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Effects of Ginsenosides on the Mechanism of Histamine Release in the Guinea Pig Lung Mast Cells Activated by Specific Antigen-Antibody Reactions

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We previously reported that some components of ginsenosides decreased mediator releases evoked by the activation of mast cells with specific antigen-antibody reactions. This study aimed to assess the effects of ginsenosides (Rb_2, Re) on the mechanism of histamine release in the mast cell activation. We partially purified guinea pig lung mast cells by using enzyme digestion, the rough and the discontinuous percoll density gradient method. Mast cells were sensitized with IgG_1 and challenged with ovalbumin (OA). Histamine was assayed by fluorometric analyzer, leukotrienes by radioimmunoassay. Phospholipase D (PLD) activity was assessed more directly by the production of [^{3}H]phosphatidylbutanol (PBut) which was produced by PLD-mediated transphosphatidylation in the presence of butanol. The amount of 1,2-diacylglycerol (DAG) were measured by the [^{3}H]DAG labeled with [^{3}H]palmitic acid or [^{3}H]myristic acid. Pretreatment of Rb_2 (300 μg) significantly decreased histamine release by 60%, but Re (300 μg) increased histamine release by 34%. Leukotrienes release in Rb_2 was decreased by 40%, Re was not affected in the leukotrienes release during mast cell activations. An increasing PLD activity during mast cell activation was decreased by the dose-dependent manner in the pretreatment of Rb_2, but Re pretreatment facilitated the increased PLD activity during mast cell activation. The amount of DAG produced by phospholipase C (PLC) activity was decreased by Rb_2 pretreatment, but Re pretreatment was not affected. The amount of mass DAG was decreased by Rb_2 and Re pretreatment during mast cell activation. The data suggest that Rb_2 purified from Korean Red Ginseng Radix inhibits the DAG which is produced by the activation of mast cells with antigen-antibody reactions via both phosphatidylinositide-PLC and phosphatidylcholine-PLD systems, and then followed by the inhibition of histamine release. However, Re increases histamine release by stimulation of DAG production, which is mediated by phosphatidylcholine-PLD system rather than by phosphatidylinositide-PLC system, but inhibits the mass DAG production. Thus, it could be inferred that other mechanisms play a role in the increase of histamine release during mast cell activation.

Key Words: Mast cell, Histamine, Leukotrienes, Phospholipase D, Phospholipase C, 1,2- diacylglycerol, Phosphatidylbutanol

INTRODUCTION

Korean herb medicine, Panax ginseng, has been observed to have a strong anti-inflammatory component in the chloroform extracts and in the single component of ginseng (Park et al, 1980; Cho, 1981; Kim et al, 1990; Toda et al, 1990).

It has also been reported that 20 (s) G-Rg_3 strongly suppressed histamine release from mast cells due to stimulation with compound 48/80 and substance P (Sugiyama et al, 1991). We reported that ginseng saponins such as total saponin, protopanaxadiol, and

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protopanaxatriol inhibited in part the mediator releases in antigen-induced airway smooth muscle contraction (Ro et al, 1993). We also reported that single components such as Rg_1, Rg_2, and Rc inhibited the releases of histamine and leukotrienes during the activation of guinea pig lung mast cells. These results supported that single component of ginsenosides decreased histamine release by the inhibition of phospholipase D (PLD) activity during mast cell activation (Ro & Kim, 1994). In our laboratory, it has also been observed that single components of ginsenosides such as Rb_1, Rb_2 decreased histamine and leukotriene release, but Re increased only histamine release during mast cell activation. Furthermore, it has been suggested that Rb_1 or Rb_2 decreased leukotriene release by the inhibition of enhanced phospholipid methylation during mast cell activation (Ro & Kim, 1995).

Allergic reactions and asthmatic disorders are caused by the mediator release during the activation of mast cells and basophils by cross-linkage of plasma membrane-bound IgG_1 or IgE-Fc receptors (Ishizaka & Ishizaka, 1978). When mast cell membrane receptors are activated by cross-linkage with antigen-antibody, the enzyme system in the cell membrane are activated. These enzymes such as tyrosine kinase (Eiseman & Bolen, 1992), phospholipase C (PLC) (Cunha-Melo et al, 1987), PLD (Yang et al, 1967; Gruchalla et al, 1990; Lin et al, 1991; Lin et al, 1992; Lin et al, 1994), phospholipase A_2 (PLA_2) (Daeron et al, 1982), adenylate cyclase (Ishizaka et al, 1980), methyltransferase (MT) (Kennerly et al, 1979; Ishizaka et al, 1983; Takei et al, 1990) etc are activated. This process is intimately related to the activation of a variety of phospholipid metabolic pathway and the generation of a number of second messengers such as 1,2-diacylglycerol (DAG) (Kennerly, 1987; Lin et al, 1992), cAMP, and Ca^{++} (Ishizaka et al, 1980; Beaven et al, 1984; Beaven and Cunha-Melo, 1987;. The results lead to exocytosis of preformed inflammatory mediators and synthesis of newly formed mediators (Cockcroft and Gomperts, 1979; Beaven et al, 1987).

In the present study we examined the inhibitory or stimulatory mechanism of Rb_2 or Re on the histamine release in the guinea pig lung mast cell activated with specific antigen-antibody reactions.

METHODS

Materials

Ovalbumin (fraction V), complete freunds adjuvant, anti-IgG_2 affinity column, collagenase (type I), elastase (type I, porcine pancreatic), phosphatidylserine, 1-oleoyl-2-acetyl-sn-glycerol, tyrphostin from Sigma; percoll from Pharmacia Fine Chemicals AB; phosphatidylbutanol from Avanti Polar Lipids; LK 5DF silica gel, LK6D silica gel from Whatmann Inc.; [^{3}H]palmitic acid (s.a., 50Ci/mmol), [^{3}H]myristic acid (s.a., 51Ci/mmol), leukotriene D_4 kit from Amersham. Rb_2 and Re extracted from Korean Red Ginseng Radix were supplied from Korean & Tobacco Research Institute (Taijeon, Korea). Several chemicals used in these studies and other reagents were of the best grade.

Active sensitization protocol (anti-OA production)

Twenty outbred female guinea pigs were first immunized by foot pad injections of mixture of 50 μg ovalbumin (OA) and complete freund's adjuvant. One week after that, animals received intradermal injections of 100 μg OA at one side back and 200 μg of OA at the other side back. Animals were sacrificed one week later and the sera were stored in aliquots at −70°C until the time of use (Andersson, 1980). The quantity of serum antibody titers by passive cutaneous anaphylaxis (PCA) were determined as described in previous articles (Graziano et al, 1984; Undem et al, 1985; Ro et al, 1991).

Serum IgG_1 antibody was separated by affinity column chromatography. Guinea pig blood serum was applied to anti-IgG_2 affinity column and 0.1 M citric acid (pH 2.1) was used to wash the column. IgG_1 was passed through and the absorbed IgG_2 antibody was rinsed with 0.2M sodium carbonate (pH 11.3). The separated IgG_1 was concentrated under pressure for the experiment (Andersson, 1980). The titers of anti-OA were 1,600-3,200. The sera were used for the preparation of passively sensitized mast cells.

Guinea pig lung mast cell preparations

Guinea pig lung mast cells were isolated and purified using techniques similar to the method previously reported (Undem et al, 1985). Briefly described

here, lungs obtained from 16 unsensitized guinea pigs were perfused with 50 ml of the modified Tyrode buffer (TGCM buffer) consisting of NaCl 137 mM, NaH_2PO_4 0.36 mM, KCl 2.6 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1.5 mM, $NaHCO_3$ 119 mM, glucose 5.5 mM, 0.1% gelatin, pH 7.4. After removing large airways and blood vessels, the lungs were minced with a Mcllwain tissue chopper (The Mickle Laboratory Engineering Co. LTD, Gomshall, Surrey, England). Pooled tissue was treated three times with 125 U/g tissue and 5 U/g tissue of collagenase and elastase, respectively. Times (min) of each consecutive exposure of lung fragments to the enzymes were 15, 15 and 25 min, respectively. Cells were separated from residual tissue by filtration through a Nytex mesh (100 μm). The resulting cell population was washed with Tyrode buffer without $CaCl_2$ and $MgCl_2$ containing gelatin (TG buffer) and layered over gradients consisting of 10 ml of Percoll (density, 1.045 g/ml), and centrifuged at 1400 rpm for 20 min. Pelleted cells (containing mast cells) were resuspended in TG buffer, and applied for further purification utilizing a continuous percoll density gradient (consisting of densities 1.06, 1.07, 1.08, 1.09, and 1.10 g/ml). This gradient was centrifuged at 1400 rpm for 20 min (3.5×10^8 cells applied). The cell band obtained between the 1.09 and 1.10 g/ml densities contained the highest purity and number ($1\text{-}2 \times 10^8$) of mast cells. This gradient band was removed, washed with TGCM buffer and designated partially purified mast cell preparation. Mast cell counts were obtained using alcian blue staining and cell viability was determined using trypan blue exclusion. Cell viability was consistently greater than 98%. The purity range of partially purified mast cells was from 60 to 70%.

Mediator release from mast cell

The partially purified mast cells were passively sensitized with anti-OA serum ($1ml/10^6$ cells) in a shaking water bath (45 min at 37°C). After this incubation period, the cells were washed, resuspended in TGCM buffer and challenged with 0.1 μg/ml of OA for 10 min. Polystyrene tubes were used for all cell incubations. Unless stated otherwise, each tube contained 4×10^5 mast cells suspended in 1 ml of TGCM buffer. The mediator release reactions were terminated by placing the tubes in an ice bath. Supernatant obtained after centrifugation was taken for determination of histamine and leukotrienes. In the supernatant for measurement of leukotrienes, 0.1% gelatin (final concentration) was added because leukotrienes were decomposed in air. In experiments utilizing ginsenosides, cells were first incubated for 45min at 37°C concomitantly with anti-OA and ginsenosides, and also incubated for 10 min concomitantly with ginsenosides and OA(1.0 μg/ml).

Histamine assay

Histamine was analyzed by the fluorometric method described by Siraganian (1974). The sensitivity of the assay was approximately 5 ng/ml of histamine. The amount of histamine released was expressed as the percentage of the total histamine present in unstimulated cells.

Leukotriene radioimmunoassay(RIA)

The leukotriene content of each cell supernatant was determined by RIA as described previously (Aharony et al, 1983). The leukotriene antibody was diluted in buffered saline (5 mM MES, HEPES adjusted to pH 7.4 with 1 N NaOH) containing 0.1% gelatin. Each assay tube contained 100 μl of supernatant, antibody(50 μl of a 1:1000 dilution), and 50 μl of [^{3}H]leukotriene D_4 (LTD_4, 2,500 to 3,000 cpm) in buffered saline. Incubations were for two h at 4°C and the reaction was terminated by the addition of 0.5 ml dextran coated charcoal (200 mg charcoal and 20m g dextran mixed with 100 ml buffered saline). After five min incubation the mixture was centrifuged at 3,000 rpm at 4°C and 0.4 ml of the supernatant was added to Aquasol(NEN Research Products) for counting by liquid scintillation spectrometry (Packard, Model 3225). Standard curves were constructed in the presence of antigen using LTD_4. The detection limit of the assay was 0.045 pmole LTD_4. Leukotriene release was expressed as $pmole/4 \times 10^5$ cells.

Determination of phospholipase D activity

In order to label mast cell phospholipid, purified cells were prelabeled with [^{3}H]palmitic acid (PlA). Purified cells ($1\text{-}2 \times 10^7$) were suspended in a final volume of 1 ml TGCM and [^{3}H]PlA (at final concentration of 3.3 μM; 200 μCi/ml), and incubated at 37°C for 1 h. Cells were washed twice and resuspended in

TGCM before use in cell activation.

Prelabeled cells ($0.75\text{-}1.25\times10^6$) were sensitized by IgG_1 antibody (anti-OA antibody, 1ml antibody/ 10^6 cells) at 37°C for 45 min, and washed and resuspended in TGCM. Prelabeled and sensitized cells were stimulated at 37°C for 10 min by OA(0.1 μg/ml) and then was added phosphatidylserine (PS, 15 μg/ml) or PS alone for a final volume of 200 μl in 5ml polypropylene tubes. Butanol (0.5% v/v) was added before stimulation. Reactions were stopped by adding 2 ml cold TGCM and centrifuged for 10 min at 1,400 rpm. Cellular lipids were extracted from the cell pellet by using a modification (Gruchalla et al, 1990; Ro & Kim, 1994) of the Bligh & Dyer procedure (1959).

The standards (phosphatidic acid, PA and phosphatidylbutanol, PBut) with the extracted samples for the measurement of [^{3}H]PBut were applied to the oxalated-treated silica gel thin layer chromatography (TLC, LK6D silica gel 60 Whatmann) plates (presorbed-TLC, 5 g potassium oxalate dissolved in 250 ml H_2O, make up to 500 ml with methanol) and developed to the top of the TLC plates with ethylacetate/acetic acid/trimethylpentane (9/2/5), and visualized standards with iodine staining. PA had an Rf value of 0.46, and that for PBut was 0.81. The plates were scraped, and counted. Butanol as the alcohol of choice for this experiments was used because of the fivefold lower potency of ethanol as an acceptor in the transphosphatidylation reaction.

The standard(DAG) with the extracted samples for the measurement of [^{3}H]DAG by using 100 mM butanol were applied to the presorbed-TLC(LK6D silica gel 60) plates and developed by diethyl ether/hexane/acetic acid (70/30/1). The only complication for the DAG assays based on labeling of phospholipids applied in this way is that it is necessary that phosphatidylalcohols as well as PA do not interfere in the separation system used. Both PA and PBut in the these sample mixture remain at the same place applied without migration. An Rf value for DAG was 0.71.

Determination of mass 1,2-diacylglycerol (DAG)

Mast cells (1×10^6) were sensitized with anti-OA antibody and were labeled in [^{3}H]palmitic acid (0.8 μM, 50 μCi/ml) or [^{3}H]myristic acid (1 μM, 1 μCi/ml) for 1h at 37°C. Cells were rinsed and resuspended with TGCM buffer and stimulated with 0.1 μg/ml OA for 15 min . The reactions were stopped by adding 1 ml of methanol. The labeled lipids were extracted by Bligh and Dyer's method (1959). [^{3}H]DAG from lipids was applied to the presorbed-TLC(LK6D silica gel 60, Whatman), and developed to the half of plates with ethylacetate/acetic acid/trimethylpentane(9/2/5). The TLC plates after air dry were developed to the top of the TLC plates with hexane/diethyl ether/methanol/acetic acid(90/20/3/2)(Lin et al, 1991). The location of [^{3}H]DAG from standard DAG was checked by exposure to iodine vapour. An Rf value for DAG was 0.55. The TLC plate was scratched to measure radioactivity. Ginsenosides was added during all procedures to ginsenoside groups.

Statistic analysis

Experimental data were shown as mean ± S.E.M.S. An analysis of variance (ANOVA) was used for statistical analysis. An analysis of significance between each control group and experimental group was carried out with the Scheffe method. When P values were less than 0.05, or 0.01 it was considered significant.

RESULTS

The effects of ginsenosides on the mediator release during mast cell activation

We examined the effect of ginsenosides on the histamine and leukotriene release from guinea pig lung mast cells activated with specific antigen-antibody reactions (Table 1). When the mast cells sensitized with IgG_1 antibody (anti-OA) were challenged by 0.1 μg/ml OA, histamine release from mast cells, after pretreatment of Rb_2 (300 $\mu g/4\times10^5$ cells), was 9.9 ± 1.92% and that showed a decrease of over 60% when compared with the challenge of antigen alone which was 27.9 ± 2.19%. Histamine release after Re pretreatment (300 $\mu g/4\times10^5$) was 34.4 ± 3.53% and showed a 23.3% increase when compared with the control group. The amount of leukotrienes released from Rb_2 pretreatment was 10.4 ± 4.12 pmole/4×10^5 cells which was approximately a 60% decrease when compared with the 25.7 ± 2.12 pmole/4×10^5 cells of antigen alone challenge, but Re pretreatment did not affect the release of leukotrienes(Table 1). We also

Table 1. Effects of ginsenosides and tyrphostin on the histamine and leukotrienes releases induced by ovalbumin in guinea pig lung mast cells sensitized with anti-ovalbumin[a]

Pretreatment	Histamine (%)[b]	Leukotreines (pmole/4 × 10^5cells)
OA alone	27.9±2.19	25.7±2.12
Rb_2[c]	9.9±1.92*	10.4±4.12*
Re[c]	34.4±3.53+	23.5±2.77
Tyrphostin	10.6±2.62*	21.6±7.54

[a]: Guinea pig lung mast cells (4 × 10^5cells) were passively sensitized by anti-ovalbumin (anti-OA), and challenged with ovalbumin (OA), 0.1 μg/ml, at 5 min after each ginsenoside pretreatment.

[b]: Histamine released is expressed as a percentage of the total histamine

[c]: The concentration of ginsenosides is 300 μg/ 4 × 10^5cells.

*: P<0.01 by comparison with OA alone.

+: P<0.05 by comparison with OA alone.

observed that tyrphostin(10^{-4}M) pretreatment, which is known as a tyrosine kinase inhibitor, inhibited histamine release up to 60% (10.6±2.62%), compared to the challenge of antigen alone (27.9±2.19%), but the tyrphostin pretreatment was not effective in the leukotrienes release(Table 1).

The effects of gensenosides on the phospholipase D activity during mast cell activation

When the mast cells purified from guinea pig lung tissues were activated by specific antigen-antibody reactions, the phospholipase D(PLD) activity increased. Therefore, the effect of ginsenoside on increasing PLD activity caused by mast cell activation were studied. The activity of PLD was determined in the process that phospholipid, in the presence of butanol, changed into transphosphatidylation reaction involving the transfer of the phosphatidyl moiety of the phospholipid substrate to aliphatic alcohol(in this experiment butanol was used) thereby producing phosphatidylbutanol (PBut). As shown in Fig. 2 and Table 2, in the mast cells stimulated only with 0.1 μg/ml of OA, the production of PBut increased remarkably from 2114±56 cpm to 5564±146 cpm, but the production of PBut decreased at the level of non-antigen challenge in the pretreated groups with Rb_2 (300 μg/ml), and tyrphostin (10^{-4}M) (from 5564±149 cpm to 2021 ±384 cpm, and 2213±71 cpm, respectively)

However the pretreatment of Re increased the production of PBut (from 5564±149 cpm to 7304± 230 cpm) during mast cell activation in the presence of butanol (Fig. 2 and Table 2)

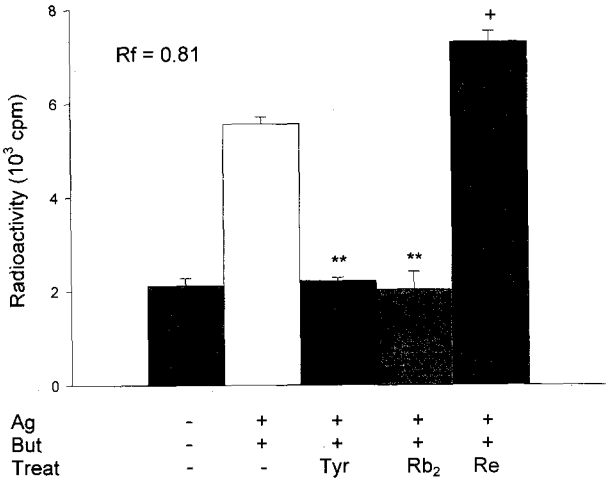

Fig. 1. Butanol-dependent PBut formation in the activation of purified guinea pig lung mast cells sensitized with anti-OA. Purified mast cells (1-2 × 10^7) were prelabeled with [^{3}H]palmitic acid (50 μCi), and washed extensively. Cells were sensitized with anti-OA antibody (1 ml/10^6 cells) in the presence of Rb_2, Re (300 μg/ml) and Tyr (10^{-4}M) for 45 min. Five minutes before OA (0.1 μg/ml) challenge, 0.5% butanol was added. Ten minutes after challenge, lipids were extracted and chromatographically separated by TLC. The radioactivity incorporated into PBut was determined by liquid scintillation spectrophotometry. Results are the mean±SEMS of duplicate determinations from five separate experiments. Asterisks and "+" symbol indicate statistically significant differences (** and +, P<0.01) compared to antigen alone. The abbreviations used are : Ag, antigen; But, butanol; Treat, treatment; Tyr, tyrphostin. The data is shown in Table 2.

The effect of ginsenosides on the production of 1,2-diacylglycerol during mast cell activation

As Rb_2 decreased PLD activity caused by activating mast cell with specific antigen-antibody reactions, Re increased, we examined the effect of these ginsenosides on DAG production which is a second-

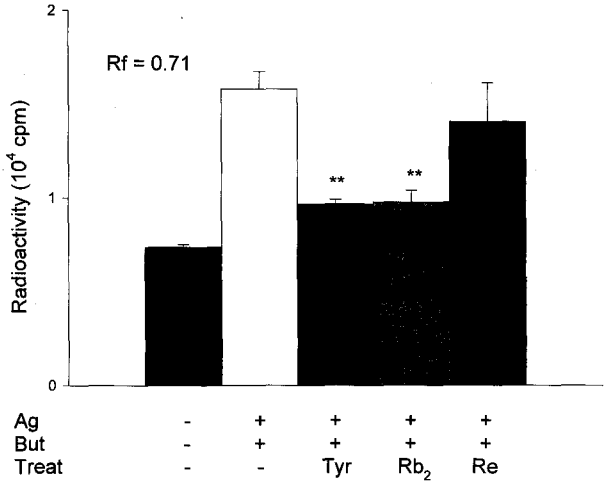

Fig. 2. Effects of ginsenosides and tyrphostin on the DAG formation in the butanol during the activation of mast cells sensitized with anti-OA. Purified mast cells (1×10^6) were prelabeled, sensitized the same as described in Fig. 1. Five minutes before OA (0.1 μg/m) challenge, 100 mM butanol was added. Ten minutes after challenge, lipids were extracted and chromatographicaly separated by TLC precoated with potassium oxalate. The radioactivity incorporated into [^{3}H]DAG was determined by liquid scintillation spectrophotometry. Results are the mean± SEMS of duplicate determinations from five separate experiments. Asterisks indicate statistically significant differences ($P<0.01$) compared to antigen alone. The abbreviations used are the same as described in Fig. 1. The data is shown in Table 2.

ary messenger related with histamine release. The mast cells(1×10^6 cells) were labelled with [^{3}H]palmitic acid, sensitized with ant-OA, challenged with OA, and the labelled phospholipdis were extracted by the same method as described in the measurement of PLD activity, except for the use of high concentration of butanol during mast cell activation because the PA produced by all PLD activities are converted into PBut, and [^{3}H]DAG produced is yielded by only PLC activity. TLC was also developed by diethyl ether/ hexane/acetic acid instead of ethylacetate/ acetic acid/ trimethylpentane which are used to measure the PLD activity. The diethyl ether/hexane/acetic acid migrated the DAG produced by the activation of mast cells, but PA and PBut did not migrate. The Rf value of DAG was 0.71. The amount of DAG produced by PLC activity increased remarkably from 7357±193 cpm to 15787±951 cpm (Fig. 2 and Table 2). These results showed that the amount of DAG produced with only antigen(0.1 μg/ml OA) challenge increased from 2.0 to 2.5 times, compared to non-antigen challenge. Rb_2(300 $\mu g/4 \times 10^5$ cells) pretreatment remarkedly decreased the amount of DAG increased by the activation of mast cells(from 15787±951 cpm to 9750±651cpm), but Re(300 $\mu g/4 \times 10^5$ cells) did not decrease(14050±2068 cpm). However, tyrphostin(10^{-4}), which is known to inhibit only PLD activity by the inhibition of tyrosine kinase activity in cultured cell

Table 2. Butanol-dependent phosphatidybutanol and 1,2-diacylglycerol formation, and mass 1,2-diacylglycerol production in the activation of purified guinea pig lung mast cells with anti-OA[a]

	(+) Butanol[b]		Mass DAG (cpm)[c]	
	PBut (cpm)	DAG (cpm)	30 sec incubation	10 min incubation
(−) OA	2114±56	7357±193	5585±371	3828±76
OA alone	5564±149	15787±951	14214±958	11518±12
Rb_2	2021±384**	9750±651**	6446±326**	6058±1711**
Re	7304±230+	14050±2068	7700±189**	5575±1916**
Tyrphostin	2213± 71**	9672±243**	10214±203*	7561±469**

[a]: Mast cells ($1\text{-}2 \times 10^7$cells) were prelabeled with [^{3}H]palmitic acid in the presence of butanol or with [^{3}H]myristic acid in the absence of butanol. The prelabeled, sensitized cells were challenged by ovalbumin (OA), 0.1 μg/ml, in the absence or presence of ginsenosides.

[b] and [c]: The labeled phospholipids were extracted by Bligh & Dyer method, and separated by TLC, developing the different solvents and TLC types. [^{3}H]phosphatidylbutanol (PBut) and [^{3}H]1,2-diacylglycerol (DAG) were counted.

*: $P<0.05$, **: $P<0.01$ by comparison with OA alone.

+: $P<0.05$ by comparison with OA alone.

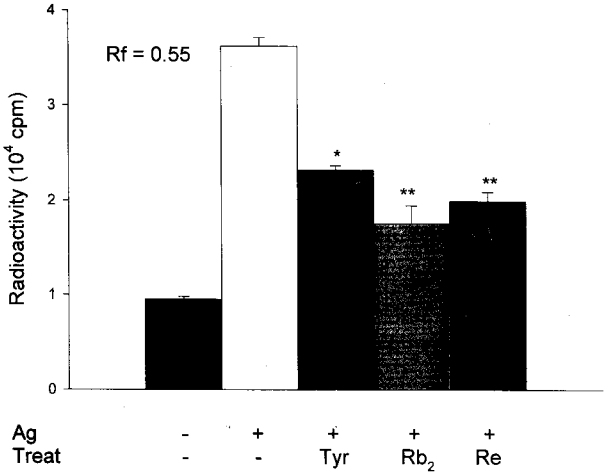

Fig. 3. Effects of ginsenosides and tyrphostin on the mass DAG formation during the activation of mast cells sensitized with anti-OA. Purified mast cells (1×10^6) were sensitized with anti-OA antibody (1 ml/10^6 cells) in the presence of Rb_2, Re (300 μg/ml) and tyrphostin (10^{-4}M) for 45 min, labeled with [^{3}H]palmitic acid (0.8 μM, 50 μCi) for 1hr, and then challenged with OA (0.1 μg/ml) for 10 min. [^{3}H]DAG was extracted and separated from other lipids as described in the Methods. Results are the mean±SEMS of duplicate determinations from five separate experiments. Asterisks indicate statistically significant differences (*, $P<0.05$; **, $P<0.01$) compared to antigen alone. The abbreviations used are the same as described in Fig. 1.

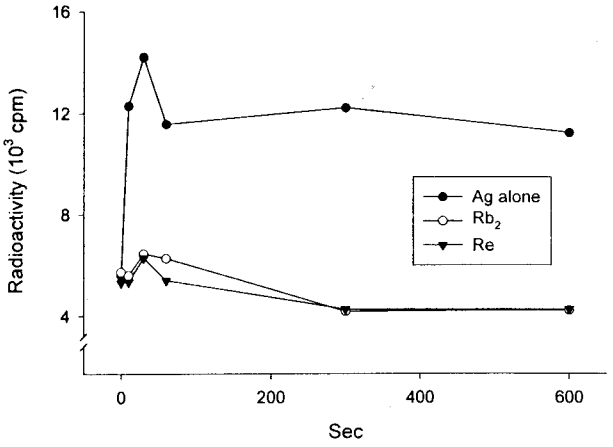

Fig. 4. Effects of ginsenosides on the biphasic increase in DAG formation during mast cell activation. Purified mast cells (1×10^6) were sensitized with anti-OA antibody (1 ml/10^6 cells) in the presence of Rb_2, Re (300 μg/ml) and tyrphostin (10^{-4}M) for 45 min, labeled with [^{3}H]myristic acid (1 μCi) for 1hr, and then challenged with OA (0.1 μg/ml) for the time period indicated. The [^{3}H]DAG was extracted and separated from the other lipids as described in the Methods. Results are the mean of duplicate determinations from one experiment.

line also unexpectedly decreased the amount of DAG, which may be produced by PLC activity(from 157871 ±951 cpm to 9672±243 cpm).

The effect of ginsenosides on the mass 1,2-diacylglycerol during mast cell activation

In order to measure the mass DAG amount, mast cells were sensitized with anti-OA antibody and then prelabeled with [^{3}H]palmitic acid , then stimulated by 0.1 μg/ml OA. The standard Rf of DAG was 0.55. The mass DAG amount produced when stimulated with only an antigen was 3.8 times greater (36223± 912 cpm) than when not stimulated with an antigen (9513±311 cpm). The increased mass DAG amount was inhibited by 52% (17532±1917 cpm) when pretreated with Rb_2 (300 μg/4×10^5), and was inhibited by 45% (19923±983 cpm) when pretreated with Re. The increased mass DAG amount was also decreased by 36% when pretreated with tyrphostin (10^{-4}M) (23191±487 cpm) (Fig. 3).

The effect of ginsenosides on biphasic nature of mass 1,2-diacylglycerol production

As seen above, the production of DAG decreased when pretreated with Re as well as when treated with Rb_2. So the effect of this phenomenon on the biphasic nature of DAG production was checked. The fact that mast cells stimulated with antigen-antibody reactions show a biphasic nature in the DAG production, the early increase(30~60 sec) and continual increase(1~ 30 min), has already been reported and this was also reconfirmed in this study(Fig. 4, Table 2). In short, when mast cells are stimulated, DAG production reaches its climax at 30 sec and there is a decrease of DAG production at 1~2 min and this continues for 30 min (Fig. 4 showed up to 10 min although observations continued for 30 min). When mast cells are stimulated by specific antigen-antibody reaction the primary increase(30 sec) of the mass DAG production is over 2.5 times greater (14214±958 cpm) than when compared with the non-antigen stimulated

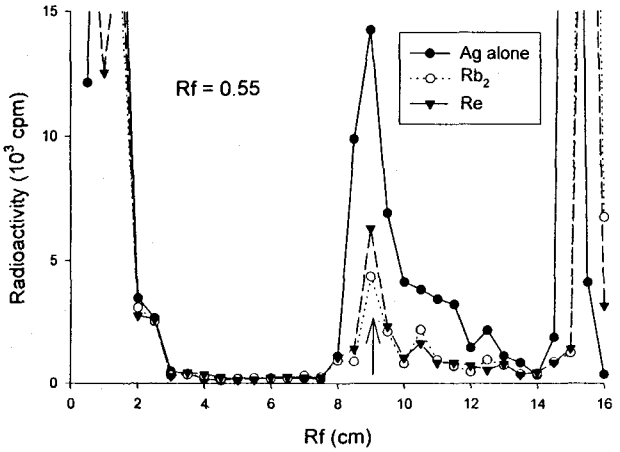

Fig. 5. Effects of ginsenosides on the DAG formation in the mast cell activation for 30 sec. The experemental protocols are the same as described in Fig 4, except to the challenge of OA (0.1 μg/ml) for 30 sec. Results are the mean of duplicate determinations from one experiment. The data is shown in Table 2. An arrow shows DAG peak.

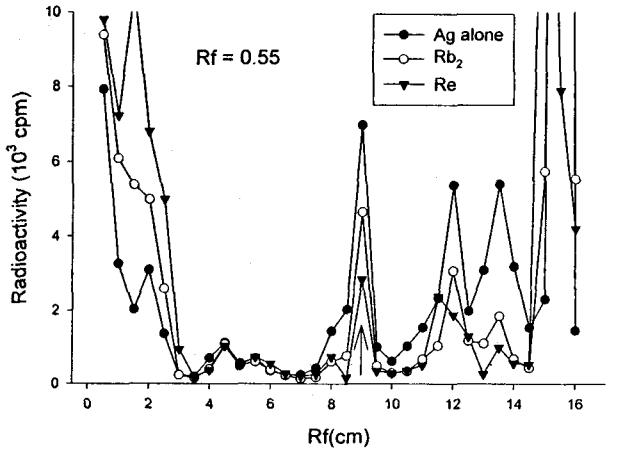

Fig. 6. Effects of ginsenosides on the DAG formation in the mast cell activation for 10 min. The experimental protocols are the same as described in Fig. 4, except to the challenge of OA (0.1 μg/ml) for 10 min. Results are the mean of duplicate determinations from one experiment. The data is shown in Table 2. An arrow shows DAG peak.

mast cells (5585±371 cpm). The increased mass DAG amount is reduced by 54.7% and 45.8% with pretreating of Rb_2 and Re, respectively. However, tryphostin pretreatment decreased by 28.1% decrease in mass DAG. But, the continual increase, at 10 min of the mass DAG amount was inhibited by 47.4%, 51.6%, and 34.4%, respectively, with Rb_2, Re, and tyrphostin pretreatment(Table 2). These results are related with the inhibition of histamine release, the inhibition of stimulated PLD activity and the inhibition of increased DAG production by Rb_2 pretreatment. However, Re results show different results under different conditions of experiments, and so further research is needed.

The inhibitory effect on DAG production evoked by ginseng single components showed that Rb_2, Re, tyrphostin, in this order, inhibited DAG production at the early stages, but Re, Rb_2, tyrphostin, in this order, at the latter stages (Fig. 5, 6).

DISCUSSION

Allergic hypersensitivity reactions and bronchial asthma are caused by the release of mediators from the activation of various enzymes during IgG_1 or IgE cross linking high affinity receptors on the surface of mast cells. That is, enzymes such as PLC, PLD, DAG, MT, PLA_2, adenylate cyclase etc are stimulated and in turn release mediators (Yang et al, 1967; Ishzaka et al, 1980; Cunha-Melo et al, 1987; Kennerly, 1987; Eiseman and Bolen, 1992; Lin et al, 1992; Sato et al, 1993; Lin et al, 1994).

There are several single component (Rg_1, Rg_2, Rg_3, Rc, Rb_1, Rb_2, Rb_3, Re etc) extracted from Korean Red Ginseng Radix. We previously reported that a few single components of ginsenosides (Rg_1, Rg_2, Rg_3, and Rc) inhibited or stimulated the releases of histamine and leukotrienes during antigen-antibody reactions in the guinea pig lung and tracheal tissues (Ro et al, 1993). We also reported that single component of ginsenosides (Rg_1, Rg_2, Rc) decreased histamine and leukotrienes releases during mast cell activation in guinea pig lung tissues, but Rg_3 increased only histamine release (Ro & Kim, 1994). These results have previously been suggested that Rg_1, Rg_2 and Rc inhibited histamine release by partially reducing of DAG production during mast cell activation, which is mediated via PLD system (Ro et al, 1993; Ro & Kim, 1994), that single component of ginsenosides, Rb_1, Rb_2, Rc reduced the phosphatidylcholine (PC) production by inhibiting of the methyltransferase (MT) during mast cell activation, which decrease the conversion of PC into arachidonic acid

and inhibit the production of LT (Ro & Kim, 1995). However the mechanism of ginseng single components, Rb_1 and Rb_2 which belongs to the protopanaxadiol, or Re which belongs to the protopanaxatriol, caused the decrease or the increase of histamine release (Table 1) and its co-relation has not been reported. Therefore, this study tried to uncover the inhibitory or stimulating mechanism of Rb_2 or Re during mast cell activation.

DAG is produced by receptors-mediated enzyme activity and is a secondary messenger which is related to histamine release. DAG is produced from phospholipids either directly by the action of PLC (Cunha-Melo et al, 1987) or indirectly by a PLD-initiated pathway (Gruchalla et al, 1990; Lin et al, 1992; Ro & Kim, 1995). PLC-mediated PIP_2 is directly hydrolyzed to DAG and 1,4,5 inositol-triphosphate (IP_3). In the indirect pathway the last phosphodiester bond of PC is broken by PLD activation, and then phosphatidic acid (PA) is produced. The subsequent action of PA by PA PHase enzyme causes DAG production. The produced DAG acts in concert with calcium, mobilized from intracellular stores, to promote the activation of the protein kinase C, and causes granules and mast cells to fuse, and so shows degranulation (Bell & Burns, 1991; Lin et al, 1991). The granulated histamine and other mediators are released by the degranulation of mast cells. Recently it has been reported that the amount of DAG produced during the activation of rat peritoneal mast cells is much greater when mediated by PLD enzymes than by PLC (Gruchalla et al, 1990). Therefore this study focused on PLD activity in order to discover the inhibitory or stimulating mechanism of Rb_2, or Re on the mediator releases during mast cell activation.

PBut is produced under the presence of butanol which is an index for measuring PLD activity. Rb_2 and tyrphostin pretreatment inhibited PBut production increased only by antigen challenge. This inhibition made the PBut production with Rb_2 pretreatment the same amount as that with non-antigen challenge. but when Re was pretreated, PBut production increased more than when only stimulated by antigen (Fig. 1, Table 2). When Rb_2 was treated at 5 min after tyrphostin pretreatment during mast cell activation, PBut production inhibited the same rate as when treated only tyrphostin (not shown data). Therefore, these results can be indirectly inferred that Rb_2 inhibits PLD activity via inhibiting of tyrosine kinase, but Re accelerates PLD activity via activating of tyrosine kinase.

As the decrease or the increase of PLD activity evoked by the pretreatment of single components was observed, the effect of single components on the indirect DAG production from phospholipids by PA PHase enzyme activity via PLD activation and on the direct DAG production by action of PLC during antigen-antibody reactions in mast cells was examined. DAG is a secondary messenger related to the histamine release caused by the stimulated mast cells. There are many reported methods to measure for DAG (Lee et al, 1991; Huang et al, 1992; Lin et al, 1994). First, when mast cells are stimulated by antigen-antibody reactions, the DAG produced by PLD activity is totally turned into PBut under the influence of a high concentration of butanol (100 mM). Then the DAG amount directly produced by PLC activity can be measured. Rb_2 pretreatment reduced the DAG produced when challenged by antigen alone. However, Re pretreatment did not reduce the DAG produced by stimulating the mast cells with antigen-antibody reactions (Fig. 2, Table 2). These results show that Rb_2 inhibit DAG production via PLC activity but Re does not affect PLC activity. However, tyrphostin pretreatment also inhibits PLC activity. These results were different from reports (Lin et al, 1991; Lin et al, 1992) that tyrphostin blocked tyrosine kinase in RBL 2H3 cultured cells and in turn PLD activity and then DAG production was decreased but didn't affect DAG production caused by PLC activity (early stages).

These different results may be due to the three events. First, the difference evoked by tyrphostin is probably due to the differences in RBL 2H3 cultured cell line and mast cells of guinea pig lung tissues. Second PLC γ_1 and PLC γ_2 is made into phosphorylation by *syk* kinase activity. PLC, phosphorylated and activated by *syk* kinase, hydrolyzes the phospholipids (phosphatidylinositide) to DAG and IP_3 (Jouvin et al, 1994; Blank et al, 1995). As a result, it may be inferred that tyrphostin inhibits DAG production through the inhibition of PLC phosphorylation. The third, it may be due to the existence of PI-PLD subtypes in the guinea pig lung mast cells. Although studies of the hydrolysis of PIP_2 constitute the mainstay of work in the area, recent research has revealed that the lipid-related events of cell signal transduction are not restricted to the participation of

PIP_2-specific phospholipase C. Although most studies have focused on PC hydrolysis by PLD, the results of two earlier works suggested that PI could be a substrate in mammalian systems (Hokin-Neaverson et al, 1975; Cockcroft, 1984). Recently, it has been reported that Madin-Darby canine kidney cells contained two PLD subtypes including PC-PLD and PI-PLD activities located in different subcellular fractions (Huang et al, 1992). It has also been reported that a several mammalian tissues contained glycosylphosphatidylinositol (GPI)- specific PLD activity, and GPI-specific PLD mRNA was present in mast cells of the adrenal gland, lung, and liver (Stadelmann et al, 1993; Xie and Low, 1994; Rose et al, 1995; Andrew et al, 1996). Therefore, the reduction of DAG obtained by tyrphostin pretreatment in the butanol could be explained by PI-PLD subtype rather than that by PI-PLC activity. However, it remains to be studied much more.

The effect of single components on mass DAG production through the actions of PLC and PLD was also observed. The mass DAG was determined by the modification of Lin et al (1991) method. The pathway of mass DAG production was checked in cultured cells by biphasic nature. That is, the increases of DAG production in the early stages caused by other phospholipase (ex. PLC) and in the continual increase by tyrosine kinase activity causing PLD activity (Lin et al, 1992) were checked in the mast cells of guinea pig lung tissues. The mass DAG produced in guinea pig mast cell activation was found to have biphasic nature (Fig. 4, Table 2), and the results are the same as the report that IgE dependant DAG production in RBL 2H3 cultured cells have a biphasic nature (Lin et al, 1992). Therefore, it can be inferred that the nature of DAG production, which is a secondary messenger, which is related with histamine release caused by immune reaction, is the same regardless of the distributions of tissues or species. When Rb_2, Re or tyrphostin is pretreated, both DAG amount produced from early stage caused by PLC activity and continual increase caused by PLD activity are blocked (Fig. 4, Table 2). In the pretreatment of single components the inhibitory degree of initial DAG production showed greater in Rb_2 (Fig. 5, Table 2), the inhibitory effectiveness of continual DAG production was greatest with Re (Fig. 5, Table 2). From these results it can be inferred that Rb_2 inhibits DAG production via both phosphatidylinositide-PLC and PC-PLD pathways. As a results, histamine release is reduced. But the result that tyrphostin inhibits DAG production in the early stages (by PLC activity) as well as continual stages (by PLD activity) determined only DAG production caused by indirect PLC activity in the presence of butanol. Therefore, these results, as mentioned above, can be explained by the differences in species, or by $PLC_{\gamma 1}$ and $PLC_{\gamma 2}$ activated by syk kinase, or by PI-PLD subtype. But more study is needed.

The result of Re was unexpected. But, it can be suggested that the increase of Ca^{++} concentration in cytoplasm evoked by Re pretreatment is stronger than Rb_2, and that Ca^{++} concentration in cytoplasm increases more than amount of DAG during mast cell activation. Study on measurement of Ca^{++} concentration and other mechanisms of Re pretreatment is needed.

From these results, it can be inferred that Rb_2 inhibit histamine release by inhibiting DAG production from phospholipids during mast cell activation via phosphatidylinositide-PLC and PC-PLD systems or via the systems of PI-PLD and PC-PLD subtypes. It can also be inferred that PLD or PLC system is related with tyrosine kinase activity. However, it can also be inferred that Re increases histamine release by stimulation of DAG production, which is mediated by PC-PLD system rather than by phosphatidylinositide-PLC system, and Re inhibits the mass DAG production. Therefore, it can inferred that other mechanisms play a role.

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