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Inhibitors of the tyrosine kinase signaling cascade attenuated thrombin-induced guinea pig airway smooth muscle cell proliferation

Fai Tsang, Hui Hwa Choo, Gavin S. Dawe, and W.S. Fred Wong*

Department of Pharmacology, Faculty of Medicine, National University of Singapore, MD2 18 Medical Drive, Singapore 119260, Singapore

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Abstract

Airway remodeling is one of the major hallmarks of asthma. The present study examined the effects of tyrosine kinase inhibitors on thrombin-induced guinea pig ASM cell proliferation, in comparison with inhibitors of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K). The ASM cells expressed smooth muscle α-actin and myosin, and responded to thrombin by increasing cytosolic Ca^{+2}. Thrombin (1–10 U/ml) induced [^{3}H]thymidine incorporation into ASM cells. Tyrphostin 47, a broad-spectrum tyrosine kinase inhibitor, PP2, a Src-specific inhibitor, and piceatannol, a Syk-selective inhibitor, significantly attenuated thrombin-induced [^{3}H]thymidine incorporation. In addition, the tyrosine kinase inhibitors significantly reduced thrombin-induced cyclin D_1 expression in ASM cells. PD098059 and U0126, two MAPK kinase inhibitors, and LY294002, a PI3K inhibitor, significantly blocked thrombin-induced [^{3}H]thymidine incorporation and cyclin D_1 expression in ASM cells. Our data show that inhibitors of Src and, probably Syk, can modulate thrombin-induced ASM cell proliferation, which may have therapeutic potential for asthma.

Keywords: Src; Syk; Mitogen-activated protein kinase; Phosphatidylinositol 3-kinase; Cyclin D_1; Focal adhesion kinase; [^{3}H]Thymidine

ASM cell hyperplasia and hypertrophy have been observed in asthmatics [1]. Aside from epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), thrombin has been reported to increase ASM cell proliferation. It is both a mitogen and a contractile agonist, it is abundant in bronchoalveolar lavage fluid and in plasma exudates at the sites of inflammation, and its receptor family has recently been identified [2–4].

Thrombin, a serine protease, binds to a novel family of G-protein-coupled receptors (GPCRs) termed as protease-activated receptors (PARs). Among the four PARs identified, thrombin can activate PAR1, PAR3, and PAR4 by cleaving the amino-terminal extracellular domain at a specific site of each receptor. Proteolytic cleavage unmasks a new receptor amino terminus that acts as a tethered ligand specific for the receptor subtype to elicit transmembrane signaling and biological responses [4]. It has been shown that p42/p44 mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol 3-kinase (PI3K) pathway play a major role in thrombin-induced ASM cell proliferation [3]. Activation of p42/p44 MAPK enhances activities of nuclear transcription factors Elk-1 and activator protein-1, and expression of cyclin D_1 which is important for initiation of DNA synthesis (S phase) of the cell cycle in ASM cells [3,5,6]. On the other hand, thrombin-induced activation of PI3K in ASM cells has been shown to increase the activity of 70 kDa S6 protein kinase ($p70^{S6k}$), a critical enzyme for translational control of mRNA transcripts during the G_1 phase of the cell cycle [7,8].

Since GPCRs do not have intrinsic tyrosine kinase activity, it is believed that thrombin-induced PAR activation recruits and activates tyrosine kinases, leading to mitogenic responses of ASM cells. While the role of MAPK and PI3K in thrombin-induced ASM cell proliferation has been confirmed, the upstream tyrosine kinases that relay the activation of PARs to the downstream MAPK and PI3K signaling in ASM cells remain undefined. The purpose of the present study was to

* Corresponding author. Fax: +65-6873-7690.
E-mail address: phcwongf@nus.edu.sg (W.S. Fred Wong).

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compare the effects of inhibitors of tyrosine kinases to those of inhibitors of MAPK and PI3K on thrombin-induced ASM cell proliferation. Our results show that tyrphostin 47, PP2, and piceatannol significantly blocked [^{3}H]thymidine incorporation into guinea pig ASM cells and cyclin D_1 expression. These findings implicate that thrombin activates Src and, probably Syk, to stimulate MAPK and PI3K signaling pathways and ASM proliferation.

Materials and methods

Materials. The following drugs and chemicals were used: bovine serum albumin (BSA), collagenase (type II), elastase (type IV), soybean trypsin inhibitor, fluorescein isothiocyanate (FITC)-conjugated mouse anti-α-smooth muscle actin antibody, alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody, tyrphostin 47 and piceatannol (Sigma); mouse anti-cyclin D_1 antibody (BD PharmMingen); Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), penicillin, streptomycin, fetal bovine serum (FBS), trypan blue, and trypsin (0.05%)-EDTA (0.53 mM) (Life Technologies); mouse anti-human smooth muscle-specific myosin heavy chain antibody and FITC-conjugated rabbit anti-mouse immunoglobulins (DAKO); human thrombin, PP2, PD098059, U0126, LY294002, and Fluo-3/AM (Calbiochem); and [*methyl*-^{3}H]thymidine (2 Ci/mmol) (NEN Life Science). Thrombin was dissolved in sterile PBS and all inhibitors in DMSO, and were stored at −20 °C.

Guinea pig ASM cell culture. Male Hartley guinea pigs (Interfauna, East Yorkshire, England) weighing 350–450 g were sacrificed by cervical dislocation. Trachealis muscle was isolated and then enzymatically digested in 2 ml of DMEM containing 1 mg/ml collagenase (type II), 0.2 mg/ml elastase (type IV), 50 μg/ml soybean trypsin inhibitor, 0.53 mM EDTA, 200 U/ml penicillin, and 0.2 mg/ml streptomycin, for 2 h in a shaking water bath at 37 °C. The dissociated ASM cells were grown in culture flasks in DMEM supplemented with 10% FBS, 200 U/ml penicillin, and 0.2 mg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells between passages 2 and 7 were used for experiments.

Characterization of guinea pig ASM cells. To confirm the expression of native contractile proteins in the primary ASM cells, immunocytochemical identification of smooth muscle α-actin and myosin was performed. ASM cells were grown on glass coverslips and were fixed and permeabilized in methanol/acetone (1:1) at −20 °C for 10 min. FITC-conjugated mouse anti-human α-smooth muscle actin antibody (1:250 dilution) or mouse anti-human smooth muscle myosin (1:50 dilution) was incubated with the cells for 2 h. Coverslips were then mounted on glass slides and examined using a confocal laser scanning microscope system (Olympus) with excitation wavelength at 488 nm and emission range at 510–550 nm. In order to confirm that the primary ASM cells retain functional cell-excitation coupling systems, thrombin-induced changes in cytosolic Ca^{+2} was determined [9]. ASM cells were grown on a chambered coverglass and were loaded with intracellular Ca^{+2} indicator dye Fluo-3/AM (4 μM) for 30 min at 37 °C in 2 ml of Ca^{+2}-free DMEM supplemented with 10% FBS. The medium was then replaced with modified Kreb's buffer (pH 7.4) 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; $MgSO_4 \cdot 7H_2O$, 1.2, HEPES, 10; and dextrose, 10.0. ASM cells were stimulated with thrombin (3 U/ml) and the changes in cytosolic Ca^{+2} were measured using a Leica SP2 laser scanning confocal microscope (Leica Microsystems) with excitation wavelength at 488 nm and emission at 500–535 nm.

Proliferation assay. DNA synthesis in ASM cells was examined using [*methyl*-^{3}H]thymidine incorporation as indicator of ASM cell proliferation [3,5]. ASM cells were grown to near confluence in 24-well plates in DMEM supplemented with 10% FBS with an initial seeding density of 10^4 cells/well. Cells were then growth-arrested in serum-free DMEM for 48 h to synchronize cells in the G_0/G_1 phase of the cell cycle. At this stage, cellular uptake of [^{3}H]thymidine is minimal [3]. After starvation, cells were stimulated with thrombin (1–10 U/ml) for a total of 24 h. Cells were pulsed with [^{3}H]thymidine (1 μCi/ml) during the last 4 h of the 24-h incubation. At the end of the incubation period, nucleoproteins were precipitated with ice-cold trichloroacetic acid (10% v/v) for 30 min and then solubilized in 0.2 N NaOH (500 μl/well) overnight at room temperature. On the following day, the contents in each well were neutralized with 500 μl of 0.2 M HCl before being mixed with 4.5 ml of biodegradable counting scintillant (Amersham). Radioactivity was determined by liquid scintillation spectrometry (Beckman LS 3801). Effects of inhibitors of the tyrosine kinase signaling cascade on [^{3}H]thymidine incorporation were examined using the same protocol. The growth-arrested cells were pretreated with tyrphostin 47, piceatannol, PP2, PD 098059, U0126, or LY294002 (1, 3, and 10 μM) or DMSO for 30 min before being stimulated by 3 U/ml thrombin.

Cell viability and counting. To examine potential cytotoxicity of the inhibitors used in this study on ASM cells, cell number and viability were examined using haematocytometry and trypan blue staining. ASM cells were grown to near confluence, growth-arrested, and treated with inhibitors or DMSO for 24 h. Cells were dissociated with trypsin-EDTA and counted using a hematocytometer. Cell number was expressed as cells/well and cell viability was expressed as percent viable cells/well.

Cyclin D_1 expression. Guinea pig ASM cells were grown to near confluence and growth-arrested for 48 h in serum-free DMEM. At the end of starvation, cells were pretreated with various inhibitors (10 μM each) or DMSO for 30 min before stimulation with thrombin (3 U/ml) for 24 h. Cells were then lysed in ice-cold lysis buffer (Tris–HCl, pH 7.4, 50 mM; Triton X-100, 1%; NaCl, 150 mM; SDS, 0.1%; Na_3VO_4, 2 mM; NaF, 1 mM; PMSF, 5 mM; aprotinin, 10 μg/ml; and leupeptin, 10 μg/ml) on ice for 30 min. Proteins (10 μg per lane) were separated by SDS–PAGE in a 4–20% gradient polyacrylamide gel and then electrotransferred to a PVDF membrane (Bio-Rad). The membrane was probed with mouse anti-cyclin D_1 antibody and then with AP-conjugated goat anti-mouse IgG antibody. The blot was visualized using 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) (Life Technologies) and quantitated using Gel-Pro imaging software (Media Cybernetic).

Statistical analysis. All data are presented as means ± SEM. Statistical differences were analyzed using ANOVA followed by Student–Newman–Keuls test. The critical level for significance was set as $P < 0.05$.

Results

Characterization of ASM cells

Confluent airway smooth muscle cells aligned in parallel giving a characteristic "hill and valley" appearance. Immuncytochemical staining of guinea pig ASM cells with anti-smooth muscle α-actin antibody and anti-myosin antibody revealed filamentous contractile proteins arranged in parallel to the long axis of the cells (Fig. 1A and B). To confirm that the primary ASM cells retain functional cell-excitation coupling systems, thrombin-induced changes in cytosolic Ca^{+2} evaluated. Fig. 1C–E reveal the increase in cytosolic Ca^{+2} in a single ASM cell induced by 3 U/ml thrombin.

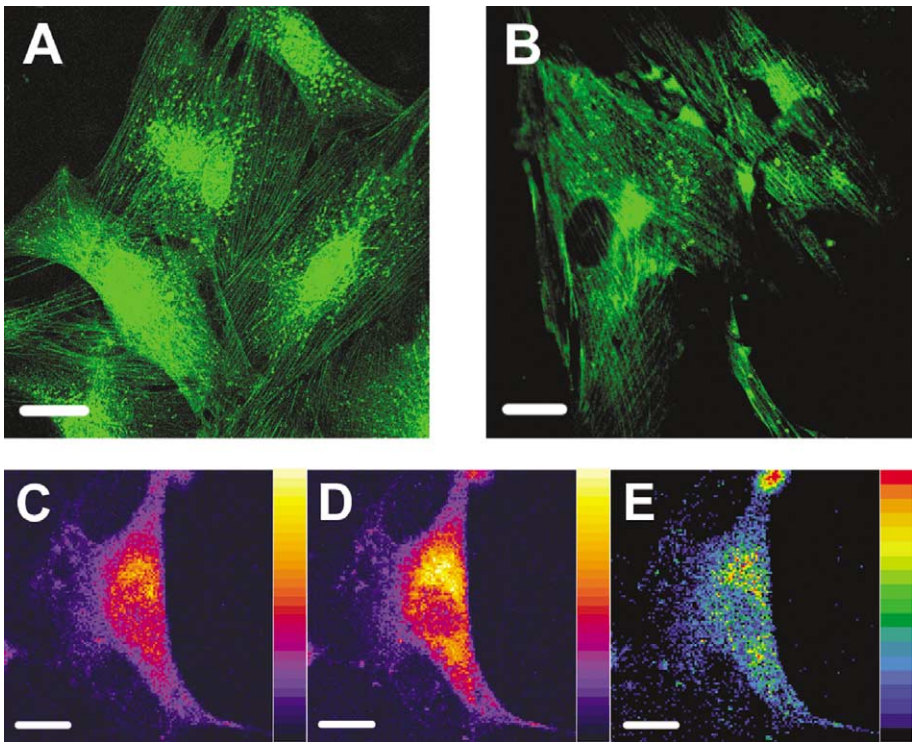

Fig. 1. Characterization of primary guinea pig ASM cell culture. Expression of smooth muscle α-actin (A) and smooth muscle-specific myosin heavy chain (B) was visualized using an Olympus confocal microscope system. ASM cells loaded with the Ca^{2+}-sensitive dye Fluo-3/AM responded to 3 U/ml thrombin, indicating that the ASM cells retained a functional cell-excitation coupling system. The response of a typical cell is illustrated by images from before (C) and after (D) application of thrombin. Each image is the average of five frames captured at a rate of one frame every 669 ms. The Ca^{+2} flux is illustrated by a pseudo-colored subtraction of the images captured before and after thrombin application (E). Scale bars represent 20 μm.

Effects of inhibitors on thrombin-induced [^{3}H]thymidine incorporation

The basal level of [^{3}H]thymidine incorporation into ASM cells was 1173 ± 358 cpm ($n = 9$) and was taken as 100%. Thrombin (1, 3, and 10 U/ml) increased [^{3}H]thymidine incorporation by 2.5-, 3.6-, and 6.3-folds, respectively (Fig. 2). In all subsequent inhibitor studies, 3 U/ml thrombin was used. Thrombin-induced [^{3}H]thymidine incorporation was markedly reduced by all three tyrosine kinase inhibitors in a concentration-dependent manner (Fig. 3A). Tyrphostin 47, a broad-spectrum tyrosine kinase inhibitor [10], at 1, 3, and 10 μM, inhibited [^{3}H]thymidine incorporation by 0%, 13.8%, and 29.4%, respectively. PP2, a Src-specific tyrosine kinase inhibitor [11], significantly reduced [^{3}H]thymidine incorporation by 13.9%, 31.2%, and 43.8% at the same increasing concentrations. Piceatannol, a Syk-selective tyrosine kinase inhibitor [12,13], suppressed uptake of [^{3}H]thymidine by 30.5%, 68.4%, and 76% at the same increasing concentrations. In comparison, PD098059 and U0126, two MAPK kinase inhibitors [14,15], at 1, 3, and 10 μM, also significantly inhibited [^{3}H]thymidine incorporation by 27.7%, 41.2%, and 55.1%, and by 50.1%, 69.8%, and 82.4%, respectively (Fig. 3B). LY294002, a PI3K inhibitor [16], markedly reduced [^{3}H]thymidine incorporation by 5.6%, 22.8%, and 50.9% at the same increasing concentrations (Fig. 3B).

Effects of inhibitors on cell viability and number

To rule out the possibility that the decrease in DNA synthesis in response to inhibitors is a result of cytotoxicity, assessments of cell number and cell viability were carried out using trypan blue stain (0.4%). After a 24-h stimulation with 3 U/ml thrombin in the presence and absence of various inhibitors, the ASM cell numbers in different treatment groups were the same as in the untreated group (Fig. 4A). None of the inhibitors of tyrosine kinase signaling cascade exhibited cytotoxicity towards ASM cells (Fig. 4B).

Effects of inhibitors on cyclin D_1 expression

Expression of cyclin D_1 in ASM cells was significantly increased by 3 U/ml thrombin. Tyrphostin 47, PP2, and piceatannol significantly inhibited thrombin-induced cyclin D_1 expression in ASM cells by 35.5%, 63%, and 62%, respectively (Fig. 5). PD 098059 and U0126 also markedly suppressed thrombin-induced cyclin D_1 expression by 52.6% and 71.0%, respectively. LY 294002

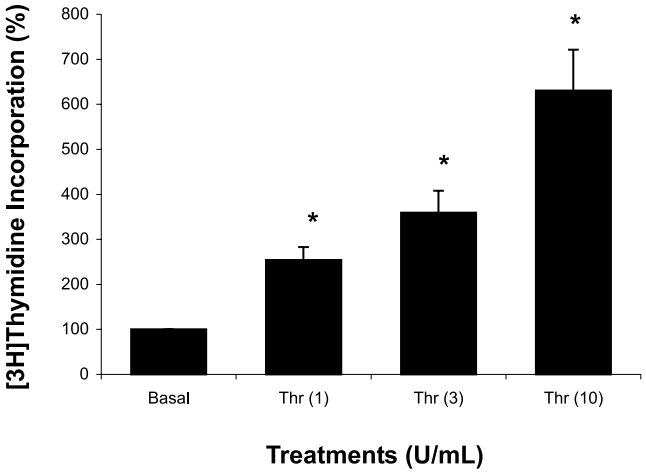

Fig. 2. Concentration-dependent effects of thrombin on guinea pig ASM cell proliferation. ASM cells were starved and then treated with the indicated concentrations of thrombin for 24 h. [^{3}H]Thymidine (1 μCi/ml) was added to the ASM cells during the last 4 h of incubation. [^{3}H]Thymidine incorporation assays were performed to assess thrombin-induced ASM cell proliferation. Each point represents the mean ± SEM of three experiments. *Significant difference from untreated control, $P < 0.05$.

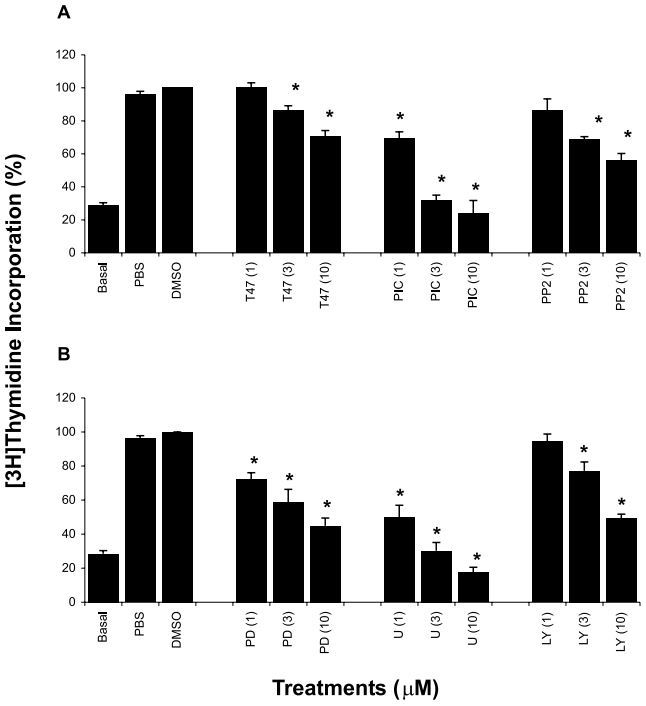

Fig. 3. Effects of (A) tyrosine kinase inhibitors and (B) MAPK kinase and PI3K inhibitors on thrombin-induced [^{3}H]thymidine incorporation into guinea pig ASM cells. Cells were growth-arrested for 24 h and inhibitors at the indicated concentrations (1–10 μM) were preincubated with the ASM cells 30 min before thrombin stimulation for another 24 h. [^{3}H]Thymidine (1 μCi/ml) was added during the last 4 h of incubation. [^{3}H]Thymidine incorporation assays were performed to assess thrombin-induced ASM cell proliferation in the presence of inhibitors. Each point represents the mean ± SEM of 4–6 experiments. *Significant difference from DMSO controls, $P < 0.05$. T47, tyrphostin 47; PIC, piceatannol; PD, PD098059; U, U0126; LY, LY294002.

blocked thrombin-stimulated cyclin D_1 expression by 60% (Fig. 5).

Discussion

Airway remodeling is one of the major pathological hallmarks of asthma manifested as airway hyperresponsiveness to histamine and methacholine. Thrombin, activated at the sites of inflammation and abundant in BAL fluid, is both a contractile agonist and a mitogen of ASM cells. Several lines of evidence demonstrate the significance of p42/p44 MAPK and PI3K pathways in mediating thrombin-induced ASM cell proliferation [3,5–8]. Nevertheless, the mechanism linking thrombin-induced PAR receptor activation to MAPK and PI3K activation is still not completely characterized. Because thrombin-induced ASM cell mitogenesis requires protein tyrosine phosphorylation, the present study explored the effects of several tyrosine kinase inhibitors on thrombin-induced guinea pig ASM cell proliferation in comparison with inhibitors of MAPK and PI3K.

At near confluence, the primary guinea pig ASM cell culture expressed both smooth muscle α-actin and smooth muscle-specific myosin heavy chain in filamentous orientation along the long axes of the cells, and showed "hill and valley" appearance, indicating the presence of contractile phenotype with well-organized ASM cell growth [17]. The ASM cells rapidly responded to thrombin stimulation by increasing the cytosolic Ca^{+2} level, indicating the PAR excitation-coupling system is functioning properly in this primary ASM cells [9].

Tyrphostin 47 (AG213/RG50864) is a derivative of the dihydroxybenzylidene malononitrile class of tyrosine kinase inhibitors that acts by competitive inhibition at the substrate site of the kinase. Tyrphostin 47 is a broad-spectrum protein tyrosine kinase inhibitor, which has been shown to inhibit a wide range of tyrosine kinases including EGFR kinase, PDGFR kinase, p210Bcr-Abl and Src kinases [10]. In this study, tyrphostin 47 significantly inhibited thrombin-induced [^{3}H]thymidine incorporation in ASM cells in a concentration-dependent manner, but the extent of inhibition was much less than that produced by PP2 and piceatannol. At 10 μM, tyrphostin 47 significantly reduced cyclin D_1 expression by 35.5%. Thrombin-induced PAR activation can transactivate EGFR via an autocrine/paracrine release of EGF-like ligands at the cell surface, which in turn, leads to c-Src activation and the downstream p21ras-dependent MAPK activation and cellular proliferation [18,19]. In addition, thrombin-induced PAR activation is associated with stimulation of Src family of kinases by the $G_{\beta\gamma}$ subunit or $G_{12\alpha}$ subunit coupled to PARs, which in turn, phosphorylates adapter protein Shc and acti-

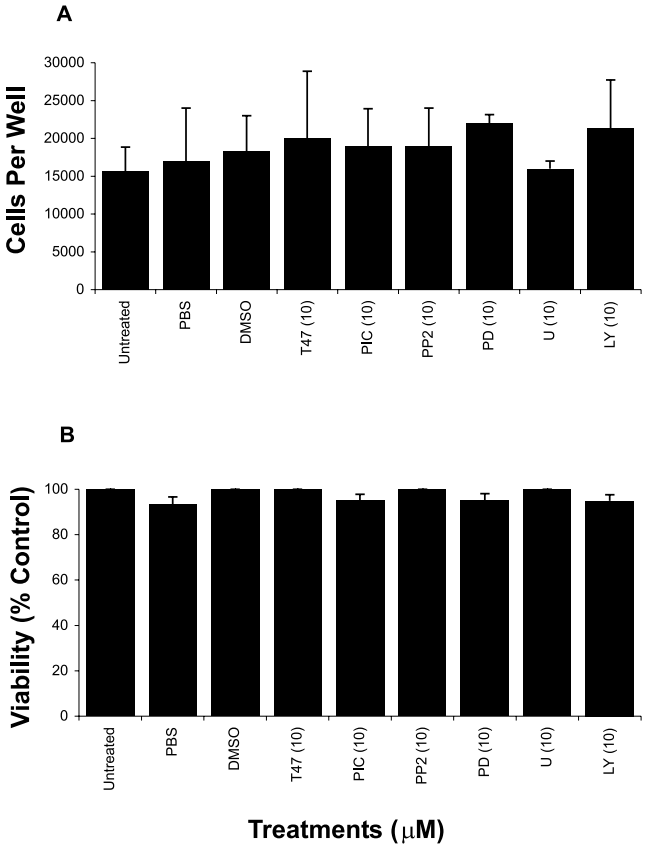

Fig. 4. Effects of inhibitors on thrombin-induced guinea pig ASM cell proliferation as assessed by cell number (A) and cell viability (B). Cells were growth-arrested, treated with various inhibitors at 10 μM, the highest concentration used in the present study, and stimulated with 3 U/ml thrombin for 24 h. Cells were collected and counted for cell number and cell viability using a hematocytometer. Each data represents the mean ± SEM of three experiments. T47, tyrphostin 47; PIC, piceatannol; PD, PD098059; U, U0126; LY, LY294002.

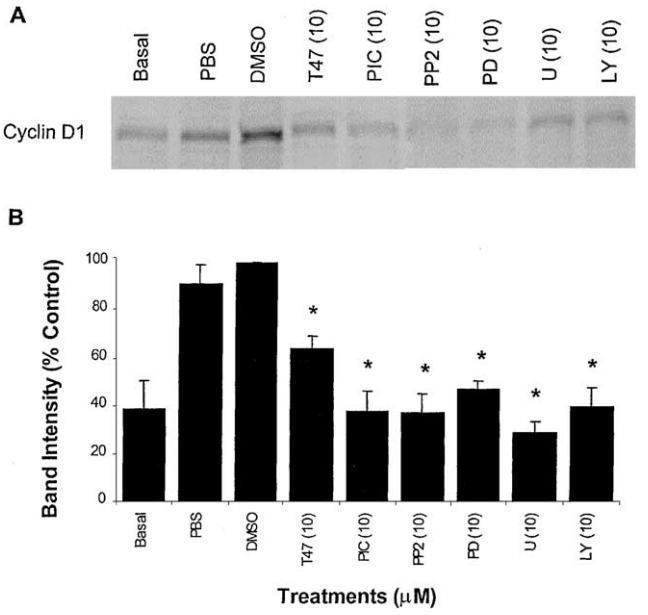

Fig. 5. (A) Immunblot showing thrombin-induced cyclin D_1 expression in guinea pig ASM cells in the presence of various inhibitors. ASM cells were growth-arrested, treated with 10 μM of various inhibitors, and stimulated with 3 U/ml thrombin for 24 h. (B) Protein expression of cyclin D_1 in ASM cells in the presence of various inhibitors was analyzed using a Gel-Pro imaging system. Band intensity for DMSO control was expressed as 100%. Each point represents the mean ± SEM of three experiments. *Significant difference from DMSO control, $P < 0.05$. T47, tyrphostin 47; PIC, piceatannol; PD, PD098059; U, U0126; LY, LY294002.

vates p21ras-dependent MAPK activation and mitogenesis [20,21]. Taken together, the inhibitory effects of tyrphostin 47 on thrombin-induced ASM cell proliferation may be due to a combined inhibition of the transactivation of EGFR kinase and c-Src kinase.

PP2, a Src family-selective inhibitor, is essentially inactive against ZAP-70, JAK2 and protein kinase A at concentrations that exhibit potent Src kinases inhibition [11]. In this study, PP2 concentration-dependently (1–10 μM) inhibited thrombin-induced [^{3}H]thymidine incorporation in ASM with a remarkable 43.8% inhibition at 10 μM. At this concentration, PP2 substantially blocked cyclin D_1 expression by 63%. These findings further support an important role of Src in thrombin-induced ASM cell proliferation. Recent findings revealed that activation of GPCRs recruits and activates Src kinases via a family of adapter protein β-arrestins, previously known to regulate receptor desensitization and internalization. β-Arrestin can act as a scaffold protein that assembles Src and p42/p44 MAPK to facilitate sequential protein activation, leading to mitogenic responses [18]. The detailed mechanism by which thrombin-induced Src kinase activation in ASM cells remains to be determined. Nevertheless, the inhibitory effects of PP2 on thrombin-induced ASM cell proliferation is mainly due to c-Src kinase inhibition.

Piceatannol, a putative Syk-selective inhibitor [12,13], markedly inhibited thrombin-induced [^{3}H]thymidine incorporation in a concentration-dependent manner (1–10 μM) in ASM cells, with a substantial 76% inhibition at 10 μM. Piceatannol showed the most potent inhibitory effects among the three tyrosine kinase inhibitors used. At this concentration, piceatannol abated cyclin D_1 expression by 62%. A recent study showed that Syk expression is not restricted to hematopoietic cells, but instead, it is expressed in a wide variety of tissues including fibroblast, epithelial cells, heptatocytes, neuronal cells, and endothelial cells [22]. However, it remains to be determined if Syk is expressed in ASM cells. Aside from Syk, piceatannol has been observed to inhibit c-Src and focal adhesion kinase (FAK) with slightly lower potency in platelets [23]. FAK has been shown to be expressed in ASM cells [24] and to be pivotal in mediating thrombin-induced p42/p44 MAPK activation in several anchorage-dependent cell lines [25]. Therefore, the anti-mitogenic effects of piceatannol on

thrombin-induced ASM proliferation can be due to a combined inhibition of Src, FAK, and probably Syk.

PD098059 and U0126, two MAPK kinase-specific inhibitors [14,15], remarkably inhibited thrombin-induced ASM cell proliferation and cyclin D_1 protein expression by 55% and 82%, and 53% and 71%, respectively. This is in line with other previous studies [3,5,6] supporting the critical role of MAPK in ASM cell proliferation. On the other hand, LY294002, a PI3K-specific inhibitor [16], suppressed [^{3}H]thymidine incorporation and cyclin D_1 protein expression in ASM cells by 51% and 60%, respectively, indicating that PI3K also plays an important role in thrombin-induced ASM cell proliferation. It is believed that thrombin-induced PAR activation in ASM cells triggers sequential activation of Src, Shc, p21ras, and finally PI3K [3]. This, in turn, activates multiple downstream substrates including $p70^{S6k}$, Rho family kinases and atypical PKCζ isozyme to regulation cell proliferation [7,26,27].

Tyrosine kinases, such as Src family kinases and Syk kinase, play an important role in the activation of mast cells and T lymphocytes [28,29]. Our previous studies show that tyrosine kinase inhibitors can abrogate antigen-induced guinea pig bronchial contraction and mediator release from chopped lung preparations [30,31]. The present study demonstrates that inhibitors of Src family kinases, FAK, and probably Syk can also attenuate thrombin-induced ASM cell proliferation, an indicator of airway hyperplasia occurring in asthma [1,3]. Taken together, selective inhibitor of the tyrosine kinase signaling cascade may have therapeutic potential for the treatment of asthma.

Acknowledgments

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