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## PKC $\delta$ mediates thrombin augmented fibroblast-mediated collagen gel contraction

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### Abstract

Fibroblast-mediated collagen gel contraction has been used as an *in vitro* model of tissue remodeling. Thrombin is one of the mediators present in the milieu of airway inflammation and may be involved in airway tissue remodeling. We have previously reported that thrombin stimulates fibroblast-mediated collagen gel contraction partially through the PAR1/PKC $\epsilon$  signaling pathway (Fang et al, ERJ, 2004; 24: 918-924). Here we further report that the delta-isoform of PKC (PKC $\delta$ ) is also activated by thrombin and involved in the thrombin-mediated augmentation of collagen gel contraction. Thrombin (10nM) significantly increased PKC $\delta$  activity (over 5-fold increase after 15-30 min stimulation) and stimulated phosphorylation of PKC $\delta$ . Rottlerin, a PKC $\delta$  inhibitor, completely inhibited activation of PKC $\delta$  and partially blocked collagen gel contraction stimulated by thrombin. Similarly, PKC $\delta$ -specific siRNA significantly inhibited PKC $\delta$  activation without affecting PKC $\epsilon$  expression and activation. Furthermore, suppression of PKC $\delta$  by siRNA resulted in partial blockade of thrombin-augmented collagen gel contraction. These results suggest that thrombin contributes to the tissue remodeling in inflammatory airways and lung diseases at least partially through both PKC $\delta$  and PKC $\epsilon$  signaling.

### Introduction

The architectural changes in lung and bronchi are thought to contribute to the loss of lung function in many chronic airway and lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD) and interstitial lung disease. Fibroblast proliferation and migration and extracellular matrix accumulation within the airway wall are characteristic pathologic changes in airway remodeling [1-4]. Fibroblast-mediated collagen gel contraction is recognized as an *in vitro* model of tissue remodeling, specifically of the contraction that characterizes both fibrotic scar tissue and normal wound healing. Many mediators including thrombin, platelet-derived growth factor (PDGF), transforming growth factor (TGF) and prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) that are believed to be involved in the tissue repair processes simulate

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collagen gel contraction [5-8]. However, the mechanisms that mediate collagen gel contraction are not fully understood.

Thrombin is a serine protease activated from pro-thrombin as part of the clotting cascade. In addition to its proteolytic effect, thrombin can initiate many cellular effects through activating protease-activated receptors [9-11]. We have previously reported that thrombin, through binding to its receptor PAR1, stimulated collagen gel contraction partially through the novel  $\epsilon$ -isoform of protein kinase C (PKC $\epsilon$ ) [5].

The family of PKC enzymes regulates diverse cellular functions in a variety of tissues including lungs. Twelve different isozymes in 3 groups have been described so far [12]. These PKC isozymes have been reported to play roles not only in maintaining normal lung structure and function, but also in the pathophysiology of many lung diseases including pulmonary edema, adult respiratory distress syndrome, interstitial lung disease, asthma and lung cancer [12-16]. PKC $\delta$  is one isozyme of the novel PKC subfamily that includes PKC $\delta$ ,  $\epsilon$ ,  $\eta$  and  $\theta$ . Recent studies suggest that PKC $\delta$  plays an important role in many cellular functions including cell survival and apoptosis, cell proliferation and migration, T cell functions, wound repair and fibrotic tissue formation [17-19]. The current study, therefore, was designed to determine whether PKC $\delta$  also regulates thrombin-augmented collagen gel contraction by lung fibroblasts.

## Materials and methods

### Materials

Type I collagen was extracted from rat tail tendons (RTTC) as previously described [20].

Thrombin from human plasma was purchased from Sigma (St. Louis, MO). Rottlerin was obtained from Calbiochem (San Diego, CA, USA). Tissue culture supplements, fetal calf serum (FCS) and media were purchased from GIBCO (Life Technologies, Grand Island, NY). SMARTpool siRNA targeting PKC $\delta$  was purchased from Dharmacon.

### Cell culture

Human fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Rockville, MD) and were cultured as described [5]. Fibroblasts used in these experiments were between cell passage 14 and 19.

### Collagen gel preparation and contraction assay

Collagen gels were prepared and contraction assays performed as described previously [20]. The areas of floating gels were measured using an image analyzer (Optomax, Burlington, MA). Each experiment included triplicate gels and each experiment was performed on no less than three separate experiments for each unique parameter.

### Transfection of siRNA targeting PKC $\delta$

Small interfering RNA (siRNA) targeting PKC $\delta$  was purchased from Dharmacon (Lafayette, CO). Transfection with *TransIT*-TKO (Mirus Corporation, Madison WI, USA) was performed as described previously [5]. Twenty-four hours after transfection, cells were used for either assessing the efficacy of silence by Western blot, or PKC activity and collagen gel contraction assays.

### Cell viability and toxicity assay

Cell viability was evaluated by calcein AM and ethidium homodimev-1, a two-color fluorescence-based method, using the LIVE/DEAD Kit, following the manufacturer's instructions (Invitrogen, Eugene, Oregon).

### Immunoblot

Immunoblotting was performed as previously described [5] and probed with anti-PKC $\delta$ , anti-PKC $\epsilon$  or anti-p-PKC $\delta$  mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti- $\beta$ -actin antibody (Sigma, St. Louis, MO). Target proteins were subsequently detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG in conjunction with enhanced chemiluminescence detection system (ECL; GE Life Sciences Products, Piscataway, NJ).

### PKC $\delta$ and PKC $\epsilon$ activity assay

Enzymatic activity of PKC $\delta$  and PKC $\epsilon$  was determined in crude whole-cell fractions. The assay employed was a modification [21] of procedures previously described [22] using 900  $\mu$ M PKC $\delta$  or PKC $\epsilon$  specific substrate peptide (Calbiochem), 8  $\mu$ M phosphatidyl-L-serine, 24  $\mu$ g/ml phorbol myristate acetate, 30 mM dithiothreitol, 150  $\mu$ M ATP, 45 mM magnesium acetate, and 10  $\mu$ Ci/ml [ $\gamma$ - $^{32}$ P] ATP in a Tris-HCl buffer (pH7.5). Kinase activity was expressed in relationship to total cellular protein assayed and calculated in picomoles phosphate transferred to the peptide substrate per minute per mg of total cellular protein assayed (pmol/min/mg). All samples were assayed in triplicate, and each experiment was repeated on no less than three separate occasions.

### Statistical analysis

Individual experiments included triplicate gels within an experiment for all experimental conditions. Results were always confirmed by repeating each experiment on separate occasions at least three times. Statistical comparisons were made from all experiments, including both the within and between group variance. PKC $\delta$  and PKC $\epsilon$  activity were expressed as fold change in activation compared to control, untreated cells for all experiments. Group data were analyzed by one-way ANOVA. Differences between series of data that appeared statistically different were corrected by Tukey's test.  $P < 0.05$  was considered significant.

## Results

### Thrombin stimulates activation and phosphorylation of PKC $\delta$

Two separate approaches were used to evaluate the effect of thrombin on PKC $\delta$  activation in human lung fibroblasts. First, PKC $\delta$  catalytic activity was determined in response to thrombin stimulation of lung fibroblasts cultured in monolayer. Thrombin (10nM) stimulated PKC $\delta$  activity in a time-dependent manner (Fig 1A). After 15min and 30min of thrombin stimulation, PKC $\delta$  activity was greatly increased ( $6.2 \pm 1.0$  and  $6.4 \pm 1.0$  fold of control,  $p < 0.01$ , Fig 1A). After 60 minutes thrombin stimulation, however, the PKC $\delta$  activity had declined to baseline levels (Fig 1A). Second, PKC $\delta$  activation was also evaluated by immunoblotting phosphorylated PKC $\delta$ . Consistent with the observed increase in kinase activity, phosphorylation of PKC $\delta$  was significantly stimulated by thrombin (10nM) and the maximum phosphorylation was observed at 15min and 30 min after stimulation with thrombin (Fig 1B). After 60min of stimulation, PKC $\delta$  phosphorylation was reduced although it was still higher than that of baseline (Fig 1B).

### Rottlerin and PKC $\delta$ siRNA inhibit thrombin-induced PKC $\delta$ activation, but not PKC $\epsilon$ activation

Both a pharmacologic inhibitor of PKC $\delta$  and RNA interference targeting of PKC $\delta$  were used to suppress PKC $\delta$  activation in response to thrombin stimulation in human lung fibroblasts.

Rottlerin (1  $\mu\text{M}$ ) significantly inhibited 10nM and 20nM thrombin-induced PKC $\delta$  activity ( $p < 0.01$ , Fig 2A), but had no effect on PKC $\epsilon$  activity (Fig 2B). To confirm the specificity of rottlerin as a pharmacologic kinase inhibitor in our assay, molecular interference with siRNA was also used to suppress PKC $\delta$ . PKC $\delta$ -specific siRNA significantly decreased both baseline PKC $\delta$  activity as well as thrombin-stimulated PKC $\delta$  activity (Fig 2C,  $p < 0.001$ ), but tended to slightly increase PKC $\epsilon$  activity both in the presence and absence of thrombin, although this effect was not statistically significant (Fig 2D,  $p > 0.05$ ). Neither rottlerin nor PKC $\delta$  siRNA altered cell viability assessed by LIVE/DEAD assay (data not shown).

### **Inhibition of PKC $\delta$ by rottlerin or by siRNA results in partial blockade of thrombin-augmented collagen gel contraction**

Fibroblast-populated collagen gels were allowed to contract in the presence or absence of thrombin (10nM), one of the factors known to stimulate collagen gel contraction by fibroblasts [5]. Two approaches were used to evaluate the role of PKC $\delta$  in mediating thrombin-augmented collagen gel contraction.

First, the effect of rottlerin, an inhibitor of the PKC $\delta$  isozyme, on thrombin-augmented collagen gel contraction was investigated. Thrombin significantly stimulated collagen gel contraction mediated by HFL-1 fibroblasts in a time-dependent manner (Fig 3A). Rottlerin (1 $\mu\text{M}$ ) alone had no effect on gel contraction on day 1, 2 and 3, but significantly inhibited on day 4 ( $85.1 \pm 0.2\%$  vs  $71.2 \pm 0.1\%$  of control,  $p < 0.05$ ) and day 5 ( $86.1 \pm 1.0\%$  vs  $69.1 \pm 0.2\%$  of control,  $p < 0.01$ , Fig 3A). In the presence of thrombin, rottlerin significantly inhibited thrombin-augmented collagen gel contraction from day 2 ( $78.5 \pm 0.8\%$  vs  $62.5 \pm 1.3\%$  of thrombin,  $p < 0.01$ , Fig 3A). The inhibitory effect of rottlerin on thrombin-augmented collagen gel contraction was also concentration-dependent (Fig 3B). Rottlerin at concentrations of 1, 2.5 and 5 $\mu\text{M}$  significantly inhibited thrombin-augmented collagen gel contraction; the gel size was  $73.5 \pm 1.5\%$ ,  $79.3 \pm 0.4\%$  and  $87.7 \pm 0.7\%$  of original gel size, respectively, ( $p < 0.05$  or  $0.01$ , compared to the corresponding gel size stimulated by thrombin, Fig 4B). Rottlerin at its maximum concentration (10 $\mu\text{M}$ ) completely inhibited collagen gel contraction stimulated by thrombin ( $99.0 \pm 0.8\%$  vs  $62.5 \pm 0.5\%$  of thrombin alone,  $p < 0.01$ , Fig 3B).

Second, expression of PKC $\delta$  was suppressed by PKC $\delta$  targeting siRNA in human lung fibroblasts following which the fibroblasts were cast into collagen gels and allowed to contract in the presence or absence of thrombin (10nM). PKC $\delta$ -specific siRNA significantly suppressed PKC $\delta$  expression as determined by immunoblot (Fig 4A), but had no effect on PKC $\epsilon$  expression (Fig 4A). In addition, consistent the effect of rottlerin on collagen gel contraction, suppression of PKC $\delta$  by siRNA resulted in partial blockade of collagen gel contraction in response to thrombin stimulation ( $p < 0.01$ , Fig 4B).

## **Discussion**

The current study investigated the role of PKC $\delta$  in thrombin-induced collagen gel contraction as mediated by human lung fibroblasts. Thrombin significantly stimulated both PKC $\delta$  and PKC $\epsilon$  activity, and rottlerin specifically inhibited PKC $\delta$  activity, but not PKC $\epsilon$  activity. Thrombin-augmented collagen gel contraction mediated by fibroblasts was partially, but significantly, inhibited by rottlerin. Furthermore, suppression of PKC $\delta$  expression by siRNA resulted in partial blockade of thrombin-augmented collagen gel contraction. These results indicate that PKC $\delta$  mediates thrombin-augmented collagen gel contraction by human lung fibroblasts, and by this mechanism, PKC $\delta$  may be involved in tissue repair and remodeling following airways inflammatory injury.

Thrombin is formed in the blood coagulation cascade, which is commonly activated following tissue injury, and during inflammation and repair. Through activating the protease-activated

receptor1 (PAR1), thrombin has been shown to stimulate cell proliferation and affect extracellular matrix deposition and degradation. Our previous study has shown that activation of PKC $\epsilon$ , downstream of PAR1, is one mechanism that contributes to thrombin-induced collagen gel contraction mediated by HFL-1 fibroblasts. In the current study, we further investigate another novel class isozyme of PKC, the delta isoform (PKC $\delta$ ), in mediating collagen gel contraction in response to thrombin stimulation. Similar to the effect of PKC $\epsilon$ , PKC $\delta$  also partially modulates fibroblast-mediated collagen gel contraction in response to thrombin.

Whether PKC $\delta$  plays a role in “constitutive” contraction of collagen gels mediated by fibroblasts was not assessed in the current study. A modest, but significant effect was observed with rottlerin under baseline conditions. While this could be due to “constitutive” PKC $\delta$ , it is not possible to rule out a non-specific effect. No effect was observed with siRNA under baseline conditions. However, like most siRNAs, suppression was not complete. Thus, the lack of effect of the siRNA does not rigorously exclude a role for PKC $\delta$  under baseline conditions. While the role of PKC $\delta$  under baseline conditions remains undefined, a role in the thrombin stimulated contraction is supported by the consistent inhibition by both rottlerin and siRNA for PKC.

PKC is comprised of a family of serine/threonine kinases that play important roles in variety of cell functions including proliferation, apoptosis and differentiation. There are 9 PKC genes, coding 11 PKC isoforms, which are classified into 3 subfamilies: classical PKCs (cPKCs: PKC $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$ ); novel PKCs (nPKCs: PKC $\epsilon$ ,  $\delta$ ,  $\eta$ ,  $\theta$ ); and atypical PKC (aPKCs: PKC $\zeta$  and  $\tau$ ) [23]. The mechanisms of PKC activation in a variety of cell types have been extensively studied [24,25]. PKC activity is regulated by several mechanisms including phosphorylation [26]. In the present study, activation of PKC $\delta$  was associated with enzyme phosphorylation. In general PKC activity is transient. There are several mechanisms for inactivation, including dephosphorylation, translocation, proteolysis and formation of covalent linkage on the active site of the enzyme [27-30]. While the loss of PKC $\delta$  catalytic activity at 60 minutes with preservation of phosphorylation, the mechanisms of this phenomenon were not directly evaluated in the current study.

Both PKC $\delta$  and PKC $\epsilon$  belong to the novel PKC (nPKC) subfamily and they are expressed in human lung fibroblasts [14,31]. We have reported that thrombin stimulates human lung fibroblast-mediated collagen gel contraction partially through PKC $\epsilon$  signaling [5]. Here we found that both a PKC $\delta$  inhibitor (rottlerin) and PKC $\delta$  specific siRNA partially blocked fibroblast-mediated collagen gel contraction in response to thrombin, indicating that PKC $\delta$  has an effect similar to that previously shown for PKC $\epsilon$  in mediating collagen gel contraction, an *in vitro* model of tissue repair. In contrast to the parallel effects observed in the current study, the biological functions of these two isozymes in cancer or cardiac injury are opposite. PKC $\delta$  is a pro-apoptotic or growth inhibitory PKC, and many types of apoptotic stimuli can induce PKC $\delta$  translocation to mitochondria, leading to cytochrome *c* release and caspase-3 activation [23,32]. In contrast, PKC $\epsilon$  promotes cell survival in many cell types through activation of Akt pathways and up-regulation of anti-apoptotic proteins [23,33]. In a model of myocardial ischemia/reperfusion injury, PKC $\delta$  and PKC $\epsilon$  play opposing roles. Activation of PKC $\delta$  during reperfusion induces cell death through the mechanism of mitochondria-mediated apoptosis, whereas activation of PKC $\epsilon$  before ischemia protects mitochondrial function and inhibits cell death [34].

Fibroblast-mediated collagen gel contraction is considered to be an *in vitro* model of wound healing and tissue remodeling [20,35]. The current study demonstrated that PKC $\delta$  mediates thrombin signaling in fibroblasts cultured in the three-dimensional collagen gels. This finding suggests that PKC $\delta$  signaling plays a role in wound repair and tissue remodeling. Consistent with our findings, recent studies reported that asbestos-induced peribronchiolar cell



proliferation, cytokine release (IL-4, IL-6 and IL-13) and inflammatory cells were reduced in PKC $\delta$  knock out mice compared to wild type mice [15]. Similar to the seemingly redundant PKC $\delta$  and PKC $\epsilon$  effects on gel contraction found in our studies, PKC $\epsilon$  signaling also plays an important role in lung cytokine release [21]. These results indicate that both PKC $\delta$  and PKC $\epsilon$  can have multiple, and sometimes overlapping, effects on pro-inflammatory and pro-fibrotic reactions in the lungs.

In summary, thrombin is capable of activating PKC $\delta$  in human lung fibroblasts cultured in three dimensional collagen gels. Rottlerin and PKC $\delta$  specific siRNA partially blocked thrombin-augmented collagen gel contraction. These results support the concept that thrombin could contribute to the pathogenesis of fibrotic tissue formation in a variety of lung and airway diseases through activating PKC $\delta$  signaling. Thus, targeted interruption of PKC $\delta$  may provide a potential therapeutic option for the blockade of peri-bronchiolar fibrosis in lung diseases such as asthma and COPD.

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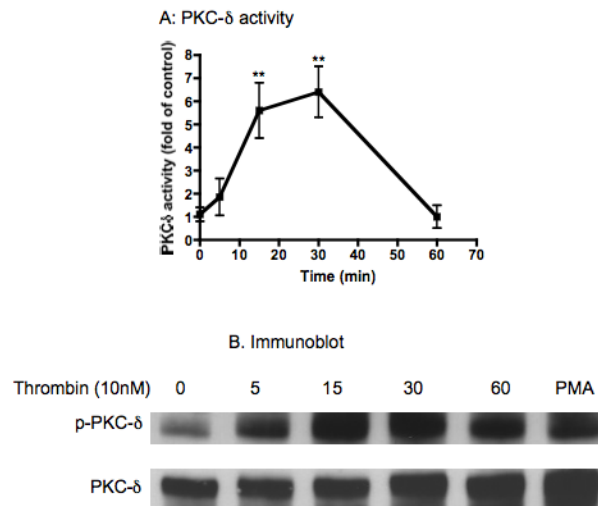
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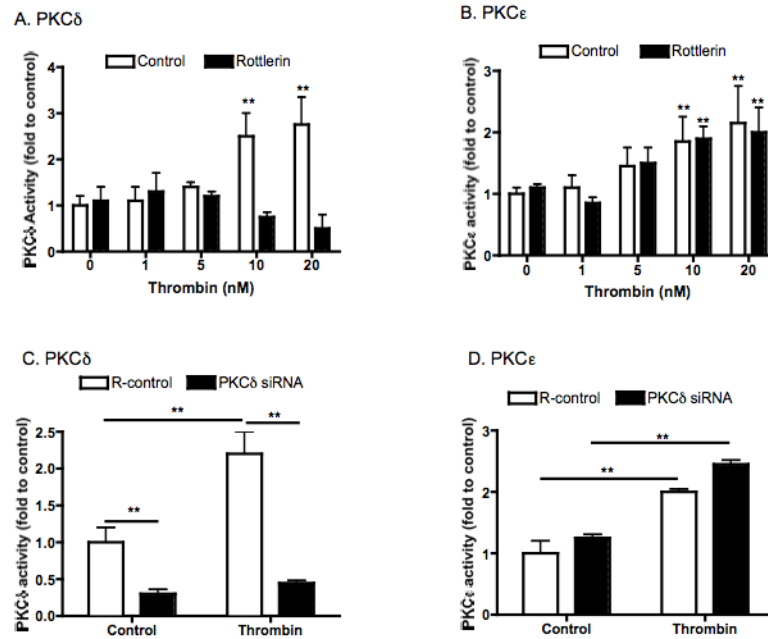
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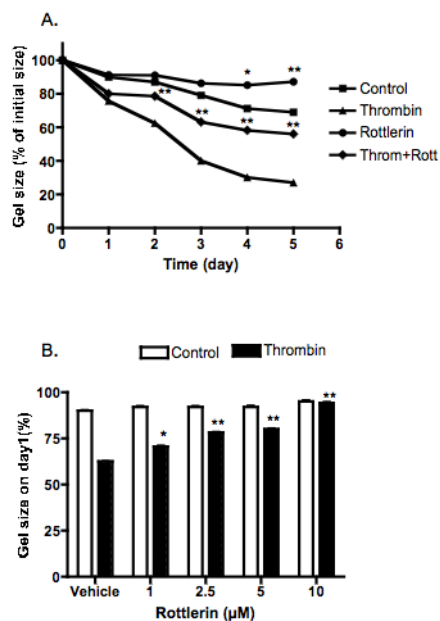


**Figure 1. Effect of thrombin on PKC $\delta$  activity and phosphorylation**

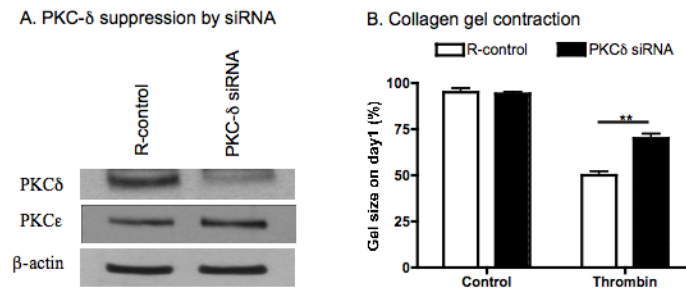
HFL-1 fibroblasts were stimulated with thrombin (10nM) for up to 60min. PKC $\delta$  activity was measured as described in methods (**Panel A**) and PKC $\delta$  phosphorylation was evaluated by immunoblot (**Panel B**). **Panel A**: *Vertical axis*: PKC $\delta$  activity (fold of control). *Horizontal axis*: Time (min). \*\*  $p < 0.01$  compared to time 0. Data presented are the mean  $\pm$  standard error of the mean for three separate experiments (n=3). **Panel B**: p-PKC $\delta$ : phosphorylated PKC $\delta$ . PMA: phorbol 12-myristate 13-acetate (100nM, 30min) was used as positive control.



**Figure 2. Effect of inhibition of PKC $\delta$  on thrombin-induced PKC $\delta$  and PKC $\epsilon$  activity**  
**Panel A and B: Pharmacologic inhibition with rottlerin.** HFL-1 fibroblasts were stimulated with varying concentrations of thrombin with or without rottlerin (1 $\mu$ M) for 30min. PKC $\delta$  (**Panel A**) and PKC $\epsilon$  (**Panel B**) activity was measured as described in methods. *Vertical axes:* PKC $\delta$  or PKC $\epsilon$  activity (fold of control). *Horizontal axes:* thrombin concentration (nM). \*\*  $p < 0.01$  compared to 0 nM thrombin. **Panel C and D: RNAi suppression.** Sub-confluent HFL-1 fibroblasts were transfected with or without specific siRNA that targeting PKC $\delta$ . After 24 hours, cells were then treated with thrombin for 30min. PKC $\delta$  (**Panel C**) or PKC $\epsilon$  (**Panel D**) activity was then measured as described in the methods. *Vertical axes:* PKC $\delta$  or PKC $\epsilon$  activity (fold of control). *Horizontal axes:* thrombin treatment (10nM) \*\*  $p < 0.01$ . Data presented are the mean  $\pm$  standard error of the mean for three separate experiments (n=3).



**Figure 3. Effect of PKC $\delta$  inhibitor (rottlerin) on thrombin-induced collagen gel contraction**  
**Panel A: Time-dependence.** Fibroblasts were cast into collagen gels which were released and cultured in serum-free DMEM with or without thrombin (10nM) and /or rottlerin (1 $\mu$ M) for 5 days. Gel size was measured daily with an image analyzer. *Vertical axis:* gel size expressed as percentage of initial gel size (%). *Horizontal axis:* Time (days). **Panel B: Concentration-dependence.** Gels were released into serum-free DMEM with varying concentrations of rottlerin as indicated in the presence or absence of thrombin (10nM). Gel size was measured on day 1 with an image analyzer. *Vertical axis:* gel size expressed as percentage of initial gel size (%). *Horizontal axis:* Rottlerin concentration ( $\mu$ M). Data presented are the mean  $\pm$  standard error of the mean for three separate experiments, each of which included triplicate gels for each condition. \*  $p < 0.05$ , \*\*  $p < 0.01$  compared to Thrombin or control alone.



**Figure 4. PKC $\delta$  suppression by siRNA**

Sub-confluent HFL-1 cells were treated with transfection reagent only (R-control) or transfected with PKC $\delta$  specific siRNA. **Panel A:** Suppression of PKC $\delta$  expression was assessed by immunoblot using anti-PKC $\delta$  or anti-PKC $\epsilon$  antibodies. Blots were probed using anti- $\beta$ -actin as a loading control. **Panel B: Effect on collagen gel contraction.** After transfection of PKC $\delta$  siRNA, the cells were cast into collagen gels and allowed to contract in the presence or absence of thrombin (10nM). Gel size was measured on day 1. *Vertical axis:* gel size expressed as percent of initial size (%). *Horizontal axis:* treatment.