British Journal of Pharmacology (1998) 125, 61–68
http://www.stockton-press.co.uk/bjp

Effects of mitogen-activated protein kinase kinase inhibitor PD 098059 on antigen challenge of guinea-pig airways *in vitro*

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1 It has been shown that activation of protein tyrosine kinases is the earliest detectable signalling response to FcεRI cross-linking on mast cell. Following tyrosine kinase activation, a family of mitogen-activated protein kinases (MAPKs) was found to be activated as well. The present study examined the role of MAPK signalling cascade in *in vitro* model of allergic asthma using a specific MAPK kinase inhibitor PD 098059.

2 Guinea-pigs were passively sensitized with IgG antibody raised against ovalbumin (OA). Effects of PD 098059 on OA-induced anaphylactic contraction of isolated bronchi and release of histamine and peptidoleukotrienes from chopped lung preparations were studied.

3 PD 098059 (10–50 μM) produced only minor reduction of maximal OA-induced bronchial contraction. In contrast, the rate of relaxation of OA-induced bronchial contraction was markedly faster in the presence of PD 098059 than the vehicle control in a concentration-dependent manner.

4 These observations corroborate well with the inability of PD 098059 (5–50 μM) to substantially block the OA-induced release of histamine and with marked inhibition of OA-induced release of peptidoleukotrienes from lung fragments in the presence of PD 098059. Exogenous arachidonic acid-induced release of peptidoleukotrienes from lung fragments was not blocked by PD 098059.

5 In immunoblotting study, we found that $p42^{MAPK}$ was constitutively expressed in guinea-pig bronchi. However, treatment with OA, histamine or LTD_4 did not cause activation of $p42^{MAPK}$. These findings together with the lack of inhibitory effects of PD 098059 on bronchial contraction induced by histamine or LTD_4 suggest that histamine- and LTD_4-induced bronchial contractions are not mediated by $p42^{MAPK}$ activation.

6 Taken together, our findings show that inhibition of MAPK signalling cascade by PD 098059 significantly reduced the OA-triggered release of peptidoleukotrienes leading to rapid relaxation of anaphylactic bronchial contraction. On the other hand, $p42^{MAPK}$ did not play a role in histamine- or LTD_4-induced bronchial smooth muscle contraction suggesting that PD 098059 exerts its inhibitory effects on OA-induced bronchial contraction primarily through inhibition of peptidoleukotrienes release from mast cells.

Keywords: bronchi; ovalbumin; histamine; peptidoleukotrienes; LTD_4; Schultz-Dale reaction; mast cell; mitogen-activated protein kinase (MAPK); extracellular signal-regulated kinase (ERK); mitogen-activated protein kinase/extracellular signal-regulated activating kinase (MEK).

Introduction

Cumulating evidence obtained from rat basophilic mast cell line (RBL-2H3) and bone marrow-derived mast cells showed that activation of non-transmembrane protein tyrosine kinases (PTKs) is the earliest detectable signalling response to FcεRI cross-linking. This is followed by downstream signalling events such as activation of PLCγ (Li *et al*., 1992), increase in inositol 1,4,5-trisphosphate and intracellular Ca^{+2} levels, and enhanced protein kinase C activity, and it eventually leads to mast cell degranulation (Beaven & Metzger, 1993; Scharenberg & Kinet, 1994). Inhibitors of PTK have been shown to block antigen-induced activation of PTK and histamine release from mast cells (Kawakami *et al*., 1992; Oliver *et al*., 1994). In an *in vitro* model of allergic asthma, PTK inhibitors were found to block antigen-induced airway smooth muscle contraction and release of histamine and peptidoleukotrienes from lung fragments (Wong *et al*., 1997).

Downstream from tyrosine kinase activation, a family of serine/threonine mitogen-activated protein kinases (MAPKs) was found to be activated upon FcεRI cross-linking on mast cell (Ishizuka *et al*., 1996; Zhang *et al*., 1997). Among the members of MAPK family, $p44^{MAPK}$ and $p42^{MAPK}$, also known as extracellular signal regulated kinase-1 (ERK1) and ERK2, respectively, are the most well-characterized kinases (Seger & Krebs, 1995; Robinson & Cobb, 1997). Activation of $p44^{MAPK}$ and $p42^{MAPK}$ requires phosphorylation on both tyrosine and threonine residues by the dual-specific action of MAPK kinase (MAPKK), also known as MEK (MAPK/ERK) (Lange-Carter *et al*., 1993; Cobb & Goldsmith, 1995). MAPKK is believed to be activated by Raf-1 which serves as an intermediate signalling molecule connecting the upstream PTKs and $p21^{ras}$ and the downstream MAPK pathway (Catling *et al*., 1995; Marais *et al*., 1995). With the availability of a specific MAPKK inhibitor PD 098059 (Alessi *et al*., 1995; Dudley *et al*., 1995), the present study was conducted to determine the role of the MAPK signalling cascade in an *in vitro* model of allergic asthma.

The Schultz-Dale reaction (Schultz, 1910; Dale, 1913; Chand & Eyre, 1978) has been used extensively to study anaphylactic contraction of airway tissue preparations such as the trachea, bronchi and lung parenchymal strips. Cumulative evidence showed that peptidoleukotrienes and histamine are

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the two major mast cell-derived mediators responsible for the anaphylactic contraction of the airways from both human and guinea-pig (Bjorck & Dahlen, 1993; Jonsson & Dahlen, 1994; Roquet *et al*., 1997). In guinea-pigs, both IgE and IgG are able to sensitize mast cells to specific antigen (Undem *et al*., 1985; Ro *et al*., 1991) and cross-linking of their corresponding FcεRI and FcγR leads to mast cell degranulation. FcγR (e.g. FcγRIII) and FcεRI are structurally and functionally related, and both belong to a family of multi-subunit antigen receptors (Alber *et al*., 1992; Bolen, 1995). It has been shown that engagement of these cell surface receptors activates PTKs and MAPK pathway for successful signal propagation and cellular activation (Bolen, 1995; Rose *et al*., 1997; Zhang *et al*., 1997).

In this study, we passively sensitized guinea-pigs with IgG antibody raised against OA and examined the effects of MAPKK inhibitor PD 098059 on OA-induced anaphylactic contraction of the bronchi and release of histamine and peptidoleukotrienes from chopped lung preparations. Our findings show that PD 098059 only slightly reduced maximal OA-induced contraction of isolated bronchi; however, the anaphylactic contraction relaxed rapidly in the presence of PD 098059 in a concentration-dependent manner. Bronchial contraction induced by OA, histamine or LTD_4 did not require activation of $p42^{MAPK}$. The rapid relaxation of anaphylactic contraction is likely to be linked to the profound inhibition of OA-induced release of peptidoleukotrienes from mast cells by PD 098059.

Methods

Sensitization procedures

Guinea-pigs were passively sensitized by a single i.p. injection of 1 mg kg^{-1} rabbit IgG antibody against OA (Wong *et al*., 1997). The animals were killed 2 days after injection.

Preparation of bronchial rings

Male Hartley guinea-pigs (Interfauna, U.K. Ltd. England) weighing 350–450 g were sacrificed by CO_2 asphyxiation and subsequent decapitation. After thoracotomy, heart and lung were excised *en bloc* and perfused with 50 ml of Krebs-bicarbonate solution *via* the pulmonary artery. Lung lobes were isolated for studies on the release of histamine and peptidoleukotrienes. Bronchial rings ($\sim$3 mm in length) were obtained from the hilar bronchi and cleaned of any parenchyma. Ring preparations were then suspended isometrically under an optimum resting load of 2 g in organ baths containing 10 ml of Krebs-bicarbonate solution, aerated with 95% O_2 and 5% CO_2 at 37°C, of the following composition (mM); NaCl, 118.2; KCl, 4.6; $NaHCO_3$, 24.8; $CaCl_2{\cdot}2H_2O$, 2.5; KH_2PO_4, 1.2; $MgSO_4{\cdot}H_2O$, 1.2 and dextrose, 10.0. Contractile responses were monitored using force-displacement transducers (Grass FT-03) coupled to a MacLab/8 data-recording system (ADInstruments, Castle Hill, Australia).

Contractile studies

The contraction caused by 60 mM KCl was defined as the maximum tissue response to which all subsequent anaphylactic contractions were compared. For antigen challenge studies, ring preparations were pre-incubated with indomethacin (4 μM) for 30 min before addition of OA. Indomethacin has been shown to reduce the production of relaxant prostanoids (e.g. PGE_2 and PGI_2) capable of attenuating the anaphylactic contraction (Abela & Daniel, 1994; Ro *et al*., 1991). To evaluate the role of MAPKK in mediating anaphylactic bronchial smooth muscle contraction, increasing concentrations of MAPKK inhibitor PD 098059 were pre-incubated with bronchial rings 30 min before addition of OA. The effects of PD 098059 were compared to those of control ring preparations in the absence of the inhibitor. To confirm that the inhibitory effects of PD 098059 on anaphylactic contraction were primarily mediated by mast cell stabilization, we examined the effects of PD 098059 on bronchial contraction induced by increasing concentrations of histamine or LTD_4. In the LTD_4-induced bronchial ring contraction study, 4 μM indomethacin and 5 mM L-cysteine were pre-incubated with the tissue for 30 min. It has been reported that very minute amounts of relaxant prostanoids (e.g. PGE_2 and PGI_2) were able to mask LTD_4-induced canine bronchial contraction and that the addition of indomethacin restored the LTD_4 effect (Abela & Daniel, 1994). L-cysteine is an inhibitor of an aminopeptidase that converts LTD_4 to less potent LTE_4. Adding L-cysteine has been shown to enhance the smooth muscle contractile response to LTD_4 (Abela & Daniel, 1994). To ascertain that the inhibitory effect of PD 098059 on OA-induced bronchial contraction was not mediated by non-selective inhibition of calcium channels, the inhibitor was pre-incubated with bronchial rings 30 min before the challenge with 60 mM KCl.

Immunoblot analysis of $p42^{MAPK}$ in bronchial rings

Bronchial rings were prepared and contracted in response to 1 μg ml^{-1} OA, 30 μM histamine or 0.1 μM LTD_4 as described above. At the peak of the contraction, rings were removed from the tissue baths and immediately frozen in liquid nitrogen. Frozen bronchial rings were later thawed, minced and homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.1% SDS; 1 mM activated sodium orthovanadate; 1 mM sodium fluoride; 2 mM phenylmethylsulphonyl fluoride (PMSF); 5 μg ml^{-1} aprotinin, leupeptin and pepstatin; and 50 μg ml^{-1} benzamidine). Lysates were incubated on ice for 30 min before centrifugation and the supernatants were then assayed for protein concentrations using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.). The supernatants were then mixed with 2 $\times$ sample buffer (125 mM Tris-HCl, pH 6.8; 20% glycerol; 10% 2-mercaptoethanol; 4% SDS and 0.1% bromophenol blue) and boiled for 5 min. Proteins (about 10 μg per lane) were separated by SDS–PAGE in a 4–20% gradient polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, U.S.A.), and then electrotransferred to a nitrocellulose membrane (0.45 μm; Nitrocellulose-1, Life Technologies, Gaithersburg, MD, U.S.A.). After blocking with 4% bovine serum albumin (BSA) in TTBS (20 mM Tris-HCl, pH 7.5; 500 mM NaCl and 0.05% Tween-20), the membrane was probed with either mouse pan ERK antibody (1 : 5000 dilution) or rabbit phospho-specific (activated) MAPK antibody (1 : 1000 dilution), and then with alkaline phosphatase (AP)-conjugated goat anti-mouse antibody (1 : 2000 dilution) or horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1 : 2000 dilution), respectively. The blot was visualized using chromogenic substrates 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium (Life Technologies, Gaitherburg, MD, U.S.A.) for AP-conjugated antibody or 4-chloro-1-napthol (Sigma Chemical Co., St. Louis, MO U.S.A.) and H_2O_2 for HRP-conjugated antibody. To ensure that $p42^{MAPK}$ activation can be observed under our assay conditions, a positive control derived from monoblastic

leukaemic U937 cells treated with 50 nM phorbol 12-myristate 13-acetate (PMA) was examined in parallel with the bronchial lysates. Furthermore, phosphorylated (activated) and unphosphorylated p44/42^{MAPK} proteins were used as additional controls to ensure that the phospho-specific MAPK antibody is specific for the activated MAPK.

Release of mediators from chopped lung preparations

Lung lobes obtained from sensitized guinea-pigs were cut into approximately 1 mm^3 pieces using a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY, U.S.A.). Fragmented lung preparations were washed thoroughly with oxygenated Krebs-$NaHCO_3$ buffer before incubation. Duplicate aliquots of 200 mg lung fragments were weighed and placed in plastic scintillation vials containing 2 ml of oxygenated Krebs solution in the presence of 4 μM indomethacin (for histamine release) plus 5 mM L-cysteine (for release of peptidoleukotrienes). Lung samples were then incubated in a shaker bath at 37°C for 45 min before they were challenged with OA for 3 min (histamine release) or 10 min (release of peptidoleukotrienes) (Wong *et al*., 1992). To determine the mast cell-stabilizing effects of PD 098059, various concentrations of the inhibitor were pre-incubated with the lung preparation for 30 min before antigen challenge. To determine if PD 098059 has any non-selective inhibitory effect on the release of peptidoleukotrienes such as inhibition of 5-lipoxygenase activity, lung fragments were challenged with either 70 μM arachidonic acid (AA) alone (Kumlin & Dahlen, 1990) or a combination of 70 μM AA and 1 μg ml^{-1} OA for 10 min. Diffusates were then collected and stored at −70°C until assay.

Histamine radioenzymatic assay

Histamine release from lung samples in response to OA was determined *via* a radioenzymatic assay as previously described with minor modification (Wong *et al*., 1992). Briefly, a total incubation volume of 60 μl was prepared by sequential addition of 10 μl of samples (or H_2O for the blank), 25 μl of H_2O (or H_2O containing 500 pg of histamine as internal standard) and 25 μl of reaction reagent into 12 × 75 mm polypropylene culture tubes. Reaction reagent contained 21 μl of 0.4 M potassium phosphate/0.1% BSA, pH 7.8, 2 μl of histamine N-methyl transferase (HNMT), and 2 μl of tritiated S-adenosylmethionine. After incubating for 1 h in a shaker bath at 2°C, the enzymatic reaction was terminated by the addition of 75 μl of 2.5 M potassium borate, pH 11; 4 ml of toluene-isoamyl alcohol (3 : 1, v/v) was then added to each tube. After centrifugation (1300 *g*) for 3 min, 3.8 ml of the organic phase containing the reaction product, tritiated N-τ methylhistamine ([^{3}H]-τ-MHm) was transferred to another set of tubes containing 500 μl of 0.5 M HCl for back extraction into the aqueous phase. Tubes were centrifuged as before, and the organic phase was aspirated and discarded. The aqueous phase was mixed with 1.25 ml of toluene-isoamyl alcohol and the procedure repeated. An aliquot of 300 μl of the final aqueous phase was transferred to a scintillation vial containing 8 ml of biodegradable counting scintillant (Readysafe, Beckman, Fullerton, CA, U.S.A.). Radioactivity was quantitated by liquid scintillation spectrometry (Beckman LS 3801, Beckman Instruments, Inc., Fullerton, CA, U.S.A.).

Leukotrienes radioimmunoassay

The release of peptidoleukotrienes from chopped lung preparations in response to OA was quantitated by radioimmunoassay (Amersham Life Science, Buckinghamshire, U.K.). Briefly, a total incubation volume of 400 μl was prepared by sequential addition of 100 μl of samples or LTC_4 standard, 100 μl of [^{3}H]-LTC_4, 100 μl of peptidoleukotriene-specific antiserum (cross-reactivity for $LTC_4/D_4/E_4$ = 100%/100%/41%), and 100 μl of assay buffer (pH 7.4) in polypropylene tubes. Antigen-antibody competition reaction was allowed to take place overnight at about 4°C. Dextran-coated charcoal suspension (250 μl) was then added to each reaction tube to adsorb any unbound leukotrienes. After centrifugation, the supernatants were transferred to scintillation vials containing 10 ml of scintillant and radioactivity determined by liquid scintillation spectrometry as described.

Data analysis

All data are presented as means ± s.e.mean. Statistical differences were analysed using ANOVA followed by Student-Newman-Keuls test (Armitage and Berry, 1987). The critical level for significance was set at $P < 0.05$.

Materials

The following drugs and chemicals were used in this study: ovalbumin (OA, grade V), histamine dihydrochloride, indomethacin, L-cysteine, BSA, sodium orthovanadate, sodium fluoride, PMSF, aprotinin, leupeptin, Tween 20, arachidonic acid, HRP-conjugated goat anti-rabbit antibody (Sigma Chemical Co., St. Louis, MO, U.S.A.), toluene, isoamyl alcohol, boric acid, potassium phosphate, bromophenol blue (Merck, Darmstadt, Germany), Triton X-100, dimethyl sulphoxide (DMSO), glycerol (BDH Laboratory Supplies, Poole, U.K.), sodium dodecyl sulphate (SDS), Tris, 2-mercaptoehtanol, AP-conjugated goat anti-mouse IgG (Life Technologies, Gaithersburg, MD, U.S.A.), rabbit IgG fraction to chicken egg albumin (OA) (Organon Teknicka Corp., Durham, NC, U.S.A.), PD 098059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] (Research Biochemicals International, Natick, MA, U.S.A.), LTD_4 (Cayman Chemical Co., Ann Arbor, MI, U.S.A.), tritiated S-adenosyl-L-[methyl-^{3}H]-methionine (60 − 85 Ci mmol^{-1}) (Amersham Life Science, Buckinghamshire, U.K.), mouse pan ERK mAb (Transduction Laboratories, Lexington, KY, U.S.A.), rabbit phospho-specific MAPK polyclonal antibody (New England Biolabs, Inc., Beverly, MA, U.S.A.) and HNMT (New England Nuclear, Boston, MA, U.S.A.). Rabbit anti-OA IgG antibody was stored in sterile H_2O, and PD 098059 in DMSO at −20°C. Indomethacin was dissolved in 4.2% (g ml^{-1}) $NaHCO_3$ stock solution. OA and histamine were prepared fresh in deionized H_2O. All other reagents were of analytical grade and were dissolved in deionized H_2O.

Results

Effects of PD 098059 on Schultz-Dale reaction

Sensitized guinea-pig bronchial rings contracted in response to 60 mM KCl with an active force of contraction that amounted to 1.84 ± 0.11 g (n = 19). To determine the effects of MAPKK inhibitor on the Schultz-Dale reaction, PD 098059 was pre-incubated with the ring preparations for 30 min before OA challenge. Based upon our previous findings (Wong *et al*., 1997), OA at a concentration of 1 μg ml^{-1} was used to induce maximum anaphylactic bronchial smooth muscle contraction (2.23 ± 0.09 *g*, n = 7). PD 098059 produced slight reduction of

maximal OA-induced bronchial contraction by 14.8%, 27.8% and 31.3% at 10 μM, 30 μM, and 50 μM, respectively (Figure 1a). The mild inhibitory effects of the latter two concentrations were found to be statistically significant ($P<0.05$). However, the anaphylactically contracted bronchi relaxed at a substantially faster rate in the presence of PD 098059 as compared to the DMSO control (Figure 1b). In the first 10 min, OA-induced bronchial contraction relaxed by 30.0%, 55.2% and 61.4% in the presence of 10 μM, 30 μM and 50 μM PD 098059, respectively; whereas, there was only slight (2.8%) relaxation in the control anaphylactic contraction. Complete relaxation was achieved in 30 min with 30 μM or 50 μM PD 098059 treatment and in 50 min with 10 μM PD 098059. In contrast, substantial anaphylactic contraction (26.1% of maximum) was still maintained in the control bronchial preparations after 60 min.

Effects of PD 098059 on mediator-induced bronchial contraction

To determine whether the rapid relaxation of anaphylactic contraction by PD 098059 is mediated by blocking the release of mast cell-derived mediators or by attenuating bronchial smooth muscle contraction, we evaluated the effects of PD 098059 on histamine- or LTD_4-induced bronchial ring contraction. PD 098059 failed to inhibit bronchial contraction induced by increasing concentrations of histamine or LTD_4 (Figures 2a and b). In contrast, PD 098059 markedly potentiated ($P<0.05$) bronchial ring contraction induced by histamine, but not that by LTD_4. On the other hand, Figure 2c

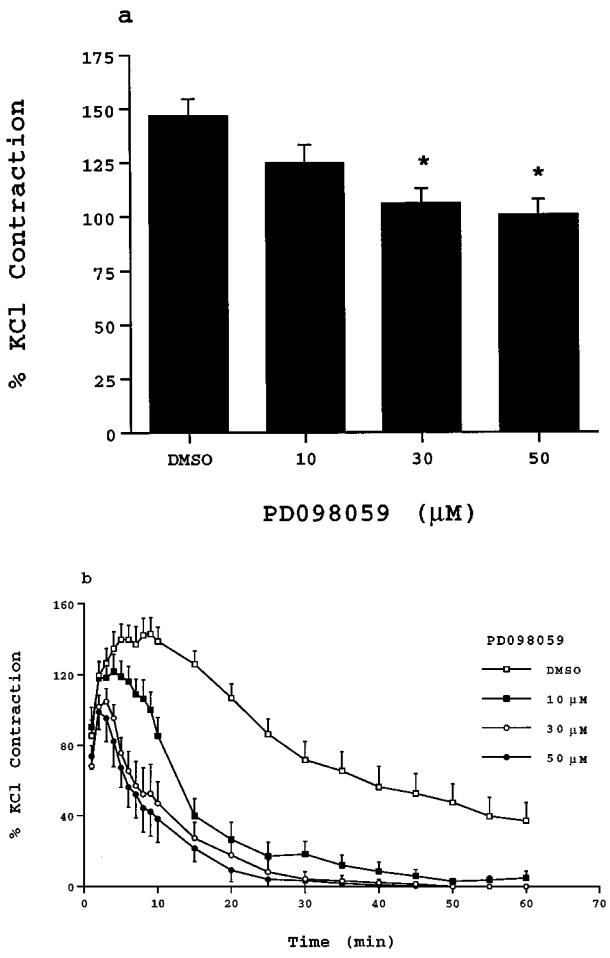

Figure 1 (a) Effects of PD 098059 on OA-induced anaphylactic contraction of guinea-pig bronchi. Bronchial rings were incubated with indicated concentrations of PD 098059 or same volume of DMSO for 30 min prior to 1 μg ml^{-1} OA challenge. Each point represents the mean$\pm$s.e.mean of four–eight experiments. (b) Time course of OA-induced anaphylactic bronchial contraction in the presence and absence of PD 098059. Each point represents the mean$\pm$s.e.mean of three–six experiments. *Significant difference from DMSO vehicle controls, $P<0.05$.

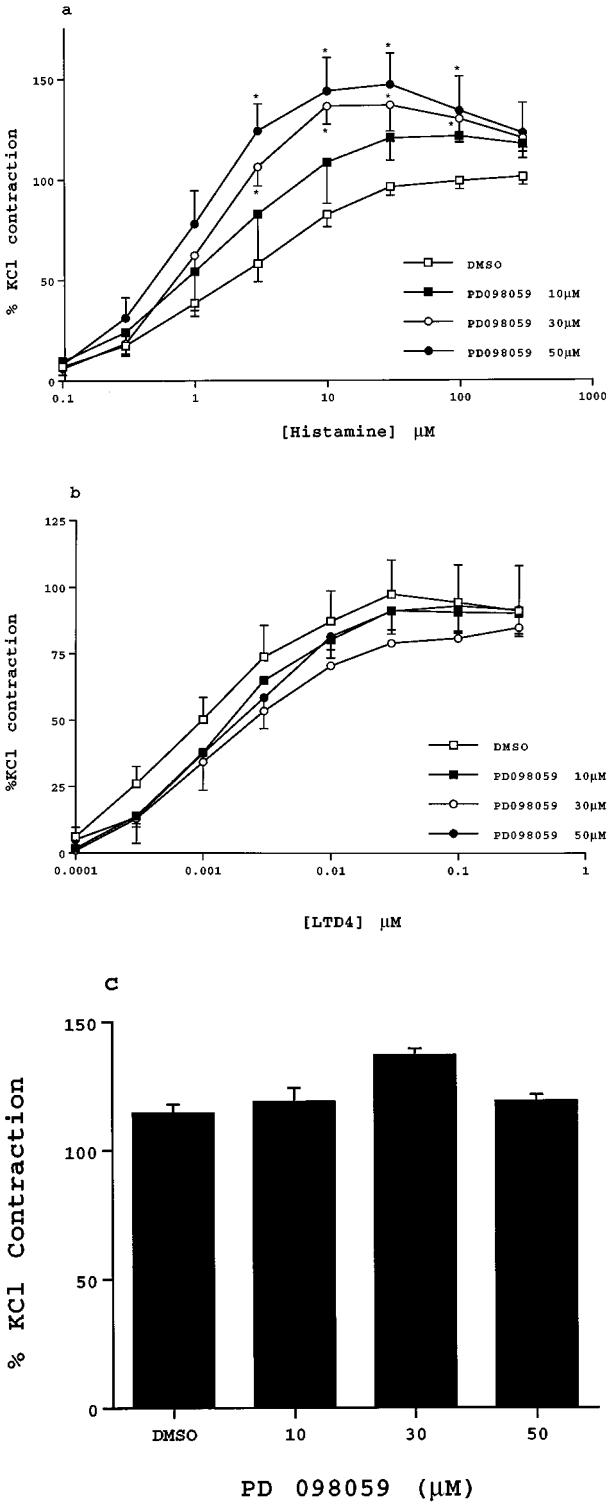

Figure 2 (a) and (b) Concentration-response curves of histamine- and LTD_4-induced guinea-pig bronchial contractions in the presence of PD 098059 (10–50 μM). (c) Effect of PD 098059 on 60 mM KCl-induced bronchial contraction. Each point represents the mean$\pm$s.e.mean of four–seven experiments. *Significant difference from DMSO vehicle controls, $P<0.05$.

shows that PD 098059 did not inhibit 60 mM KCl-induced bronchial contraction, indicating that PD 098059-mediated rapid relaxation of anaphylactic contraction was not due to non-selective activity such as inhibition of voltage-dependent calcium channels.

Effects of PD 098059 on p42^{MAPK} in whole bronchi

Figure 3 (upper panel) shows that p42^{MAPK}(ERK2) was constitutively expressed in guinea-pig bronchi (lanes 1–4) and in U937 cells (lane 5) which were used as a positive control. Treatments of the bronchi with OA, histamine or LTD_4 did not cause activation of p42^{MAPK} (Figure 3, lower panel, lanes 1–4). Whereas, U937 cells treated with PMA produced activation of p42^{MAPK} protein which is consistent with the results reported by Franklin & Kraft (1997). The phosphorylated (activated) ERK (lane A), but not the unphosphorylated ERK (lane N), was also detected by the phospho-specific ERK antibody. These findings suggest that histamine- and LTD_4-induced bronchial contractions are not mediated by p42^{MAPK} activation, which is further supported by the lack of inhibitory effect of PD 098059 on their contractions (Figures 2a and b).

Effects of PD 098059 on the release of mediators

Chopped lung preparations released histamine from undetectable level at resting condition to 318.9 ± 92.6 ng g^{-1} tissue ($n=4$) in response to 1 μg ml^{-1} OA challenge. On the other hand, low level of peptidoleukotrienes (<1 ng g^{-1} tissue, $n=4$) was released spontaneously from the lung fragments, and upon 1 μg ml^{-1} OA challenge, the release of peptidoleukotrienes was significantly increased by 34-fold (34.4 ± 8.5 ng g^{-1} tissue, $n=4$). PD 098059 failed to signifi-

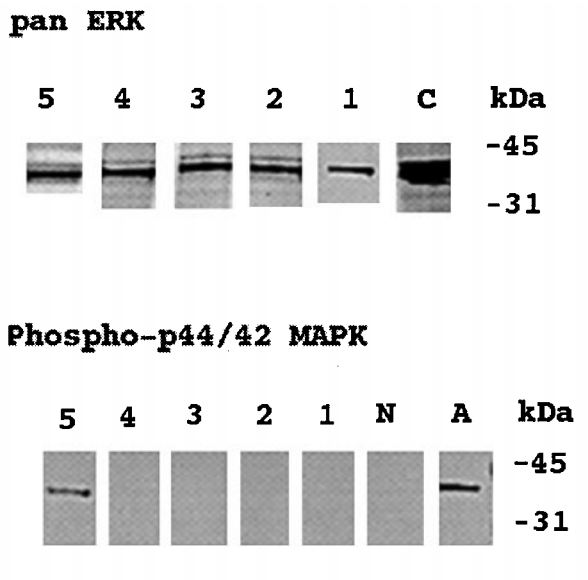

Figure 3 Immunoblots of tissue lysates extracted from bronchial rings that were prepared at their maximal contractions in response to 1 μg ml^{-1} OA (lane 2), 30 μM histamine (lane 3), or 0.1 μM LTD_4 (lane 4). Lane 1 represents untreated bronchial ring. Lane 5 represents positive control lysate prepared by stimulating U937 cells with 50 nM phorbol 12-myristate 13-acetate. C represents rat pituitary lysate provided by the manufacturer as positive control. A and N represent phosphorylated (activated) and nonphosphorylated MAPK controls respectively provided by the manufacturer. Lysates were probed with mouse pan ERK mAb (upper panel) or phospho-specific MAPK antibody (lower panel). Each immunoblot is representative of three experiments.

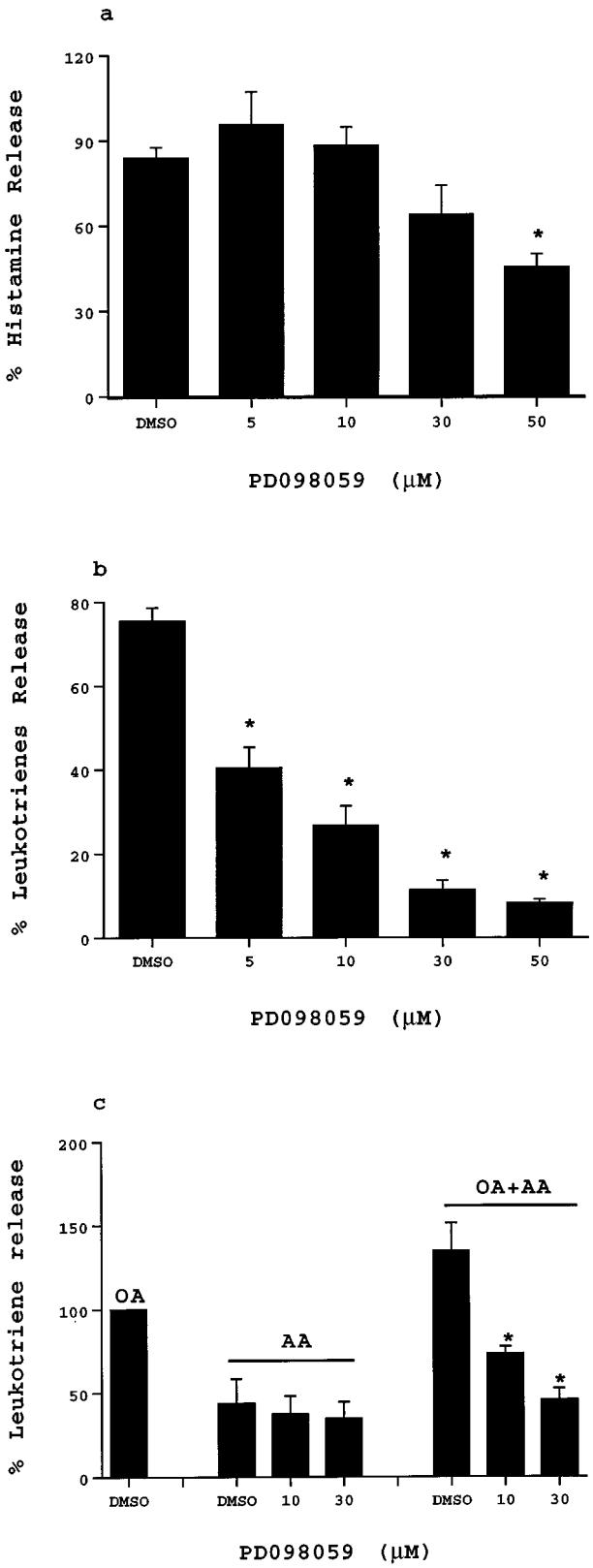

Figure 4 Effects of PD 098059 on the release of histamine (**a**) or peptidoleukotrienes in the absence (**b**) and presence (**c**) of 70 μM arachidonic acid (AA) from OA-stimulated guinea-pig chopped lung preparations. PD 098059 at various concentrations (5,10,30, and 50 μM) were incubated with the lung preparations in the presence of 4 μM indomethacin and 5 mM L-cysteine for 30 min before OA or combined OA and AA stimulation. Each point is the mean ± s.e.mean of three–five experiments. *Significant difference from DMSO vehicle controls, $P<0.05$.

cantly block OA-induced histamine release from lung fragments at concentrations of 5–30 μM. However, at 50 μM, PD 098059 elicited a 40% inhibition ($P<0.05$) from the lung fragments in response to OA (Figure 4a). In contrast, PD 098059 concentration-dependently inhibited OA-induced release of peptidoleukotrienes by 46, 65, 85 and 89% at 5 μM, 10 μM, 30 μM and 50 μM, respectively ($P<0.05$) (Figure 4b). Figure 4c shows that exogenous AA (70 μM) alone stimulated release of peptidoleukotrienes from lung fragments by $43.8\pm14.4\%$ ($n=4$) as compared to OA control (100%, $n=4$). PD 098059 (10 and 30 μM) failed to block AA-induced release of peptidoleukotrienes. This finding indicates that PD 098059 did not inhibit the conversion of AA to peptidoleukotrienes *via* 5-lipoxygenase. Addition of OA and AA together to lung fragments produced an additive effect on the release of peptidoleukotrienes ($134.6\pm16.8\%$, $n=4$). PD 098059 significantly reduced ($P<0.05$) the component of peptidoleukotriene release induced by OA in a concentration-dependent manner, leaving the residual peptidoleukotriene release induced by exogenous AA intact (Figure 4c).

Discussion

PD 098059 is believed to be a selective inhibitor of the activation of MAPKK by Raf-1 and is reported to have no significant direct inhibitory effect on other serine/threonine kinases such as $p42^{MAPK}$, JNK and myosin light chain kinase (Dudley *et al*., 1995; Alessi *et al*., 1995). It blocks the inactive (dephosphorylated) form of MAPKK in a non-competitive manner with respect to ATP-binding. By doing so, it prevents subsequent activation of $p42^{MAPK}$ and all the downstream signalling events. It has been established that MAPK signalling pathway is regulated by protein tyrosine kinases *via* the intermediate signalling molecule Raf-1 kinase in a variety of cell types including mast cell (Song *et al*., 1996). Aggregation of FcεRI and FcγR has been shown to induce protein tyrosine kinase activity and subsequently, tyrosine phosphorylation and activation of $p42^{MAPK}$ (Durden *et al*., 1995; Hirasawa *et al*., 1995; Rose *et al*., 1997).

Schultz-Dale reaction manifested in sensitized airways from both human and guinea-pig has been shown to be mediated by two major mast cell-derived mediators, namely, histamine, a preformed biogenic amine, and peptidoleukotrienes, biosynthetic metabolites of AA (Bjorck & Dahlen, 1993; Roquet *et al*., 1997). Histamine appears to be relatively more important as mediator during the initial peak phase of anaphylactic contraction, whereas peptidoleukotrienes appear to have a dominant role in maintaining the plateau phase of the contraction (Jonsson & Dahlen, 1994). Biochemical analysis of OA-induced release of histamine and LTC_4 from lung fragments showed similar characteristics with histamine level peaked in 3 min and LTC_4 level in 5–10 min after exposure to the antigen (Wong *et al*., 1992). The present finding shows that PD 098059 did not substantially inhibit the amplitude of OA-induced bronchial contraction with maximum inhibition of only 30% achieved at 50 μM, the highest concentration of the inhibitor used (Figure 1a). This observation corroborates well with the 40% attenuation ($P<0.05$) of OA-induced histamine release from chopped lung preparation at the same high concentration of PD 098059 while at lower concentrations, PD 098059 failed to substantially block the histamine release (Figure 4a). In line with these findings, Zhang *et al*. (1997) reported that PD 098059 failed to block the release of preformed mediator hexosaminidase from mast cells upon FcεRI aggregation. Partial inhibition ($<30\%$) of hexosaminidase release was achieved only at 50 μM PD 098059. These findings further confirm the fact that release of primary mediators such as histamine and hexosaminidase resulting from mast cell degranulation is not regulated by MAPK signalling pathway.

In contrast, OA-induced bronchial contraction relaxed markedly faster in PD 098059-pretreated bronchi in a concentration-dependent manner as compared to the control (Figure 1b). This increased relaxation rate is not secondary to the reduced peak OA-induced bronchial contraction since at 10 μM PD 098059, the peak contraction was not significantly lower than that of the control (Figure 1a). The rapid relaxation caused by PD 098059 might be due to either potential smooth muscle relaxant effect or inhibition of secondary lipid mediator release (i.e. peptidoleukotrienes) upon antigen challenge. Several lines of evidence demonstrated that vascular smooth muscle contraction induced by phenylephrine, KCl, histamine or serotonin was partly mediated through tyrosine phosphorylation and activation of $p42^{MAPK}$, and inhibition of MAPKK by PD 098059 attenuated the agonist-induced contraction (Katoch & Moreland, 1995; Jin *et al*., 1996; Watts, 1996). These results led to a generalization that MAPK pathway is activated in response to all forms of contractile stimulation (Katoch & Moreland, 1995). Our results reveal that $p42^{MAPK}$ was constitutively expressed in guinea-pig bronchi (Figure 3, upper panel). These findings are consistent with those reported by Pyne *et al*. (1996) that guinea-pig airway smooth muscle cells expressed $p42^{MAPK}$ under resting condition. However, our immunoblot studies show that bronchial smooth muscle contraction induced by either OA challenge, histamine or LTD_4 was not associated with activation of $p42^{MAPK}$ (Figure 3, lower panel). Phosphorylation of both tyrosine and threonine residues is required for $p42^{MAPK}$ activation (Cobb & Goldsmith, 1995). Under the same assay conditions, we observed that PMA activated $p42^{MAPK}$ in U937 cells (Figure 3, lower panel, lane 5), which is in line with the report by Franklin & Kraft. (1997). The phospho-specific MAPK antibody also selectively detected the phosphorylated (activated) MAPK in lane A (Figure 3, lower panel). These findings suggest that histamine- and LTD_4-induced bronchial contractions are not mediated through $p42^{MAPK}$ activation, which is further supported by the results that PD 098059 did not inhibit bronchial contraction induced by either of the mast cell-derived inflammatory mediators (Figures 2a and b). A recent study demonstrated that LTD_4-stimulated intracellular Ca^{++} increase in guinea-pig tracheal smooth muscle cells is mainly mediated through extracellular Ca^{++} influx through receptor-operated calcium channels (Dumitriu *et al*., 1997). Our finding indicates that PD 098059 did not affect the receptor-operated calcium channels stimulated by LTD_4. Furthermore, PD 098059 did not affect bronchial contraction induced by KCl (Figure 2c), suggesting that the rapid relaxation of OA-induced anaphylactic bronchial contraction by the MAPKK inhibitor is not mediated by other non-specific activity such as inhibition of voltage-dependent calcium channels.

Unexpectedly, histamine-induced bronchial contraction was significantly potentiated by PD 098059 (Figure 2a). Interaction of H_1-receptor with histamine stimulates phosphoinositide-specific phospholipase C (PLC) activity, which mediates the release of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers the release of intracellular Ca^{++}, which causes the transient airway smooth muscle contraction, and the receptor-mediated Ca^{++} influx contributes to the sustained contraction (Kotlikoff *et al*., 1987; Hoiting *et al*., 1996). On the other hand, DAG activates protein kinase C (PKC) which has been shown to negatively modulate the formation of IP_3 and

the release of intracellular Ca^{++} by a direct effect on PLC (Kotlikoff *et al*., 1987; Murray *et al*., 1989). The potentiation effect of PD 098059 on histamine-induced bronchial contraction might be due to certain 'cross-talk' between PLC signalling pathway and MAPK pathway or to non-specific activity of the inhibitor. In addition to blocking MAPKK activation by Raf, PD 098059 has also been found to enhance the activity of Raf (Alessi *et al*., 1995). It remains to be determined if Raf could regulate the activity of PLC or PKC.

On the other hand, PD 098059 markedly inhibited the release of peptidoleukotrienes from OA-challenged lung fragments in a concentration-dependent manner (Figure 4b). It has been demonstrated that cytosolic phospholipase A_2 ($cPLA_2$) can be phosphorylated and activated by $p42^{MAPK}$, leading to enhanced release of AA from the phospholipid membrane (Lin *et al*., 1993). In turn, the AA is converted to peptidoleukotrienes by the action of 5-lipoxygenase (Hay *et al*., 1995; Lepley & Fitzpatrick, 1996). Therefore, inhibition of the MAPK pathway is expected to result in a reduction in the biosynthesis of peptidoleukotrienes. Study from Zhang *et al*. (1997) also showed that PD 098059 dose-dependently inhibited AA release from mast cells upon FcεRI engagement. In addition, PD 098059 has recently been shown to block the translocation and activation of 5-lipoxygenase (5-LO) in HL-60 cells resulting in decreased 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) formation. However, PD 098059 did not directly inhibit the activity of recombinant purified 5-LO (Lepley & Fitzpatrick, 1996). In the present study, we also examined potential non-specific inhibitory effect of PD 098059 on peptidoleukotriene production such as inhibition of 5-lipoxygenase. Our results showed that PD 098059 did not block the exogenous AA-induced release of peptidoleukotrienes from lung fragments (Figure 4c) suggesting that the inhibitor does not have direct effect on 5-lipoxygenase activity. Therefore, our present finding of marked inhibition of OA-induced release of peptidoleukotrienes by PD 098059 is likely mediated through reduction in $cPLA_2$ activation *via* inhibition of $p42^{MAPK}$ signalling pathway. Furthermore, the substantial inhibition of release of peptidoleukotrienes by PD 098059 is likely linked to the rapid relaxation of the OA-induced anaphylactic bronchial contraction.

Aside from being potent bronchoconstrictors, peptidoleukotrienes are also responsible for other pathological processes that contribute to the pathogenesis of asthma, which include eosinophil chemotaxis, hypertrophy of mucosal glands and enhanced secretion of mucus, and proliferation of airway smooth muscle cells (Cohen *et al*., 1995; Hay *et al*., 1995). Our present study demonstrates that PD 098059 produced substantial inhibition of release of peptidoleukotrienes from sensitized lung fragments and rapid relaxation of anaphylactic bronchial contraction induced by OA. In addition, Zhang *et al*. (1997) showed that PD 098059 concentration-dependently inhibited tumour necrosis factor-α production from mast cell line upon FcεRI cross-linking. Taken together, these findings implicate that inhibition of MAPK signalling pathway in lung inflammatory cells (e.g. mast cells) may have therapeutic potential in the treatment of allergic diseases such as asthma.

The authors wish to thank Dr Yap Eu Hian for the generous gift of HRP-conjugated goat anti-rabbit antibody and Miss Li Hui for preparing the U937 cells. This work was supported by grant NMRC/0169/1996 of the National Medical Research Council of Singapore (W.S.F.W.)

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(Received December 12, 1997
Revised June 5, 1998
Accepted June 11, 1998)