}]i$ was not necessarily important in the enhanced inhibition of Ag-mediated degranulation by PMFs-combination. Further study to clarify the mechanism underlying the degranulation inhibition by the PMFs should be needed.

4. Materials and methods

4.1. Reagents and materials

Polymethoxy flavones isolated from *C. reticulate* include PMF-1: 6,7,4′,5′-tetramethoxy-5-monohydroxyflavone, PMF-2: 5,6,8,3′,6′-pentamethoxy flavone, and PMF-3: 5,6,7,3′,4′,5′-hexamethoxy flavone. Monoclonal mouse IgE anti-dinitrophenol (DNP) was purchased from Yamasa Co. Ltd (Tokyo, Japan). The 25 × Complete®, a mixture of protease inhibitors mixture was from Roche (Penzberg, Germany). The phosphatase Inhibitor Cocktail® 1 and 2 was from Sigma (St. Louis, MO, USA). The antibodies to anti-rat p44/42 MAP kinase (ERK), anti-rat phospho-p44/42 MAPK (Thr202/Tyr204) (p-ERK), anti-rat SAPK/JNK (JNK), anti-rat phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK), anti-rat p38 MAP kinase (p38), anti-rat phospho-p38 MAP kinase (Thr180/Tyr182) (p-p38), anti-rat Lyn, anti-rat phospho-Lyn, anti-rat $cPLA_2$, anti-rat phosphor-$cPLA_2$, anti-rat phosphor-PLCγ1, anti-rat phosphor-PLCγ2, and anti-phospho-tyrosine (pTyr) were from Cell Signaling Technology (MA, USA). The antibodies to anti-rat Syk were from Santa Cruz Biotechnology (CA, USA). The antibodies to anti-rat β-actin were from Sigma. FcεRIβ antibody was kindly provided by Dr. J. Rivera (NIH, molecular immunology and inflammation branch). Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit were obtained from GE Healthcare Sci. (Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, England). The other reagents were purchased from standard sources and were of molecular biology grade or higher.

4.2. Cell culture

RBL-2H3 cells were obtained from Health Science Research Resource Bank (Tokyo, Japan). Cells were grown in Eagle's minimum

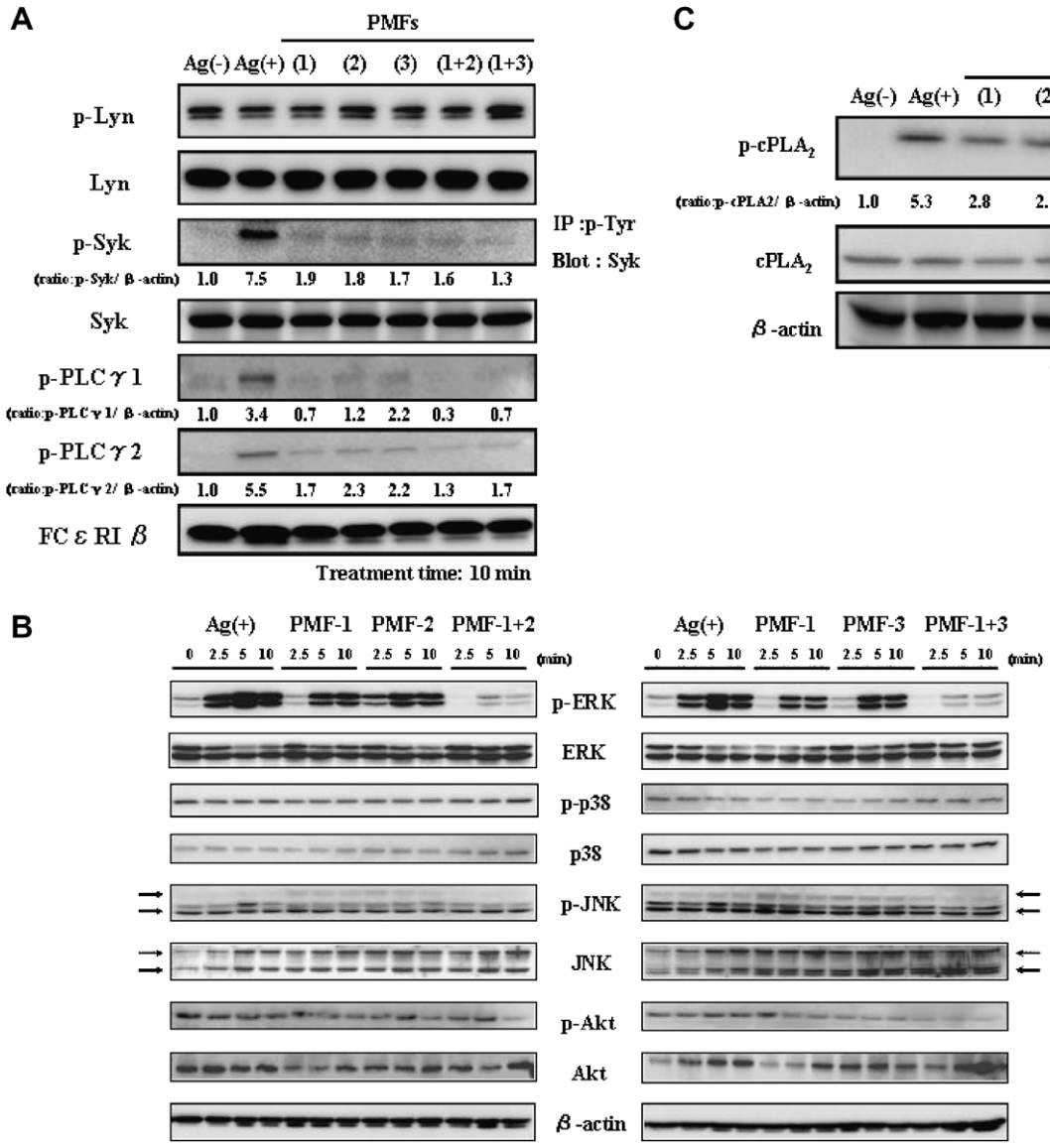

Figure 5. Effect of PMFs on the signaling pathway in Ag-stimulated RBL-2H3 cells. PMFs-treated cells (single: 50 μM, combination: 25 + 25 μM) were stimulated with DNP-BSA for 10 min and were resuspended in RIPA buffer. Twenty micrograms of protein of each cell lysate was separated by SDS–PAGE and electroblotted onto a PVDF membrane for Western blotting. (A) Phosphorylation of Syk, Lyn, and PLCγs. (B) Phosphorylation of ERK1/2, JNK1/2, p38, and PI3K/Akt in Ag-mediated activation. (C) Phosphorylation of p-cPLA$_2$ in Ag-mediated activation.

essential medium (Gibco, MD, USA) containing 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

4.3. Histamine release assay

As a marker of degranulation, we measured the release of histamine.[20] RBL-2H3 cells were inoculated into 24-well plates (5×10^5 cells/ml, 400 μl/well; Nunc, Roskilde Denmark) and cultured for 1 h. Then, monoclonal mouse IgE anti DNP was added to cultured medium at a concentration of 0.45 μg/ml and incubated for 24 h. Treated cells were washed twice by Siraganian buffer (in mM: NaCl 119, KCl 5, $MgCl_2$ 0.4, PIPES 25, NaOH 40, pH 7.2), and then 160 μl of Siraganian buffer was added containing 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA. After incubation at 37 °C for 10 min, the IgE-sensitized cells were treated with 25 μM or 50 μM of MFs (20 μl) at 37 °C for 30 min. Subsequently, 20 μl of DNP-labeled bovine serum albumin (DNP-BSA) was added to cultured medium at a concentration of 10 μg/ml and was incubated for 10 min. To terminate the reaction, the treated cells were kept on ice for 10 min, and then centrifuged at 300g at 4 °C for 10 min.

To measure histamine release, 75 mg of NaCl, 50 μl of 1 N NaOH, 500 μl of a mixture 3:2 (v/v) of *n*-butanol and chloroform were added to 200 l of Ag-stimulated Siraganian buffer and mixed for 5 min. The organic layer was recovered and mixed with 15 μl of 1 N NaOH and 10 μl of 0.2% *o*- pthalaldehyde, and kept for 5 min at room temperature. This reaction terminated by adding 15 μl of 0.5 N H_2SO_4, and then the fluorescence intensity was measured by fluorocount microplate reader (MTP-600F, CORONA ELECTRIC Co. Ltd Hitachinaka, Japan, excitation wavelength 360 nm, emission wavelength 450 nm). The percentage inhibition of histamine release was calculated as follows: inhibition of histamine release (%) = [1 − (test − negative control)/(positive control − negative control)] × 100.

4.4. Measurement of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} level was determined with Calcium Kit-Fluo 3™ (Dojindo Laboratories, Kumamoto, Japan). RBL-2H3 cells

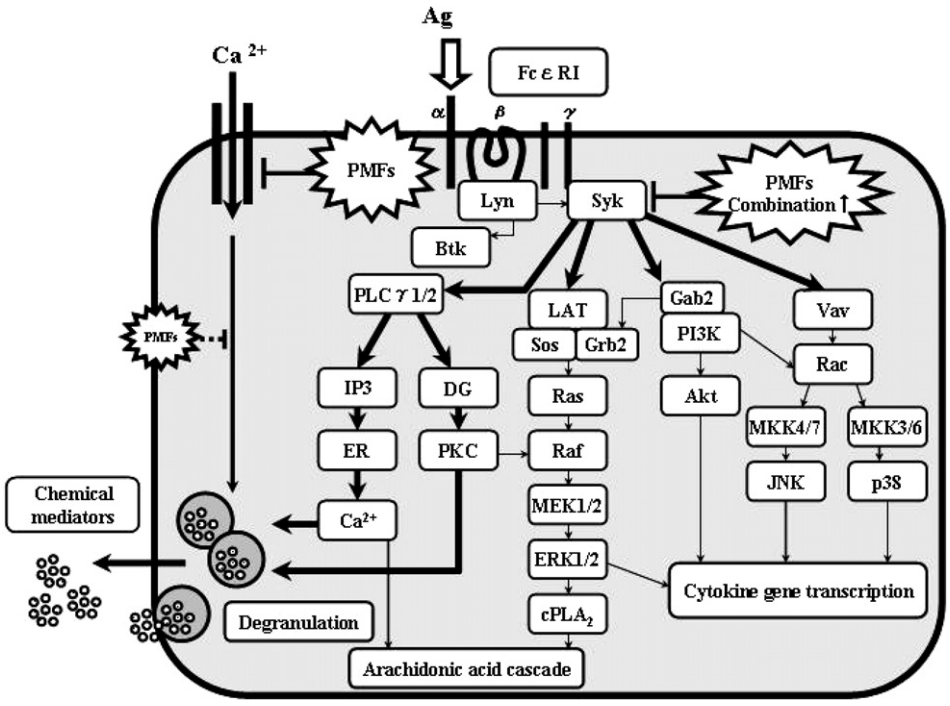

Figure 6. A scheme showing the inhibitory effect on degranulation by the polymethoxy flavones in RBL-2H3 cells. Ag-stimulated FcεRI on mast cells leads to transphosphorylation of the β- and γ-chain ITAMs by Src family protein tyrosine kinase (PTK) Lyn. The protein tyrosine kinase Syk was recruited to the phosphorylated ITAMs through its tandem Src homology 2 region (SH2) domains. Activated-Syk leads to tyrosine phosphorylation of other proteins such as LAT, PLCγs, and SLP-76. These initial signaling events result in degranulation, cytokine gene transcription, and arachidonic acid cascade. PMFs suppressed the degranulation by the inactivation of Syk, the suppression of Ca^{2+} influx and the inhibition Ca^{2+}-dependent degranulation process. Interestingly, PMF-combination treatment significantly enhanced Syk inactivation.

(5×10^4 cells/well) were seeded into 94-black walled-bottom clear micro plates and incubated for 1 h. After incubation, monoclonal mouse IgE anti DNP was added to cultured medium at a concentration of 0.45 μg/ml and incubated for 24 h. The IgE-sensitized cells were washed twice with PBS and incubated with 100 μl of loading buffer containing Fluo-3AM (Calcium Kit-Fluo 3™) for 1 h. The treated cells were washed with PBS and incubated with 90 μl of loading buffer (Calcium Kit-Fluo 3™) including 50 μM of PMFs for 30 min. Changes in intracellular Ca^{2+} concentration induced by DNP-BSA (10 μg/ml) were measured with excitation at 480 nm and emission at 530 nm with a fluorometric imaging plate reader (Flexstation 3; Molecular Devices Corp., CA, USA).

4.5. Measurement of intracellular ROS level by CM-H_2DCF-DA fluorescent probe

Amount of intracellular ROS was measured by using 5-(and-6-)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA). CM-H_2DCF-DA is a fluorogenic freely permeable tracer specific for ROS assessment. It is deacetylated by intracellular esterases to the non-fluorescent 2′,7′-dichlorohydrofluorescein (DCFH), which is oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by ROS. The IgE-sensitized RBL-2H3 cells (5×10^4 cells/well) were incubated with 10 μM CM-H_2DCF-DA for 30 min at 37 °C. Cells were washed twice with PBS to remove the excess of CM-H_2DCF-DA. CM-H_2DCF-DA-loaded cells were incubated with 50 μM PMFs for 30 min. CM-H_2DCF-DA-PMF treated cells were stimulated by DNP-BSA (10 μg/ml) and then measured with excitation at 490 nm and emission at 530 nm with a fluorometer (MTP-600F, CORONA ELECTRIC Co. Ltd Hitachinaka, Japan).

4.6. Measurement of DPPH radical-scavenging activity

To measure antioxidant activity, a DPPH radical-scavenging assay was carried out according to the previous method with a slight modification.[21] Briefly, the DPPH radical-scavenging activity was measured in a reaction mixture containing 0.5 mM DPPH radical solution 0.1 ml, 99% ethanol 0.8 ml, and 0.1 ml of PMF solutions. The solution was rapidly mixed and the scavenging capacity was measured by monitoring the decrease in absorbance at 517 nm. Vitamin C (L-ascorbic acid), vitamin E (α-tocopherol), and EGCG were used as positive controls.

4.7. Immunoprecipitation and immunoblot analysis

For preparation of cell lysate, RBL-2H3 cells were washed twice with PBS and harvested. The cell pellet was resuspended in RIPA buffer containing 25 × Complete®, and Phosphatase Inhibitor Cocktail® (Roche). Protein content was measured with a DC Protein assay kit (BIO RAD, Hercules, CA). Whole cell lysates were incubated with Protein A agarose beads (Roche) overnight at 4 °C. After centrifugation, cell lysate was incubated with protein A agarose beads bound with the pTyr antibody for 3 h at 4 °C. The beads were washed three times with lysis buffer, and then resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer containing 2% 2-mercaptoethanol, and boiled for 5 min. The beads were removed by centrifugation, and supernatants were subjected to Western blot analysis. After blockage of nonspecific binding sites for 1 h by 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4 °C with Syk antibody. Moreover, other proteins (20 μg of protein of each cell lysate) were separated by SDS–PAGE and electroblotted onto a PVDF membrane (Du Pont, Boston, MA). The membrane was then washed three times with TPBS, incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody or anti-rabbit antibody at room temperature, and then washed three times with TPBS. Proteins were detected with enhanced ECL kit and chemiluminescence detector (LAS-1000, Fuji, Japan).

4.8. Statistical analysis

All data were analyzed first by one-way ANOVA, and subsequently by Fisher's-multiple range test. The differences among the means were considered significant at $p < 0.05$.

5. Conclusions

In this study, we have demonstrated that polymethoxy flavones isolated from *C. reticulate*, significantly suppressed the degranulation in Ag-mediated activation of FcεRI in RBL-2H3 cells. It was strongly suggested that inhibition of degranulation by the polymethoxy flavone was mainly due to inhibition of the Syk/PLCγs/PKC pathway and the elevation of $[Ca^{2+}]i$. Interestingly, the combination of PMFs enhanced the inhibition of degranulation induced by Ag-stimulation. As a mechanism, enhancement of the inhibition of degranulation by the combined treatment of PMFs was mainly ascribed to inhibition of Syk/PLCγs/PKC pathway.

Acknowledgments

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