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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 [^{3}H]PIP$_2$, phospholipase C (PLC) activity was assessed by the production of [^{3}H]insitol phosphates. Protein kinase C (PKC) activity was assessed by measuring the protein phosphorylated from mast cells prelabeled with [γ-^{32}P]ATP, and Phospholipase A$_2$ (PLA$_2$) activity by measuring the lyso-phosphatidylcholine produced from mast cell prelabeled with 1-palmitoyl-2-arachidonyl phosphatidyl-[^{14}C]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 PLA$_2$ activity was increased in the activated mast cells. The pretreatment of cromakalim did not significantly decrease PLA$_2$ 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 PLA$_2$ activity related to leukotriene release.

Key Words: Cromakalim, Mast cell, Histamine, Leukotrienes, Phospholipase C, Phospholipase A$_2$, 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 (Grissmer 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-sesitive 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 brochi, 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 freunds adjuvant, anti-IgG_2 affinity column (type I), collagenase, elastase (type I, porcine pancreatic), phosphatidylserine, 1-palmitoyl-2-arachidonyl-phosphatidylcholine (PAPC), phosphatidylcholine, lyso-phosphatidylcholine, phosphatidylinositol-4, 5-bisphosphate (PIP_2), histone, GTPγ-S, tetra-sodium pyrophosphate, arachidonic acid from Sigma; percoll from Pharmacia Fine Chemicals AB; LK 5DF silica gel, LK6D silica gel from Whatmann Inc. [^{3}H]PIP_2(s.a., 2-10Ci/mmol), PAP[^{14}C]C(s.a., 30-60Ci/mmol), [γ-^{32}P]ATP(s.a., 10Ci/mmol), leukotriene D_4 kit from New England Nuclear. Glass-fiber disk from Whatman, GF/B, Maistone, 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 μg ovalbumin (OA) and complete freund's adjuvant. one week after that, animals received intradermal injections of 100 μg OA at one side back and 200 μg of OA at the other side back. Animals were sacrificed one week later and the sera were stored in aliquots at -70°C until the time of use (Andersson, 1980). The 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 IgG_1 antibody was separated by affinity column chromatography. Guinea pig blood serum was

applicated to anti-IgG_2 affinity column and 0.1 M citric acid (pH 2.1) was used to wash the column. IgG_1 was passed through and the absorbed IgG_2 antibody was rinsed with 0.2M sodium carbonate (pH 11.3). The separated IgG_1 was 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; 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 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 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 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 ($1\text{-}2 \times 10^8$) 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 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 at 37°C for 45 min concomitantly with anti-OA and cromakalim, and also incubated for 10min concomitantly with cromakalim and OA (0.1 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 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 $\mu\ell$ of supernatant, antibody (50 $\mu\ell$ of a 1:1000 dilution), and 50 $\mu\ell$ of [^{3}H]leukotriene D_4 (LTD_4, 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 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 C activity

The purified mast cells (1×10^{6} cells) were incubated with [^{3}H]PIP$_2$ (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 IgG$_1$(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 μg/4×10^5 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 [^{3}H]inositol phosphates in aquaous layer were separated by adding miture (0.5 ml) of chloroform and 0.25 M HCl. One ml of aquaous layer was dried under N_2 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 IgG$_1$ antibody (1 ml antibody/1×10^6 cells) were preincubated by adding histone (0.2 mg/ml), phosphatidylserine (40 μg/ml), and [γ-^{32}P]ATP(1 μM) at 30°C for 5 min. The cells were challenged with OA (0.1 μg/4×10^5 cells) for 10 min. Staurosporine (0.5 nM) or calphostin C (50 nM) were added 5 min before OA challege. 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 filtrated 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 A_2 activity

The phospholipase A_2 (PLA$_2$) activity was assessed by measuring the labeled phospholipid(lyso-phosphatidylcholine) released by activation of PLA$_2$ enzyme (Reynolds et al, 1991; Hirasawa et al, 1995). The mast cells (1×10^6 cells) sensitized with IgG$_1$ 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 μg/4×10^5 cells) for 10 min. cromakalim (2×10^6 M) were added 5 min before OA challege. 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 vaccum. 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 PLA$_2$ activity was idendified 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 IgG$_1$ antibody (anti-OA) were challenged by 0.1 µg/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 $\pm$ 6.4 pmole/1×10^6cells in the cromakalim pretreated group, which shows a 33% decrease, when compared with the 84.8 $\pm$ 9.8 pmole/1×10^6cells 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. Efeect 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±1.3*	52.4±6.4*
1× 10^{-5}	18.7±1.9*	49.5±8.2*

[a], Purified mast cells(4× 10^5) were passively sensitized with anti-OA and challenged with 0.1 μg/ml OA in the absence or presence of cromakalim. Histamine in supematents was determined by fluorometric analyzer, leukotrienes by radioimmunoassay.
*, $P<0.05$: Ag(antigen) alone vs cromakalim pretreatment.

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

We examined the phodpholipase 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 μg/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 also examined the effect of cromakalim on the PKC activity. When the sensitized (anti-OA) and prelabeled mast cells (1× 10^6 cells) were challenged

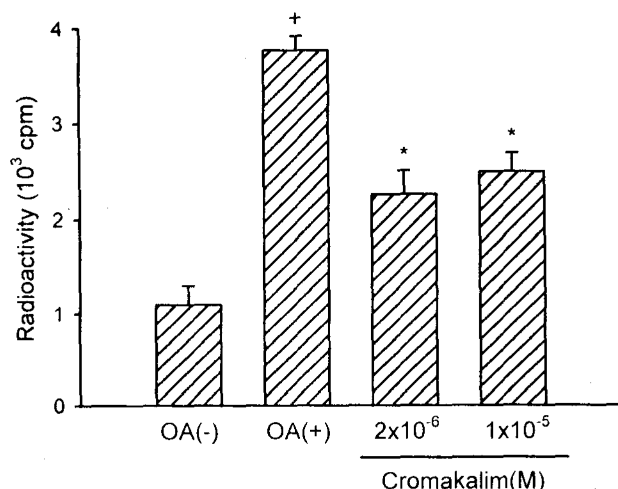

Fig. 1. Effect of cromakalim on the phospholipase C activity in guinea pig lung mast cells activated with specific antigen-antibody reactions. Purified mast cells(1× 10^6) were relabeled with [^{3}H]PIP_2(100 μCi), passively sensitized with anti-OA, and challenged with 0.1 μg/ml OA in the absence or presence of cromakalim(1× 10^{-6}). The phospholipase C activity was determined by [^{3}H]inositol released into cytosol. The results are means±S.E.M of 6 experiments. $^+$, $P<0.05$: non-antigen vs Ag(antigen) alone. *, $P<0.05$ Ag alone vs cromakalim pretretment.

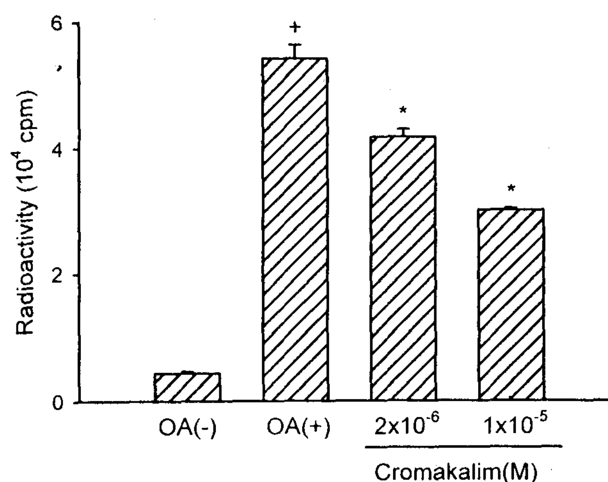

Fig. 2. Effect of cromakalim on the protein kinase C activity in guinea pig lung mast cells activated with specific antigen-antibody reactions. Purified mast cells (1× 10^6) were passively sensitized with anti-OA, labeled with [γ-^{32}P]ATP(1 μM), challenged with 0.1 μg/ml OA. The radioactivity of incorporated 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±S.E.M of 6 experiments. $^+$, $P<0.05$: non-antigen vs Ag(antigen) alone. *, $P<0.05$: Ag alone vs cromakalim pretreatment.

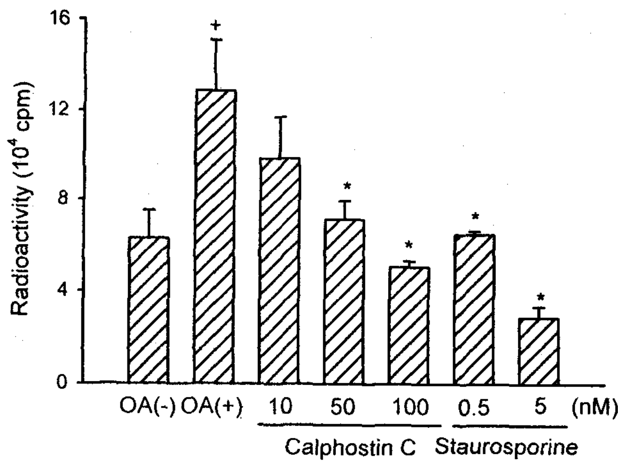

Fig. 3. Effect of staurosporine or calphostin C on the protein kinase C activity in guinea pig lung mast cells activated with specific antigen-antibody reactions. Pirified mast cells (1×10^6) were passively sensitized, labeled, and challenged as described in Fig. 2, except the addition of staurosporine (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.

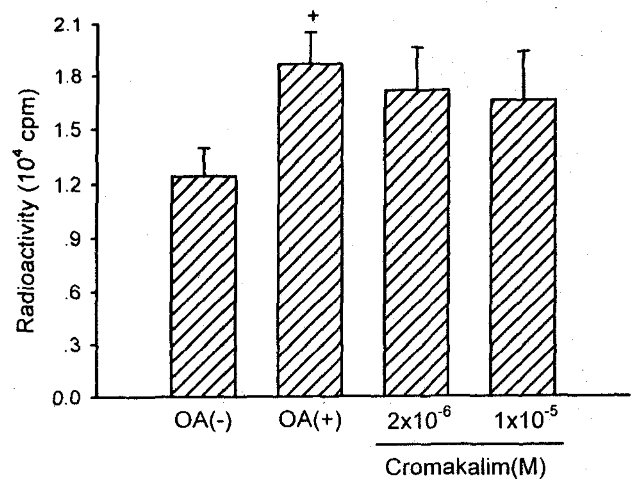

Fig. 4. Effect of cromakalim on the phospholipase A_2 activity in guinea pig lung mast cells activated with specific antigen-antibody reactions. Purified mast cells (1×10^6) were passively sensitized with anti-OA, labeled with 1-palmitoyl-2-arachidonyl phosphoatidyl- [^{14}C]choline (1 μM) and cold substrate, and challenged with 0.1 μg/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.

with 0.1 μg/ml OA, the PKC activity was increased over 10 times (54198$\pm$2170 cpm), compared with non-antigen challenge (4390$\pm$276 cpm). The pretreatment of staurosporine (0.5 nM) and calphostin C (50 nM), inhibitors of PKC, inhibited the increased PKC avtivity from 128705$\pm$22253 cpm to 64721$\pm$1558 cpm, and from 128705$\pm$22253 cpm to 71526$\pm$8297 cpm, respectively (Fig. 3). The pretreatment of cromakalim (2×10^{-6}M) significantly also decreased PKC activity from 54198$\pm$2170 cpm to 41787$\pm$ 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 A_2 activity

We observed whether the PLA_2 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 μg/mlOA, the PLA_2 activity assessed by measuring lyso-PC released was increased up to 40% from 12404$\pm$1533 cpm to 18631 $\pm$1851 cpm. The pretreatment of cromakalim (1 ± 10^{-5}M) showed the pattern of decrease (16537$\pm$2811 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 miced 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 reacctions.

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 PLA_2 (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 (IP_3) 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 PIP_2-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 PIP_2 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 PIP_2 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 PIP_2 to DAG and IP_3, 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 PLA_2, 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 IgG_1-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 PLA_2 activity in the low level of leukotriene synthesis. The PLA_2 activity is significantly increased by antigen-antibody reactions. This result of the increased PLA_2 activity agrees with several reports that the PLA_2 is activated by a various mast cell lines (Lin et al, 1992b; Murakami et al, 1992a; Hirasawa et al, 1995). However, this increased PLA_2 activity is not significantly reduced by cromakalim pretreatment (Fig. 4). As a result, it can be inferred that cromakalim do not affect the PLA_2 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 PLA_2 activity related to leukotriene release.

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