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Inhibitory effect of xanthones isolated from the pericarp of *Garcinia mangostana* L. on rat basophilic leukemia RBL-2H3 cell degranulation

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Abstract—Mangostin, *Garcinia mangostana* L. is used as a traditional medicine in southeast Asia for inflammatory and septic ailments. Hitherto we indicated the anticancer activity induced by xanthones such as α-, β-, and γ-mangostin which were major constituents of the pericarp of mangosteen fruits. In this study, we examined the effect of xanthones on cell degranulation in rat basophilic leukemia RBL-2H3 cells. Antigen (Ag)-mediated stimulation of high affinity IgE receptor (FcεRI) activates intracellular signal transductions resulting in the release of biologically active mediators such as histamine. The release of histamine and other inflammatory mediators from mast cell or basophils is the primary event in several allergic responses. These xanthones suppressed the release of histamine from IgE-sensitized RBL-2H3 cells. In order to reveal the inhibitory mechanism of degranulation by xanthones, we examined the activation of intracellular signaling molecules such as Lyn, Syk, and PLCγs. All the xanthones tested significantly suppressed the signaling involving Syk and PLCγs. In Ag-mediated activation of FcεRI on mast cells, three major subfamilies of mitogen-activated protein kinases were activated. The xanthones decreased the level of phospho-ERKs. Furthermore, the levels of phospho-ERKs were observed to be regulated by Syk/LAT/Ras/ERK pathway rather than PKC/Raf/ERK pathway, suggesting that the inhibitory mechanism of xanthones was mainly due to suppression of the Syk/PLCγs/PKC pathway. Although intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was elevated by FcεRI activation, it was found that α- or γ-mangostin treatment was reduced the $[Ca^{2+}]_i$ elevation by suppressed Ca^{2+} influx.

1. Introduction

Recently, the number of allergic patients is increasing all over the world. Especially this tendency is high in advanced countries. Such an increase in an allergy disease is considered to be due to the influence of various environmental factors (e.g., eating habits, stress, living environment.).[1]

Allergy is classified into five type models (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), type V; stimulative type (e.g., Graves' disease)). At type I allergy, binding of antigen to the high affinity IgE receptor (FcεRI) on the surface of mast cell and basophils induces the release of preformed

Abbreviations: AA, arachidonic acid; Ag, antigen; A23187, calcimycin, calcium ionophore; Btk, bruton's tyrosine kinase; ERK, extracellular signal-regulated kinase; EGCG, (−)-epigallocatechin gallate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; IgE, immunoglobulin E; SAPK/JNK, stress-activated protein kinace/c-jun-N-terminal kinase; LAT, linker for activation of T cells; Lyn, Src family protein kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PVDF, polyvinylidene fluoride; Syk, Syk/Zap-70 family protein kinase, spleen tyrosine kinase; SLP-76, SH-2-containing leukocyte-specific protein of 76 kDa; SNARE, soluble NSF attachment protein receptor; SNAP-23, synaptosome-associated protein of 23 kDa; VAMPs, vesicle-associate membrane protein.

Keywords: Allergy; Rat basophilic leukemia RBL-2H3 cells; Xanthone; Degranulation; IgE.

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intragranular mediators such as histamine, arachidonic acid metabolites, proteases, serotonin, and heparin.[2–4] Thus, mast cell is bearing the important key in the allergic reaction.

Moreover, it was reported in the latest researches that several natural products inhibited degranulation from mast cells.[5–7] As the inhibitory mechanisms, suppression of FcεRI expression, inhibition of intracellular signaling through FcεRI activation, etc., have been considered. Therefore, these various natural products have been known to be effective on prevention of anti-allergic reaction.

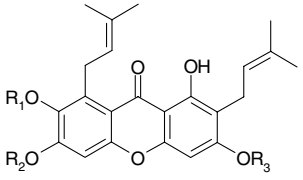
Xanthones are natural organic compounds isolated from the pericarps of mangosteen (*Garcinia mangostana* L.). Among the xanthones, α-, β-, and γ-mangostin were found to have various functions, such as anti-bacteria,[8] anti-inflammatory,[9] anti-cancer,[10] and inhibition of prostaglandin E2 synthesis.[11]

In this study, we investigated effects of α-, β-, and γ-mangostin on activation of intracellular signaling pathways leading to the release of histamine in rat basophilic leukemia RBL-2H3 cells induced FcεRI stimulation. These xanthones were found to suppress the degranulation from Ag-induced activation of FcεRI in RBL-2H3 cells, which is mediated via the signaling cascade involving Syk (see Fig. 1).

2. Results

2.1. Inhibition of histamine release by xanthones

We examined the effects of the xanthones (α-, β-, and γ-mangostin) on the histamine release in IgE-sensitized RBL-2H3 cells. Release of histamine by DNP-BSA stimulation for 10 min in Siraganian buffer was inhibited by xanthones in a dose-dependent manner (Fig. 2). On the other hand, we examined the effect of xanthones on calcium ionophore A23187-induced degranulation. Calcium ionophore A23187 plays as a divalent cation ionophore, elevates the intracellular calcium concentration, and induces degranulation. But these xanthenes treatment did not inhibit A23187-induced deregulation (data not shown). These results suggested that these xanthones suppressed the upstream of the degranulation process in RBL-2H3 cells. We also examined the synergistic effect on the inhibition of degranulation in the combined treatments of xanthones. However, the synergistic effects were not indicated by the combined treatments compared with the single treatment with each xanthone 20 μM.



α-Mangostin: R_1=CH_3, R_2=R_3=H

β-Mangostin: R_1=R_3=CH_3, R_2=H

γ-Mangostin: R_1=R_2=R_3=H

Figure 1. Chemical structures of xanthones.

2.2. Suppression of intracellular Ca^{2+} mobilization by xanthones

To gain more information regarding the suppression mechanism of degranulation by the xanthones, we examined intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in Ag-mediated stimulation of mast cells. This $[Ca^{2+}]_i$ elevation was abolished in the presence of EGTA in the medium, indicating the Ca^{2+} influx from the outside of the cell as the major source of $[Ca^{2+}]_i$ elevation. To examine the suppression of $[Ca^{2+}]_i$ mobilization by xanthones, we measured the $[Ca^{2+}]_i$ mobilization by fluorometric analysis and found that these xanthones suppressed $[Ca^{2+}]_i$ elevation in the IgE-sensitized RBL-2H3 cells (Fig. 3). Especially, α-mangostin completely blocked $[Ca^{2+}]_i$ elevation. It was suggested that xanthones suppressed various intracellular signals, which are associated with the elevation of $[Ca^{2+}]_i$.

2.3. Effects of xanthones on FcεRI-triggered ROS production in RBL-2H3 cells

It was reported that the RBL-2H3 mast cell line produces ROS by FcεRI cross-linking and releases ROS into the extracellular medium.[12] Moreover, FcεRI activation-induced intracellular ROS was involved in the regulation of calcium influx and mediator release.[12] In order to examine the intracellular ROS production by xanthone treatments, intracellular ROS levels were measured by using CM-H_2DCF-DA fluorescent probe. DCF oxidation was gradually increased by Ag treatment. α- and β-mangostin treatments did not reduce intracellular ROS level, but γ-mangostin significantly reduced it (Fig. 4A). We also measured the radical-scavenging activity of xanthones by the DPPH radical-scavenging method. As shown in Figure 4B, γ-mangostin exhibited a strong radical-scavenging activity which was nearly equal to that of vitamin C. However, α- and β-mangostin did not show a significant radical-scavenging activity. These results suggested that suppression of intracellular ROS production by γ-mangostin was due to the strong anti-oxidant activity.

2.4. Xanthones suppresses activation of Syk and PLCγ1/2 in IgE-sensitized RBL-2H3 cells

To disclose the mechanism of inhibitory effect of degranulation by the xanthones, we examined the early intracellular signaling events. FcεRI stimulation activates non-receptor-associated protein tyrosine kinase such as Lyn and Syk.[13,14] And the activation of Lyn and Syk induces the degranulation. As shown in Figure 5A, Lyn phosphorylation was not affected by the xanthones. However, phosphorylation of Syk, PLCγ1, and -γ2 was remarkably suppressed by the xanthones.

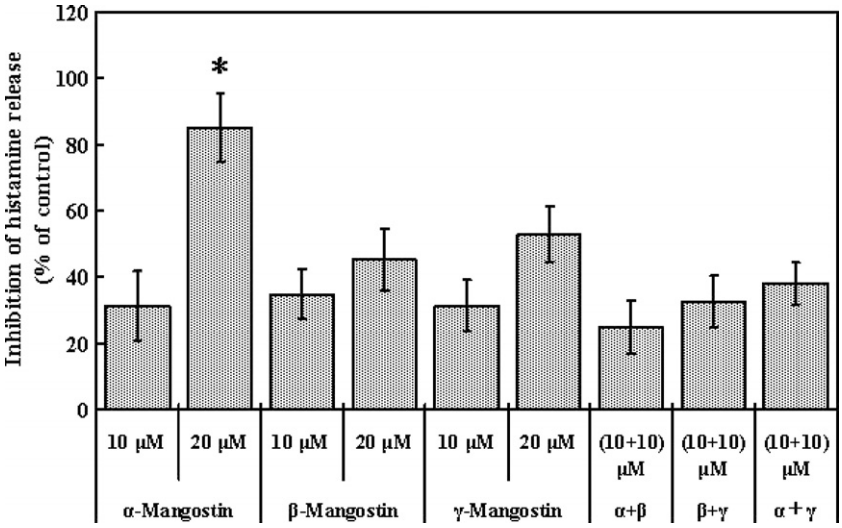


Figure 2. Inhibitory activity of the xanthones on histamine release from rat basophilic leukemia RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were stimulated with DNP-BSA in the presence of the each xanthone or the mixture of two xanthones between the three xanthones. As a marker of degranulation, we examined the release of histamine was measured as follows: 75 mg of NaCl and 50 μl of 1 N NaOH, 500 μl of a 3:2 (v/v) mixture of *n*-butanol and chloroform were added to 200 μl 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 of 0.2% *o*-phtalaldehyde (10 μl), and stayed for 5 min. This 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. Those not sharing a common superscript letter are significantly different at $p < 0.05$ by Fisher's- multiple range test.

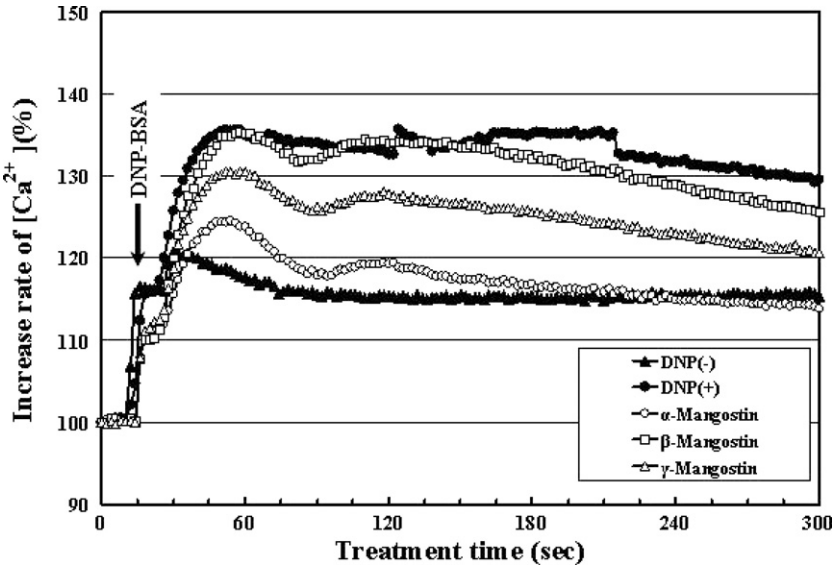


Figure 3. Effect of the xanthones on the [Ca^{2+}] mobilization in Ag-mediated activation of in RBL-2H3 cells. The intracellular Ca^{2+} mobilization was measured with Calcium Kit-Fluo 3™ (Dojindo Laboratories, Kumamoto, Japan). IgE-sensitized RBL-2H3 cells (5×10^4 cells/well) were incubated with 100 μl of loading buffer including Fluo-3AM (Calcium Kit-Fluo 3™) for 1 h. The treated cells were incubated with 90 μl of loading buffer (Calcium Kit-Fluo 3™) including 20 μM of α-, β-, or γ-mangostin for 30 min. Changes in intracellular Ca^{2+} concentration induced by DNP-BSA (10 μg/ml) were measured with a fluorometric imaging plate reader (Flexstation 3; Molecular Devices Corp., CA, USA). Each value represents the means with SEM ($n = 32$).

In Ag-mediated stimulation of mast cells, three major subfamilies of mitogen-activated protein kinases (MAPKs; ERKs, JNKs, p38) were activated.[15–20] As shown in Figure 5B, the phosphorylation of ERK1/2 was completely suppressed by the xanthones. On the other hand, the phosphorylation of JNK1/2 and p38 MAP kinase was not changed by the same treatment. Additionally, the serine/threonine protein kinase, Akt/Protein kinase B, which is known as one of the main downstream effectors of PI3K that promotes cell proliferation and survival,[21,22] is also activated positively by Syk and Btk and inhibited by Lyn.[23] In this study, phospho-Akt was slightly suppressed by the xanthones. These results indicated that the reduction of the level of phospho-Syk by the xanthones could act as a suppressor in degranulation.

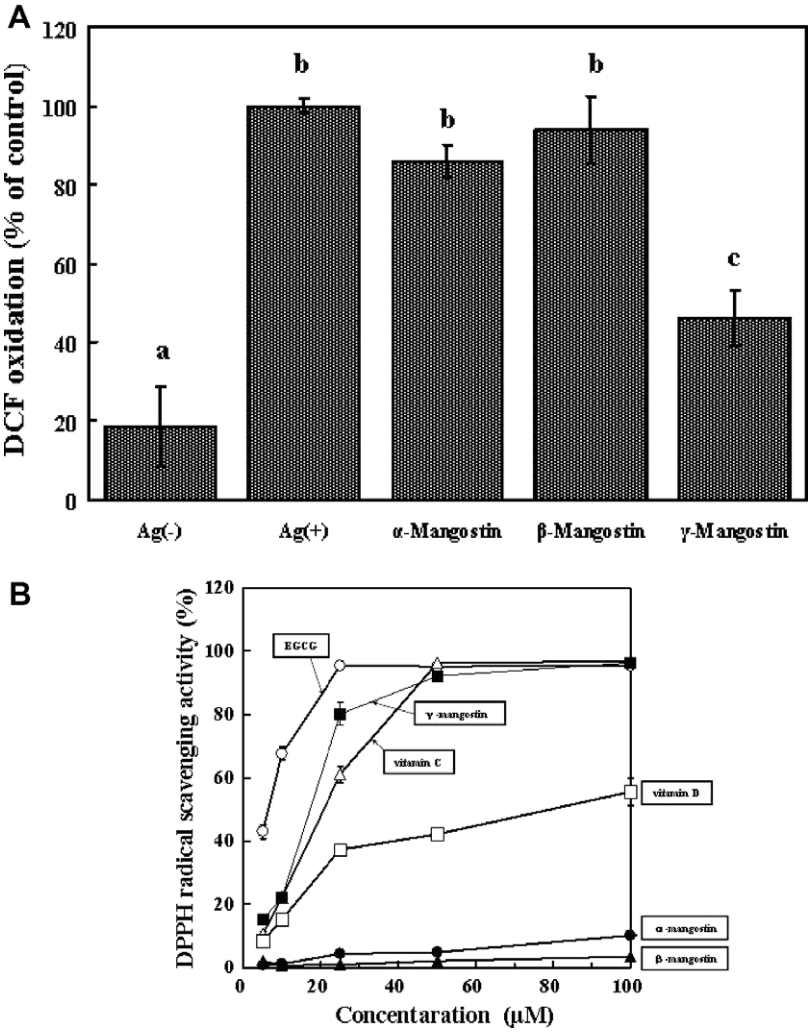


Figure 4. DPPH radical-scavenging activity of xanthones. (A) FcεRI-triggered intracellular ROS production in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were pre-incubated with 20 μM of the each xanthone and 10 μM of CM-H_2DCF-DA for 30 min, washed twice with PBS, and resuspended in Siraganian buffer. CM-H_2DCF-DA-loaded cells were stimulated with Ag for 2.5 min, and ROS-mediated DCF oxidation was measured by microplate fluorometer. The data are expressed as a percent of control value, where DCF oxidation in the cells stimulated with Ag alone is 100%, and represented as the means ± SEM of three separate experiments with similar results. Those not sharing a common superscript letter are significantly different at $p < 0.05$ by Fisher's- multiple range test. (B) The reaction mixture contained 0.1 ml of 1 mM DPPH radical solution, 0.8 ml of 99% ethanol, and 0.1 ml of xanthones (α- (●), β- (▲), and γ-mangostin (■)), a reference drug, EGCG (○), vitamin C (△), and -E (□). The solution was rapidly mixed and scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. All data are expressed as means ± SEM ($n = 12$) of the inhibition of DPPH radical (% inhibition).

2.5. Xanthones suppresses arachidonic acid cascade by activation of ERK in IgE-sensitized RBL-2H3 cells

Cytosolic phospholipase A_2 (cPLA$_2$) is activated by the increase of Ca^{2+} mobilization and ERK1/2 activation in Ag-mediated stimulation of mast cells.[24–27] cPLA$_2$ releases arachidonic acid (AA) from membrane phospholipids, which is further catalyzed by cyclooxygenase, 5- and 12/15-lipooxygenase, which produce in turn, the lipoxyn, leukotriens, and prostaglandins. We examined whether the AA cascade is involved in xanthone treatments and found that phosphorylations of ERK and cPLA$_2$ induced by Ag-treatment were remarkably decreased by the xanthones (Fig. 5B). ERK and cPLA$_2$ act as downstream regulators of Syk. Thus, it was assumed that the suppression of levels of p-ERK and p-cPLA$_2$ by xanthone was led by Syk inactivation. It was thus assumed that the suppression of level of Syk by the xanthones was also involved in AA cascade.

2.6. Ag-mediated PKC/ERK signal in our assay system was not regulated by PKC and PI3K

RBL-2H3 cells express the isozymes of PKC.[28,29] In Ag-mediated stimulation, PKC regulates the ERK1/2 activation which is related to the AA cascade as well as degranulation.[30] To disclose whether phosphorylations of ERK1/2 are induced by PKC in our assay system, we examined the phosphorylation after the treatment with PKC, PI3K, and MEK inhibitors. As shown in Figure 6, treatment with Gö 6893 (pan-PKC inhibitor), Rottlerin (PKCδ and -θ inhibitor), Calphostin C (DAG-dependent PKC inhibitor), and Ro-32-0432 (PKCα, -βI, -βII, -γ, and -ε inhibitor) did not suppress the phosphor-

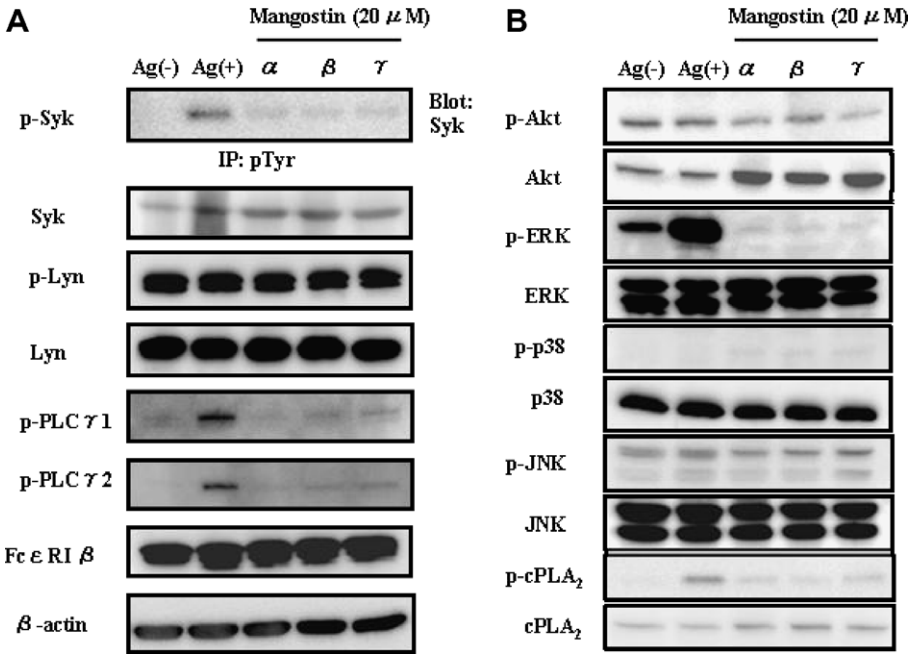


Figure 5. Xanthones suppress the signaling pathway in Ag-mediated aggregation of FcεRI cross-linking on RBL-2H3 cells. Xanthone-treated cells were stimulated with DNP-BSA for 10 min. Reacted cells were resuspended in RIPA buffer. Twenty micrograms of protein of each cell lysate was separated by SDS–PAGE and electroblotted onto a PVDF membrane. (A) Effect of xanthones on Ag-induced tyrosine phosphorylation of Syk, Lyn, and PLCγs. (B) Effect of xanthones on ERK1/2, JNK1/2, p38, and PI3K/Akt in Ag-mediated activation.

ylation of ERK1/2. These results indicated that the Syk/LAT/Ras pathway in the phosphorylation of ERK plays a more important role than PKC.

Additionally, it has been reported that PLCγ1 is regulated by PI3K.[31,32] In our study, a PI3K specific inhibitor, LY294002, blocked the degranulation, but MAP kinase pathway had no effect.

3. Discussion

In this study, we examined the inhibitory mechanism of degranulation by the xanthones which were isolated from the pericarp of *G. mangostana* L. in RBL-2H3 cells. The inhibition of degranulation was found to be mainly due to the inactivation of Syk and the suppression of $[Ca^{2+}]_i$ elevation.

As shown in Figure 7, when IgE-antigen binds to FcεRI, the high affinity IgE receptor is activated, resulting in the release of biologically active mediators that cause several allergic reactions such as degranulation (releasing allergic mediators such as histamine, serotonin, β-hexosaminidase, and mast cell-specific proteases.), production of leukotriens and prostaglandins, and expression and secresion of cytokines.[33] The FcεRI consists of an α-chain, which binds the IgE, a β-chain and two γ-chains. β- and γ-chains are associated with intracellular signal transduction.[34] Both β- and γ-chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) within their intracellular domains. Ag-mediated aggregation of FcεRI on mast cells leads to transphosphorylation of the β- and γ-chain ITAMs by Src family protein tyrosine kinase Lyn. Furthermore, the protein tyrosine kinase Syk is recruited to the phosphorylated ITAMs through its tandem Src homology 2 region (SH2) domains. This interaction also activates Syk, leading to tyrosine phosphorylation of other proteins such as LAT, PLCγs, SLP-76, and Vav.[35–37] Activated-Syk is associated with the action of phospholipases C and D. Tyrosine phosphorylated PLCγ1 and -γ2 catalyzes the hydrolysis of PIP_2, resulting in the generation of IP_3 and DG. These second messengers induce release of Ca^{2+} from internal stores and activate protein kinase C (PKC), respectively. Therefore, activation of Bruton's tyrosine kinase (Btk), SLP-76, and LAT plays an important role for the generation and/or continuance of Ca^{2+} influx. Phosholipase D also regulates FcεRI-induced mast cell degranulation.[38,39] Phospholipase D catalyzes hydrolysis of phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline. PA is rapidly converted to the second messenger DG through the action of phosphatidate phosphohydrolase (PAP). Thus, PLD sustained activation of PKC. It was reported that PKC and calcium are related to the activation of ERK1/2, JNK, and p38 MAP kinase.[40–42] Moreover, the activated Syk also regulates small GTPases such as Rac, Ras, and Rho. These signaling also activate downstream factors such as ERK, JNK, and p38 MAP kinase, which were associated with AA cascade and cytokine gene transcription. From the above-mentioned, the Syk/PLCγs/PKC pathway coupled with Ca^{2+} influx plays an important role in degranulation.

The degranulation induced by Ag-mediated activation of FcεRI is also dependent on the influx of extracellular

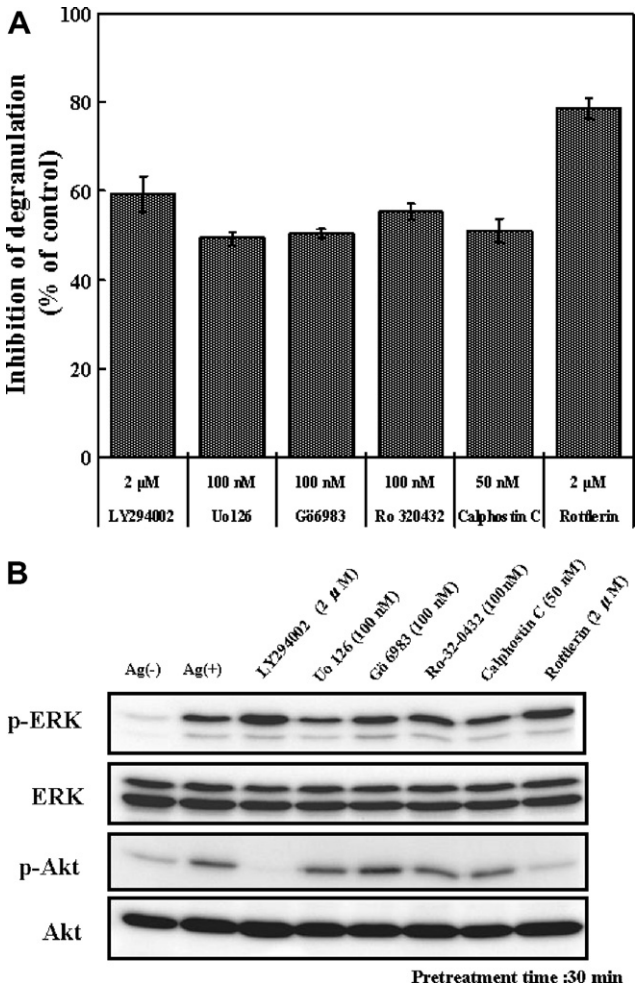


Figure 6. Effect of inhibitors for PI3K, ERK, and PKC on degranulation. IgE-sensitized RBL-2H3 cells were pre-treatmented with PI3K, MEK, and PKCs inhibitors for 30 min and then stimulated with DNP-BSA for 10 min. (A) The levels of degranulation were measured by histamine release assay as described in Section 5. Data are expressed as the means ± SEM of three separate experiments with similar results. (B) Inhibitors were added 30 min before the addition of DNP-BSA. After treatment, washed twice with PBS and then added to Siraganian buffer. Treated cells were stimulated with DNP-BSA for 10 min. Reacted cells were resuspended in RIPA buffer. Twenty micrograms of protein of each cell lysate was separated by SDS–PAGE by using an adequate percent of polyacrylamide in the gel and electroblotted onto a PVDF membrane. Phospho-ERKs, ERKs, phospho-Akt, and Akt were detected with enhanced ECL kit and chemilluminescence detector.

Ca^{2+} across the cell membrane.[43] Moreover, recent studies reported that elevation of $[Ca^{2+}]_i$ was associated with the members of the SNARE family, including SNAP-23, synaptotagmin, syntaxin, and molecules of the VAMPs family which regulates granule-to-plasma or granule-to-granule membrane fusion process.[44] From these observations, $[Ca^{2+}]_i$ is very important in FcεRI-mediated degranulation. Intracellular Ca^{2+} concentration was also regulated by not only Syk/PLCγs/PKC pathway but also sphingosine kinase (SPHK). SPHK, which is downstream of PLD, phosphorylates sphingosine to generate sphingosine-1-phosphate (S1P). S1P plays an important role of Ca^{2+} mobilization from intracellular stores.[45] Moreover, it was reported that the first elevation of $[Ca^{2+}]_i$ is triggered by sequential activation of PLD1 and SPHK1, and the elevation of $[Ca^{2+}]_i$ is triggered by PLCγ1.[45] Thus, activation of PLCγ is a critical event for the induction of store-operated calcium entry. We reported here that the xanthones significantly suppressed Syk/PLCγs/PKC pathway but we did not examine the activation of PLD1 and SPHK. Since PLD is regulated by Fyn/PI3K in Ag-mediated activation of FcεRI, it is necessary to verify further involvement of PLD.[46] In the present study, we examined $[Ca^{2+}]_i$ and intracellular ROS level. Several reports indicated that endogeneous ROS is a critical regulator of mast cell response.[12,47,48] Intracellular ROS is produced by FcεRI activation, which regulates calcium influx and mediator release.[12,46] Furthermore, the ROS production by FcεRI activation was blocked by antioxidants such as EGCG.[46] In our present data, γ-mangostin blocked ROS production but α- and β-mangostin did not show. These results suggested that the suppression of $[Ca^{2+}]_i$ elevation by the xanthones was not regulated only by suppression of ROS production. With regard to calcium influx regulation factors, it is well known that lipooxygenease pathway and mitochondria play an important role in Ca^{2+}-release activated Ca^{2+} channels (CRAC).[49] Especially, mitochondrial permeability transition pore (mPTP) is involved in the regulation of Ca^{2+} signaling and ROS plays a critical role in mPTP opening.[44,50] Suzuki et al. also indicated that ebselen, the selective scavenger for peroxides including H_2O_2, can abolish mitochondrial Ca^{2+} release and $[Ca^{2+}]_i$ elevation.[51] Furthermore, Ca^{2+} influx through CRAC is also activated $cPLA_2$, LTC_4 secretions.[52] In our present study, xanthones remarkably suppressed the activation of $cPLA_2$ and ERKs. Thus, it was suggested that these xanthones regulate Ca^{2+} influx through CRAC. To disclose this mechanism, we need to study further.

In summary, this study has shown that these xanthones mainly suppressed the Syk/ PLCγs/PKC pathway and Ca^{2+} influx in Ag-mediated activation of FcεRI in RBL-2H3 cells. In recent studies, Syk-deficient mast cells were shown to fail to degranulate, to secrete LTs and cytokines when stimulated by FcεRI clustering.[53] Furthermore, the FcεRI-induced rise in intracellular Ca^{2+}, and activation of the ERK and JNK MAP kinase pathways were completely abrogated in the absence of Syk.[54] From these reports, it is very clearly that Syk plays the important role in Ag-mediated mast cell activation. Thus, these xanthones suppressed initial stage of the FcεRI-mediated events in RBL-2H3 cells. However, we could not disclose the mechanism underlying the suppression of $[Ca^{2+}]_i$ elevation.

4. Conclusion

In this study, we have demonstrated that xanthones, isolated from mangosteen, significantly suppressed the degranulation in Ag-mediated activation of FcεRI in RBL-2H3 cells. It was strongly suggested that inhibition of degranulation by the xanthone was mainly due to inhibition of the Syk/ PLCγs/PKC pathway and Ca^{2+} in-

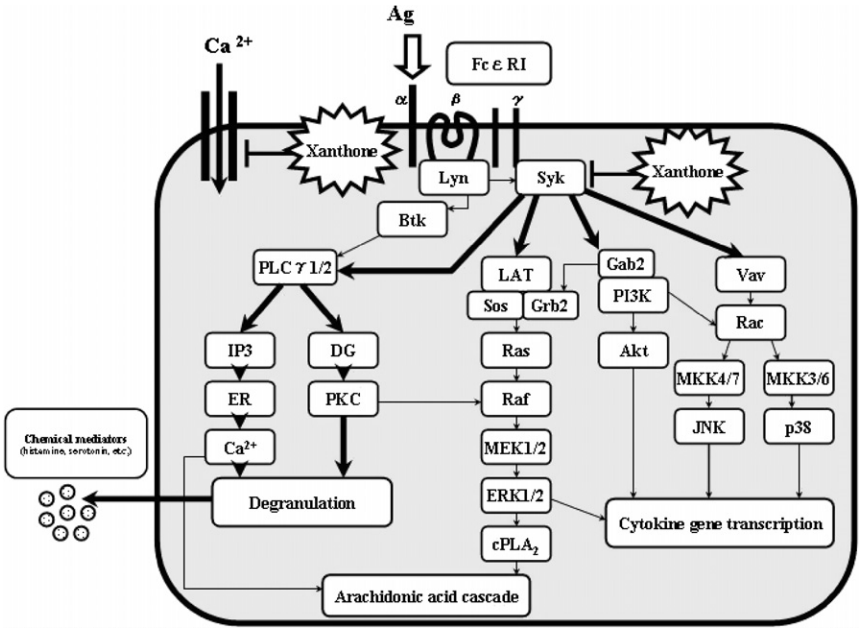


Figure 7. A scheme showing the degranulation mechanism by the xanthones in RBL-2H3 cells. Ag-mediated aggregation of FcεRI on mast cells leads to transphosphorylation of the β- and γ-chain ITAMs by Src family protein tyrosine kinase (PTK) Lyn. The protein tyrosine kinase Syk was recruited to the phosphorylated ITAMs through its tandem Src homology 2 region (SH2) domains. Activated-Syk leads to tyrosine phosphorylation of other proteins such as LAT, PLCγs, SLP-76, and so on. These initial interactions finally lead to degranulation, cytokine gene transcription, and arachidonic acid cascade. Xantnones significantly suppressed Syk activation and Ca^{2+} influx, resulting in degranulation.

flux. Although we could not make clear the suppression of $[Ca^{2+}]_i$ elevation, it was presumed that the xanthones must be regulating Ca^{2+} influx through CRAC.

5. Materials and methods

5.1. Reagents

Xanthones used in this study were purified as described in our previous study.[55] Monoclonal mouse IgE anti-dinitrophenol (DNP) was purchased from Yamasa Co. Ltd. (Tokyo, Japan). The 25× Complete®, a mixture of protease inhibitors mixture and the phosphatase Inhibitor Cocktail® was from Roche (Penzberg, Germany). The antibodies to anti-rat Lyn, anti-rat phospho-Lyn (Tyr507) (p-Lyn), anti-rat phospho-PLCγ1 (Tyr783) (p-PLCγ1), anti-rat phospho-PLCγ2 (Tyr1217) (p-PLCγ2), anti-rat $cPLA_2$, anti-rat phospho-PLA_2 (Ser505) (p-$cPLA_2$), anti-rat p44/42 MAP kinase (ERK), anti-rat phospho-p44/42 MAPK kinase (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), and anti-phosphotyrosine (pTyr) were from Cell Signaling Technology (MA, USA). The antibody to anti-rat Syk was from Santa Cruz Biotechnology (CA, USA). The anti-rat FcεRIβ chain mAb (the hybridoma (clone JRK) was kindly gifted by Dr. Juan Rivera at NIH) was prepared in our laboratory. The antibodies to anti-human β-actin were from Sigma. Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit was obtained from GE Healthcare Sci. (Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, England). The other reagents were of the highest quality available.

5.2. Cell culture

RBL-2H3 cells were purchased from Health Science Research Resource Bank (Tokyo, Japan). Cells were grown in Eagle's minimum essential medium (Gibco, MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

5.3. Histamine releases assay

As a marker of degranulation, we examined the release of histamine and was measured.[56] RBL-2H3 cells were inoculated into 24-well plate (5×10^5 cells/ml, 400 μl/well; Nunc, Roskilde Denmark) and cultured for 1 h. After incubation, monoclonal mouse IgE anti DNP was added to the 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 added 160 μl of Siraganian buffer including 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA). After incubation at 37 °C for 10 min, the IgE-senstized cells were treated with 20 μM of α-, β-, and γ-mangostin (20 μl) at 37 °C for 30 min. As the next operation, 20 μl of DNP-labeled BSA (DNP-BSA) was added to cultured medium at a concentration of 10 μg/ml and was incubated for 10 min correctly. To terminate the reaction, the treated cells were stayed on ice for

10 min. The reacted buffer was collected in a 1.5 ml tube and centrifuged at 300*g* at 4 °C for 10 min.

To measure histamine release, 75 mg of NaCl and 50 μl of 1 N NaOH, 500 μl of a 3:2 (v/v) mixture of *n*-butanol, and chloroform were added to 200 μl 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*-phthalaldehyde, and stayed for 5 min at room temperature. This reaction was terminated by adding 15 μl of 0.5 N H_2SO_4, and then the fluorescence intensity was measured by fluorocount microplate reader (MTP-600 F, 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.

5.4. Measurement of intracellular Ca^{2+} mobilization

The intracellular Ca^{2+} mobilization was measured with Calcium Kit-Fluo 3™ (Dojindo Laboratories, Kumamoto, Japan). RBL-2H3 cells (5×10^4 cells/well) were seeded into 94-black walled-bottom clear micro plate and pre-incubated for 1 h. After incubation, monoclonal mouse IgE anti DNP was added to the cultured medium at a concentration of 0.45 μg/ml and incubated for 24 h. The IgE-senstized cells were washed twice with PBS and incubated with 100 μl of loading buffer including 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 20 μM of α-, β-, and γ-mangostin 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).

5.5. 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 diacetylated 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-senstized RBL-2H3 cells (5×10^5 cells/ml, 400 μl/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 20 μM xanthones for 30 min. CM-H_2DCF-DA-xanthone treated cells were stimulated by DNP-BSA (10 μg/ml) and incubated for 10 min. After incubation, 100 μl of lysis buffer was added to the cell layer and stayed for 5 min on ice and then measured with excitation at 490 nm and emission at 530 nm with a fluorometer (MTP-600F, CORONA ELECTRIC Co. Ltd. Hitachinaka, Japan).

5.6. 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.[57] 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 xanthone 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) and vitamin E (α-tocopherol), and EGCG were used as positive controls.

5.7. 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 including 25× Complete®, and Phosphatase Inhibitor Cocktail® (Roche). Protein content was measured with a DC Protein assay kit (BIO RAD, Hercules, CA). Whole cell lysate was incubated the Protein A agarose beads (Roche) over night at 4 °C. After centrifugation, cell lysate was incubated with protein A agarose beads bound with the anti-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. Twenty micrograms of protein of each cell lysate was separated by SDS–PAGE by using an adequate percent of polyacrylamide in the gel and electroblotted onto a PVDF membrane (Du Pont, Boston, MA). After blockage of non-specific binding sites for 1 h by 5% non-fat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4 °C with various antibodies. 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 chemilluminescence detector (LAS-1000, Fuji, Japan).

5.8. 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 $p < 0.05$.

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