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SPECIAL ARTICLE

Effects of Inhibitors of the Tyrosine Kinase Signaling Cascade on an *In Vitro* Model of Allergic Airways

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SUMMARY It has been shown that activation of protein tyrosine kinases (PTKs) is the earliest detectable signaling response to FcεRI cross-linking on mast cells. Following tyrosine kinase activation, a family of mitogen-activated protein kinases (MAPKs) was found to be activated as well. Activation of this PTK signaling cascade will lead to mast cell degranulation. This review summarizes our recent studies on the role of PTK signaling cascade in an *in vitro* guinea pig model of allergic asthma using PTK inhibitors, genistein and tyrphostin 47, and MAPK kinase inhibitor, PD098059. Inhibitors of the PTK and MAPK signaling pathways significantly attenuated the ovalbumin (OVA)-induced bronchial anaphylactic contraction and enhanced relaxation of constricted airways, respectively, and substantially blocked the release of histamine and peptidoleukotrienes from chopped lung preparations induced by OVA. Based upon their substantial inhibitory effects on the Schultz-Dale reaction, further examination on the potential anti-asthmatic effects of PTK cascade inhibitors in an *in vivo* model of allergic asthma is recommended.

Asthma is a chronic airway inflammatory disease characterized by airway hyperresponsiveness and recurrent reversible airway obstruction. The prevalence and mortality rate of asthma have been rising for the past decade despite the fact that our understanding of the pathogenesis of this airway disease has advanced substantially.[1] The mast cell has been implicated to play a pivotal role in asthma because mast cell degranulation induced by cross-linking of high-affinity Fc receptors (FcεRI) releases a wide array of inflammatory mediators such as histamine, leukotrienes and cytokines.[2] Recently, the precise molecular signaling pathways responsible for mast cell degranulation have been delineated (Fig. 1). Cumulating evidence obtained from a rat basophilic mast cell line (RBL-2H3) and bone marrow-derived mouse mast cells showed that activation of non-transmembrane protein tyrosine kinases (PTKs) is the earliest detectable signaling response to FcεRI cross-linking.[3] Specific PTKs such as src-related kinase Lyn,[4] 72-kDa Syk[5] and 77-kDa Btk[6] have been shown to be activated rapidly after FcεRI aggregation. This is followed by downstream signaling events such as activation of phospholipase Cγ (PLCγ), increase in inositol 1,4,5-trisphosphate (IP_3) and intracellular Ca^{++} levels, enhanced protein kinase C activity and activation of mitogen-activated protein kinase (MAPK), and eventually leads to mast cell degranulation.[7-10]

PTK inhibitors have been shown to block antigen-induced activation of PTKs, related downstream signaling events (eg. IP_3 production) and histamine release from mast cells.[11,12] On the other hand, MAPK kinase inhibitor was found to inhibit tumor necrosis factor-α production from a mast cell line upon FcεRI cross-linking.[13]

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Since mast cell degranulation is the hallmark of immediate-type hypersensitivity reaction occurring in asthma, it is essential to examine the potential anti-asthmatic effects of PTK inhibitors on an *in vitro* model of allergic asthma.

In vitro guinea pig model of allergic asthma

The Schultz-Dale reaction[14] has been used extensively to study anaphylactic contraction of airway tissue preparations such as trachea, bronchi and lung parenchymal strips.[15,16] In guinea pigs, both IgE and IgG are able to sensitize mast cells to specific antigen, and cross-linking of their corresponding Fcε RI and FcγR leads to mast cell degranulation.[17] FcεRI and FcγR are structurally and functionally related, and both belong to a family of multi-subunit antigen receptor.[18] It has been shown that engagement of these cell surface receptors activates PTKs and MAPK pathways for successful signal propagation and cellular activation.[13,18] Among a wide array of mast cell-derived inflammatory mediators such as thromboxane A_2, prostaglandin D_2 and platelet-activating factor, peptidoleukotrienes and histamine have been shown to be the major mediators responsible for the anaphylactic contraction of the airways.[19,20] A combination of histamine (H_1) receptor antagonist and peptidoleukotriene receptor antagonist has been shown to block substantially the anaphylactic contraction of airway tissue preparations from both human and guinea pig.[20] Recently, we have examined the effects of PTK inhibitors and MAPK kinase inhibitor on antigen-induced bronchial smooth muscle contraction and release of inflammatory mediators from sensitized lung fragments.[21,22]

Inhibitors of PTK cascade on anaphylactic airway contraction

We passively sensitized guinea pigs with IgG raised against ovalbumin (OVA) and studied the effects of two PTK inhibitors, genistein[23] and tyrphostin 47,[24] and a MAPK kinase inhibitor, PD098059[25] on OVA-induced anaphylactic contraction of isolated guinea pig bronchi. Genistein and tyrphostin 47 are structurally and functionally unrelated inhibitors of PTK. Genistein is an isoflavone compound that inhibits PTK activity of the epidermal growth factor receptor and $pp60^{v\text{-}src}$ via competitive inhibition at the ATP-binding domain of the kinases.[23] Tyrphostin 47 is a derivative of the dihydroxybenzylidene malononitrile classs of PTK inhibitors that acts by competitive inhibition at the substrate site of the kinase.[24] Their relative potencies against non-transmembrane PTKs (eg. Syk, Lyn, and Btk) remain to be determined. PD098059 is a selective inhibitor of the activation of MAPK kinase by Raf-1 and blocks the inactive (dephosphorylated) form of the MAPK kinase in a non-competitive manner with respect to ATP-binding.[25] By doing so, it prevents subsequent activation of $p42^{MAPK}$ and all the downstream signaling events.

OVA-induced anaphylactic contraction of the guinea pig bronchi was significantly inhibited by either genistein or tyrphostin 47 in a concentration-dependent manner (Fig. 2). At 50 μM, both genistein and tyrphostin 47 markedly suppressed bronchial anaphylactic contraction by at least 70%. In contrast, 50 μM daidzein, an analogue of genistein that has no inhibitory activity on PTK,[23] failed to alter the anaphylactic contraction. These findings are consistent with those reported by Iwagoe *et al.*[26] that herbimycin A and genistein, two PTK inhibitors, significantly inhibited antigen-induced guinea pig tracheal contraction *in vitro*. At the concentration that significantly inhibited OVA-induced contraction, neither genistein nor tryphostin 47 altered histamine-induced bronchial contraction; whereas only genistein significantly suppressed leukotriene D_4 (LTD_4)-induced bronchial contraction (data not shown).[21] The smooth muscle relaxant effect of genistein might be due to inhibition of voltage-operated calcium channel currents as demonstrated in vascular smooth muscle.[27] However, we still do not know whether the inhibition of calcium channel is a direct blockade by genistein or a result of PTK inactivation. These findings indicate that tyrphostin 47 inhibited OVA-induced bronchial contraction mainly via stabilization of mast cell while the inhibitory effect of genistein is partly mediated by mast cell stabilization and partly through smooth muscle relaxation.

In contrast, PD098059 did not substantially inhibit the amplitude of OVA-induced bronchial contraction with maximum inhibition of only 30% achieved at 50 μM (Fig. 3a). However, the OVA-induced bronchial contraction relaxed markedly faster in PD098059-pretreated bronchi in a concentration-dependent manner (Fig. 3b). The rapid relaxation caused by PD098059 might be due to either potential smooth muscle relaxant effect or inhibition of secondary lipid mediator release (eg. peptido-

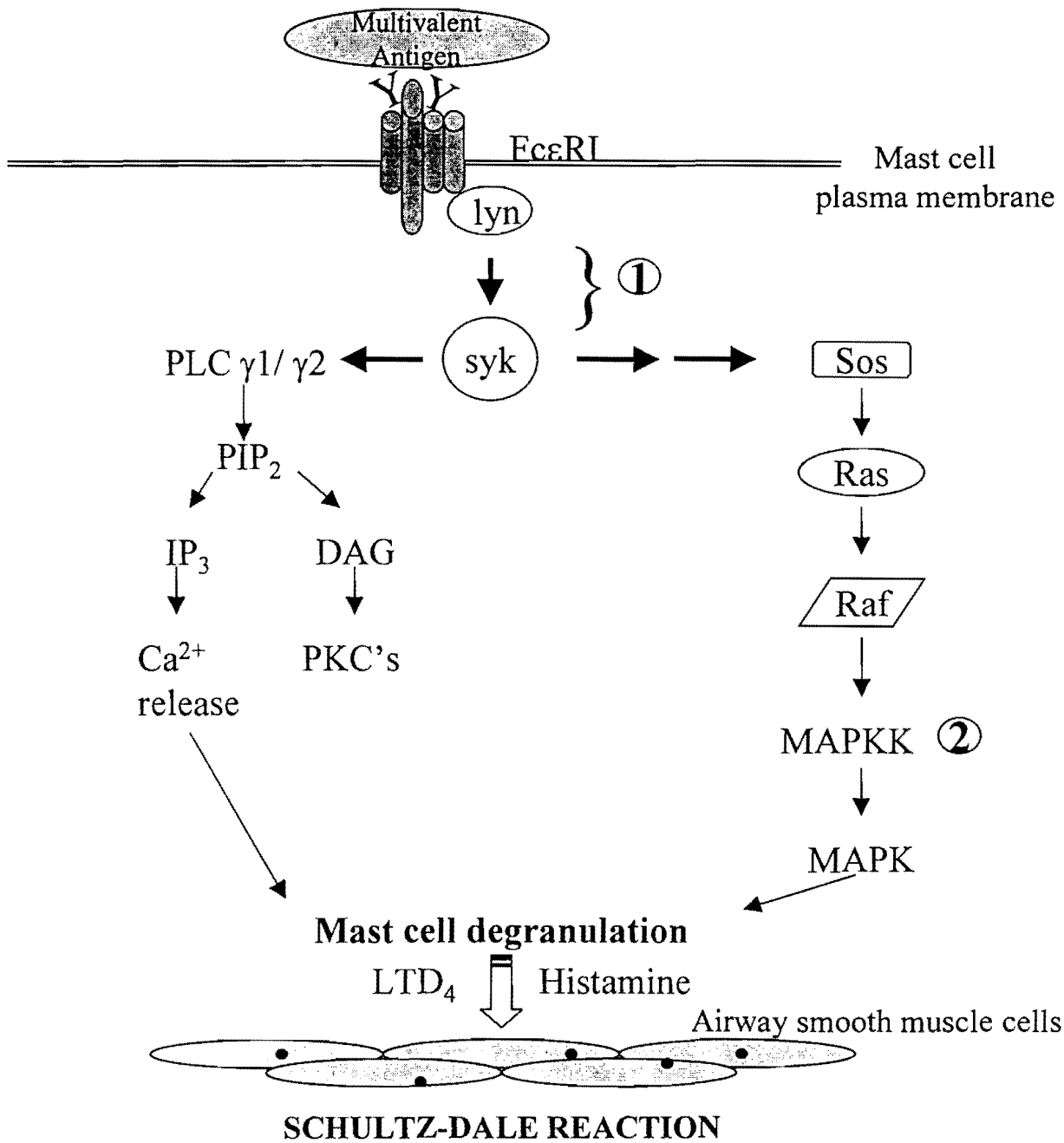

Fig. 1 Schematic diagram showing the tyrosine kinase signaling cascade in mediating Schultz-Dale reaction. Upon cross-linking of high affinity FcεRI, specific non-transmembrane protein tyrosine kinases such as Lyn and Syk are activated. This is followed by downstream signaling events such as activation of phospholipase Cγ (PLCγ), increase in inositol 1,4,5-trisphosphate (IP_3) and intracellular Ca^{++} levels, enhanced protein kinase C activity and activation of mitogen-activated protein kinase (MAPK), and eventually leads to mast cell degranulation. 1, inhibition of tyrosine kinases by genistein or tyrphostin 47; 2, inhibition of MAPK kinase by PD 098059.

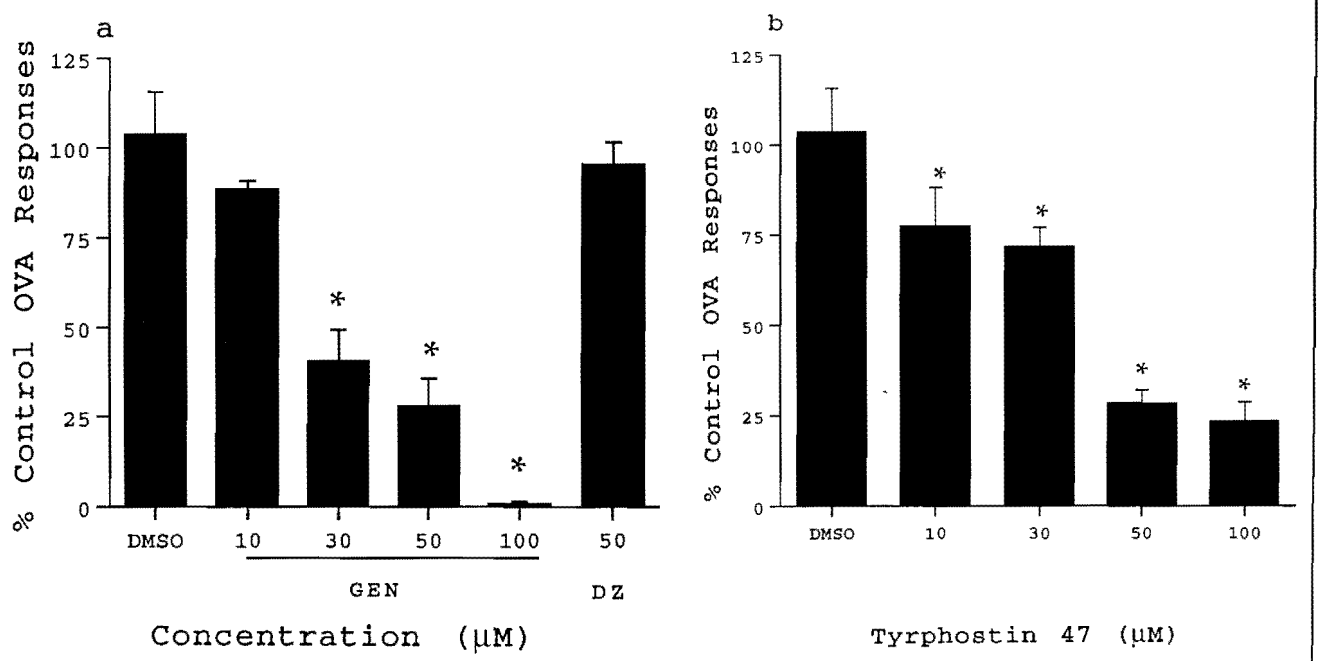

Fig. 2 Effects of genistein (GEN)/daidzein (DZ) (panel a) and tyrphostin 47 (panel b) on OVA-induced anaphylactic contraction of guinea pig bronchi. Bronchial rings were incubated with indicated concentrations of inhibitors or same volume of DMSO for 30 minutes prior to 1 μg/ml OVA challenge. Each point represent the mean ± S.E.M. for 3-10 experiments. *Significant differences from DMSO vehicle controls, $P < 0.05$. (Reproduced from Wong *et al.*[21] with permission of the American Society of Pharmacology and Experimental Therapeutics, copyright 1997)

leukotrienes) 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 MAPK kinase by PD098059 attenuated the agonist-induced contraction.[28-30] However, our results showed that PD098059 did not inhibit bronchial contraction induced by KCl, histamine or LTD_4, and these inflammatory mediators failed to activate $p42^{MAPK}$ in the bronchi (data not shown).[22] In fact, we observed that PD098059 concentration-dependently enhanced histamine-induced bronchial contraction. The potentiation effect of PD098059 might be due to certain "cross-talk" between phospholipase C signaling pathway and MAPK pathway or to non-specific activity of the inhibitor. These findings suggest that PD098059-mediated rapid relaxation of OVA-induced bronchial contraction is likely associated with inhibition of secondary lipid mediator release.

Inhibitors of PTK cascade on the release of inflammatory mediators

Guinea pig chopped lung preparations released low levels of histamine and peptidoleukotrienes spontaneously. Upon OVA challenge, the release of histamine and that of peptidoleukotrienes from lung fragments were dramatically increased by about 25-folds and 60-folds, respectively.[21] Both genistein and tyrphostin 47 concentration-dependently inhibited OVA-induced release of histamine and peptidoleukotrienes (Fig. 4). At 50 μM, genistein attenuated the release of histamine and peptidoleukotrienes by 54% and 58%, respectively; whereas, tyrphostin 47 inhibited the release by 41% and 92%, respectively. In addition, Iwagoe *et al.*[26] have also reported that both herbimycin A and genistein

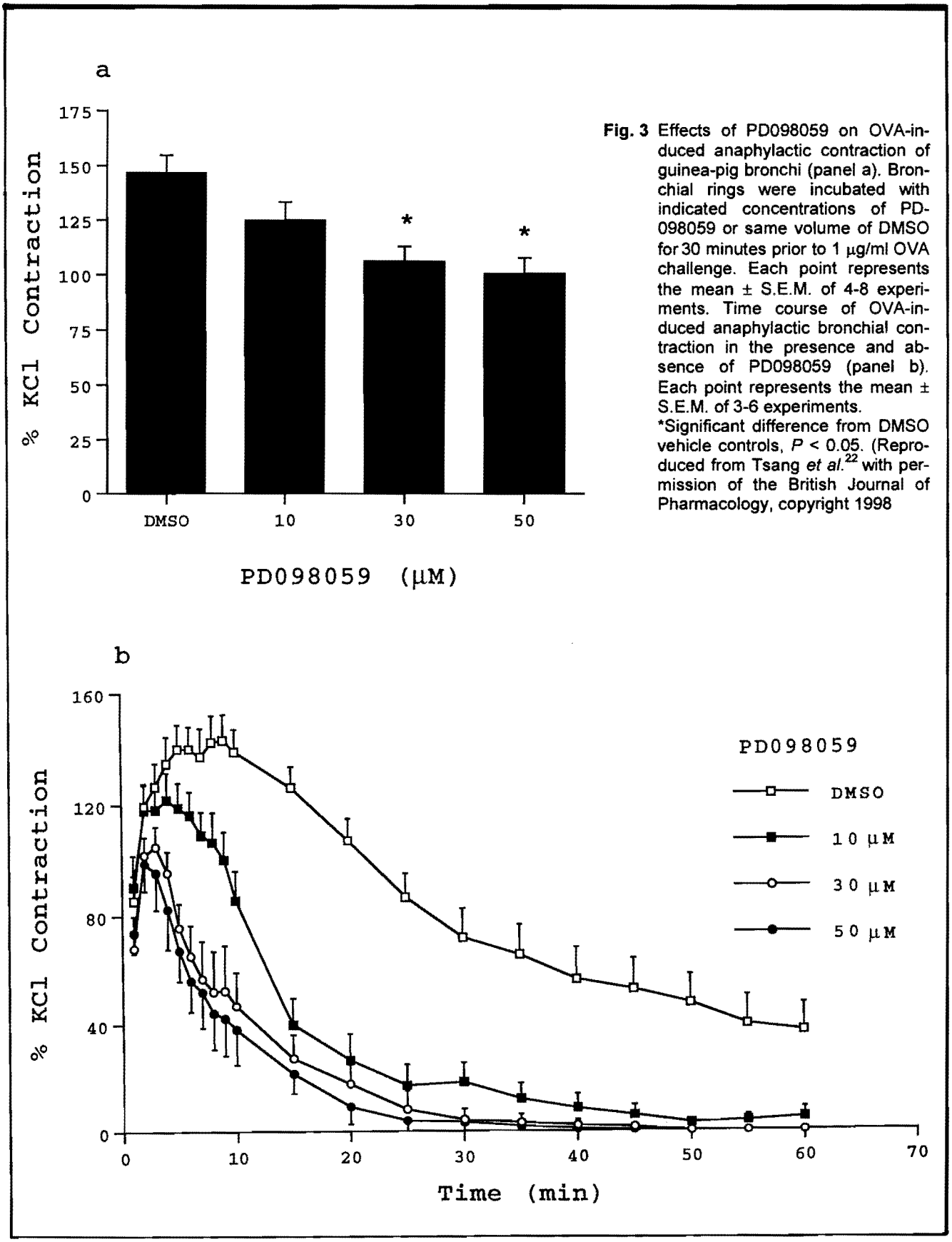

Fig. 3 Effects of PD098059 on OVA-induced anaphylactic contraction of guinea-pig bronchi (panel a). Bronchial rings were incubated with indicated concentrations of PD-098059 or same volume of DMSO for 30 minutes prior to 1 μg/ml OVA challenge. Each point represents the mean ± S.E.M. of 4-8 experiments. Time course of OVA-induced anaphylactic bronchial contraction in the presence and absence of PD098059 (panel b). Each point represents the mean ± S.E.M. of 3-6 experiments.
*Significant difference from DMSO vehicle controls, $P < 0.05$. (Reproduced from Tsang *et al.*[22] with permission of the British Journal of Pharmacology, copyright 1998

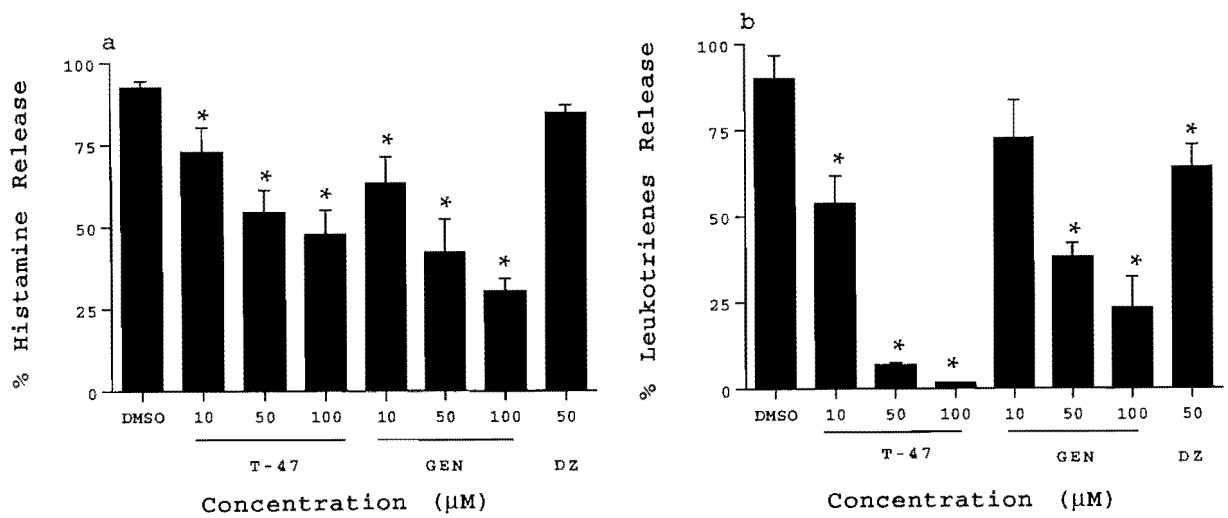

Fig. 4 Effects of tyrphostin 47 (T-47), genistein (GEN) and daidzein (DZ) on the release of histamine (panel a) or peptidoleukotrienes (panel b) from OVA-stimulated guinea pig chopped lung preparations. Inhibitors at various concentrations were incubated with the lung preparations in the presence of 4 μM indomethacin and/or 5 mM L-cysteine for 30 minutes before OVA stimulation. Each point is the mean ± S.E.M. of 3-7 experiments. *Significant differences from DMSO vehicle controls, $P < 0.05$. (Reproduced from Wong *et al.*[21] with permission of the American Society of Pharmacology and Experimental Therapeutics, copyright 1997)

concentration-dependently inhibited histamine release from guinea pig tracheal preparations. Several lines of evidence showed that genistein, tyrphostin 47 and other PTK inhibitors blocked anti-IgE-induced histamine release from rodent and human lung mast cells.[11,31,32] Since it has been shown that histamine and peptidoleukotrienes are critical for anaphylactic contraction of the airways,[20] marked inhibition of the release of both histamine and peptidoleuktrienes by genistein or tyrphostin 47 may be responsible for the inhibition of OVA-induced bronchial contraction.

In contrast, PD098059 failed to substantially block OVA-induced histamine release from lung fragments at lower concentrations. At 50 μM, PD098059 elicited a 40% inhibition of histamine release from the lung fragments in response to OVA (Fig. 5a). The extent of inhibition of histamine release produced by PD098059 resembles that by tyrphostin 47. However, PD098059 could only demonstrate 30% inhibition of OVA-induced bronchial anaphylactic contraction as compared to 70% attenuation produced by tyrphostin 47. This can be explained by the fact that PD098059, but not tyrphostin 47, significantly potentiated histamine-induced bronchial contraction.

On the other hand, PD098059 markedly inhibited the release of peptidoleukotrienes from OVA-challenged lung fragments in a concentration-dependent manner (Fig. 5b). It has been demonstrated that cytosolic phospholipase A_2 ($cPLA_2$) can be phosphorylated and activated by p42MAPK, leading to enhanced release of arachidonic acid (AA) from the phospholipid membrane.[33] In turn, the AA is converted to peptidoleukotrienes by the action of 5-lipooxygenase (5-LO).[34] Therefore, inhibition of the MAPK pathway is expected to result in a reduction in the biosynthesis of peptidoleukotrienes. Study from Zhang *et al.*[13] showed that PD098059 concentration-dependently inhibited AA release from mast cells upon FcεRI engagement. We have also showed that PD098059 did not block the exogenous AA-induced release of peptidoleukotrienes from lung fragments (Fig. 5c) suggesting that the inhibitor does not have direct effect on 5-LO activity. This is consistent with another study reporting that PD098059 does not have direct inhibitory effect on recombinant

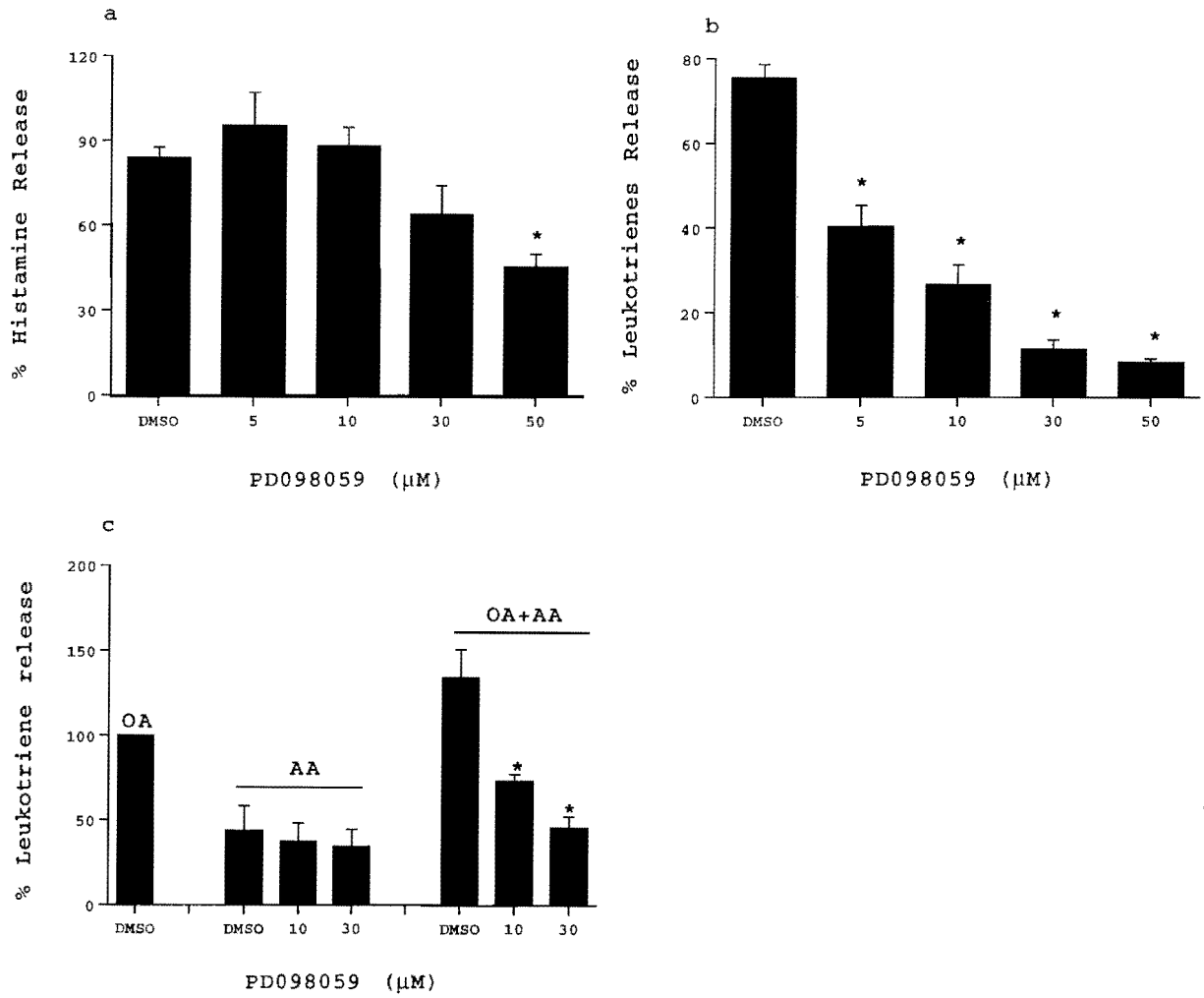

Fig. 5 Effects of PD098059 on the release of histamine (panel a) or peptidoleukotrienes in the absence (panel b) and presence (panel c) of 70 μM arachidonic acid (AA) from OVA-stimulated guinea pig chopped lung preparations. PD098059 at various concentrations were incubated with the lung preparations in the presence of 4 μM indomethacin and/or 5 mM L-cysteine for 30 minutes before OVA or combined OVA and AA stimulation. Each point is the mean ± S.E.M. of 3-5 experiments. *Significant difference from DMSO vehicle controls, $P < 0.05$. (Reproduced from Tsang *et al.*[22] with permission of the British Journal of Pharmacology, copyright 1998)

purified 5-LO.[34] Therefore, our finding of marked inhibition of OVA-induced release of peptidoleukotrienes by PD098059 is likely mediated through reduction of cPLA2 activation via inhibition of p42MAPK signaling pathway. Furthermore, the substantial inhibition of the release of peptidoleukotrienes by PD098059 is likely linked to the rapid relaxation of the OVA-induced anaphylactic bronchial contraction.

Conclusions

Our findings suggest that PTK and MAPK are involved in the *in vitro* model of allergic asthma and the inhibitors of PTK and MAPK kinase interrupt the early signaling pathway of mast cell activation and, therefore, attenuate the anaphylactic bronchial contraction or facilitate relaxation of constricted airways by preventing the release of mast cell-derived inflam-

matory mediators such as histamine and peptidoleukotrienes. To further explore the therapeutic potential of inhibitors of the PTK signal cascade for the treatment of asthma, examination of this class of inhibitors in an *in vivo* model of allergic asthma is recommended.

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