Inhibitory Mechanism of Aloe Single Component (Alprogen) on Mediator Release in Guinea Pig Lung Mast Cells Activated with Specific Antigen-Antibody Reactions

JAI YOUL RO, BYUNG CHUL LEE, JI YOUNG KIM, YEAN JUN CHUNG, MYUNG HEE CHUNG, SEUNG KEE LEE, TAE HYUNG JO, KYUNG HWAN KIM, and YOUNG IN PARK

Department of Pharmacology, Yonsei University College of Medicine (J.Y.R., J.Y.K., K.H.K.); Graduate School of Biotechnology, Korea University (B.C.L., Y.J.C., Y.I.P.); Department of Pharmacology, Seoul National University College of Medicine (M.H.C.); Department of Biochemistry, Seoul National University College of Pharmacy (S.K.L.); and Life Science Institute, Nam Yang Aloe Company (T.H.J.), Seoul, Korea

Accepted for publication August 16, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

We previously reported that the glycoprotein extracted from aloe strongly inhibited the mediator releases caused by the activation of guinea pig lung mast cells. Therefore, this study aimed to purify a single component that has an antiallergic effect from crude aloe extract and then to assess the effects of aloe single component (alprogen) on the mechanism of mediator releases caused by the mast cell activation. We purified aloe extracts by using various columns. We also purified mast cells from guinea pig lung tissues by using enzyme digestion, rough and discontinuous density Percoll gradient. Mast cells were sensitized with IgG1 (anti-ovalbumin) and challenged with ovalbumin. Histamine was assayed by using a fluorometric analyzer and leukotrienes by radioimmunoassay. [Ca$^{2+}$], level was analyzed by using a confocal laser scanning microscope. Protein kinase activity was determined by the protein phosphorylated with [γ-32P]ATP. The phospholipase D activity was assessed by the labeled phosphatidylalcohol. The amount of mass 1,2-diaclylglycerol (DAG) was measured by the [3H]DAG produced when prelabeled with [3H]myristic acid. Phospholipase A$_2$ activity was determined by measuring the lyso-phosphatidylincholines released from the labeled phospholipids. Alprogen significantly decreased histamine and leukotriene releases and blocked completely Ca$^{2+}$ influx during mast cell activation. The protein kinase C and phospholipase D activities were decreased by alprogen in dose-dependent manner. Alprogen inhibited mass DAG formation and the phospholipase A$_2$ activity during mast cell activation. The data suggest that alprogen purified from aloe inhibits multiple signals as well as blocking Ca$^{2+}$ influx caused by mast cells activated with specific antigen-antibody reactions and then the inhibition of histamine and leukotriene release follows.

Plant medicines have been commonly used in basic health care in many countries throughout the centuries. One of the fairly well documented preparations in traditional medicine is the whole or parenchymous leaf gel of aloe vera (Grindlay and Reynolds, 1986). Aloe vera is a complex plant containing many biologically active substances (Klein and Penneys, 1988). It has been reported that glycoprotein extracted from aloe vera has a strong anti-inflammatory response (Davis, 1988; Davis et al., 1991, 1992; Shelton, 1991) and antiallergic reactions (Ro et al., 1998b), and that the polysaccharides, especially mannose-6-phosphate, in aloe vera have strong wound healing activity and an anti-inflammatory response (Davis et al., 1994a). It has also been reported that sterols extracted from aloe vera have good anti-inflammatory activity (Davis et al., 1994b). Mast cells and basophils play a pivotal role in the pathogenesis of allergic reactions such as asthma. These reactions are the consequence of the release of granular mediators (histamine, 5-hydroxytryptamine, etc.), newly synthesized mediators (leukotrienes, prostaglandins, platelet-activating factor, etc.), and cytokines such as interleukins (Plaut et al., 1989) and tumor necrosis factor (Ohno et al., 1990). When these cells are activated, the initial event in degranulation is the cross-linking of receptor-bound IgE antibody by a specific antigen. The activation of tyrosine kinase (Jouvin et al., 1994; Blank et al., 1995), phospholipase C (PLC) (Berridge and Irvine, 1984), phospholipase D (PLD) (Gruchalla et al., 1989; Lin et al., 1991, 1992; Stadelmann et al., 1993), adenylate cyclase (Hirata et al., 1979), phospholipase

ABBREVIATIONS: PLD, phospholipase D; OA, ovalbumin; PBut, phosphatidylbutanol; DAG, 1,2-diaclylglycerol; PKC, protein kinase C; PLA$_2$, phospholipase A$_2$; PC, phosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonyl phosphatidyl-[14C]choline; S.A., specific activity.
Mechanism of Action of Alprogen in Mast Cell Activation

2000

A$_2$ (PLA$_2$) (Hirasawa et al., 1995), and Ca$^{2+}$ influx (Weintraub et al., 1994) follows. The activation of these enzymes, especially PLC or PLD activation, ultimately leads to the production of second messengers such as 1,2-diacylglycerol (DAG), which is known to be produced by protein kinase activation in a rat mast cell line (Lin et al., 1994), such as protein kinase C (PKC), which is known to be activated by DAG (Altrichter et al., 1995), and Ca$^{2+}$, which is known to precede the release of mediators (Weintraub et al., 1994).

As described above, it can be inferred that the effect of aloe extracts on inflammation may have therapeutic relevance to allergic hypersensitivity and asthmatic disorders. Therefore, we first attempted to purify crude aloe vera and then examined whether the isolated single constituent of aloe vera, alprogen, inhibits the mediator releases from guinea pig lung mast cells activated by specific antigen-antibody reactions. We also examined the mechanism of alprogen on the mediator release during the mast cell activation.

Materials and Methods

Animals.

Hartley albino female guinea pigs, weighing about 200 to 250 g, were used. Animals were maintained in accordance with the National Institutes of Health Principles of Laboratory Animal Care.

Drugs and Solutions.

The following substances were used: Ovalbumin (OA), collagenase (type I), elastase (type I, porcine pancreatic), arachidonic acid, Tris-HCl, silver nitrate, a polypeptide size marker, phosphatidylcholine (PC), lyso-PC, methyl-$\alpha$-D-mannopyranoside, Fluo-3 AM, and polyvinylpyrrolidine were all purchased from Sigma Chemical Co. (St. Louis, MO); gelatin was purchased from Difco Laboratories (Detroit, MI); Percoll was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden); LK5DF and LK6D silica gel plates were purchased from Whatman (Maidstone, Kent, UK); [9,10$\alpha$-$^{3}$H]palmitic acid [specific activity (S.A.), 53.0 Ci/mmol], [H]$^{3}$myristic acid S.A., 51 Ci/mmol], [y-$^{32}$P]ATP (S.A., 3000 Ci/mmol), leucotriene D$_4$ (LTD$_4$) assay kit, and 1-palmitoyl-2-arachidonoyl phosphatidyl-[1-C]choline (PAPC; S.A., 40–60 mCi/3000 Ci/mmol) were all purchased from NEN (Seoul, Korea); and phosphatidylinositol (PPI) was purchased from Avanti Polar Lipids, (Albaster, AL). Sephadex G-25, DEAE-Sepharcyl, concanavalin A Sepharose, Superdex 75, phenyl Sepharose CL-4B, and Sephracyl-100 HR was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and POROS 50 HQ was purchased from Boehringer Mannhein Co. (Mannheim, Germany). All chemicals used in these studies were of the highest grade available.

Purification of Aloe Vera.

Fresh aloe vera leaves (10 kg) were crushed with a commercial blender (LG Electric Co., Seoul, Korea) in 1/3 volumes of extraction buffers (50 mM sodium phosphate, pH 8.0, 1.44 mM 2-mercaptoethanol, 1% polyvinylpyrrolidone, and 1 mM EDTA) and the slurries were collected. The slurries were filtered through cheesecloth and centrifuged by centrifugation at 10,000 g for 30 min at 4°C. The supernatant of the crude extracts was precipitated with 25 to 80% ammonium sulfate saturation. The precipitate was dissolved in 20 mM Tris-HCl (pH 8.0) buffer and was then desalted by application to a Sephadex G-25 column (8.0 x 80 cm) equilibrated with the same buffer. The desalted extracts were applied to a DEAE-Sephascel column (3.4 x 30 cm) equilibrated with 20 mM Tris-HCl (pH 8.0) buffer. The column was washed with the equilibration buffer. Bound proteins were eluted with 2 M NaCl in the same buffer. Fractions containing eluted protein were pooled, dialyzed, and adjusted to 0.5 M NaCl in 20 mM Tris-HCl (pH 7.4).

The eluted proteins were chromatographed through a column of concanavalin A-Sepharose (3.4 x 26 cm) equilibrated and washed with 20 mM Tris-HCl (pH 7.4) buffer containing 0.5 M NaCl. The eluates (negative charged proteins in the pH 8.0 precipitate) were recovered in the pass-through fraction and concentrated by ultrafiltration and dialyzed by 20 mM Tris-HCl (pH 7.4) buffer. Bound glycoproteins were eluted with a 0.5 M methyl-$\alpha$-D-mannopyranoside in the same buffer, dialyzed against 20 mM Tris-HCl (pH 7.4) buffer, and applied to a POROS 50 HQ anion exchange column that had been equilibrated with the same buffer. After washing with the same buffer, proteins were eluted at the flow rate of 4 ml/min with a 400-mL linear gradient of 0.0 to 0.5 NaCl in the same buffer. Fractions containing antihistamine activity were collected and concentrated to 1.5 ml using and Amicon Centriplus 10 concentrator (Amicon, Beverly, MA). The preparation was filtered through Superdex 75 column with 20 mM Tris-HCl (pH 7.4) buffer containing 50 mM NaCl. Flow rates were 3.0 ml/min. Fractions containing antihistamine activity were applied to a phenyl Sepharose CL-4B hydrophobic interaction chromatography column (2 ml of total volume) that had been equilibrated with 20 mM Tris-HCl (pH 7.4) buffer containing 0.5M (NH$_4$)$_2$SO$_4$. After washing with the same buffer until only basal levels of proteins were detected, the column was eluted at the flow rate of 0.4 ml/min with 40 ml decreasing linear gradient of 0.5 to 0 M (NH$_4$)$_2$SO$_4$ in the buffer. Fractions containing antihistamine activity were collected, and it is coined as alprogen.

Polyacrylamide Gel Electrophoresis (PAGE).

Electrophoresis of total proteins under the denaturing condition was performed by the modified procedure described by Shaggy and Jagow (1987). SDS-PAGE gel included a 0.4% stacking gel, a 10.0% space gel, and a 16.5% separating gel, and the space and separated gels were stained with silver nitrate.

Active Sensitization Protocol (Anti-OA Production).

Ten outbred female guinea pigs were first immunized by footpad injections of mixture of 50 $\mu$g OA and complete Freund’s adjuvant. One week after that, animals received intradermal injections of 100 $\mu$g OA at one side back and 200 $\mu$g of OA at the other side back. Animals were sacrificed 1 week later and the sera were stored in aliquots at $-70^\circ$C until the time of use. The quantity of serum antibody titers by passive cutaneous anaphylaxis was determined as described in a previous article (Adersson, 1980). Serum IgG$_1$ antibody was separated by affinity column chromatography. Guinea pig blood serum was applied to anti-IgG$_2$ affinity column and 0.1M citric acid (pH 2.1) was used to wash the column. IgG$_1$ was passed through and the absorbed IgG$_2$ antibody was rinsed by 0.2 M sodium carbonate (pH 11.3). The separated IgG$_1$ was concentrated under pressure for the experiment. The titers of anti-OA were 1600 to 3200 (dilution time). 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; Ro et al., 1998a).

Brieﬂy, lungs obtained from 16 unsensitized guinea pigs were each perfused with 50 ml of the modiﬁed Tyrode’s buffer (TCGM buffer) consisting of 137 nM NaCl, 0.36 nM NaHPO$_4$, 2.6 nM KCl, 1 nM CaCl$_2$, 1.5 nM MgCl$_2$, 119 nM NaHCO$_3$, 5.5 nM glucose, and 1 g/l lactate, pH 7.4. After removing large airways and blood vessels, the lungs were minced with a McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Gosnall, England). Pooled tissue was treated three times with collagenase (125 U/g tissue) and elastase (5 U/g tissue). Time of each consecutive exposure of lung fragments to the enzymes were 15, 15, and 25 min, respectively. Freed cells were separated from residual tissue by filtration through mesh and Nytex mesh (100 $\mu$m). The cells were washed with Tyrode’s buffer without CaCl$_2$ and MgCl$_2$ containing gelatin (TG buffer) and layered over gradients consisting of 10 ml of Percoll (density, 1.045/ml), and centrifuged at 800g for 20 min. Pelleted cells (containing mast cells) were resuspended in TG buffer and applied for further purification using a discontinuous Percoll density gradient (consisting of densities 1.06, 1.07, 1.08, 1.09, and 1.10 g/ml). This gradient was centrifuged at 800g for 20 min (3.5 x 10$^6$ cells/gradient). The cell band obtained between the 1.09 and 1.10 g/ml densities contained the highest purity and number (1-2 x 10$^8$) of mast cells. This gradient band was removed, washed with TCM buffer, and designated with...
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 80 to 90%.

**Mediator Release from Mast Cells.** The purified mast cells were passively sensitized with anti-OA serum (1 ml/10^6 cells) for 45 min at 37°C in a shaking water bath. 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, and unless stated otherwise, each tube contained 4 x 10^5 mast cells suspended in 1 ml of TGCM buffer. The reaction was terminated by placing the tubes in an ice bath. Supernatants obtained after centrifugation were taken for determination of histamine and leukotrienes. In the supernatants for the measurement of leukotrienes, 0.1% gelatin (final concentration) was added because leukotrienes were decomposed in air. In experiments using alprogen (0.5, 1.0, 2.5, and 5.0 µg/4 x 10^5 cells), we conducted four experiments. In the first experiment alprogen was added to mast cells sensitized with anti-OA (1 x 10^6 cells/1 ml anti-OA antibody). In the second experiment alprogen was added to mast cells 5 min before OA challenge (0.1 µg/ml). In the third experiment alprogen was added to mast cells activated with OA and anti-OA reactions. In the fourth experiment alprogen was concomitantly added to mast cells with OA antigen (Undem et al., 1985; Ro et al., 1991).

**Histamine Assay.** Histamine was analyzed by the automated fluorometric method (with a dialyzer) as described by Siragianin (1974). The sensitivity of the assay is 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.** The leukotriene content of each cell supernatant was determined by radioimmunoassay as described previously (Aharony et al., 1983; Ro et al., 1991). The leukotriene antibody was diluted in buffered saline (5 mM 2-(N-morpholino)ethanesulfonic acid and HEPES adjusted to pH 7.4 with NaOH containing 0.1% gelatin). Each assay tube contained 100 µl of sample supernatant, antibody (50 µl of a 1:1000 dilution), and 50 µl of [3H]LTD4 (2500–3000 cpm) in buffered saline. Incubations were for 2 h at 4°C and the reaction was terminated by addition of 0.5 ml dextran-coated charcoal (200 mg charcoal and 20 mg dextran mixed with 100 ml buffered saline). Five minutes after incubation, the mixture was centrifuged at 800 g at 4°C and 0.4 ml of the supernatant was added to Aquasol (NEN Research Products) for counting. The radioactivity was measured with a liquid scintillation counter (Hirasawa et al., 1995). The standards (phosphatidic acid and PBut) with extracted samples were applied to the oxalated-treated thin-layer chromatography (TLC) plates (LK60 silica gel 60; Whatman) plates (presorbed-TLC, 5 g potassium oxalate dissolved in 250 ml H2O, made up to 500 ml with methanol), developed to the top of the TLC plates with ethyl acetate/acidic acid/2,4,2,4-trimethylpentane (9:2:5) and visualized with iodine staining. The RF value for PBut was 0.81. TLC plates were scraped and counted. Butanol was used because of the 5-fold lower potency of ethanol as an acceptor in the transphosphodiesterification reaction.

**Determination of Mass DAG during Mast Cell Activation.** Prelabeled cells (1–1.25 x 10^6) with [3H]myristic acid (0.1 nM, 1.0 µCi) were sensitized by anti-OA (1 ml/10^6 cells) at 37°C for 45 min and stimulated with 0.1 µg/ml OA at 37°C for 10 min (Lin et al., 1994). The reaction was stopped by adding 1 ml of methanol. The labeled lipids were extracted by the Bligh and Dyer method (1959). The standard and extracted samples was applied to the presorbed-TLC plates (LK5DF) and developed up to the half of the TLC plates with ethyl acetate/acidic acid/triethylpentane (9:2:5:3). After air drying the TLC plates were filled to the top in a second system with hexane/diethylether/methanol/acidic acid (90:20:3:2). The location of [3H]DAG was checked by exposure to iodine vapor. The TLC plates were scraped to measure radioactivity. Alprogen was added at 5 min before antigen challenge. The RF value for DAG was 0.55.

**Determination of PLA2 Activity.** Sensitized mast cells (1 x 10^6) were prelabeled with [3H]PACPC (200 µCi, 1 µM) and phospholipid at 37°C for 5 min. Mast cells prelabeled were washed twice and resuspended in TGCM buffer. The cells were sensitized and stimulated, and the reaction was stopped by the addition of 1 N HCl. The cell suspension was mixed with 0.2 ml of [3H]myristic acid (0.1 nM, 1.0 µCi) in cell pellet by using a modification (Gruchalla et al., 1990) of the Bligh and Dyer procedure (1959). In experiments using alprogen, cells were preincubated with alprogen for 5 min before challenge of 0.1 µg/ml OA.

**Statistical Analysis.** Experimental data are shown as mean ± S.E. An ANOVA was used for statistical analysis. An analysis of significance between each control group and experimental group was carried out with the Scheffe’s method. p values < .05, .01, .001 were considered significant.

**Results**

**Purification of Single Component, Alprogen, from Crude Aloe Extracts.** We attempted to purify aloe vera as described in Materials and Methods. We obtained alprogen that showed as a single band by SDS-PAGE and silver stain-
ing, and we assumed it was glycoprotein. The molecular mass of alprogen was estimated to be approximately 10.0 kDa on SDS-PAGE (Fig. 1). The isoelectric point (pI) value for alprogen has been estimated to be 6.0 by using ampholine polyacrylamide gels. However, further studies on the characteristics of alprogen are needed. The N-terminal 15-amino acid sequence analysis of alprogen was NEVPYLRTGEVLGPN.

The protein was found not to be homologous by homology search in GenBank, although it is similar to vesicular γ-aminobutyric acid transporter, sulfate transporter, aminopeptidase, etc.

**Effect of Alprogen on Mediator Releases during Mast Cell Activation.** To examine whether alprogen itself has the mediator releasing effect on hypersensitivity reactions, unsensitized mast cells were challenged by OA, 0.1 μg/ml after the pretreatment of the varying concentrations of alprogen (0.5, 1.0, 2.5, 5.0, or 10.0 μg/ml). The mast cells sensitized with anti-OA antibody were also challenged by varying concentrations of alprogen (0.5, 1.0, 2.5, 5.0, or 10.0 μg/ml). The results showed that alprogen itself did not affect the mediator releases (data not shown).

We conducted three experiments. In the first experiment alprogen was added to mast cells sensitized with anti-OA. In the second experiment alprogen was added to mast cells challenged with OA. In the third experiment alprogen was added to mast cells activated with OA and anti-OA reactions. In all three experiments, histamine release was similar: 19.5 ± 0.66% (with anti-OA), 18.8 ± 0.87% (with OA), and 19.3 ± 0.28% (OA-anti-OA), respectively. This means that the decrease of histamine release was 32.7, 35.2, and 33.4%, respectively, compared with the OA alone which was 29.0 ± 1.0%. Leukotriene release was also affected similarly to the histamine release in all three experiments. However, when the alprogen was concomitantly added with OA challenge (fourth experiment), the inhibitory effect of mediators reduced by approximately 15%. Therefore, alprogen was added to mast cells 5 min before OA challenge.

When the mast cells sensitized with anti-OA antibody were challenged by 0.1 μg/ml OA after the pretreatment of 1.0 μg/ml alprogen, histamine release was 18.6 ± 1.90% and that showed a 35.9% decrease when compared with the OA alone, which was 29.0 ± 1.10%. The amount of leukotriene released by 1.0 μg/ml alprogen pretreatment was 29.8 ± 5.6 pmol/10⁶ cells, which was a 30.5% decrease compared with the 40.5 ± 7.50 pmol/10⁶ cells of OA alone group (Table 1). The inhibitory effect of both mediator releases by alprogen pretreatment showed the dose-dependent manner. In this study, the 1.0- and 5.0-μg doses of alprogen were used in each experiment, because in both mediators, release of the activated mast cells was decreased by over 35 and 50%, respectively.

When mast cells were activated with a calcium ionophore (0.5 and 1.0 μM), histamine release was 47.7 ± 1.5% and 62.6 ± 1.2%, respectively. Leukotriene release by a calcium ionophore (0.5 and 1.0 μM) was 39.6 ± 5.69 pmol/4 × 10⁶ cells and 52.7 ± 8.56 pmol/1 × 10⁶ cells, respectively. However, alprogen (5 μg) did not inhibit histamine and leukotriene releases evoked by a calcium ionophore (0.5 and 1.0 μM).

**Effects of Alprogen on Influx of Ca²⁺ during Mast Cell Activation.** It has been reported that the increase of [Ca²⁺]i, and the activation of PKC are necessary for degranulation of preformed inflammatory mediators in mast cells (Takata et al., 1994). Therefore, the effects of alprogen on [Ca²⁺]i, were examined in a single mast cell and visualized by using a confocal laser scanning microscope through fluorescence intensity (optical density, A). [Ca²⁺]i, in a single mast cell reached a plateau 14 s (from 20,022 ± 75 to 25,291 ± 951) in the presence of external Ca²⁺ (Fig. 2a). In the buffer without Ca²⁺, however, [Ca²⁺]i, was slowly decreased by OA challenge for 10 min (from 20,956 ± 956 to 12,981 ± 19) (Fig. 2d). Furthermore, [Ca²⁺]i, was significantly decreased by alprogen in dose-dependent manner (from 20,033 ± 156 to 19,633 ± 630 for 1.0 μg and from 20,191 ± 90 to 17,517 ± 762 for 5.0 μg at 14 s). No fluorescent signal could not be detected after 2 or 3 min (Fig. 2).

Furthermore, [Ca²⁺]i, was increased by calcium ionophore in dose-dependent manner (from 18,579 ± 115 to 28,850 ± 362 for 0.5 μM and from 18,179 ± 137 to 28,141 ± 462 for 1.0 μM). However, alprogen (5 μg) did not decrease [Ca²⁺]i, which was increased from 18,179 ± 137 to 28,141 ± 462 by a calcium ionophore (1.0 μM; fluorescent data not shown).

**TABLE 1**

**Effect of alprogen on mediator releases from passively sensitized (anti-OA) lung mast cell activation caused by 0.1 μg/ml OA**

Guinea pig lung mast cells were isolated and purified by digestion and rough and continuous Percoll density gradient method. Mast cells (4 × 10⁶ cells) were passively sensitized by anti-OA antibody and challenged by 0.1 μg/ml OA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Histamine %</th>
<th>Leukotrienes pmol/4 × 10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA alone</td>
<td>29.0 ± 1.10</td>
<td>42.9 ± 5.40</td>
</tr>
<tr>
<td>Alprogen (μg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>23.5 ± 1.21*</td>
<td>40.5 ± 7.53</td>
</tr>
<tr>
<td>1.0</td>
<td>18.6 ± 1.90**</td>
<td>29.8 ± 6.52**</td>
</tr>
<tr>
<td>2.5</td>
<td>11.1 ± 6.12***</td>
<td>25.2 ± 7.85***</td>
</tr>
<tr>
<td>5.0</td>
<td>7.5 ± 7.50***</td>
<td>23.2 ± 6.23***</td>
</tr>
</tbody>
</table>

* p < .05; ** p < .01; *** p < .001 compared with OA alone.
Effect of Alprogen on PKC Activity during Mast Cell Activation. Because \([\text{Ca}^{2+}]_i\) is decreased by the treatment of alprogen, alprogen may also influence PKC activity. Phosphorylated protein was monitored to measure the effects of alprogen on the activity of PKC. Phosphorylated proteins activated by OA and anti-OA reactions increased by approximately 7.8 times (from 27,793 ± 10,200 to 217,755 ± 20,334 cpm). In addition, 1 μg of alprogen decreased the activity of PKC by 37.4% (from 217,755 ± 20,334 to 136,232 ± 9,432 cpm; Fig. 3).

Effect of Alprogen on PLD Activation during Mast Cells Activation. An increase of membranous PLD activity during mast cell activation evoked by specific antigen-antibody reactions ultimately leads to the release of mediators from mast cells. Therefore, the effects of alprogen on increasing PLD activity in mast cells activated by OA and anti-OA antibody reactions were studied. The production of PBut in the activated mast cell increased remarkably from 3,237 ± 669 to 11,555 ± 570 cpm, but with alprogen (0.5 μg) pretreatment, the production of PBut was decreased remarkably from 11,555 ± 570 to 6,592 ± 659 cpm. In the alprogen (0.5 μg) pretreatment, the PLD activity was decreased by 43% when compared with antigen alone (Fig. 4). The PLD activity was decreased by alprogen in dose-dependent manner.

Effect of Alprogen on Production of Mass DAG during Mast Cell Activation. PKC activity is well known to be activated by DAG. The mass DAG is formed by the activation of phospholipases in lung mast cells activated by OA and anti-OA reactions. Therefore, the effect of alprogen on the formation of mass DAG in the lung mast cells activated by OA and anti-OA reactions was investigated. Mass DAG production decreased by 40% after the treatment with alprogen.
(1.0 μg) (from 36,200 ± 900 to 21,900 ± 30 cpm; Fig. 5). In the treatment of alprogen (5.0 μg), the formation of mass DAG is completely blocked.

**Effect of Alprogen on Activation of PLA2 during Mast Cell Activation.** The increase of [Ca\(^{2+}\)] has a number of effects on mast cells. One of the major effects is the induction of the association of PLA2 with membranes to facilitate the synthesis of lipid mediators such as leukotrienes and prostaglandins. Because the release of arachidonic acid is responsible, in part, for type I hypersensitivity reactions, we investigated whether alprogen inhibits PLA2 activity. As shown in Fig. 6, the activity of PLA2 increased by about 41% in mast cells activated by 0.1 μg/ml OA challenge (from 8,237 ± 554 to 11,617 ± 769 cpm). However, the activity of PLA2 decreased by 26.7% (from 11,617 ± 769 to 8,520 ± 1,144) by the treatment with 5.0 μg/ml alprogen compared with that of OA challenge alone.

**Discussion**

It has been reported that aloe vera has a stimulatory system that enhances antibody production (Shelton, 1991), and an inhibitory system that influences both inflammatory and immune responses (Davis et al., 1991, 1992, 1994a). The interaction between these two systems is referred to as biological modulation. In this study we focused on the inhibitory system of aloe vera.

A single inhibitory component in aloe vera was purified by various processes. We obtained alprogen that showed a single band by using SDS-PAGE (Fig. 1), and we assumed it to be glycoprotein.

In this experiment, we observed that alprogen strongly inhibits histamine and leukotriene releases during the activation of mast cells by specific antigen-antibody reactions (Table 1). The inhibitory effects of mediator releases by alprogen has a kind of anti-inflammatory activity found in the extracts of aloe vera (Davis et al., 1991, 1992, 1994a, b).

To examine the binding sites of alprogen, we conducted three different experiments as described in **Effect of Alprogen on Mediator Releases during Mast Cell Activation in Results.** We found similar results in all three experiments except one (fourth experiment). From these results, it can be inferred that alprogen binds to the mast cell surface differently from the antibody binding site, and that it then blocks the movement of antibody-bound receptor to cross-link to antigen. It can also be inferred that alprogen binds to a variable region of IgG1 antibody and then blocks the binding of antigen to the variable region.

We first attempted to confirm the inhibitory mechanism of alprogen on the mediator release caused by mast cells activated with specific antigen-antibody reactions. When mast cell membrane receptors are activated by antigen-antibody reactions, Ca\(^{2+}\) long has been recognized as essential for secretion from mast cells. [Ca\(^{2+}\)] increased remarkably in the presence of external Ca\(^{2+}\) after stimulation with antigen-antibody reactions (Fig. 2a). This is the same result reported previously in a rat mast cell line (Hide and Beaven, 1991). The increase in [Ca\(^{2+}\)] due to influx from extracellular Ca\(^{2+}\) by OA and anti-OA reactions was blocked by alprogen (Fig. 2, c–d), but the increase in [Ca\(^{2+}\)] by a calcium ionophore was not blocked. Although Ca\(^{2+}\) alone is not sufficient to trigger exocytosis, an increase in [Ca\(^{2+}\)] is prominent signal in mast cells. Therefore, it can be inferred that alprogen inhibits, in part, the release of mediators by blocking Ca\(^{2+}\) influx caused by specific antigen-antibody reactions but does not block the mediators by nonimmunological reactions (with a calcium ionophore). Also, alprogen moves out Ca\(^{2+}\) more rapidly than in the absence of external Ca\(^{2+}\). This result needs more study.

There are a number of reports that PKC is an essential transducer of signals for secretion in human and RBL-2H3 mast cells (Warner and MacGlashan, 1990; Ozawa et al.,...
1993). We previously reported that protein is phosphorylated by PKC in the guinea pig lung mast cell activation (Ro et al., 1998b). Some PKC isoforms are activated by the increase in [Ca\(^{2+}\)], and by DAG, which is the result from cell activation. Therefore, the decrease in [Ca\(^{2+}\)], caused by alprogen may influence the PKC activity. Alprogen remarkably inhibited PKC activity (Fig. 3). As a result, it can be inferred that alprogen blocks external Ca\(^{2+}\) influx, followed by the inhibition of PKC activity.

When mast cell membrane receptors are activated by antigen-antibody reactions, Ca\(^{2+}\)-dependent enzyme systems in the cell membrane are activated. The activated enzymes (PLD, PLA\(_2\), etc.) are intimately related to the generation of DAG, PKC, etc.). This leads to exocytosis of preformed inflammatory mediators and synthesis of newly formed mediators, which then can induce asthma and allergic hypersensitivity. The most common second messenger that is related to histamine release is DAG. DAG can be formed either from PC indirectly by a PLD-initiated pathway or directly from other phospholipids by the activation of PLC with receptor-mediated cell activation (Gruchalla et al., 1990; Lin et al., 1992). Recently, it has been reported that the amount of DAG produced by PLD activity during the activation of rat peritoneal mast cells was greater than that by PLC activity (Gruchalla et al., 1990; Andrew et al., 1996). Therefore, we focused on PLD activity to examine the inhibitory mechanism of alprogen on the histamine release. It has previously been reported that PLD activity increased two to three times during guinea pig lung mast cell activation (Lin et al., 1992; Ro et al., 1998a). This increased PLD activity is decreased by alprogen pretreatment (Fig. 4). From these results it can be inferred that alprogen inhibits PLD activity during the activation of mast cells sensitized with specific antigen-antibody reactions. The inhibition of PLD activity was stronger than the inhibition of histamine release. Lin et al. (1992) suggested that the activation of PLD may be regulated by both the activation of PKC and a rise in [Ca\(^{2+}\)]. Therefore, it can be inferred that both the decrease of PKC and [Ca\(^{2+}\)], caused by alprogen inhibits more PLD activity. However, this result needs more study.

Because the inhibition of PLD activity by alprogen was observed, the effects of alprogen on the mass DAG production was examined. Mass DAG produced by the activation of phospholipases are completely blocked by alprogen (Fig. 5). This indicates that alprogen inhibits DAG production via phospholipase pathways. As a result, histamine release is reduced.

The increase of [Ca\(^{2+}\)], has a number of effects on mast cells. One of the major effects is the induction of the association of PLA\(_2\) with membranes to facilitate the synthesis of lipid mediators such as leukotrienes and prostaglandins. That is, synthesis of leukotrienes is mediated by PLA\(_2\) and Ca\(^{2+}\) mobilization, but they are not dependent on the activity of PKC (Hirasawa et al., 1995). Alprogen inhibited the activity of PLA\(_2\) mediating the synthesis of arachidonic acid. The release of arachidonic acid is responsible, in part, for type I hypersensitivity. It can be suggested that because alprogen reduces histamine release and synthesis and secretion of leukotrienes simultaneously, it has potential for as an anti-allergic agent because it blocks multiple signals as well as Ca\(^{2+}\) influx in mast cells. Our data suggest that alprogen purified from aloe inhibits multiple signals as well as blocking of Ca\(^{2+}\) influx caused by mast cells activated with specific antigen-antibody reactions, and the inhibition of histamine and leukotriene releases follows.

Acknowledgments
We thank all the members of Creation of Aloe Pharmaceuticals Institute of Nam Yang Aloe Co. for insightful advice.

References
Re JC, Buckner CR, Bredel JK, Fishleder R and Graziano FK (1991) Influence of
indomethacin and L-cysteine on histamine and peptidoleukotriene release from superfused trachea taken from guinea pig passively sensitized with IgG1 and IgE antibodies. J Allergy Clin Immunol 87:1150–1160.

Send reprint requests to: Dr. Jai Youl Ro, Department of Pharmacology, Yonsei University College of Medicine, CPO Box 8044, Seoul, Korea, 120-752.
E-mail: JYRO426@yumc.yonsei.ac.kr