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The Effects of Cromakalim on the Mediator Releases from Guinea Pig Lung Mast Cell Activated by Specific Antigen-Antibody Reactions

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The inhibitory effect of cromakalim on the mediator release from mast cells caused by antigen-antibody reactions was in controversy with the specific antigen used. However, it has recently been observed that cromakalim inhibits the release of mediators from superfused tracheal and parenchymal strips or lung mast cells after passive sensitization with the IgG_1 antibody. An attempt, therefore, was made to determine the inhibitory mechanisms of cromakalim on the release of mediators such as histamine and leukotriene released by the activation of enzymes during mast cell activation. Guinea pig lung mast cells were purified through enzyme digestion, rough percoll and continuous percoll density gradients. The purified mast cells were prelabeled with [^{3}H]palmitic acid. PLD activity was assessed more directly by the production of labeled phosphatidylethanol by PLD-mediated transphosphatidylation in the presence of ethanol. In the cells labelled with [^{3}H]myristic acid, [^{3}H] DAG production was measured. The methyltransferase activity was assessed by measuring the incorporation of [^{3}H]methyl moiety into phospholipids in sensitized mast cells labelled with L-[^{3}H] methylmethionine. cAMP level was measured by radioimmunoassay. Cromakalim resulted in a decrease in the amount of histamine and leukotrienes releases by 30% in the ovalumin-induced mast cell. Cromakalim had little effect on phospholipase D activity enhanced by the activated mast cell. Cromakalim inhibited the initial increase of diacylglycerol production during mast cell activations. Cromakalim inhibited the phospholipid methylation increased in the activated mast cell. These results show that cromakalim decreases histamine release by inhibiting the initial increase of 1,2-diacylglycerol during the mast cell activation, which is mediated via the phosphatidylinositide-phospholipase C system rather than the phosphatidylcholine-phospholipase D system. Furthermore, cromakalim reduces phosphatidylcholine production by inhibiting the methyltransferase, which decreases the conversion of phosphatidylcholine into arachidonic acid and inhibits the production of leukotrienes.

Key Words: Mast cell, histamine, leukotrienes, phospholipase D, 1,2-diacylglycerol, methyl-transferase

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Potassium(K^+) channels existing in various kinds of smooth muscle cells play an important role in the repolarization or hyperpolarization of excitable cells (Latorre and Miller, 1983; Cook, 1988). Much attention has been focussed on K^+ channels, with the development of new drug which open up K^+ channels and accelerate the movement of the K^+ ions (McCann and Welsh, 1986; Mondot *et al.* 1988; Small *et al.* 1988; Cook, 1990; Thomas *et al.* 1988). Recently, the strongest and the most se-

lective among compounds which act on the K^+ channels in smooth muscle cell membranes has been known to be cromakalim (BRL 34915). Cromakalim causes an efflux of K^+ ions as K^+ channels in cell membranes are opened, resulting in hyperpolarization of cell membranes and relaxation of the smooth muscles cells and heart muscle cells (Hamilton *et al.* 1986; Escande *et al.* 1988; Beech and Bolton, 1989; Hamilton and Weston, 1989).

On the other hand, it has been reported that contractions of tracheal and broncheal muscle in guinea pig and human by agonists such as histamine and acetylcholine are reduced by cromakalim, and that several K^+ channel blockers reduce the effect of cromakalim(Allen *et al.* 1986; Arch *et al.* 1988; Baird *et al.* 1988; Murray *et al.* 1989; Judith *et al.* 1990; Nielsen-Kudsk *et al.* 1990; Raeburn and Brown, 1991; Nagai *et al.* 1991). It has also been reported that cromakalim could prevent nocturnal asthma(Williams *et al.* 1988). Therefore, allergic hypersensitivity such as asthma may be among the potential therapeutic targets for cromakalim (Williams *et al.* 1988).

There have been a few reports on the effects of cromakalim on allergic reactions. They report that experimental asthma caused by the IgE antibody(anti-benzyl penicilloyl bovine γ-globulin, anti-BPO-BGG) and antigen (benzyl penicilloyl bovine serum albumin, BPO-BSA) system in guinea pigs is inhibited by cromakalim, that antigen-induced contraction of isolated sensitized guinea pig tracheal muscle is relaxed by cromakalim, but that antigen-induced histamine release from sensitized guinea pig lung monodispersed cells is not effected by cromakalim (Nagai *et al.* 1991). However, Ro and Kim (1993) reported that cromakalim reduced histamine and leukotriene release from purified guinea pig lung mast cells activated by specific antigen-antibody reactions, and that this effect can be partially inhibited by glibenclamide which blocks ATP-dependent K^+ channels or by non-specific K^+ channel blockers such as tetraethylammonium. As allergic mediator releases were not completely modified by K^+ channel blockers, it is possible that mechanisms other than K^+ channel opening may participate in the action of cromakalim.

We examined the inhibitory mechanism of cromakalim on the mediator releases caused by specific antigen-antibody reactions in the guinea pig lung mast cells.

MATERIALS AND METHODS

Materials

Ovalbumin (fraction V), complete Freunds adjuvant, anti-IgG2 affinity column (type I), collagenase, 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; Kodak AR film from Estman Kodak; phosphatidylethanol, phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine from Avanti Polar Lipids; LK 5DF silica gel, LK6D silica gel from Whatmann Inc.; [^{3}H] palmitic acid (s.a., 50 Ci/mmol), [^{3}H]myristic acid (s.a., 51 Ci/mmol), L-[^{3}H]methymethionine (s.a., 83 Ci/mmol), En^3 Hance R, cAMP kit, leukotriene D_4 kit from Amersham. Cromakalim was a gift from Smith Kline Beecham Pharmaceuticals. 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. 1 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 1 week later and the sera were stored in aliquots at -70℃ until the time of use (Andersson, 1980). The quantitation of serum antibody titers by passive cutaneous anaphylaxis (PCA) were performed 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 applicated to anti-IgG_2 affinity col-

umn 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 under pressure concentrated 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 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 McIlwain 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, 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 ($1 \sim 2 \times 10^8$) 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 60~70%.

Mediator release from mast cell

The partially purified mast cells were passively sensitized with anti-OA serum (1 ml/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 1.0 μg/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. In experiments utilizing cromakalim, cells were first incubated for 45 min at 37℃ concomitantly with anti-OA and cromakalim, and also incubated for 10 min concomitantly with cromakalim and OA (1.0 μg /ml).

Histamine assay

Histamine was analyzed by the automated fluorometric method (with dialyzer) 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, 2500 to 3000 cpm) in buffered saline. Incubations were for 2 h at 4℃ and the reaction was terminated by the addition of 0.5 ml dextran coated charcoal (200 mg charcoal and 20mg dextran mixed

with 100 ml buffered saline). After 5min incubation the mixture was centrifuged at 3000 rpm 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 ($1\sim2\times10^7$) 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℃ for 1 hr. Cells were washed twice and resuspended in TGCM before use in cell activation.

Prelabeled cells($0.75\sim1.25\times10^6$) were sensitized by IgG_1 antibody (anti-Ox antibody, 1 ml antibody/10^6 cells) at 37℃ for 45 min, and washed and resuspended in TGCM. Sensitized cells were stimulated at 37℃ for 10 min by OA(1.0 μg/ml) and then was added phosphatidylserine (PS, 15 μg/ml) or PS alone for a final volume of 200 μg in 5ml polypropylene tubes. Ethanol (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 and Kim, 1994) of the Bligh and Dyer procedure (1959).

A double one-dimensional TLC (precoated silica gel plates, Macherey-Nagel, Postfach, Germany) was used to separate the phospholipids of interest from the extracted lipids and from the neutral lipids. A series of samples and standard (phosphatidylethanol) were spotted 12 cm from the bottom of the plate and developed in hexane/ether/acetic acid (30/70/1) to 20 cm in order to resolve labeled neutral lipids from phospholipids that remained at the origin. Plates were then cut at 0.8 cm above the origin, rotated 180° and developed to the top with chloroform/methanol/ammonium hydroxide (65/35/5). After they had dried, one of the plates was sprayed with En^{3}Hance, and autoradiography was performed by using Kodak XAR film for 1 week. The other plates were exposed with iodine vapour. Radioactive bands were removed from the silica plates by gentle scraping, and were placed and counted in scintillation vials containing 5 ml of scintillation cocktail. The identities of labeled band were determined based on Rf values obtained for phospholipids visualized by iodine staining.

Determination of 1,2-diacylglycerol (DAG)

Mast cells (1×10^6) were sensitized with anti-OA antibody for 45 mim, and were labeled in [^{3}H]myristic acid (1 μM, 1 μCi/ml) for 1hr at 37℃. Cells were rinsed and resuspended with TGCM buffer and stimulated for 15 min with 1.0 μg/ml OA. 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 separated by TLC (LK6D silica gel, Wattman) (Lin *et al.* 1991). The location of [^{3}H]DAG from standard DAG was checked by exposure to iodine vapour. The TLC plate was scraped to measure radioactivity. Cromakalim was added during all procedures to cromakalim groups.

Determination of phospholipid methylation

Phospholipid methylation was determined as described by Ishizaka *et al*(1980). The purified mast cells sensitized with anti-OA($\sim4\times10^5$ cells) were incubated (25 min, 37℃) with L-[^{3}H-methyl]methionine (2 μM, 165 μCi/ml), and washed twice. The resuspended cells were challenged with 1.0 μg/10 μl OA for the stated times. The reaction was stopped by the addition of 900 μl of ice-cooled 10% TCA containing 10 mM L-methionine, and tubes were centrifuged at 2,500 rpm for 10 min 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 1.5 ml of 0.1M KCl in 50% methanol. A 1 ml fraction of the chloroform phase was transferred to a counting vial and evaporated to dryness in an oven at 70℃. The residue was counted.

Identification of methylated phospholipid was carried out by TLC as described by

Hirata *et al*(1978). The chloroform layer obtained from the chloroform/methanol extracts were evaporated under the N_2 gas, and the residue was dissolved in 50 μl of chloroform/methanol (2:1 v/v). Then 30 μl 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 LPC) were scraped into scintillation vials, and dispersed by sonication with 400 μl of methanol and 5 ml of scitillation solution, and radioactivity was counted.

Determination of cAMP

Mast cells($\sim 4\times10^5$) were incubated with anti-OA (10^6 cells/1 ml) and cromakalim (45min, 37℃), and then washed and suspended with buffer. Cells were challenged with 1.0 μg/10 μl OA for the stated times. The reactions were quenched with 900 μl ice-cold ethanol with vortex mixing. Samples were centrifuged (2500 rpm, 20 min, 4℃) to precipitate denatured protein. The supernatants were added with 4 mM EDTA, a known inhibitor of the phosphodiesterase enzyme. The supernatants were evaporated to dryness in a heating block (80℃, 10 min). cAMP in the residue was quantified by RIA (Stein *et al.* 1972; Ishizaka, 1980).

Statistic analysis

Experimental data was 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, it was considered significant. Least Squares method was used on regression.

RESULT

The effects of cromakalim on mediator releases from mast cell activation

We have previously reported that cromakalim inhibited histamine and leukotiene releases during mast cell activation (Ro and Kim, 1993). Because the batch of cromakalim was different, we examined the effect of cromakalim itself on non-sensitized mast cells challenged with 1.0 μg/ml of OA. It was confirmed that the concentration of cromakalim (10^{-6}, 2×10^{-6}, 10^{-6} and 2×10^{-6}M) had no effect on mast cells (data not shown).

We also examined the effect of cromakalim on the release of histamine and leukotriene from guinea pig lung mast cells activated with specific antigen-antibody reactions. When the mast cells sensitized with IgG_1 antibody (anti-OA) were challenged by 1.0 μg/ml OA, histamine release from mast cells after pretreatment of cromakalim, 2×10^{-6}M, was 29.5±2.1% which was a 35% decrease when compared to the control group which was 46.3±3.2 %. The amount of leukotriene released was 25.2±2.9 pmole/4×10^5 cells in the cromakalim pretreated group, which was a 33% decrease when compared to the 37.4±1.3 pmole/4×10^5 cells of the control group (Table 1). However, the inhibition of mediator releases evoked by cromakalim pretreatment did not increase with the increase in the concentration of cromakalim. Therefore, most of these experiments were performed with 2×10^{-6}M of cromakalim. These results reconfirmed the

Table 1. Effect of cromakalim on the antigen-induced release of histamine and leukotrienes in guinea pig lung mast cells sensitized with antibody[a]

Cromakalim (M)	Histamine (%)	Leukotrienes (pmole/4×10^5 cells)
Ag alone	46.3±3.2	37.4±1.3
1×10^{-6}	36.5±3.5	29.9±2.7
2×10^{-6}	29.5±2.1*	25.2±2.9*
1×10^{-5}	34.7±1.9*	26.1±1.6*
2×10^{-5}	35.5±4.2	25.8±2.4*

[a]: Guinea pig lung mast cells were isolated and purified by digestion and rough and continuous percoll density gradient method. Mast cells(4×10^5) were passively sensitized by anti-OA antibody and challenged by OA, 1.0 μg/ml.

*: $P<0.05$: Ag(antigen) alone vs. cromakalim pretreatment

results of earlier documented reports.

The effect of cromakalim on the phospholipase D activity during mast cell activation

When mast cells of guinea pig lung tissues were activated by specific antigen-antibody reactions, it has already been reported that phospholipase D (PLD) activity was increased up to 3~5 times. Therefore, the effect of cromakalim on increasing PLD activity caused by mast cell activation was studied. PLD activity was measured as the transfer of the phosphatidyl moiety of the phospholipid substrate to ethanol thereby producing phosphatidylethanol (PEt). However, the production of PEt which is the index of mast cell PLD activity did not show a significant decrease statistically after cromakalim (2×10^{-6}M) pretreatment (Fig. 1). As shown in Fig. 2, the mast cells were sensitized only with 1.0 μg/ml of OA, the production of PEt was increased remarkably (from 205±56 to 533±85) and the production of phosphatidic acid (PA) was decreased (from 401±90 to 215±38).

Effect of cromakalim on the production of 1,2-diacylglycerol

As cromakalim did not influence PLD activity caused by activating the mast cell with specific antigen-antibody reactions, we examined the effect of cromakalim on 1,2-diacylglycerol (DAG) production which is a secondary messenger related with histamine release. When mast cells (1×10^6 cells) sensitized with anti-OA were labeled with [^{3}H]myristic acid, and challenged with OA, 1.0 μg/ml, DAG produc-

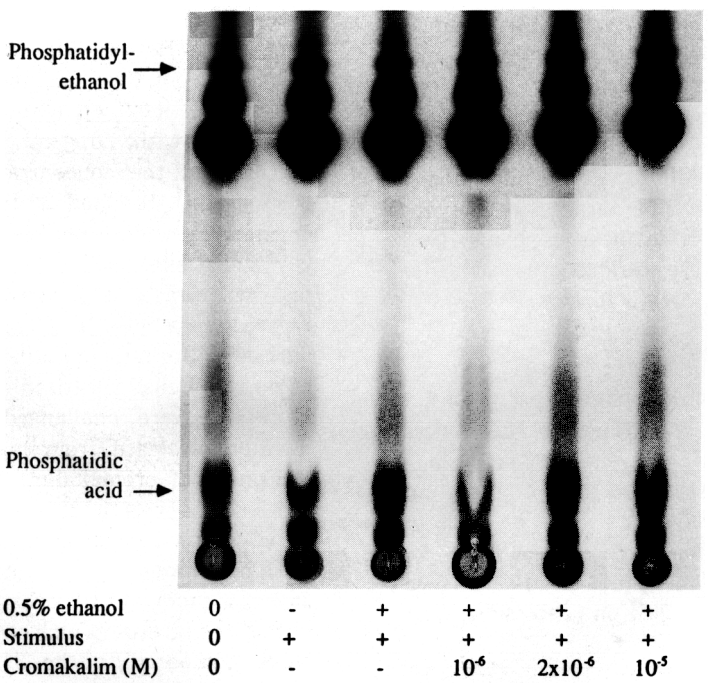

Fig. 1. Autoradiogram of ethanol-dependent phosphatidylethanol formation in the activated mast cells after cromakalim pretareatment. Purified mast cells ($1\text{-}2\times10^7$) were prelabeled with [^{3}H]palmitic acid(50 μCi), and washed extensively. The prelabelled cells were sensitized with anti-OA antibody (1 ml/10^6 cells) in the absence or presence of cromakalim for 45 min. Five minutes before OA challenge, 0.5% ethanol was added. Ten minutes after challenge, lipids were extracted, phospholipids resolved by double one-dimensional TLC, autoradiography performed.

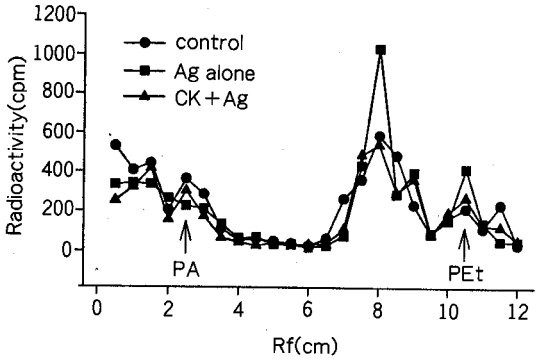

Fig. 2. *Ethanol-dependent phosphatidylethanol formation in activated mast cells. Purified mast cells (1-2×10⁷) were prelabeled with [³H]palmitic acid(50 μCi), and washed extensively. Cells were sensitized with anti-OA antibody (1 ml/10⁶ cells) in the absence(■) or presence(▲) of cromakalim (2×10⁻⁶M) for 45min. Five minutes before OA challenge, 0.5% ethanol was added. Ten minutes after challenge, lipids were extracted and chromatographically separated by TLC. The radioactivity incorporated into PEt was detemined by liquid scintillation spectrophotometry. The control (●) indicates no antigen challenge. The abbreviations used are: CK, cromakalim; PA, phosphatidic acid; PEt phosphatidylethanol.*

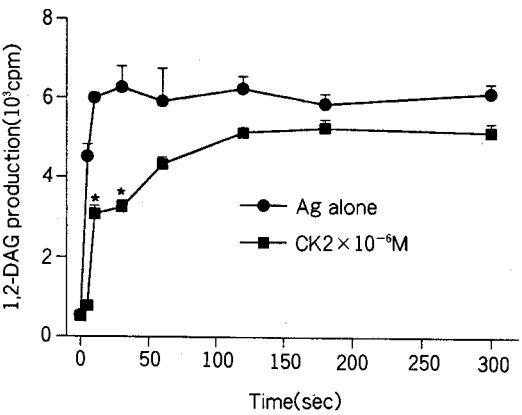

Fig. 3. *Effect of cromakalim on the 1,2-diacylglycerol formation in the mast cell activated by antigen-antibody reactions. Purified mast cells (1×10⁶) were sensitized and labeled with [³H]myristic acid(1 μCi), and then preincubated for 5min in the absence(●) or presence(■) of cromakalim (2×10⁻⁶M) before challenge with OA (1.0 μg/ml) for the time period indicated. The [³H]DAG was then extracted and separated from other lipids as described in the Methods. Vertical lines represent the SEM of mean from 6 experiments.*

tion started to increase after 5sec (from 534± 46 cpm to 4505±311 cpm) and continued for 30min (5940±591 cpm) (Fig. 3). Cromakalim (2 ×10⁻⁶M) pretreatment could only inhibit initial increases of DAG productions (Fig. 3)

When tyrphostin (10⁻⁴M), which is known to inhibit the secondary (continuous) increase of DAG, was used to pretreat mast cell activated by specific antigen-antibody reactions, it was not able to affect the initial increase in DAG production but it was able to inhibit the secondary increase of DAG production (Fig. 4).

Effect of cromakalim on adenylate cyclase activity

When mast cells are activated by specific antigen-antibody reactions, cAMP in the cytoplasm transiently increases due to the increase of adenylate cyclase activity. Therefore, cAMP in the cytoplasm was measured after mast cells were activated by specific antigen-antibody reactions. The amount of cAMP in the cytoplasm was 4.28±0.06 pmole/4 ×10⁵ cells in 5sec which showed a transient increase, compared to 2.38±0.06 pmole/4×10⁵ cells before challenge, and returned to control level after 30sec (2.93±0.18 pmole/4×10⁵ cells) (Table 2). However, each concentration of cromakalim did not decrease this transient increase of cAMP in the cytoplasm (Table 2).

Effect of cromakalim on phospholipid methylation of mast cells

After labeling mast cells sensitized by specific antibody (anti-OA) with [³H]methylmethionine, the effect of cromakalim on the increase of antigen-stimulated phospholipid methylation was studied. The incorporation of the [³H]methyl moiety into mast cell membrane phospholipids reached its peak at 17088 ±119 cpm at 15sec after mast cell activation, which was a 31.6% increase when compared

Table 2. Effects of cromakalim on the intracellular cAMP in the mast cells activated by antigen-antibody reactions[a]

Cromakalim(M)	Incubation Time(sec)			
	0	5	10	30
Ag alone	2.38±0.06	4.28±0.23***	3.13±0.15	2.92±0.18
1×10^{-6}	2.43±0.14	4.00±0.11**	3.48±0.23*	2.58±0.09
2×10^{-6}	2.50±0.19	4.00±0.18	2.75±0.12	2.65±0.16
1×10^{-5}	2.55±0.10	3.88±0.10	2.63±0.05	2.58±0.05

[a]: Purified mast cells(4×10^5) were sensitized with anti-OA antibody in the absence or presence of cromakalim for 45 min, and challenged with OA for the time periods indicated.

*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$: zero time vs. incubation time

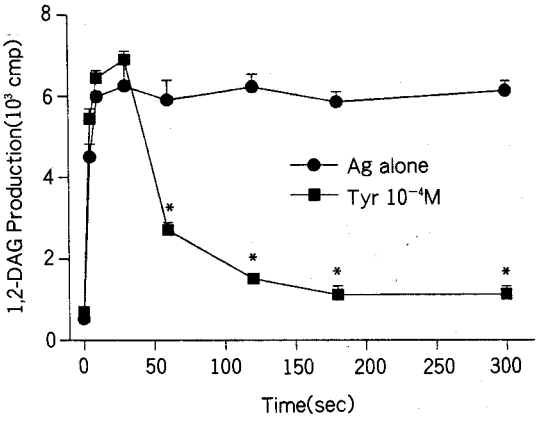

Fig. 4. *Effect of tyrphostin on the 1,2-diacylglycerol formation in the mast cell activated by antigen-antibody reactions. Purified mast cells (1×10^6) were sensitized and labeled with [^{3}H]myristic acid(1 μCi), and then preincubated for 5min in the absense(●) or presence(■) of tyrphostin (10^{-4}M) before challenge with OA (1.0 μg/ml) for the time period indicated. The [^{3}H]DAG was then extracted and separated from the other lipids as described in the Methods. Vertical lines represent the SEM of mean from 6 experiments.*

with 12981±293 cpm before stimulation. With the cromakalim (2×10^{-6}M) pretreatment, the incorporation of the methyl moiety into membrane phospholipids was 11920±352 cpm, which is about a 30.2% decrease (Table 3, Fig. 5). However, this inhibitory effect on cell membrane phospholipid methylation of cromakalim

Table 3. Effects of cromakalim on the antigen-induced ^{3}H-methyl incorporation and mediator release in guinea pig lung mast cells sensitized with antibody[a]

Cromakalim (M)	Phsopholipd Methylation (cpm)	Histamine (%)	Leukotrienes (pmole/4×10^5 cells)
Ag alone	17083±119	46.3±3.2	37.4±1.3
1×10^{-6}	13314±548***	36.5±3.5	29.9±2.7
2×10^{-6}	11920±353***	29.5±2.1*	25.2±2.9*
1×10^{-5}	12129±200***	34.7±1.9*	26.1±1.6*
2×10^{-5}	11989±301***	35.5±4.2	25.8±2.4*

[a]: Purified most cell(1×10^6) were passively sensitized, labelled, and challenged in the absence or presence of cromakalim as described in Fig. 5 and Table 1.

*: $P<0.05$, ***: $P<0.001$: Ag(antigen) alone vs. cromakalim pretreatment

did not show a dose-dependent response.

The main products of phospholipid methylation after activation of mast cells with antigen-antibody reactions were also separated by TLC. The main products of phospholipids methylated by mast cell activation showed significant increase, such as increase of lyso-PC from 1161±160 cpm to 2498±463, of PC from 3413±181 cpm to 6540±947 cpm, of PDME from 5504±328 cpm to 9665±627 cpm, and of PMME from 3926±161 cpm to 4249±132 cpm, respectively (Fig. 6). After cromakalim (2×10^{-6}M) pretreatment, PC produced by antigen stimulation decreased from 6540±947 cpm to

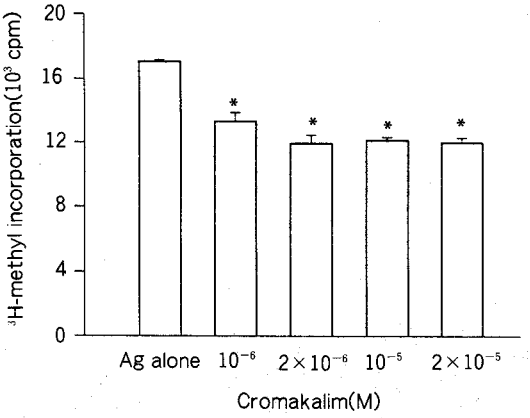

Fig. 5. *Effects of cromakalim on the phospholipid methylation in activated mast cells. Purified mast cells (1×10^6) were sensitized with anti-OA in the absence or presence of cromakalim for 45min and labeled with [^{3}H] methylmethionine(165 μCi) for 25min. Fifteen seconds after the OA challenge lipids were extracted, and the radioactivities incorporated into lipids was determined. The data is summarized in Table 3.*

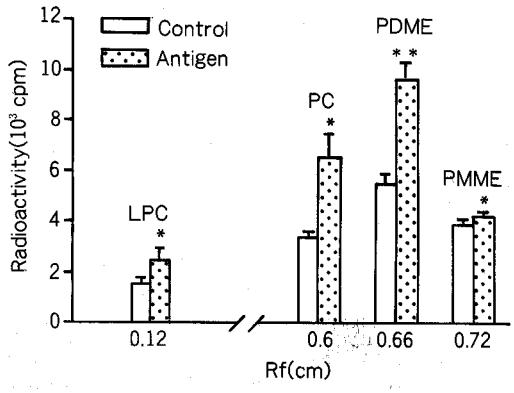

Fig. 6. *TLC chromatogram of the ^{3}H-methylated phospholipids. Purified mast cells (2×10^6) were sensitized with anti-OA in the absence or presence of each cromakalim for 45min and labeled with [^{3}H]methylmethionine (165 μCi) for 25min. Fifteen seconds after the OA challenge lipids were extracted, the ^{3}H-methylated phospholipids were chromatographically separated by TLC and each phospholipid was counted. The abbreviations used are LPC, lyso-phosphatidylcholine; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PMME, phosphatidylmonomethylethanolamine; CK, cromakalim. The data is shown in the text.*

3647±228 cpm, PDME from 9665±627 cpm to 5768±156 cpm, and PMME from 4249±132 cpm to 2713±166 cpm (Fig. 7).

It was also examined whether there was a definite correlation between the effect of inhibiting histamine and leukotrienes releases and decreasing phospholipid methylation by cromakalim when mast cells are activated by specific antigen-antibody reactions. The results showed that there was a very significant correlation between the effect of inhibiting leukotriene release and decreasing phospholipid methylations (Fig. 8, $\gamma^2=0.92671$, $P<0.05$). There also was a significant correlation between the effect of reducing histamine release and decreasing phospholipid methylation (Fig. 9, $\gamma^2=0.794936$, $P<0.05$).

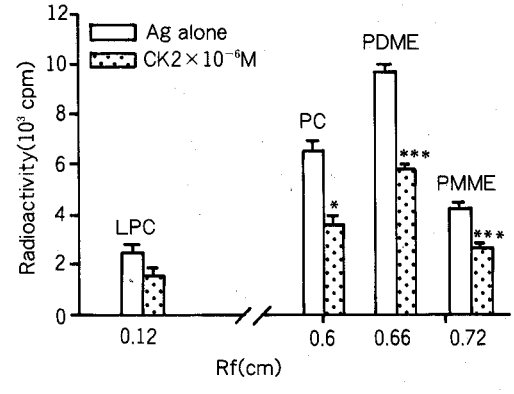

Fig. 7. *Effect of cromakalim on the TLC chromatogram of the ^{3}H-methylated phospholipids. The experimental protocols ad abbreviations are the same as described in Fig.6. The data is shown in the text.*

DISCUSSION

Recently, it has been reported that cromakalim, which is a K^+ channel opener, has an effect on the relaxation of several smooth mus-

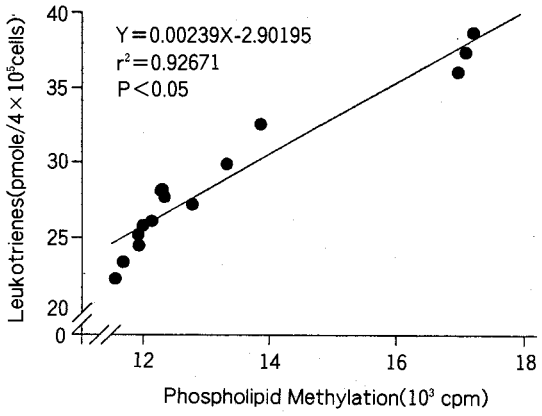

Fig. 8. *Correlation between the leukotriene release and phospholipid methylation during mast cell activation in the presence of cromakalim. Each points represents the value of one sample. The line was calculated by the least squares method.*

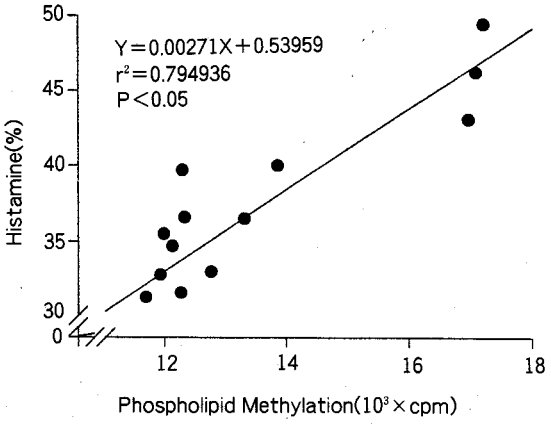

Fig. 9. *Correlation between the histamine release and phospholipid methylation during mast cell activation in the presence of cromakalim. Each points represents the value of one sample. The line was calculated by the least squares method.*

cles (especially tracheal and broncheal smooth muscles) and so much attention has been placed on it to clinically cure asthma and hypersensitivity reaction. It has also been reported that cromakalim had no effect on the antigen (BPO-BSA) -induced release of histamine from monodispersed mast cells of guinea pig lung tissues (Nagai *et al.* 1991). However, we reported that cromakalim could inhibit mediator releases in the purified guinea pig lung mast cell activations caused by antigen-antibody (OA) reactions (Ro and Kim, 1993). In this experiment, we observed again that cromakalim could inhibit these mediators by 30% (Table 1). Therefore, this study was attempted to understand the inhibitory mechanism of cromakalim on the histamine and leukotriene releases caused by mast cells activated with specific antigen-antibody reactions.

When mast cell membrane receptors are activated by antigen-antibody reactions, the enzyme systems within the cell membrane are activated: enzymes such as phospholipase C (PLC), PLD, PLA_2, adenylate cyclase, methyltransferase, etc. are activated (Ishizaka *et al.* 1980; Cunha-Melo *et al.* 1987; Kennerly, 1987; Lin *et al.* 1992). 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 which can induce asthmatic symptoms and allergic reactions.

With receptor-mediated cell activation, DAG can be formed from PC or other phospholipids either directly by the action of PLC or indirectly by a PLD-initiated pathway (Yang *et al.* 1967; Cunha-Melo *et al.* 1987; Gruchalla *et al.* 1990; Lin *et al.* 1992; Ro and Kim, 1995). In the indirect pathway PC is hydrolyzed to PA by PLD and the subsequent action of PA by PA-PHase yields DAG. DAG then activates protein kinase C, and causes mast cells and granules to fuse, and causes degranulation to occur (Lin *et al.* 1991). 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). Therefore, this study put its emphasis on PLD activity in order to understand the inhibitory mechanism of cromakalim on the release of mediators from guinea pig lung mast cells.

In the presence of ethanol, which is an index for measuring PLD activity, PEt is pro-

duced, but when cells were treated with a variety of concentrations of cromakalim and autoradiographed, there was little difference in PEt production (Fig. 1). After separation by TLC, also checked with iodine vapour, the result of counting showed an increase in PLD activity when antigen alone was used to stimulate. However, the production of PEt which increased with antigen challenge was significantly not reduced by cromakalim pretreatment (Fig. 1, 2). Therefore, it is suggested that cromakalim has little effect on PLD activity.

The most common secondary messenger related with histamine release is DAG. Therefore, it was studied if the DAG production increased by mast cell activation is decreased in the pretretment of cromakalim. It has also been proposed that the pathway of DAG production has a biphasic nature in other cultured cells, in other words the initial rise in the DAG production is caused by a phospholipase (ex., PLC) activation, while continuous increase is caused by tyrosine kinase activity which causes PLD activity (Lin *et al.* 1991; Eiseman and Bolen, 1992). Therefore, DAG production was studied in guinea pig lung mast cell. Cromakalim pretreatment was able to inhibit the initial increase in DAG production at an early stage (Fig. 3). Tyrphostin, which is an antagonist of tyrosine kinase, inhibited the continuous increase in DAG production (Fig. 4). These results can infer that when the guinea pig lung mast cells are activated by a specific antigen-antibody reaction, the produced DAG has a biphasic nature. It is also supposed that the inhibition of histamine release caused by cromakalim is due to the inhibition of the initial increase of DAG production during the mast cell activation, which is mediated via the phosphatidylinositol-phospholipase C (PI-PLC) pathway (Fig. 3, 4). These results are the same as the reports that the IgE-dependent DAG production is of a biphasic nature in the rat RBL 2H3 cells (Lin *et al.* 1991). Therefore it can be inferred that the biphasic property of DAG prduction is not different for different species, mast cell distributed organs, or tissues.

If mast cells are also activated with antigen-antibody reactions, the amount of cAMP in the cytoplasm is transiently increased because of the activation of adenylate cyclase (Ishizaka *et al.* 1983) and the degranulation of cells occuring due to the activity of protein kinase A (Ishizaka *et al.* 1988). This study showed that cromakalim has little effect on the cAMP level in cytoplasm. Therefore, cromakalim has no relation with adenylate cyclase activity (Table 2).

When mast cells are activated by antigen-antibody reactions, leukotrienes, which are a synthetized and released, are much more powerful than histamine, and are the strong causal material of asthma and allergic responses. We attempted to confirm the inhibitory mechanism of leukotriene release by cromakalim in mast cell activations. It has been suggested that mast cell membrane receptor activation causes phospholipid methylation by activation of the methyltransferase I and II (MT I and MT II) enzymes , the result is a rise in Ca^{++} influx which increases the activity of PLA_2, and leads to the hydrolysis of PC with subsequent arachidonic acid and lyso-PC releases. This arachidonic acid is what makes leukotrienes (Hirata *et al.* 1978; Hirata *et al.* 1979; Hirata and Axelrod, 1980; Daeron *et al.* 1982; Baries *et al.* 1983; Beaven *et al.* 1984; Beaven and Cunha-Melo, 1988). However, there is a report that there is no relation between membrous phospholipid methylation and mediator releases(Benyon *et al.* 1988). Due to such conflicting reports a few researchers questioned if phospholipid methylation is associated with mediator release during mast cell activations. However, it has been reconfimed that antigen, anti-IgE, and concanavalin A induce both the incorporation of the ^{3}H-methyl moiety into membrous phospholipids and histamine release(Takei *et al.* 1990). We also reported recently that IgG_1-or IgE-dependent activation of purified guinea pig lung mast cells by using [^{3}H]methylmethionine was associated with increased ^{3}H-methyl incorporation after the challenge of specific antigens (Ro and Kim, 1995). It has also been observed that this phospholipid methylation is inhibited by 3-deazaadenosine which inhibits methylation.

In this study, it was seen that phospholipid methylation increased greatly when mast cells were activated by antigen-antibody reactions (Fig. 5). The methylated phospholipids separated by TLC were mainly lyso-PC, PC, PDME, and PMME (Fig. 6). Phospholipid methylation also decreased a great deal when cells were pretreated with cromakalim (Fig. 5, 7), and the important phospholipid methylations were for PC, PDME, and PMME (Fig. 7).

The results of the study were also examined to see if there was a correlation between the inhibitory effect of cromakalim on the phospholipid methylation and leukotirene release, and it was seen that there was very significant correlation (Fig 8, $\gamma^2 = 0.92675$, $P < 0.05$). Therefore, cromakalim reduced the production of PC and arachidonic acid successively by inhibiting both MT I and II enzyme activity when the mast cells were activated. It can ultimately be inferred that this reduces the release of leukotriene which is produced via the arachidonic acid metabolic pathway. However, it can be supposed that the differences between these results and other laboratory results(Benyon *et al.* 1988) were because antigens or cell types which were used to stimulate mast cells differed from each other.

Furthermore, a correlation between the inhibitory effect of cromakalim on the phospholipid methylation and histamine release was statistically significant (Fig. 9, $\gamma^2 = 0.794936$, $P < 0.05$). PC produced by membranous phospholipid methylation increases the entry of Ca^{++} into the cells due to the enhancement of the fluidity of the cell membranes. The Ca^{++} influxing into cells and Ca^{++} secreted from storage in cytoplasm, and protein kinase C activated by DAG concomitantly make myosin light chains into becoming phosphorylated and then the permeability of granules to increase. It is supposed that this in turn releases histamine. Another suggestion is that an increase of Ca^{++} in cells through the production of PC accelerates the assembly of microtubules and contraction of microfilaments, which migrate the granules to the cell membrane and cause degranulation, followed by histamine release. Therefore, it can be suggested that there is a correlation between inhibitions of phospholipid methylation and histamine release.

These results show that cromakalim decreases histamine release by inhibiting the initial increase of 1,2-diacylglycerol during mast cell activation, which is mediated via the phosphatidylinositide-phospholipase C system rather than the phosphatidylcholine-phospholipase D system. Furthermore, cromakalim reduces the phosphatidylcholine productioin by inhibiting the methyltransferase, which decreases the conversion of phosphatidylcholine into arachidonic acid and inhibits the production of leukotrienes.

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