

Cromakalim Blocks Membrane Phosphoinositide Activated Signals in the Guinea Pig Lung Mast Cells Stimulated with Antigen-Antibody Reactions

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Cromakalim (BRL 34915), known as an airway smooth muscle relaxant, inhibited the releases of mediators in the antigen-induced mast cell activation. It has been suggested that cromakalim, in part, inhibited mediator releases by inhibiting the initial increase of 1,2-diacylglycerol (DAG) produced by the activation of the other phospholipase system which is different from phosphatidylcholine-phospholipase D pathway. The aim of this study is to further examine the inhibitory mechanism of cromakalim on the mediator release in the mast cell activation. Guinea pig lung mast cells were purified by using enzyme digestion and percoll density gradient. In purified mast cells prelabeled with [³H]PIP₂, phospholipase C (PLC) activity was assessed by the production of [³H]inositol phosphates. Protein kinase C (PKC) activity was assessed by measuring the protein phosphorylated from mast cells prelabeled with [γ -³²P]ATP, and Phospholipase A₂ (PLA₂) activity by measuring the lyso-phosphatidylcholine produced from mast cell prelabeled with 1-palmitoyl-2-arachidonyl phosphatidyl-[¹⁴C]choline. Histamine was assayed by fluorometric analyzer, and leukotrienes by radioimmunoassay. The PLC activity was increased by activation of the passively sensitized mast cells. This increased PLC activity was decreased by cromakalim pretreatment. The PKC activity increased by the activation of the passively sensitized mast cells was decreased by calphostin C, staurosporine and cromakalim, respectively. The PLA₂ activity was increased in the activated mast cells. The pretreatment of cromakalim did not significantly decrease PLA₂ activity. These data show that cromakalim inhibits histamine release by continuously inhibiting signal transduction processes which is mediated via PLC pathway during mast cell activation, but that cromakalim does not affect PLA₂ activity related to leukotriene release.

Key Words: Cromakalim, Mast cell, Histamine, Leukotrienes, Phospholipase C, Phospholipase A₂, Protein kinase C

INTRODUCTION

Cromakalim (BRL 34915), which is known to selectively modulate ATP-activated K⁺ channel, has been reported to produce the relaxation of various smooth muscles including airway smooth muscles (Allen et al, 1986; McCann & Welsh, 1986; Escande et al, 1988; Mondot et al, 1988; Cook, 1990). It has

also been reported that cromakalim might be useful for the treatment of immune, cardiac disorders (Grismer et al, 1994), airway hypersensitivity, and human ureteric colic (de Moura & de Lemos, 1996).

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 ATP-sensitive K⁺ channel blockers reduce the effect of cromakalim (Baird et al, 1988; Murray et al, 1989; Judith et al, 1990; Nielsen-Kudsk et al, 1990; Raeburn & Brown, 1991; Nagai et al, 1991). It has also been reported

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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.

There have been a few reports on the effects of cromakalim on allergic reactions. It has been reported 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 was inhibited by cromakalim. It has also been reported that antigen-induced contraction of isolated sensitized guinea pig tracheal muscle was relaxed by cromakalim, but that antigen-induced histamine release from sensitized guinea pig lung monodispersed cells was not affected by cromakalim (Nagai et al, 1991). It has also been reported that cromakalim significantly inhibited ovalbumin (OA)-induced bronchoconstrictor response and plasma leakage in trachea and main bronchi, and inhibited exogenous histamine- and leukotrienes-induced bronchoconstriction and microvascular leakage, but cromakalim did not affect OA-induced histamine release from minced lung tissues in sensitized guinea pigs (Ichinose et al, 1994; Ichinose et al, 1995). However, Ro & 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 could be partially inhibited by glibenclamide which blocks ATP-dependent K^+ channels or by non-specific K^+ channel blockers such as tetraethylammonium. Since allergic mediator releases were not completely modified by K^+ channel blockers, it was considered possible that mechanisms other than K^+ channel opening might be participated in the action of cromakalim. Therefore, we previously examined the other inhibitory mechanism of cromakalim on the mediator releases caused by specific antigen-antibody reactions in the guinea pig lung mast cells. The results observed that cromakalim decreased histamine release by inhibiting the initial increase of 1,2-diacylglycerol (DAG) during mast cell activation, and reduced the phosphatidylcholine production by inhibiting the methyltransferase, which decreases the conversion of phosphatidylcholine into arachidonic acid and inhibited the production of leukotrienes (Ro et al, 1996). It has previously been reported that DAG during the activation of mast cell evoked by antigen-antibody reactions is produced from phospholipids either directly by the action of

phospholipase C (PLC) (Cunha-Melo et al, 1987) or indirectly by a phospholipase D (PLD)-initiated pathway (Lin et al, 1992a; Ro & Kim, 1993). However, cromakalim did not influence DAG production which is mediated via the phosphatidylcholine-PLD pathway caused by activating the mast cells with specific antigen-antibody reactions (Ro et al, 1996). Therefore, in this study, we examined that other inhibitory mechanism of cromakalim on the mediator releases caused by specific antigen-antibody reactions.

METHODS

Materials

Ovalbumin (fraction V), complete Freund's adjuvant, anti-IgG₂ affinity column (type I), collagenase, elastase (type I, porcine pancreatic), phosphatidylserine, 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (PAPC), phosphatidylcholine, lyso-phosphatidylcholine, phosphatidylinositol-4, 5-bisphosphate (PIP₂), histone, GTP γ -S, tetra-sodium pyrophosphate, arachidonic acid from Sigma; percoll from Pharmacia Fine Chemicals AB; LK 5DF silica gel, LK6D silica gel from Whatmann Inc. [³H]PIP₂(s.a., 2-10Ci/mmol), PAP[¹⁴C]C(s.a., 30-60Ci/mmol), [γ -³²P]ATP(s.a., 10Ci/mmol), leukotriene D₄ kit from New England Nuclear. Glass-fiber disk from Whatman, GF/B, Maidstone, UK. 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 μ g ovalbumin (OA) and complete Freund's adjuvant. One week after that, animals received intradermal injections of 100 μ g OA at one side back and 200 μ g of OA at the other side back. Animals were sacrificed one week later and the sera were stored in aliquots at -70°C until the time of use (Andersson, 1980). The quantitation of serum antibody titers by passive cutaneous anaphylaxis (PCA) were performed as described in previous articles (Undem et al, 1985; Ro et al, 1991).

Serum IgG₁ antibody was separated by affinity column chromatography. Guinea pig blood serum was

applied to anti-IgG₂ affinity column and 0.1 M citric acid (pH 2.1) was used to wash the column. IgG₁ was passed through and the absorbed IgG₂ antibody was rinsed with 0.2M sodium carbonate (pH 11.3). The separated IgG₁ 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 by using the techniques similar to the method previously reported (Undem et al, 1985). Briefly described here, lungs obtained from 16 unsensitized guinea pigs were perfused each with 50ml of the modified Tyrode buffer (TGCM) consisting of (millimolar): NaCl, 137; NaH₂PO₄, 0.36; KCl, 2.6; CaCl₂, 1; MgCl₂, 1.5; NaHCO₃, 119; glucose, 5.5; gelatin, 1g/L, pH 7.4. After removing large airways and blood vessels, the lungs were minced with a Mellwain 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 μ m). The resulting cell population was washed with Tyrode buffer without CaCl₂ and MgCl₂ containing gelatin (TG buffer) and layered over gradients consisting of 10ml of Percoll (density, 1.045 g/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 continuous percoll density gradient (consisting of densities 1.06, 1.07, 1.08, 1.09, and 1.10 g/ml). This sample (cells) applied on top of the 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 ($1-2 \times 10^8$) of mast cells. This cells contained 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 antibody/ 10^6 cells) in a shaking water bath (45 min at 37°C). After this incubation period, the cells were washed, resuspended in TGCM buffer and challenged with 0.1 μ g/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 at 37°C for 45 min concomitantly with anti-OA and cromakalim, and also incubated for 10min concomitantly with cromakalim and OA (0.1 μ g/ml).

Histamine assay

Histamine was analyzed by the automated fluorometric method (with dialyzer) described by Siraganian (1974). 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 (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 μ l of supernatant, antibody (50 μ l of a 1:1000 dilution), and 50 μ l of [³H]leukotriene D₄ (LTD₄, 2500 to 3000 cpm) in buffered saline. Incubations were at 4°C for 2 h and the reaction was terminated by the addition of 0.5 ml dextran coated charcoal (200 mg charcoal and 20 mg dextran mixed with 100 ml buffered saline). After 5min incubation the mixture was centrifuged at 3000 rpm at 4°C and 0.4 ml of the supernatant was added to Aquasol (NEN Research Products) for counting by liquid scintillation spectrometry (Packard, Model 3225). Standard curves were constructed in the presence of antigen using LTD₄. The detection limit of the assay was 0.045 pmole LTD₄. Leukotriene release was expressed as pmole/ 4×10^5 cells.

Determination of phospholipase C activity

The purified mast cells (1×10^6 cells) were incubated with [^3H]PIP₂ (final conc. 0.6 mM) and 2.0 mM phosphatidylinositol at 37°C for 1 hr. The cells were washed twice and suspended with 1 ml of TGCM. The prelabeled cells (1×10^6 cells) were sensitized by IgG₁ (1 ml antibody/ 1×10^6 cells) at 37°C for 45 min and then washed with TGCM. The prelabeled and sensitized cells (1×10^6 cells) were challenged by OA (0.1 $\mu\text{g}/4 \times 10^5$ cells) and GTP γ -S (1 μM) for 1 min. The reactions were stopped by adding 1.2 ml cold chloroform methanol (1/2, v/v), and the labeled [^3H]inositol phosphates in aqueous layer were separated by adding mixture (0.5 ml) of chloroform and 0.25 M HCl. One ml of aqueous layer was dried under N₂ gas, and counted the radioactivity (Atkinson et al, 1992; Atkinson & Yang, 1996)

Determination of protein kinase C activity

The protein kinase C (PKC) activity was determined by using a modification of procedure of previously described (Kikkawa et al, 1983; Lee & Bell, 1991; Hug & Sarre, 1993; Altrichter et al, 1995). The mast cells (1×10^6 cells) sensitized with IgG₁ antibody (1 ml antibody/ 1×10^6 cells) were preincubated by adding histone (0.2 mg/ml), phosphatidylserine (40 $\mu\text{g}/\text{ml}$), and [γ - ^{32}P]ATP (1 μM) at 30°C for 5 min. The cells were challenged with OA (0.1 $\mu\text{g}/4 \times 10^5$ cells) for 10 min. Staurosporine (0.5 nM) or calphostin C (50 nM) were added 5 min before OA challenge. The reactions were stopped by adding 10% TCA (1 ml) and incubated for precipitating the protein at 4°C for 30 min. The precipitated protein was filtered with glass-fiber disk (Whatman, GF/B, Maidstone, UK) for eliminating the excess of [γ - ^{32}P]ATP, and disk containing the phosphorylated protein was washed 4 times with 20 mM tetra-sodium pyrophosphate, one time with ethanol, and dried and counted.

Determination of phospholipase A₂ activity

The phospholipase A₂ (PLA₂) activity was assessed by measuring the labeled phospholipid (lyso-phosphatidylcholine) released by activation of PLA₂ enzyme (Reynolds et al, 1991; Hirasawa et al, 1995). The mast cells (1×10^6 cells) sensitized with IgG₁ antibody (1 ml antibody/ 1×10^6 cells) were incubated with 1-palmitoyl-2-arachidonyl phosphatidyl- [^{14}C]

choline (1 μM) and excess cold phospholipid (10 μM) at 37°C for 30 min. The washed cells were challenged with OA (0.1 $\mu\text{g}/4 \times 10^5$ cells) for 10 min. cromakalim (2×10^{-6} M) were added 5 min before OA challenge. The reactions were stopped by addition of 1 N formic acid, mixed with 0.2 ml n-butanol under the shaking, and then phospholipids were extracted. The organic layer containing standard phospholipids (phosphatidylcholine, lyso-phosphatidylcholine, arachidonic acid) was dried with speed vacuum. The samples were applied to TLC plate, and developed with chloroform/ethanol/water/triethylamine (30/34.8/8/35). The lyso-PC produced by activation of PLA₂ activity was identified with iodine staining, and scraped into scintillation vials. The radioactivities were measured.

Statistic analysis

Experimental data were shown as mean \pm 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.

RESULTS

The effects of cromakalim on mediator releases from activated mast cell

We have previously reported that cromakalim inhibited histamine and leukotriene releases during mast cell activation (Ro & Kim, 1993; Ro et al, 1996). Using different batch of cromakalim, we reconfirmed 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 IgG₁ antibody (anti-OA) were challenged by 0.1 $\mu\text{g}/\text{ml}$ OA, histamine release from mast cells after pretreatment of cromakalim, 2×10^{-6} M, was $20.1 \pm 1.3\%$ (a 36% decrease) when compared with the control group which was $31.2 \pm 2.4\%$. The amount of leukotriene released was 52.4 ± 6.4 pmole/ 1×10^6 cells in the cromakalim pretreated group, which shows a 33% decrease, when compared with the 84.8 ± 9.8 pmole/ 1×10^6 cells of the control group (Table 1).

However, the inhibition of mediator release evoked by cromakalim pretreatment was not increased with the increase in the concentration of cromakalim. Therefore, most of these experiments were performed with 2×10^{-6} M of cromakalim.

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/ 1×10^6 cells)
Ag alone	31.2 ± 2.4	84.8 ± 9.8
1×10^{-6}	27.2 ± 1.6	67.3 ± 5.7
2×10^{-6}	$20.1 \pm 1.3^*$	$52.4 \pm 6.4^*$
1×10^{-5}	$18.7 \pm 1.9^*$	$49.5 \pm 8.2^*$

^a, Purified mast cells (4×10^5) were passively sensitized with anti-OA and challenged with $0.1 \mu\text{g/ml}$ OA in the absence or presence of cromakalim. Histamine in supernatants was determined by fluorometric analyzer, leukotrienes by radioimmunoassay.

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

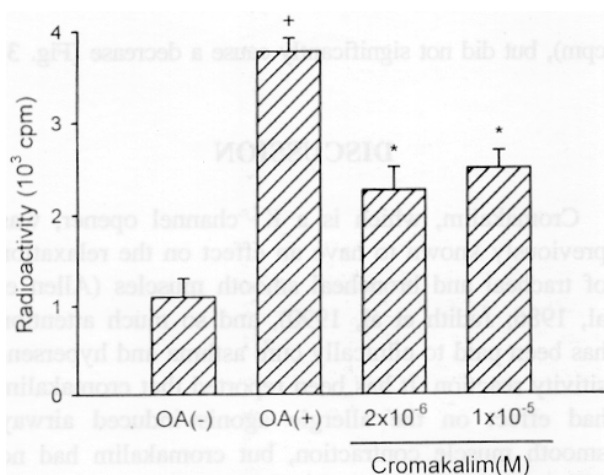


Fig. 1. Effect of cromakalim on the phospholipase C activity in guinea pig lung mast cells activated with specific antigen-antibody reactions. Purified mast cells (1×10^6) were relabeled with [^3H]PIP₂ ($100 \mu\text{Ci}$), passively sensitized with anti-OA, and challenged with $0.1 \mu\text{g/ml}$ OA in the absence or presence of cromakalim (1×10^{-6}). The phospholipase C activity was determined by [^3H]inositol released into cytosol. The results are means \pm S.E.M of 6 experiments. ⁺, $P < 0.05$: non-antigen vs Ag(antigen) alone. *, $P < 0.05$: Ag alone vs cromakalim pretreatment.

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

We examined the phospholipase C (PLC) activity evoked by mast cell activated with OA-anti-OA antibody reactions. When the mast cells (1×10^6 cells) sensitized with anti-OA were challenged with $0.1 \mu\text{g/ml}$ OA, the PLC activity was increased up to 1.5~2.0 times (3769 ± 154 cpm), compared with non-antigen challenge (1094 ± 206 cpm). This increased PLC activity was reduced to 2261 ± 249 cpm by pretreating cromakalim (2×10^{-6} M) (Fig. 1). However, this decrease caused by cromakalim pretreatment did not show a dose dependent manner.

Effect of cromakalim on the protein kinase C activity

As the activation of PLC enzyme was increased in activated mast cells with antigen-antibody reactions, we examined whether PLC activation might successively activate the protein kinase C (PKC) or not. We examined the effect of cromakalim on the PKC activity. When the sensitized (anti-OA) and prelabeled mast cells (1×10^6 cells) were challenged

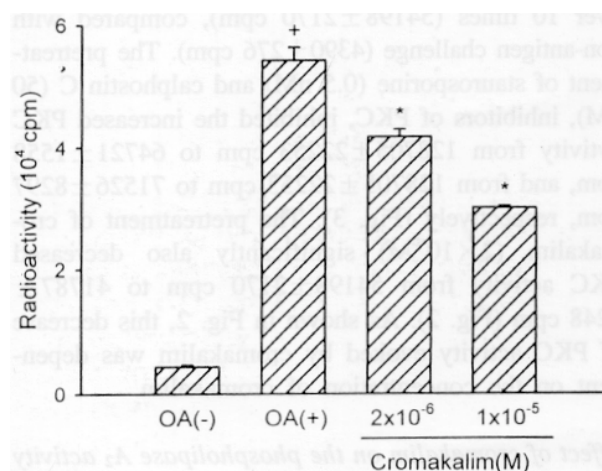


Fig. 2. Effect of cromakalim on the protein kinase C activity in guinea pig lung mast cells activated with specific antigen-antibody reactions. Purified mast cells (1×10^6) were passively sensitized with anti-OA, labeled with [γ - ^{32}P]ATP ($1 \mu\text{M}$), challenged with $0.1 \mu\text{g/ml}$ OA. The radioactivity of incorporated protein was determined after washed to eliminate the unbound isotope. Cromakalim (2×10^{-6}) was added 5 min before OA challenge. The results are means \pm S.E.M of 6 experiments. ⁺, $P < 0.05$: non-antigen vs Ag(antigen) alone. *, $P < 0.05$: Ag alone vs cromakalim pretreatment.

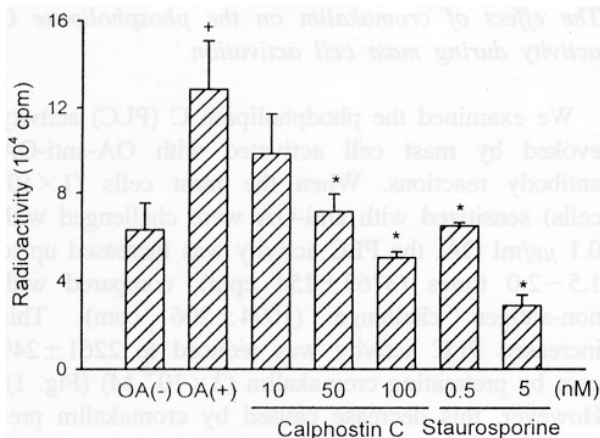


Fig. 3. Effect of staurosporine or calphostin C on the protein kinase C activity in guinea pig lung mast cells activated with specific antigen-antibody reactions. Purified mast cells (1×10^6) were passively sensitized, labeled, and challenged as described in Fig. 2, except the addition of staurosporine (0.1 nM) or calphostin C (50 nM). The results are means \pm S.E.M of 6 experiments. +, $P < 0.05$: non-antigen vs Ag (antigen) alone. *, $P < 0.05$: Ag alone vs staurosporine or calphostin C pretreatment.

with 0.1 $\mu\text{g/ml}$ OA, the PKC activity was increased over 10 times (54198 ± 2170 cpm), compared with non-antigen challenge (4390 ± 276 cpm). The pretreatment of staurosporine (0.5 nM) and calphostin C (50 nM), inhibitors of PKC, inhibited the increased PKC activity from 128705 ± 22253 cpm to 64721 ± 1558 cpm, and from 128705 ± 22253 cpm to 71526 ± 8297 cpm, respectively (Fig. 3). The pretreatment of cromakalim (2×10^{-6} M) significantly also decreased PKC activity from 54198 ± 2170 cpm to 41787 ± 1248 cpm (Fig. 2). As shown in Fig. 2, this decrease of PKC activity evoked by cromakalim was dependent on the concentration of cromakalim.

Effect of cromakalim on the phospholipase A₂ activity

We observed whether the PLA₂ activity was increased or not by the activation of mast cells in order to examine other mechanism of leukotriene release during mast cell activation. When the sensitized (anti-OA) and prelabeled mast cells (1 ± 10^6 cells) were challenged with 0.1 $\mu\text{g/ml}$ OA, the PLA₂ activity assessed by measuring lyso-PC released was increased up to 40% from 12404 ± 1533 cpm to 18631 ± 1851 cpm. The pretreatment of cromakalim (1 ± 10^{-5} M) showed the pattern of decrease (16537 ± 2811

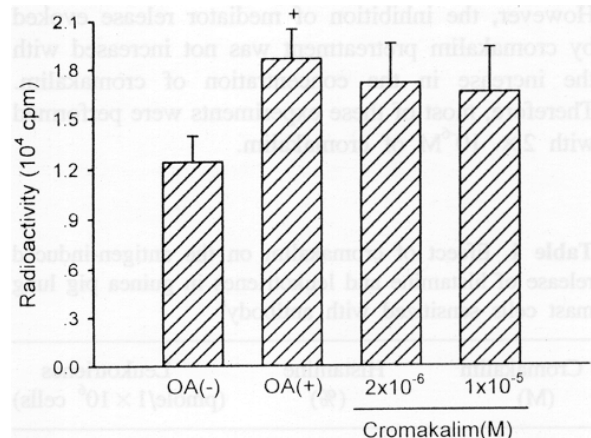


Fig. 4. Effect of cromakalim on the phospholipase A₂ activity in guinea pig lung mast cells activated with specific antigen-antibody reactions. Purified mast cells (1×10^6) were passively sensitized with anti-OA, labeled with 1-palmitoyl-2-arachidonyl phosphatidyl- [¹⁴C]choline (1 μM) and cold substrate, and challenged with 0.1 $\mu\text{g/ml}$ OA. The labeled phospholipids were by organic compounds, separated by TLC. Cromakalim (2×10^{-6}) was added 5 min before OA challenge. The results are means \pm S.E.M of 6 experiments. +, $P < 0.05$: non-antigen vs Ag(antigen) alone.

cpm), but did not significantly cause a decrease (Fig. 3)

DISCUSSION

Cromakalim, which is a K⁺ channel opener, was previously known to have an effect on the relaxation of tracheal and broncheal smooth muscles (Allen et al, 1986; Judith et al, 1990), and so much attention has been paid to clinically cure asthma and hypersensitivity reaction. It has been reported that cromakalim had effect on the allergic agonist-induced airway smooth muscle contraction, but cromakalim had no effect on the antigen-induced histamine release from monodispersed or minced mast cells of guinea pig lung tissues (Nagai et al, 1991; Ichinose et al, 1994; Ichinose et al, 1995). However, we reported that cromakalim could partially inhibit mediator releases in the purified guinea pig lung mast cell activation caused by specific antigen-antibody reactions (Ro & Kim, 1993). It can be suggested that differences between monodispersed or minced mast cells and purified mast cells on the release of mediators are due to the existence of some substances which can rapidly de-

grade mediators in monodispersed or minced cells.

In this experiment, we examined the effect of cromakalim on the mediator release because it could differ from batch to batch. We reconfirmed that cromakalim inhibited the release of these mediators by 30% (Table 1). This study attempted to understand other 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 linked with the cell membrane are activated. These enzymes are tyrosine kinase (Eiseman & Bolen, 1992), adenylate cyclase (Ishizaka et al, 1980), phospholipase C (PLC)(Cunha-Melo et al, 1987; Park et al, 1991; Atkinson & Yang, 1996), phospholipase D(PLD) (Gruchalla, et al, 1990; Lin et al, 1992a), methyltransferase (Hirata et al, 1978; Takei et al, 1990), and PLA₂ (Reynolds et al, 1991; Lin et al, 1992b; Murakami et al, 1992a; Murakami et al, 1992b; Hirasawa et al, 1995), etc. This process is related to the activation of a variety of phospholipid metabolic pathway and the generation of a number of second messengers. The intracellular second messengers are Ca⁺⁺ (Beaven et al, 1984), DAG (Gruchalla et al, 1990), and inositol triphosphates (IP₃) etc.

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 (Cunha-Melo et al, 1987; Gruchalla et al, 1990; Lin et al, 1992a). 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). We previously reported that cromakalim had little effect on PLD activity, but inhibited the production of initial DAG evoked by mast cell activation (Ro et al, 1996). Therefore, this study put its emphasis on PLC activity in order to understand the inhibitory mechanism of cromakalim on the release of mediators from guinea pig lung mast cells. When the mast cells stimulated with anti-OA antibody were challenged with OA, the PLC activity was increased up to 2.5~3.0 times. We observed a similar result as reported by many researchers previously (Cunha-Melo et al, 1987; Atkinson & Yang, 1996). Cromakalim significantly reduced the PLC activity up to 40%, but did not completely inhibit the

PLC activity (Fig. 1). As a result, it can be suggested that cromakalim reduces histamine release by inhibiting the initial DAG production during mast cell activation, which is mediated via PIP₂-PLC pathway.

The DAG production is biphasically increased in various cells (Lin et al, 1992a; Ro et al, 1996). Lin et al (1992a) demonstrated that PC-PLD activation does not initiate the IgE-dependent increase in intracellular DAG levels and PKC activation, but may play a role in the maintenance of PKC activation by producing a secondary increase in DAG levels in RBL 2H3 cells. It has also been reported that PKC, in α -thrombin-stimulated IIC9 fibroblasts, could be activated by DAG derived from PIP₂ but not derived from PC hydrolysis (Leach et al, 1991), and that ester-linked, but not ether-linked, DAG species activate PKC in a cell free assay system (Musial et al, 1995). Therefore, we first studied if the DAG production produced by activation of mast cell in our experiment system activates the PKC and then studied the effect of cromakalim. The PKC activity is remarkably increased by mast cells activated with antigen-antibody reactions (Fig. 2). This PKC activity is completely inhibited by staurosporine which is known to inhibit the secondary phase of DAG production, and to be non-selective inhibitor of PKC (Fig. 3). Calphostin C, relatively selective inhibitor of PKC, also inhibited the PKC activity during mast cell activation. This result of the increased PKC is similar to that obtained by cultured RBL 2H3 cell line activated with TNP-anti-TNP antibody reactions (Lin et al, 1992a). However, we did not examine whether the PKC is activated by DAG derived from either PIP₂ or PC, but the increased PKC is decreased in a dose-dependent manner by cromakalim (Fig. 2). It can be inferred from previous reports that when the guinea pig lung mast cells are activated by a specific antigen-antibody reactions, the activated PLC hydrolyses the PIP₂ to DAG and IP₃, and the initial produced DAG activates the PKC, and then the activated PKC phosphorylates some cytosolic protein, probably related to the histamine release. Furthermore, it can be also inferred that cromakalim inhibits signal cascade described above which is related to the histamine release.

Mast cells are activated by antigen-antibody reactions, leukotrienes, which are released from, are the strong causal material of asthma and allergic responses. It has been suggested that mast cell membrane receptor activation cause phospholipid methyl-

ation 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 PLA₂, and leads to the hydrolysis of PC with subsequent arachidonic acid and lyso-PC releases. This arachidonic acid makes leukotrienes (Hirata et al, 1978; Hirata and Axelrod, 1980; Beaven et al, 1984; Lin et al, 1992b; Hirasawa et al, 1995). We recently reported that IgG₁-or IgE-dependent activation of purified guinea pig lung mast cells was associated with the increased methyl incorporation after the challenge of specific antigens (Ro & Kim, 1995). It has also been observed that this phospholipid methylation is inhibited by cromakalim (Ro et al, 1996). Therefore, we examined if cromakalim has effect on the PLA₂ activity in the low level of leukotriene synthesis. The PLA₂ activity is significantly increased by antigen-antibody reactions. This result of the increased PLA₂ activity agrees with several reports that the PLA₂ is activated by a various mast cell lines (Lin et al, 1992b; Murakami et al, 1992a; Hirasawa et al, 1995). However, this increased PLA₂ activity is not significantly reduced by cromakalim pretreatment (Fig. 4). As a result, it can be inferred that cromakalim do not affect the PLA₂ activity, but more study needs.

These data show that cromakalim inhibits histamine release by continuously inhibiting a various signal transduction process which is mediated via PLC pathway during mast cell activation, but that cromakalim does not affect PLA₂ activity related to leukotriene release.

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REFERENCES

- Aharony D, Dobson P, Bernstein P, Kusner E, Krell R, Smith JB. Determination of SRS-A release from guinea-pig lungs by a radioimmunoassay. *Biochem Biophys Res Commun* 117: 574–579, 1983
- Allen SL, Boyle JP, Cortijo J, Foster RW, Morgan OP, Small RC. Electrical and mechanical effects of BRL 34915 in guinea-pig isolated trachealis. *Br J Pharmacol* 89: 395–405, 1986
- Altrichter J, Guse AH, Resch K, Brock J, Daeron M, Huckel C. Phosphatidylinositol hydrolysis and an increase in Ca^{++} concentration in the signal-transduction process triggered by murine Fc γ RIII are not required for protein kinase C translocation. *Eur J Biochem* 228: 587–595, 1995
- Andersson P. Antigen-induced bronchial anaphylaxis in actively sensitized guinea pigs. *Allergy* 35: 65–71, 1980
- Atkinson TP, Kaliner MA, Hohman RJ. Phospholipase C- γ 1 is translocated to the membrane of rat basophilic leukemia cells in response to aggregation of IgE receptors. *J Immunol* 148: 2194–2200, 1992
- Atkinson TP, Yang Q. Translocation of phospholipase C γ -2 induced by in vitro activation of protein tyrosine kinase activity in mast cell lysates. *Cell Signal* 8(6): 461–465, 1996
- Baird A, Hamiton TC, Richards DH, Tasker T, Williams AJ. Inhibition of histamine-induced bronchoconstriction in normal healthy volunteers by a potassium channel activator, BRL 34915. *Br J Clin Pharmacol* 25: 114–119, 1988
- Beaven MA, Rogers J, Moore JP, Hesketh TR, Smith GA, Metcalfe JC. The mechanism of the calcium signal and correlation with histamine release in RBL 2H3 cells. *J Biol Chem* 259: 7129–7136, 1984
- Cook NS. *Potassium channels structure, classification, function and therapeutic potential*. In Ellis Horwood Ltd ed. New York, Chichester, John Wiley and Sons (Halsted Press), p12–49, 1990
- Cunha-Melo JR, Dean NM, Moger JD, Maeyama K, Beaven MA. The Kinetics of phosphoinositide hydrolysis in rat basophilic leukemia(RBL-2H3) cells varies with the type of IgE receptor cross linking agent used. *J Biol Chem* 262: 11455–11464, 1987
- Eiseman E, Bolen JB. Engagement of the high-affinity IgE receptor activates *src* protein related tyrosine kinases. *Nature* 355: 78–80, 1992
- Escande D, Thuringer D, Leguern S, Cavero I. The potassium channel opener cromakalim (BRL34915) activates ATP-dependent K^{+} channels in isolated cardiac myocytes. *Biochem Biophys Commun* 154: 620–625, 1988
- Grissmer S, Nguyen AN, Aiyar J, Hanson DC, Mather RJ, Gutman GA, Karmilawicz MJ, Auperin DD, Chandy KG. Pharmacological characterization of five cloned voltage-gated K^{+} channels, types Kv 1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol Pharmacol* 45(6): 1227–1234, 1994
- Gruchalla RD, Dinh TT, Kennerly DA. An indirect pathway of receptor-mediated 1,2-diacylglycerol formation in mast cells. I. IgE receptor-mediated activa-

- tion of phospholipase D. *J Immunol* 144: 2334–2342, 1990
- Hirata F, Axelrod J. Phospholipid methylation and biological signal transduction. *Science* 209: 1082–1090, 1980
- Hirata F, Vievos OH, Diliberto EJ, Axelrod JA. Identification and properties of two methyltransferases in conversion of phosphatidylethanolamine to phosphatidylcholine. *Proc Natl Acad Sci USA* 75: 1718–1721, 1978
- Hirasawa N, Santini F, Beaven MA. Activation of the mitogen-activated protein kinase/cytosolic phospholipase A₂ pathway in a rat mast cell line. *J Immunol* 154: 5391–5402, 1995
- Hug H, Sarre TF. Protein kinase C isoenzyme: divergence in signal transduction? *Biochem J* 291: 329–343, 1993
- Ichinose M, Miura M, Takahashi T, Yamauchi H, Nakajima N, Igarashi A, Ishkawa J, Inoue H, Maeyama K, Watanabe T et al. Antigen-induced airway responses are inhibited by a potassium channel opener. *Am J Resp Crit Care Med* 150(2): 388–393, 1994
- Ichinose M, Miura M, Takahashi T, Yamauchi H, Kageyama N, Tomaki M, Endoh N, Sakurai E, Watanabe T, Shirato K. Allergic airway response and potassium channels: histamine release and airway inflammation. *Methods Find Exp Clin Pharmacol* 17(C): 36–39, 1995
- Ishizaka T, Hirata F, Ishizaka K, Axelrod J. Stimulation of phospholipid methylation, Ca⁺⁺ influx, and histamine release by bridging of IgE receptor on rat mast cells. *Proc Natl Acad Sci USA* 77: 1903–1910, 1980
- Judith LB, Carol LA, Reter RAJ, Lorraine AA, Peter JB. The Action of a potassium activator, BRL 38277 (Lemakalim) on human airway smooth muscle. *Am Rev Respir Dis* 142: 1384–1389, 1990
- Kikkawa U, Minakuchi R, Takai Y, Nishizuka Y. Calcium-activated, phospholipid-dependent protein kinase (protein kinase C) from rat brain. *Methods Enzymol* 99: 288–298, 1983
- Leach KL, Ruff VA, Wright TM, Pessin MS, Raben DM. Dissociation of protein kinase C activation and sn-1,2-diacylglycerol formation. Comparison of phosphatidyl- and phosphatidylcholine-induced diglycerides in α -thrombin-stimulated fibroblasts. *J Biol Chem* 266: 3215–3221, 1991
- Lee MH, Bell RM. Mechanism of protein kinase C activation by phosphatidylinositol 4,5-bisphosphate. *Biochem* 30: 1041–1049, 1991
- Lin P, Fung WC, Gilfillan AM. Phosphatidylcholine-specific phospholipase D-derived, 2-diacylglycerol does not initiate protein kinase C activation in the RBL 2H3 mast cell line. *Biochem J* 287: 325–331, 1992a
- Lin LL, Lin AY, Knopf JL. Cytosolic phospholipase A₂ is coupled to hormonally regulated release of arachidonic acid. *Proc Natl Acad Sci USA* 89: 6147–6151, 1992b
- McCann JD, Welsh MJ. Calcium-activated potassium channels in canine airway smooth muscle. *J Physiol* 372: 113–127, 1986
- Mondot S, Mestre M, Callard CG, Cavero I. RP49356: A vasorelaxant agent with potassium channel activating properties. *Br J Pharmacol* 95: 913–922, 1988
- de Moura RS, de Lemos NM. Effects of potassium channel modulators cromakalim, tetraethylammonium and glibenclamide on the contractility of the isolated human ureter. *J Urol* 156(1): 276–280, 1996
- Murakami MI, Kudo I, Suwa Y, Inoue K. Release of 14-kDa group-II phospholipase A₂ from activated mast cells and its possible involvement in the regulation of the degranulation process. *Eur J Biochem* 209: 257–265, 1992a
- Murakami MI, Kudo I, Umeda M, Matsuzawa A, Takeda M, Komada M, Fujimoi Y, Takahashi K, Inoue K. Detection of three distinct phospholipases A₂ in cultured mast cells. *J Biochem* 111: 175–181, 1992b
- Murray MA, Boyle JP, Small RC. Cromakalim-induced relaxation of guinea-pig isolated trachealis: Antagonism by glibenclamide and by phentolamine. *Br J Pharmacol* 98: 865–874, 1989
- Musial A, Mandal A, Coroneos E, Kester M. Interleukin-1 and endothelin stimulate distinct species of diglycerides that differentially regulate protein kinase C in mesangial cells. *J Biol Chem* 270: 21632–21638, 1995
- Nagai H, Kunihiko K, Shoichi G, Hiroshi S, Akihiko K. Effect of three novel K⁺ channel openers, cromakalim, pinacidil and nicorandil on allergic reaction and experimental asthma. *Jpn J Pharmacol* 56: 13–21, 1991
- Nielsen-Kudsk JE, Lone B, Anne MB. Glibenclamide blocks the relaxant action of pinacidil and cromakalim in airway smooth muscle. *Eur J Pharmacol* 180: 291–296, 1990
- Park DJ, Min HK, Rhee SG. IgE-induced tyrosine phosphorylation of phospholipase C γ 1 in rat basophilic leukemia cells. *J Biol Chem* 266: 24237–24240, 1991
- Raeburn D, Brown TJ. RP49356 and cromakalim relax airway smooth muscle in vitro by opening a sulfonylurea-sensitive K⁺ channel: A comparison with Nifedipine. *J Pharmacol Exp Ther* 256: 480–485, 1991
- Reynolds LJ, Washburn WN, Deems RA, Dennis EA. Assay strategies and methods for phospholipases. *Methods Enzymol* 197: 3–23, 1991
- Ro JY, Kim KH. Effects of cromakalim on the release of mediators in hypersensitivity of guinea pig. *Korean J Pharmacol* 29: 263–274, 1993
- Ro JY, Yim YN, Kim KH. The effects of cromakalim

- on the mediator releases from guinea pig lung mast cells activated by specific antigen-antibody reactions. *Yonsei Med J* 37(5): 325–338, 1996
- Siraganian RP: An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal Biochem* 56: 383–394, 1974
- Takei M, Matumoto T, Endo K, Muraratu M. Inhibition of phospholipid methylation by an anti-allergic agent, NCO-650, during histamine release. *Biochem Pharmacol* 40: 1773–1778, 1990
- Udem B, Green F, Warner T, Bucker C, Graziano FM. A procedure for isolation and partial purification guinea pig lung mast cells. *J Immunol Methods* 81: 187–197, 1985
- Williams AJ, Lee TH, Cochrane GM, Hopkirk A, Vyse T, Chiew F, Lavender E, Richards DH, Owen S, Stone P, Church S, Woodcock AA. Attenuation of nocturnal asthma by cromakalim. *Lancet* ii: 334–336, 1988
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