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해명 폐조직 비반세포가 항원-항체 반응에 의해 활성화시 매체 유리에 미치는 Ginsenoside(Rb1)의 기전 연구

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Studies of Ginsenoside on the mechanism of mediator releases in the guinea pig lung mast cells activated by specific antigen-antibody reactions

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국문요약

본 연구실은 인삼 사포닌인 ginsenosides의 여러 단일성분이 항원-항체 반응에 의해 활성화된 비반세포에서 유리되는 매개체 유리를 억제시킨다는 사실을 이미 보고하였다. 그러므로 본 연구는 비반세포 활성화시 매개체 유리 억제 효과를 가지는 Rb_1(protopanaxadiol)의 기전을 연구하는데 목적이 있다. 해명 폐조직 비반세포는 효소처리법(collagenase와 elastase)과 percoll density gradient법을 이용하여 분리하였다. 분리된 비반세포는 IgG_1(anti-ovalbumin, anti-OA) 항체(10^6 cells/l ml antibody)로 감작시킨후 OA($0.1\mu g/4\times10^5$ cells)로 자극시켰다. 이때 유리되는 histamine은 fluorometric analyzer로 측정하였으며 leukotrienes는 방사면역법(radioimmunoassay)으로 측정하였다. Phospholipase D (PLD) 활성도는 부탄올 존재하에서 인지질의 phosphatidyl기가 부탄올로 전이되어 생성되는 phosphatidylbutanol(PBut)을 유기용매로 추출하여 TLC로 분리 측정하였다.

Phospholipase C(PLC) 활성도는 과량의 부탄올(100 mM)을 사용하여 PLD-매개 1,2-diacylglycerol(DAG) 생성을 완전히 제거한 후 PLC에 의하여 생성되는 것으로 간주되는 DAG량을 TLC로 측정하는 간접법으로 측정하였으며 총 DAG량은 부탄올 부재시에 같은 방법으로 측정하였다. Methyltransferase I과 II(MT I과 II) 활성도는 인지질 메칠화를 통하여 측정하였다. Rb_1(50-300$\mu g/4\times10^5$ cells) 전처치는 비반세포 활성화시 histamine(30-57%)과 leukotrienes(26-60%) 유리량을 농도 의존적으로 억제시켰다. Rb_1($300\mu g/1\times10^6$ cells) 전처치는 항원-항체 반응에 의한 비반세포 활

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성화시 PLD 활성도를 의의있게 억제시켰다. Rb_1($300\mu g/1\times10^6$ cells) 전처치는 항원-항체 반응에 의한 비반세포 활성화시 PLC 활성도에 의해 생성되는 DAG 량을 의의있게 억제시켰다. Rb_1($300\mu g/1\times10^6$ cells) 전처치는 항원-항체 반응에 의한 비반세포 활성화시 총 DAG 생성을 억제시켰다. Rb_1($300\mu g/1\times10^6$ cells) 전처치는 항원-항체 반응에 의한 비반세포 활성화시 인지질 메칠화를 억제시켰으며 특히 phosphatidylcholine과 lyso-phosphatidylcholine 생성을 감소시켰다.

이상의 결과를 종합하면, 고려 인삼 사포닌의 단일성분인 Rb_1은 해명 폐조직 비반세포가 항원-항체 반응에 의해 활성화시 phosphatidylcholine-PLD와 phosphatidylinositol-PLC 경로를 통한 DAG 생성을 억제시켜서 histamine 유리를 감소시키는 것으로 생각된다. 또한 Rb_1은 methyltransferase I과 II 효소를 억제시켜 phosphatidylcholine 생성을 감소시키므로 결과적으로 leukotrienes의 전구물질인 arachidonic acid의 생성을 감소시키는 것으로 추정된다.

Key words : mast cell, histamine, leukotrienes, phospholipase D, 1, 2-diacylglycerol, methyltransferase

Introduction

Korean herb medicine, Panax ginseng, has been observed to have strong anti-inflammatory component in the chloroform extracts and in the single component of ginseng[1-4].

It has also been reported that 20(s) G-Rg_3 suppressed strongly histamine release from mast cells due to stimulation with compound 48/80 and substance P[5]. We reported that ginseng saponins such as total saponin, protopanaxadiol, and protopanaxatriol inhibited in part of the mediator release in antigen-induced airway smooth muscle contraction[6]. 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 PLD activity during mast cell activation[7].

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, and subsequent bridging of IgG_1- or IgE-Fc receptors[8]. 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[9-12], PLC[13], PLD[14-21], phospholipase A_2[22], adenylate cyclase[23], MT[24-29]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. The results lead to exocytosis of preformed inflammatory mediators and synthesis of newly formed mediators[20-24, 30-34].

In the present study we examined the inhibitory mechanism of Rb1 on the mediator release in the mast cell activated with specific antigen-antibody reactions.

Material and methods

Materials

Ovalbumin(fraction V), complete freunds adjuvant, anti-IgG_2 affinity column, collagenase(type I), elastase(type I, porcine pancreatic), phosphatidylserine, methionine, lyso-phosphatidylcholine, phosphatidylethanolamine, phosphatidylcholine, 1-oleoyl-2-acetyl-sn-glycerol, from Sigma; percoll from Pharmacia Fine Chemicals AB; phosphatidylbutanol, phosphatidyl- monomethylethanolamine, phosphatidyldimethylethanolamine 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), L-[^{3}H]methymethionine(s.a., 83Ci/mmol), leukotriene D_4 kit from Amersham. Rb1, single component, extracted from Korean Red Ginseng Radix was supplied from Korean & Tobacco Research Institute(Taiseon, Korea). Several chemicals used in these studies and other reagents were the best of grade.

Active sensitization protocol (anti-OA production)

Twenty outbred female guinea pigs were first immunized by foot pad injections of mixture of 50μg ovalbumin(OA) and complete freund's adjuvant. One week after that, animals received intradermal injections of 100μg OA at one side back and 200μg of OA at the other side back. Animals were sacrificed one week later and the sera were stored in aliquots at -70℃ until the time of use[35]. The quantity of serum antibody titers by passive cutaneous anaphylaxis(PCA) were determined as described in previous articles[36-38].

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[35]. 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[37]. Briefly described here, lungs obtained from 16 unsensitized guinea pigs were each perfused with 50ml of the modified Tyrode buffer (TGCM) consisting of(millimolar): NaCl, 137; NaH_2PO_4, 0.36; KCl, 2.6; $CaCl_2$, 1; $MgCl_2$, 1.5; $NaHCO_3$, 119; glucose, 5.5; gelatin, 1g/L, 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 125U/g tissue and 5U/g tissue of collagenase and elastase, respectively. Times(min) of each consecutive exposure of lung fragments to the enzymes were 15, 15 and 25, respectively. Cells were separated from residual tissue by

filtration through a Nytex mesh(100㎛). 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 10ml of percoll (density, 1.045g/ml), and centrifuged at 1400 rpm for 20min. Pelleted cells(containing mast cells) were resuspended in TG buffer, and applied for further purification utilizing a discontinuous percoll density gradient(consisting of densities 1.06, 1.07, 1.08, 1.09, and 1.10g/ml). This gradient was centrifuged at 1400 rpm for 20min(3.5×10^8 cells applied). The cell band obtained between the 1.09 and 1.10g/ml densities contained the highest purity and number($1-2\times10^8$) 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 70-80%.

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℃). After this incubation period, the cells were washed, resuspended in TGCM buffer and challenged with 0.1㎍/ml of OA. 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 reaction was terminated by placing the tubes in an ice bath. Supernatants obtained after centrifugation were taken for determination of histamine and leukotrienes[37-38]. In the supernatants for measurement of leukotrienes, 0.1% gelatin(final concentration) was added because leukotrienes were decomposed in air. In experiments utilizing Rb_1, cells were first incubated for 45min at 37℃ concomitantly with anti-OA and Rb_1, and also incubated for 10min concomitantly with Rb_1 and OA(0.1㎍/ml).

Histamine assay

Histamine was analyzed by the fluorometric method described by Siraganian[39]. The sensitivity of the assay was approximately 5ng/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[40]. The leukotriene antibody was diluted in buffered saline(5mM MES, HEPES adjusted to pH 7.4 with 1 N NaOH) containing 0.1% gelatin. Each assay tube contained 100㎕ of supernatant, antibody (50㎕ of a 1:1000 dilution), and 50㎕ of [^{3}H]leukotriene D_4(LTD_4, 2,500 to 3,000 cpm) in buffered saline. Incubations were for 2 h at 4℃ and the reaction was terminated by the addition of 0.5ml dextran coated charcoal(200mg charcoal and 20mg dextran mixed with 100ml buffered saline).

Five min after incubation the mixture was centrifuged at 800 x g at 4℃ 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/4×10^5 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($1-2 \times 10^7$) were suspended in a final volume of 1 ml TGCM and [^{3}H]PlA(at final concentration of 3.3μM; 200μCi/ml), and incubated at 37 ℃ for 1 hr. Cells were washed twice and resuspended in TGCM before use in cell activation.

Prelabeled cells($0.75-1.25 \times 10^6$) were sensitized by IgG_1 antibody(anti-OA antibody, 1ml antibody/10^6 cells) at 37℃ for 45 min, and washed and resuspended in TGCM. Prelabeled and sensitized cells were stimulated at 37℃ for 10 min by OA(0.1μg/ml) and phosphatidylserine(PS, 15μg/ml) or PS alone for a final volume of 200$\mu\ell$ in 5ml polypropylene tubes. Butanol(50 mM or 100 mM) was added at 5 min before stimulation. Reactions were stopped by adding 2ml cold TGCM and centrifuged for 10min at 800 x g. Cellular lipids were extracted from the cell pellet by using a modification[7,15] of the Bligh and Dyer procedure[41]. In the experiments utiliting Rb_1, the prelabeled and the sensitized cells were concomitantly incubated with 0.1μg/ml OA.

The standards(PA and PBut) with extracted samples were applied to the oxalated -treated silica gel TLC plates(presorbed-TLC, 5g potassium oxalate dissolved in 250ml H_2O, make up to 500ml with methanol), developed to the top of the TLC plates with ethylacetate/acetic acid/trimethylpentane(9/2/5), and visialized 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 100mM butanol was applied to the presorbed-TLC plates, and developed by diethyl ether/hexane/acetic acid(70/30/1). These developing sovents migrated the DAG, but 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) during mast cell activation

Prelabeled mast cells(1×10^6) with [^{3}H] myristic acid(1 M, 1 Ci/ml) for 1hr at 37℃ were sensitized with anti-OA antibody for 45 min. Cells were rinsed and resuspended with TGCM buffer, stimulated by 0.1g/ml OA for the time specified. The reactions were stopped by adding 1 ml of methanol.

The labeled lipids were extracted by Bligh and Dyer's method[41]. [^{3}H]DAG from lipids was applied to presorbed TLC(LK6D silica gel, Whatman), and developed to the half the TLC plates with ethylacetate/acetic acid / triethylpentane(9/2/5). The TLC plates after air dry were run to the top of the TLC plates in a second system such as hexane/diethyl ether/methanol/acetic acid(90/20/3/2)[16]. The location of [^{3}H]DAG from standard DAG was checked by exposure to iodine vapour. An Rf value for DAG were 0.55. The TLC plate was scratched to measure radioactivity. Rb1 was added during all procedures.

Determination of phospholipid methylation

Phospholipid methylation was determined as described by Ishizaka et al[23]. The mast cells sensitized with anti-OA(1×10^6 cells/ml) were incubated(25min, 37℃) with L-[^{3}H-methyl]methionine(2μM, 165μCi/ml), and washed twice. The resuspended cells were challenged with 0.1μg/10$\mu\ell$ OA for the time specified. The reaction was stopped by the addition of 900$\mu\ell$ of ice-cooled 10% TCA containing 10mM L-methionine, and tubes were centrifuged at 1,000 x g for 10min at 4℃. The precipitates were washed with 10% TCA and then extracted with 3ml of chloroform/methanol(2/1 v/v). The chloroform phase was washed twice with 1.5ml of 0.1M KCl in 50% methanol. A 1ml fraction of the chloroform phase was transferred to a counting vial and evaporated to dryness at room temperature. The residue was counted.

Identification of methylated phospholipid was carried out by TLC as described by Hirata et al[25]. The chloroform layer obtained from the chloroform/methanol extracts were evaporated under the N_2 gas, and the residue was dissolved in 50$\mu\ell$ of chloroform/methanol(2:1 v/v). Then 30$\mu\ell$ of each solution was applied to a TLC plate (LK5DF, Whatman), and developed for 4hr with n-propanol/propionic acid/chloroform/water(3:2:2:1 v/v). Lipid spots identified with iodine staining and corresponding to known Rf values of authentic standards (PMMC, PDME, PC and Lyso-PC) were scraped into scintillation vials, and dispersed by sonication with 400$\mu\ell$ of methanol and 5ml of scitillation solution, and radioactivity was counted.

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 Rb1 on the mediator releases during mast cell activation.

We examined the effect of Rb_1 on the histamine and leukotriene releases 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 0.1μg/ml OA, histamine release from mast cells after pretreatment of Rb_1 (100μg/4×10^5 cells) was 19.1±1.61%, and that showed 30% above decrease when compared to the challenge of antigen alone which was 27.9±2.19 %. The amount of leukotrienes released from Rb_1(100μg/4×10^5 cells) pretreatment was 16.8±2.39 pmole/4×10^5 cells, which was approximately a 35% decrease when compared to the 25.7±2.12 pmole/4×10^5 cells of antigen alone challenge. The inhibitory effect of both mediator releases evoked by Rb_1 pretreatment showed the dose-dependent response of Rb_1(Table 1). In this study, the concentration(300μg) of Rb_1 which decreased up to the maximum both mediator releases during mast cell activation was mainly used in each experiment.

The effects of Rb_1 on the phospholipase D activity during mast cell activation.

When the mast cell purified from guinea pig lung tissues are activated by specific antigen-antibody reactions, the phospholipase D(PLD) activity is increased. Therefore, the effect of Rb_1 on increasing PLD activity caused by mast cell activation were studied. The activity of PLD was measured by the process that in the presence of aliphatic alcohol, phospholipid 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. 1, in the mast cells stimulated only with 0.1μg/ml of OA, the production of PBut increased remarkably from 2114±56 cpm to 5564±146 cpm, but the production of PBut decreased from 5564±149 cpm to 2580±50

Table 1. Effects of Rb_1 on the histamine and leukotrienes releases induced by OA in guinea pig lung mast cells sensitzed with anti-OA[a].

Ttreatment	Histamine (%)	Leukotreines (pmole/4×10^5 cells)
OA alone	27.9±2.19	25.7±2.12
Rb_1(μg)		
50	28.5±1.57	24.9±1.99
100	19.1±1.61*	16.8±2.39*
300	11.9±1.94**	9.9±3.86**

a. Guinea pig lung mast cells(4×10^5 cells) were passively sensitized by anti-ovalbumin(anti-OA), and challenged with ovalbumin(OA), 0.1μg/ml, at 5min after each Rb_1 prtreatment.
*, $P<0.05$; **, $P<0.01$ by comparison with OA alone.

Table 2. Effects of Rb_1 on the [^{3}H]methyl incorporation, mediator releases induced by OA in guinea pig lung mast cell sensitzed with anti-OA[a].

Ttreatment	Phospholipid Methylation (cpm)	Histamine (%)	Leukotreines (pmole/4×10^5 cells)
OA alone	16233±375	27.9±2.19	25.7±2.12
Rb_1(μg)			
50	16759±413	28.5±1.57	24.9±1.99
100	11363±612*	19.1±1.61*	16.8±2.39*
300	9586±464**	11.9±1.94**	9.9±3.86**

a. Guinea pig lung mast cells(4×10^5 cells) were passively sensitized by anti-OA, prelabeled with [^{3}H]methylmethionine, and challenged with 0.1g/ml OA, at 5min after Rb_1 prtreatment.
b. Values represent maximum methylation of phospholipid at 15sec after challenge of OA.
*, $P<0.05$; **, $P<0.01$ by comparison with OA alone.

cpm at the level of non-antigen challenge in the pretreatment groups with Rb_1(300μg/ml).

The effect of Rb_1 on the production of 1,2-diacylglycerol during mast cell activation

The production of DAG which is a 2nd messenger strongly related with histamine release during mast cell activation is already known to have biphasic nature. As Rb_1 decreased PLD activity caused by activating mast cells with specific antigen-antibody reactions, it can be inferred that Rb_1 decreases the production of DAG by inhibiting the PLD activity which is known to induce the production of DAG with indirect pathway system. Therefore, we examined the effect of Rb_1 on DAG production caused by mast cell activation. When the mast cells(1×10^6 cells) were labeled with [^{3}H]palmitic acid, sensitized with anti-OA, challenged with OA for 10 min in the presence of high concentration of butanol(100 mM), the amounts of DAG, which means that the amounts of DAG produced by PC-PLD pathway were subtracted, increased remarkably from 7357

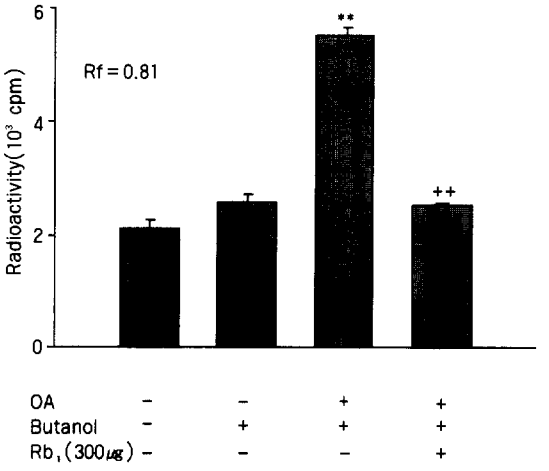

Fig. 1. Butanol-dependent PBut formation in the activation of purified guinea pig lung mast cells sensitized with antigen-antibody reactions. Purified mast cells($1\text{-}2 \times 10^7$) were prelabeled with [^{3}H]palmitic acid(0.8μM, 50μCi), and washed extensively. Cells were sensitized with anti-OA antibody(1ml/10^6 cells) in the presence of Rb_1(300μg/ml) or tyrphostin (10^{-4}M) for 45min at 37℃. Five minutes before OA(0.1μg/ml) challenge, 50 mM 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. **, $P<0.01$ by comparison without OA challenge. ++, $P<0.01$ by comparison with OA challenge in the presence of butanol(50 mM).

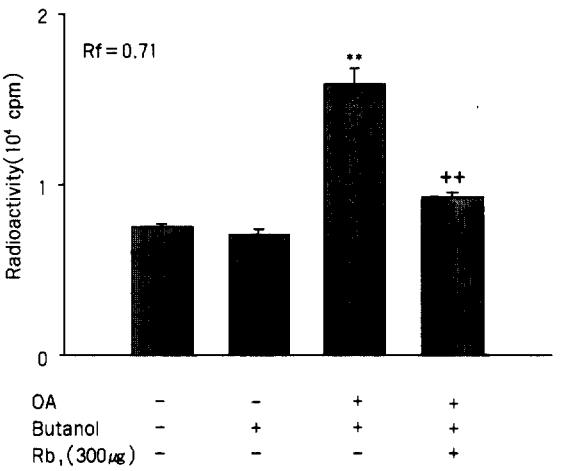

Fig. 2. Effects of Rb_1 on the DAG formation in the butanol during the activation of mast cells sensitized with antigen-antibody reactions. Purified mast cells(1×10^6) were prelabeled, sensitized the same as described in Fig 1. Five minutes before OA(0.1μg/m) challenge, 100mM butanol was added. Ten minutes 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. **, $P<0.01$ by comparison without OA challenge. ++, $P<0.01$ by comparison with OA challenge in the presence of butanol(100 mM).

±152 cpm to 15787±951 cpm(Fig. 2). These results showed that the amount of DAG produced with only antigen(0.1μg/ml OA) challenge increased from 2.0 to 2.5 times, compared to non-antigen challenge. Rb_1(300μg/ml) pretreatment almost decreased the amount of DAG increased by the activation of mast cells(from 15787± 951 cpm to 9311±171 cpm).

The effect of Rb_1 on biphasic nature of mass 1,2-diacylglycerol production during mast cell activation

As seen above, the production of DAG decreased when pretreated with Rb_1 during mast cell activation. So the effect of this phenomenon on the biphasic nature of DAG production was checked. The fact that mast cells stimulated by antigen-antibody reaction show a biphasic nature, the early increase(30-60sec) and continual increase(1-30 min), has already been reported and this was also reconfirmed in this study(Fig. 3). In short, when mast cells are stimulated, DAG production reached its climax at 30-60 sec, and there is a decrease of DAG production at 1-2 min, and this continues for 30 min(Fig. 3 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

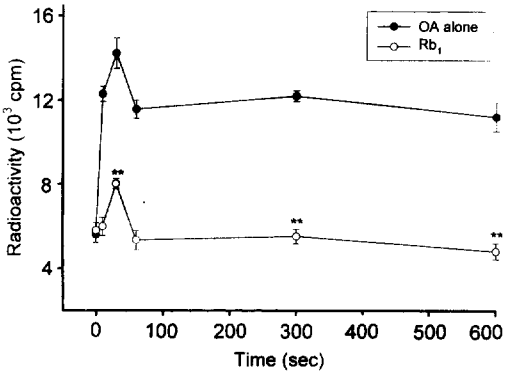

Fig. 3. Effects of Rb_1 on the biphasic increase in mass DAG formation during mast cell activation. Purified mast cells(1×10^6) were labeled with [^{3}H]myristic acid(1μM, 1μCi) for 1hr, sensitized with anti-OA antibody (1ml/10^6 cells) in the presence of Rb_1(300μg/mℓ) for 45 min at 37℃, and then challenged with OA(0.1μg/ml) for the time period indicated. The [^{3}H]DAG was extracted and separated from the other lipids as described in the Methods. **, $P<0.01$ by comparison with OA alone.

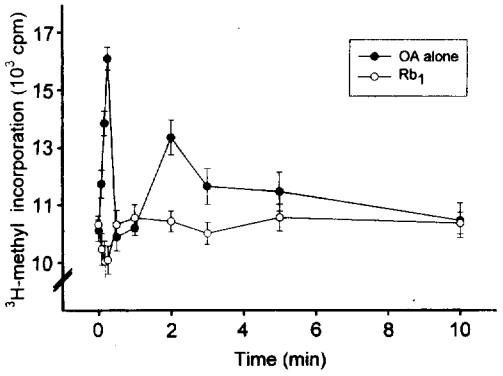

Fig. 4. Effects of Rb_1 on the kinetics of [^{3}H]methyl incorporation in the mast cell activation. Purified mast cells(1×10^6) were sensitized with anti-OA antibody(1ml/10^6 cells), labeled with [^{3}H-methyl]methionine(2μM, 165μCi, and then challenged with OA(0.1μg/ml) for 15 sec at 37 ℃ in the absence or presence of Rb_1(300μg/mℓ). The incorporation of methyl moiety of phospholipids was separated by method as described in the Material and Methods. The data in the peak of methylated phospholipids and amounts of mediator release is shown in Table 2. [^{3}H]methyl incorporation by unstimulated cells was 10556±356 cpm/4×10^5 cells. Spotaneous histamine release from mast cells was 3.2±0.13 %. Each value is the mean(±SE) of six experiments.

times(14214±958 cpm), compared with the non-antigen stimulated mast cells(5585±371 cpm). The initial increase of mass DAG amount is reduced by 44.0 % with pretreating Rb_1. But, the continual increase of the mass DAG amount was inhibited by 42.2 % with Rb_1 pretreatment. These results are related with the inhibition of histamine release.

The effect of Rb_1 on methylation of lipids during mast cell activation

The effect of Rb_1 on phospholipid methylation activity in guinea pig lung mast cells was examined after the addition of an optimal concentration of OA($0.1\mu g/4\times10^5$ cells).

The incorporation of the [^{3}H]methyl moiety into the phospholipids reached a maximum(16233±375 cpm) at 15sec after the addition of OA(Table 2), and phospholipid methylation decreased rapidly in 1min(Fig 4). But, the other peak of phospholipid methylation was observed around 2min after the addition of OA. However, the incorporation of the [^{3}H]methyl moiety into the membrane phospholipid with Rb_1($100\mu g/4\times10^5$ cells) was inhibited markedly from 16233±375 cpm to 11363±612 cpm(Fig 4, Table 2). The inhibition of methylation with Rb_1 ($300\mu g$) was 43.5% at 15sec after challenging of OA(from 16233±375 cpm to 9586±464 cpm).

We also examined each methylated products during the activation of mast cells by thin-layer chromatography. The results of maximal phospholipid methylation after challenging of OA(15sec) showed in Fig 5. As observed in the stimulated control(Fig 5a), the [^{3}H]methyl moiety was obviously incorporated into phospholipid of mast cell activated with antigen-antibody reactions(OA -anti-OA). The incorporated amount of [^{3}

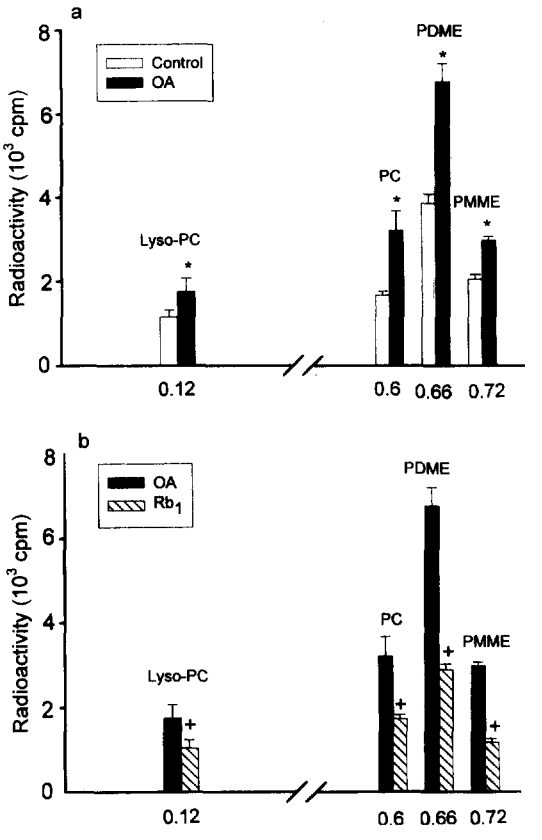

Fig. 5. Chromatographic pattern of each [^{3}H]methylated phospholipids on a TLC plate in the absence or presence of Rb_1. Mast cells were sensitized, labeled, and challenged by method as described in Fig 5. The incorporation of methyl moiety of each phospholipids were separated by TLC.(a), unstimulated vs stimulated mast cells;(b), pretreated with Rb_1($300\mu g/4\times10^5$ cells). Blank column, non-antigen challenge;black column, antigen challenge; hatched column, Rb_1 pretreatment. Values are means±SE for experiments. *, $P<0.05$ by comparison without OA challenge. +, $P<0.05$ by comparison with OA alone.

H]methyl moiety in each lipid product markedly increased in the mast cell activated with antigen, compared to the unstimulated control(Fig 5a)(Lyso-PC, from 1161±160 cpm to 1748±324 cpm; PC, from 1672±89 cpm to 3205±463 cpm; PDME, from 3854 ±230 cpm to 6766±439 cpm; PMME, from 2048±113 cpm to 2974±92 cpm). Rb_1 strongly inhibited each [^{3}H]methyl incorporation into phospholipid(Fig 5b)(Lyso-PC, from 1748±324 cpm to 1044±182 cpm; PC, from 3205±463 cpm to 1734±69 cpm; PDME, from 6766±439 cpm to 2889±132 cpm; PMME, from 2974±92 cpm to 1172± 92 cpm).

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 with high affinity receptors on the surface of mast cells. That is, enzymes such as so PLC, PLD, MT, PLA_2, adenylate cyclase, tyrosine kinase etc. are stimulated and in turn release mediators[8-18, 33-34].

There are several single component extracted from Korean Red Ginseng Radix. We have previously reported that a few single component 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[6]. We have also reported that single component of ginsenosides(Rg_1, Rg_2, Rc), belongs to the protopanaxadiol, decreased histamine and leukotrines releases during mast cell activation in guinea pig lung tissues, but Rg_3 belongs to the protopanaxatriol, increased only histamine release[7]. These results have 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[6-7]. However the mechanism affecting ginseng single components, Rb_1, caused the inhibition of histamine and leukotriene releases(Table 1) has not been reported. Therefore, this study tried to uncover the inhibitory mechanism of Rb_1 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[13] or indirectly by a PLD-initiated pathway[7, 15, 17]. PIP_2-mediated PLC 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 phosphohydrolase(PA Phase) enzyme causes DAG production. The produced DAG activates the protein kinase C, and causes granules and mast cells to fuse, and so shows degranulation[16, 42]. The granulated histamine and other mediators are released by the granulation 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[15-16, 43]. Therefore, this study focused on PLD activity in order to discover the inhibitory mechanism of Rb_1 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_1 pretreatment inhibited PBut production increased by antigen only challenge. This inhibition made the PBut production with Rb_1 pretreatment the same amount as that with non-antigen challenge (Fig. 1). Therefore, these results can be inferred that Rb_1 inhibits PLD activity caused by mast cell activation.

As the inhibition of PLD activity evoked by the pretreatment of Rb_1 was observed, the effects of Rb_1 on the direct DAG production via action of PLC during mast cell activation was examined. There are many reported methods to measure for DAG[18, 44-45]. First, when mast cells are stimulated by antigen-antibody reaction, the DAG produced by PLD activity is totally turned into PBut under the influence of a high concentration of butanol(100mM). Then, the DAG amount directly produced by PLC activity can be measured under the high concentration of butanol. In the high concentration of butanol Rb_1 pretreatment reduced the increased DAG produced when challenged by antigen alone(Fig. 2). These results showed that Rb_1 inhibited DAG production via PLC activity.

The effect of Rb_1 on mass DAG production through the actions of PLC and PLD was also observed. The mass DAG was determined by the modification of Lee et al[44] method. The pathway of mass DAG production was determined 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 stages by tyrosine kinase activity causing PLD activity[17] were checked in the mast cells of guinea pig lung tissues. The mass DAG produced by mast cell activation was found to have biphasic nature(Fig. 3), and the results are the same as the report that IgE dependant DAG production in RBL 2H3 cultured cells have a biphasic nature[17]. Therefore, it can be inferred that the nature of DAG production, which is related with histamine release caused by immune reaction, is the same regardless of the distributions of tissues or species. When Rb_1 is pretreated, DAG amount produced from early stage caused by PLC activity and continual stages caused by PLD activity are blocked(Fig. 3). From these results it can be inferred that Rb_1 inhibits DAG production via both PIP_2-PLC and PC-PLD pathways. As a results, histamine release is reduced.

It has been suggested that binding of anti-IgE to the membranous IgE receptors causes phospholipid methylation by activation of MT I and II[23, 28]. It has also been reported that IgE-dependent activation of human lung mast cells and rat peritoneal mast cells are not associated with increased phospholipid methylation[46-47]. Due to this conflicting report a few researchers questioned if phospholipid methylation is associated with mediator release from mast cells. However, Takei

et al[29] reconfirmed that antigen, anti-IgE and concanavalin A induce both the incorporation of the [^{3}H]methyl moiety into phospholipids and histamine release.

Therefore, in this study, the effect of Rb_1 on phospholipid methylation activity in guinea pig lung mast cells was examined after the challenge of OA, and the methylation and degradation products such as PMME, PDME, PC and Lyso-PC by thin-layer chromatography was identified. Based on the experimental protocol of Ishizaka et al[23], we reconfirmed a transient increase in [^{3}H] methyl incorporation at 15sec from OA-activated mast cells(Table 2). We also observed the other peak around 2min after the challenge of OA(Fig 4). This other peak may be caused by the incorporation of other granulocytes because the low purity of mast cells 70-80%) is used in this experiment. We further need to study the other peak in this regard. Rb_1 markedly inhibited the incorporation of [^{3}H]methyl moiety into phospholipid(both peaks, Fig 4). These methylation products were identified by thin-layer chromatography(Fig 5). The data obtained with TLC infer that Rb_1 may inhibit both MT I and II, and phospholipase A_2(PC hydrolysis to arachidonic acid and Lyso-PC). Therefore, these results show that Rb_1 inhibits the phospholipid methylation(especially PC product) induced by antigen, followed by inhibiting of leukotriene release.

From these results, it can be inferred that Rb_1 inhibits histamine release by inhibiting DAG production from phospholipids during mast cell activation, which is mediated via PIP_2-PLC and PC-PLD pathway systems. Furthermore, Rb_1 reduces the phosphatidylcholine production by inhibiting the methyltransferase, which decreases the conversion of phosphatidylcholine into aracidonic acid and inhibits the production of leukotrienes.

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