

# Interaction between *src* family kinases and rho-kinase in agonist-induced $\text{Ca}^{2+}$ -sensitization of rat pulmonary artery

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Protein kinases;  
Prostaglandins;  
Rho-kinase

**Aims** We investigated the role of *src* family kinases (*srcFK*) in agonist-mediated  $\text{Ca}^{2+}$ -sensitization in pulmonary artery and whether this involves interaction with the rho/rho-kinase pathway.

**Methods and results** Intra-pulmonary arteries (IPAs) and cultured pulmonary artery smooth muscle cells (PASMC) were obtained from rat. Expression of *srcFK* was determined at the mRNA and protein levels.  $\text{Ca}^{2+}$ -sensitization was induced by prostaglandin  $\text{F}_{2\alpha}$  ( $\text{PGF}_{2\alpha}$ ) in  $\alpha$ -toxin-permeabilized IPAs. Phosphorylation of the regulatory subunit of myosin phosphatase (MYPT-1) and of myosin light-chain-20 (MLC<sub>20</sub>) and translocation of rho-kinase in response to  $\text{PGF}_{2\alpha}$  were also determined. Nine *srcFK* were expressed at the mRNA level, including *src*, *fyn*, and *yes*, and  $\text{PGF}_{2\alpha}$  enhanced phosphorylation of three *srcFK* proteins at tyr-416. In  $\alpha$ -toxin-permeabilized IPAs,  $\text{PGF}_{2\alpha}$  enhanced the  $\text{Ca}^{2+}$ -induced contraction (pCa 6.9) approximately three-fold. This enhancement was inhibited by the *srcFK* blockers SU6656 and PP2 and by the rho-kinase inhibitor Y27632. Y27632, but not SU6656 or PP2, also inhibited the underlying pCa 6.9 contraction.  $\text{PGF}_{2\alpha}$  enhanced phosphorylation of MYPT-1 at thr-697 and thr-855 and of MLC<sub>20</sub> at ser-19. This enhancement, but not the underlying basal phosphorylation, was inhibited by SU6656. Y27632 suppressed both basal and  $\text{PGF}_{2\alpha}$ -mediated phosphorylation. The effects of SU6656 and Y27632, on both contraction and MYPT-1 and MLC<sub>20</sub> phosphorylation, were not additive.  $\text{PGF}_{2\alpha}$  triggered translocation of rho-kinase in PASMC, and this was inhibited by SU6656.

**Conclusions** *srcFK* are activated by  $\text{PGF}_{2\alpha}$  in the rat pulmonary artery and may contribute to  $\text{Ca}^{2+}$ -sensitization and contraction via rho-kinase translocation and phosphorylation of MYPT-1.

## 1. Introduction

$\text{Ca}^{2+}$ -sensitization in smooth muscle is the process by which increased myosin light-chain phosphorylation, and hence contractile force, occurs, generated in response to an external stimulus independently of changes in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ).<sup>1</sup> In smooth muscle, this is believed to occur principally through phosphorylation of myosin-binding subunit (or MYPT-1) and subsequent inhibition of myosin light-chain phosphatase.<sup>2</sup> MYPT-1 is phosphorylated by rho-kinase at two sites (thr-697 and thr-855 in rat), the first of which results in inhibition of phosphatase activity<sup>3</sup>

and the second results in dissociation of the phosphatase from myosin.<sup>4</sup> Rho-kinase is activated by binding to the GTP-bound form of the small G-protein RhoA and subsequent translocation.<sup>5</sup> The mechanisms by which agonist stimulation activates RhoA are not fully understood, but the involvement of guanine nucleotide exchange factors (RhoGEF) is a requirement.<sup>6</sup>

*src* family kinases (*srcFK*) are a group of closely related non-receptor tyrosine kinases, the eponymous member of which has been found to be highly expressed in vascular tissues.<sup>7</sup> *srcFK* are activated by myristoylation and/or palmitoylation,<sup>8</sup> translocation to the plasma membrane,<sup>9</sup> and subsequent auto-phosphorylation at tyr-416.<sup>10</sup> Tyrosine kinases in general are implicated in vascular smooth muscle

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contraction, both via actions upon Ca<sup>2+</sup> release and/or influx and via Ca<sup>2+</sup>-sensitization.<sup>11–15</sup> Although evidence for the latter is somewhat contradictory,<sup>11,14</sup> one study clearly shows that sphingosylphosphorylcholine-mediated contraction and translocation of rho-kinase in pig coronary artery are dependent on srcFK.<sup>16</sup> srcFK may regulate rho activation via phosphorylation of RhoGEF.<sup>6</sup>

Ca<sup>2+</sup>-sensitization and the rho/rho-kinase pathway are important components of contractile function in pulmonary artery, in response to agonists and acute or chronic hypoxia, both in normal and patho-physiological settings,<sup>17,18</sup> whereas the role(s) of srcFK are largely unexplored.<sup>19</sup> In the present study, therefore, we evaluated the role of srcFK in Ca<sup>2+</sup>-sensitization-dependent prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>)-mediated contraction in rat small distal pulmonary arteries and srcFK interaction with the rho/rho-kinase pathway.

## 2. Methods

### 2.1 Animals, tissue isolation, and cell culture

This study conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Male Wistar rats (200–250 g) were killed by lethal overdose of pentobarbital (ip). Lungs were excised and placed in cold physiological salt solution (PSS), and first to third-order branches of the intra-pulmonary artery (IPA) were dissected free of surrounding parenchyma. First, second, and third-order branches (~150–1000 μm diameter) were used for the extraction of mRNA for polymerase chain reaction (PCR) and protein for western blot experiments. Second or third-order branches (~150–600 μm diameter) were used for the preparation of pulmonary artery smooth muscle cells (PASMC) and measurement of isometric tension.

PASMC were dispersed enzymatically and grown in DMEM with 10% FCS to passage 4 or 5. Cells were then growth-arrested in serum-free media for 24 h and harvested for PCR/western blot or plated on 13 mm coverslips and then growth-arrested for staining and translocation experiments. Identification of each line of cells as smooth muscle was verified by positive staining with anti-smooth muscle α-actin, and anti-calponin antibodies (Santa Cruz Biotechnology, CA, USA).

### 2.2 Solutions, drugs, and chemicals

PSS contained (mM): NaCl 118; NaHCO<sub>3</sub> 24; KCl 4; CaCl<sub>2</sub> 1.8; MgSO<sub>4</sub> 1; NaH<sub>2</sub>PO<sub>4</sub> 0.434, glucose 5.56. Ca<sup>2+</sup>-free relaxing solution contained (mM): PIPES 30, Mg(Ms)<sub>2</sub> 5.3, KMs 46.6, K<sub>2</sub>EGTA 10, Na<sub>2</sub>ATP 5, Na<sub>2</sub> creatinine phosphate 10, and the pH was set at 7.1. Ca<sup>2+</sup>-containing intracellular solution was identical except for the substitution of CaEGTA for K<sub>2</sub>EGTA. Free [Ca<sup>2+</sup>] was adjusted by mixing the two solutions in the appropriate proportion, as calculated by WEBMAXC software (www.stamford.edu). SU6656, PP2, PP3 and Y27632 were all obtained from Calbiochem (Merck Biosciences Nottingham, UK). PGF<sub>2α</sub> (tromethamine salt) was purchased from Biomol (Exeter, UK). All other reagents were obtained from Sigma (Poole, UK) Calbiochem, Invitrogen (Paisley, UK), or Fisher (Loughborough, UK).

### 2.3 RNA isolation and reverse transcriptase–polymerase chain reaction

Total RNA was extracted from IPA or PASMC using the Qiagen RNeasy mini kit and TissueLyser (Qiagen, Crawley, UK). RNA was treated with TURBO DNase (Ambion, Austin, TX, USA) to remove any remaining contaminating DNA and then reverse-transcribed in the presence of RNAGuard (GE Healthcare, Chalfont St Giles, UK) by using random

hexamers and revert-aid reverse transcriptase (Fermentas International, York, UK). MacVector™ (version 7.2) and Ensembl Genome Browser (www.ensembl.org) were used to design RT-PCR primer pairs. Sense and antisense primers on either side of a small intron (<1 kb) were made to allow distinction from amplification of any contaminating DNA as opposed to reverse-transcribed mRNA. Primer pairs are as follows. BLK (accession no. BC098683): sense GGACAATGGAGGCTATTACATCTCG; antisense ATTCTTCGGGG CTGGGTTACAC. FGR (accession no. BC062025): sense TCTATGC TACTTGCTCACCGCAC; antisense ATAAATGGGTTCTCTGACACCAC. FRK (accession no. U09583): sense TGTGTGGTCTTTTGGAAATCTGC; antisense TTGGTCGTTGCTTGGGCTCTAC. FYN (accession no. U35365): sense GAAGAGCCCATTTACATTGTCCAG; antisense ATGAGTCCGTTCCCCACCAG. HCK (accession no. BC078890): sense CTGGACAGTGGAGGCTTCTACATC; antisense ATGGCTTCTGGGG TTTGGG. LCK (accession no. BC099218): sense TCCCCTCGTAT-CACTTTTCCCG; antisense CCCTTGCTCAGACTTTTCACTGC. LYN (accession no. AF000300): sense GACAATCTGAATGACGATGGAG; antisense CGTAGTTGCTGGGGATGAAGC. SRC (accession no. AF157016): sense TTCAAGAAAGGGGAGCGGCTGC; antisense TGTCAAAAGTCGGATACAGAGAGGC. YES1 (accession no. BC079403): sense GCAAAAATGGGAGAAAAGATGCTG; antisense TGGTCGTGATG TAGTATCCACCG.

All PCR primers were supplied by MWG Biotech (Ebersberg, Germany). PCR was carried out using 100 ng of reverse-transcribed RNA, 1× PCR II buffer, 4 mM MgCl<sub>2</sub>, 2 U Ampliqaq Gold (Applied Biosystems, Warrington, UK), 0.5 U Perfect Match (Stratagene Europe, The Netherlands), 0.25 mM dNTPs (Fermentas International, York, UK), and 1.25 μM primer pair in a final volume of 40 μL. PCR cycling conditions were 10 min 95°C followed by 4 cycles of 2 min 95°C, 10 min 57°C, 2 min 72°C and then a variable number of cycles of 2 min 95°C, 2 min 57°C, 2 min 72°C (total number of cycles indicated in figure legends). Eighty microlitres of PCR products (reaction equivalent on 20 ng reverse-transcribed RNA) were analysed by electrophoresis on 2.8% agarose gels run in 1× TAE buffer (National Diagnostics, Yorkshire, UK) with PhiX174 DNA/HinfI Marker (Fermentas International, York, UK). Gel-purified PCR fragments were sequenced to confirm identity (Geneservice, Medical Solutions plc, UK).

### 2.4 Western blot

IPA segments were treated with PGF<sub>2α</sub> (20 μM), following a 15 min equilibration period in PSS and a 15 min pre-incubation with pharmacological agents where appropriate, gassed with 5% CO<sub>2</sub>/balance air at 37°C, prior to snap-freezing. Tissue was homogenized and protein extracted in 50 μL of Tris/SDS sample buffer containing phosphatase inhibitor cocktail I and II (Sigma) and protease inhibitor cocktail I (Sigma). Protein was extracted from PASMC by the same method. Protein extracts (12–15 μL, ~10 μg, per lane) were run on SDS/PAGE gels (4–12% gradient, Invitrogen), transferred to nitrocellulose membrane, blocked with 5% skimmed milk for 1 h, probed with primary antibody (1:1000, in Tris-buffered saline with 0.1% skimmed milk) overnight at 4°C and then with horseradish-peroxidase conjugated anti-IgG secondary antibody (1:5000 in Tris-buffered saline with 1% milk) for 1 h at room temperature. For phosphorylation experiments, membranes were first probed with anti-phospho-antibodies, stripped for 1 h (Pierce stripping buffer), re-blocked, and re-probed with corresponding anti-total antibodies. Protein bands were visualized with Supersignal West Femto Maximum Sensitivity Substrate (Pierce, Cramlington, UK) or ECL western blotting detection reagent (Amersham, Bucks, UK) and exposed to photographic film. Rabbit anti-src, rabbit anti-phospho-srcFK (tyr-416), mouse anti-phospho-tyrosine, rabbit anti-myosin light-chain, mouse anti-phospho-myosin light-chain (ser-19), and mouse anti-β-actin antibodies were obtained from Cell Signaling. Sheep anti-MYPT-1, rabbit anti-phospho-MYPT-1 (thr-695: equivalent to thr-697 in rat), and rabbit

anti-phospho-MYPT-1 (thr-850: equivalent to thr-855 in rat) antibodies were obtained from Upstate (UK).

## 2.5 Force measurement and $\alpha$ -toxin permeabilization

IPAs were mounted on a Mulvany–Halpern wire myograph (DMT A/S, Aarhus, Denmark), bathed in PSS (gassed with 95% air/5% CO<sub>2</sub>, pH 7.4), and stretched to 90% of the circumference necessary to create an equivalent transmural pressure of 4.4 kPa (30 mmHg). IPAs were equilibrated with three 3 min contractions to 80 mM K<sup>+</sup>PSS and then permeabilized with  $\alpha$ -toxin as follows. First, arteries were equilibrated in relaxing solution (as mentioned earlier) and then permeabilized by incubation at pCa 6.5 with 60  $\mu$ g/mL  $\alpha$ -toxin until the resulting vasoconstriction reached a plateau. Following re-equilibration with relaxing solution, submaximal vasoconstriction was elicited with pCa 6.9, and then PGF<sub>2 $\alpha$</sub>  (100  $\mu$ M) was applied, in the presence of 1  $\mu$ M GTP to support G-protein activation. All contraction experiments were conducted at 26°C and in the presence of 10  $\mu$ M cyclopiazonic acid to remove the influence of Ca<sup>2+</sup> store release on contraction.

## 2.6 Staining and image analysis

PASMC were treated with 20  $\mu$ M PGF for 10 min in DMEM at 37°C with or without prior application of 30  $\mu$ M SU6656 for 10 min. Reactions were terminated by addition of paraformaldehyde fixative. For ROCK-2 translocation, fixed cells were permeabilized with Triton-100 and stained overnight with anti-ROCK-2 primary antibody (1:100, Santa Cruz Biotechnology) at 4°C following incubation with Alexa Fluor 488-labelled secondary antibody (1:1000, Santa Cruz) for 1 h at room temperature. Specificity of anti-ROCK-2 was confirmed by western blot (single band at 160 kDa). Staining was negative with secondary antibody alone.

## 2.7 Data analysis and statistics

Images of western blot bands of appropriate molecular weights were quantified using Image J software (rsb.info.nih.gov). Band intensity was expressed as a ratio of phospho/total for each protein, and values from each treated sample were normalized to those from control (untreated) samples run on the same gel. Two to three control samples were run on each gel and averaged. Statistical analysis was performed with SigmaStat (Systat software Inc.). Time-dependent drug responses were examined by two-way repeated measures ANOVA with appropriate post-tests. Individual comparisons (e.g. single-dose PGF<sub>2 $\alpha$</sub>  vs. control) were performed by Student's *t*-test or Mann–Whitney rank sum test, where appropriate. Comparisons of the effects of different drugs or dose-responses of single drugs against PGF<sub>2 $\alpha$</sub>  were performed by one-way ANOVA with post-tests or by Kruskal–Wallis one-way ANOVA on ranks with post-tests, where appropriate.

## 3. Results

### 3.1 Expression of *src* family kinases

PCR was performed on mRNA extracts from IPA. All nine *src* family members were expressed, and the three most abundant are shown in *Figure 1A* (see Supplementary material online, *Figure Si* for all nine). A similar expression pattern was obtained from cultured rat PASMC (see Supplementary material online, *Figure Sii*). *src*FK were also expressed at the protein level. Three bands were visualized, two at ~59/60 kDa and one at ~54 kDa. These correspond to the three most highly expressed at the mRNA level, *src*, and the closely related *fyn* and *yes*, with expected molecular weights in rat of 60, 60, and 54 kDa, respectively (as

calculated from the predicted amino acid sequence at www.bioinformatics.org). Representative blots are shown in *Figure 1B*. Similar protein expression was obtained from cultured rat PASMC (see Supplementary material online, *Figure Sii*). In order to demonstrate full kinase activity, *src*FK require auto-phosphorylation at tyr-416.<sup>10</sup> Phospho-*src*FK (tyr-416) immunoreactivity was inhibited by the *src*FK inhibitor PP2 (at 3 and 30  $\mu$ M but not at 0.3  $\mu$ M) but not by the inactive analogue PP3 (30  $\mu$ M) (*Figure 1B*), indicating in this preparation that 30  $\mu$ M PP2 is required to substantially inhibit *src*FK auto-phosphorylation, and hence *src*FK activity.

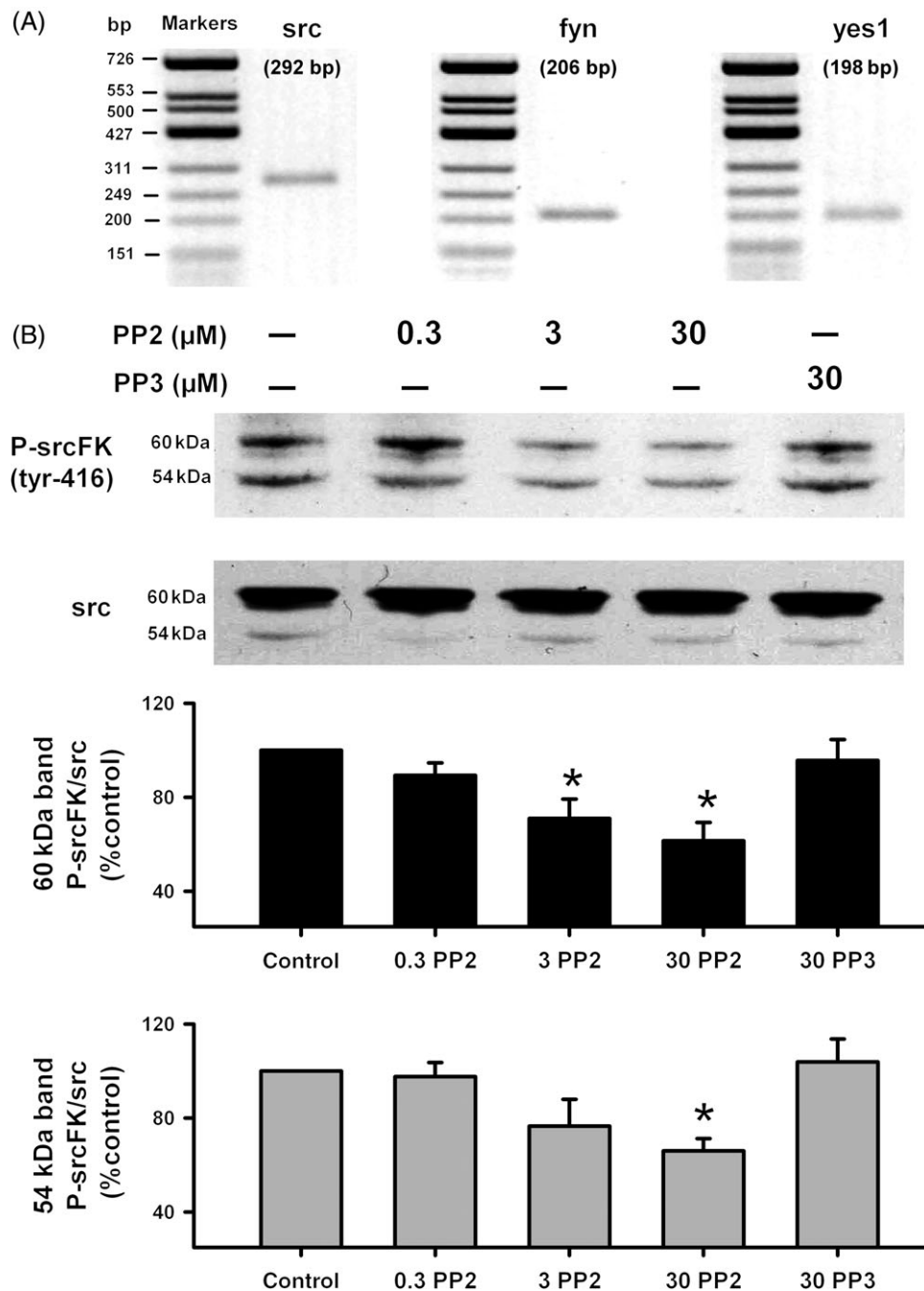
### 3.2 *src* family kinases inhibition antagonizes PGF<sub>2 $\alpha$</sub> -mediated Ca<sup>2+</sup>-sensitization

IPAs were permeabilized with  $\alpha$ -toxin and submaximally constricted with pCa clamped at 6.9. This constriction was enhanced approximately three-fold by application of PGF<sub>2 $\alpha$</sub> , reaching a maximum at 40–45 min. At this point, the *src*FK inhibitors SU6656 and PP2 or the inactive analogue PP3 was applied. These treatments were compared with parallel time controls in which drug was not applied. DMSO vehicle controls (applied in the place of drug) were indistinguishable from time controls (not shown). SU6656 (*Figure 2A*) and PP2 (*Figure 2B*) both caused significant relaxation at 3 and 30  $\mu$ M, and this was significantly greater than the small degree of spontaneous run-down shown by the time controls. PP3 also caused relaxation at 30  $\mu$ M, but this was significantly less than for PP2 (*Figure 2C*). SU6656-mediated relaxation was unaffected by de-endothelialization (*n* = 6) and was similar when SU6656 was applied prior to the PGF<sub>2 $\alpha$</sub>  (*n* = 5) as when it was added acutely (see Supplementary material online, *Figures Siii* and *Siv*, respectively).

The *src*FK inhibitors SU6656 and PP2 cannot distinguish between different members of the *src* family. However *src* may be distinguished by the manner in which it is modified for insertion into the plasma membrane, a process required for full kinase activity.<sup>8,20</sup> *src* is simply myristoylated, whereas other family members, including *fyn* and *yes*, are also palmitoylated.<sup>8,21</sup> We therefore evaluated the effects of the acylation inhibitor 2-bromopalmitate and of eicosapentaenoic acid (EPA, which selectively displaces palmitoylated proteins from the membrane) on PGF<sub>2 $\alpha$</sub> -induced Ca<sup>2+</sup>-sensitization.<sup>8,20,21</sup> When 100  $\mu$ M 2-bromopalmitate was applied to a pre-existing PGF<sub>2 $\alpha$</sub> -induced contraction, a small additional transient contraction was caused, which was followed by a large sustained relaxation (*Figure 2D*). In contrast to this, 60  $\mu$ M EPA caused a large sustained additional contraction and no relaxation (288 ± 66% increase, *P* < 0.01 vs. time controls at 90 min, *n* = 5).

### 3.3 PGF<sub>2 $\alpha$</sub> enhances *src* family kinases auto-phosphorylation and protein tyrosine phosphorylation

In order to test the hypothesis that PGF<sub>2 $\alpha$</sub> -mediated contraction was acting via *src*FK activation and protein tyrosine phosphorylation, further western blot experiments were performed in PGF<sub>2 $\alpha$</sub> -treated IPA with anti-phospho-*src*FK (tyr-416) and anti-phospho-tyrosine. As shown in *Figure 3A*, *src*FK phosphorylation at tyr-416 was enhanced by PGF<sub>2 $\alpha$</sub>  in a time-dependent manner. The two bands analysed (60 and



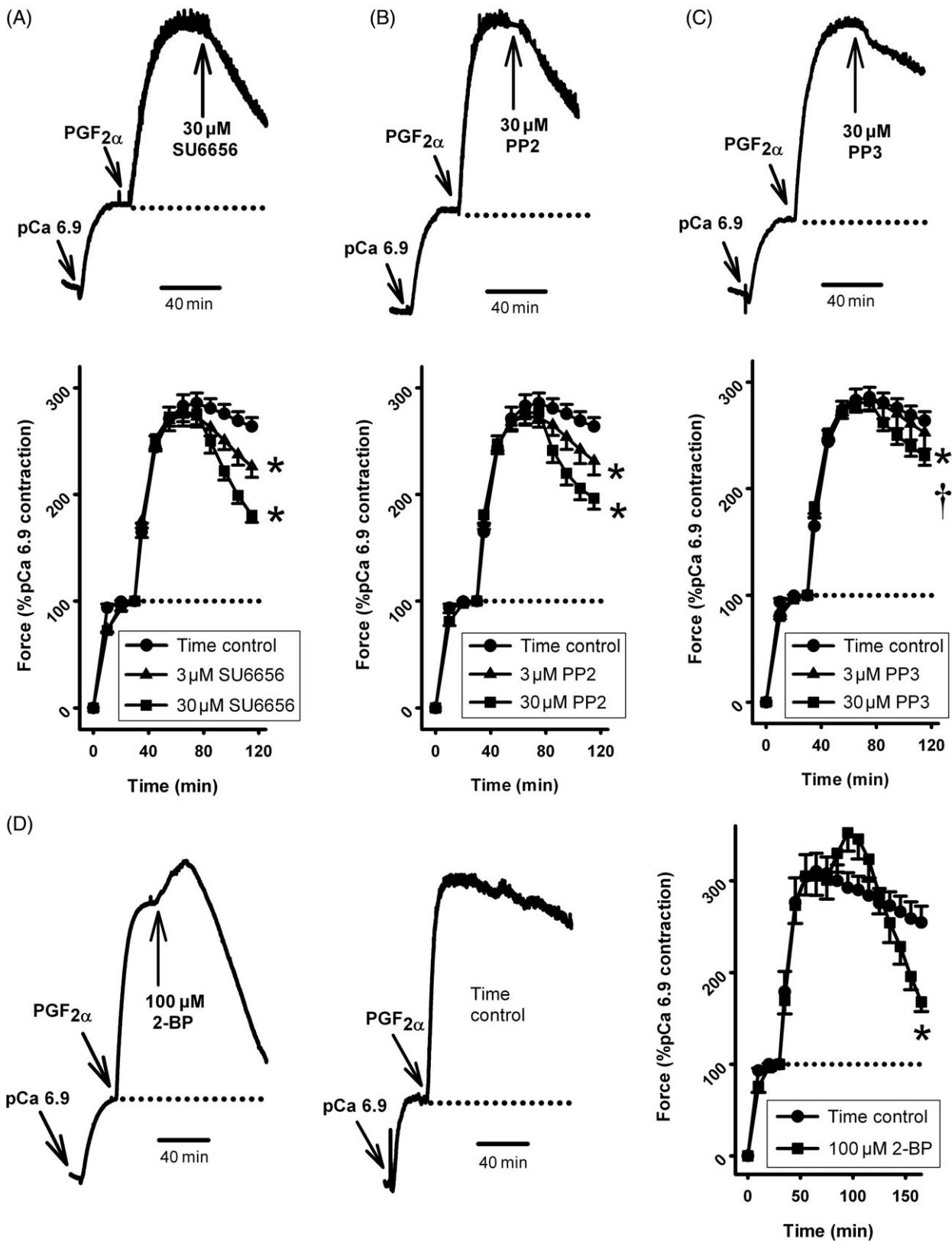
**Figure 1** Expression of *src* family kinases in intra-pulmonary artery. (A) mRNA expression by polymerase chain reaction for *src*, *fyn*, and *yes*. The examples shown are representative of triplicate determinations. Thirty-two polymerase chain reaction cycles were carried out for each cDNA. Values in parentheses indicate expected amplicon sizes in base pairs (bp). (B) Protein expression of *src* family kinases by western blot analysis of intra-pulmonary artery. Three bands (two at ~59/60 kDa, and one at 54 kDa) were visualized with anti-*src* and by anti-phospho-*src* family kinases (tyr-416). Phospho-*src* family kinases (tyr-416) immunoreactivity at 60 and 54 kDa was inhibited by PP2 (3 μM, \* $P < 0.05$ ,  $n = 8$  rats and 30 μM,  $P < 0.05$ ,  $n = 8$  rats) but not by PP3 (30 μM,  $n = 8$  rats).

54 kDa) showed significant peak enhancements at 2–5 min, whereas at 60 kDa, a significant second-phase-sustained enhancement at 30 min was apparent. Several protein bands were visualized with anti-phospho-tyrosine in IPA: a single intense band at ~120 kDa, bands at ~95 and ~75 kDa, and a close collection of bands centred at ~65 kDa (Figure 3B). This immunoreactivity was enhanced by PGF<sub>2α</sub>, significantly inhibited by PP2, but unaffected by PP3 (30 μM). Other fainter bands were present but were not quantified.

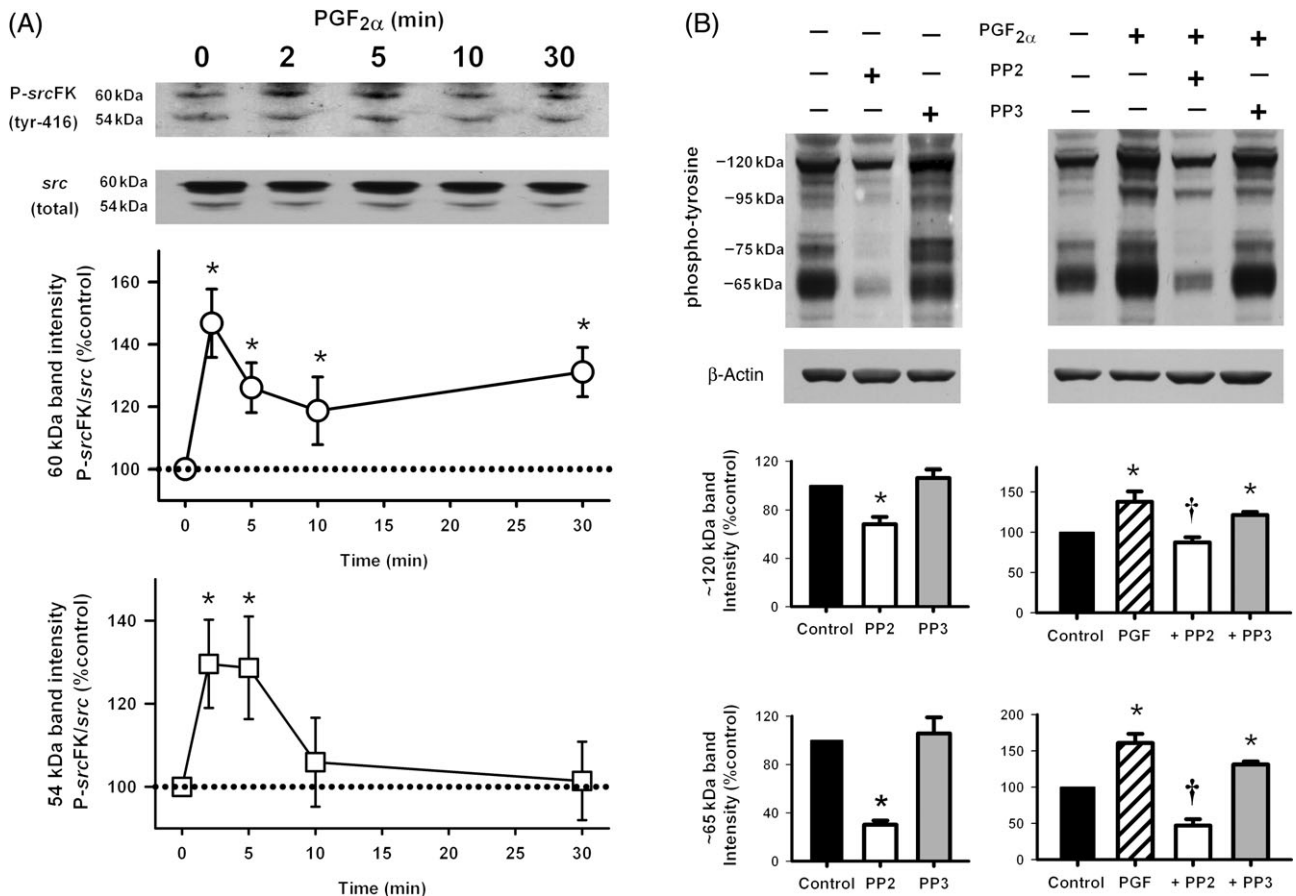
### 3.4 PGF<sub>2α</sub> mediates Ca<sup>2+</sup>-sensitization via MYPT-1 and MLC<sub>20</sub> phosphorylation

Western blot experiments were carried out for MYPT-1 (both phospho-thr-697 and phospho-thr-855) and MLC<sub>20</sub> (phospho-ser-19) in PGF-treated IPA. PGF<sub>2α</sub> significantly enhanced phosphorylation of MYPT-1 at both thr-697 (Figure 4A) and thr-855 (Figure 4B), although this effect was greater at thr-855. As expected, PGF<sub>2α</sub> also significantly enhanced ser-19 phosphorylation on MLC<sub>20</sub> (Figure 4C). With both MYPT-1 at thr-855 and MLC<sub>20</sub> at ser-19, the





**Figure 2** Effect of *src* family kinase inhibitors on  $\text{PGF}_{2\alpha}$ -induced contraction in  $\alpha$ -toxin-permeabilized intra-pulmonary artery. Intra-pulmonary arteries were pre-constricted with pCa6.9 for 30 min, prior to the addition of 100  $\mu\text{M}$   $\text{PGF}_{2\alpha}$  for 40 min (A–C) or 90 min (D). Compared with appropriate time controls ( $n = 12$  arteries), this contraction was significantly relaxed by SU6656 [(A) 3  $\mu\text{M}$ ,  $28 \pm 3\%$ ,  $*P < 0.05$ ,  $n = 8$  arteries and 30  $\mu\text{M}$ ,  $54 \pm 4\%$ ,  $*P < 0.01$ ,  $n = 8$  arteries], this contraction was significantly relaxed by SU6656 [(A) 3  $\mu\text{M}$ ,  $28 \pm 5\%$ ,  $*P < 0.05$ ,  $n = 8$  arteries and 30  $\mu\text{M}$ ,  $46 \pm 5\%$ ,  $*P < 0.01$ ,  $n = 12$  arteries]. PP3 also caused relaxation at 30  $\mu\text{M}$  [(C)  $28 \pm 2\%$ ,  $*P < 0.05$ ,  $n = 12$  arteries] but was significantly less effective than PP2 ( $\dagger P < 0.05$ ). 2-Bromopalmitate (2-BP) caused a transient contraction (non-significant) followed by significant relaxation [(D) 100  $\mu\text{M}$ ,  $71 \pm 4\%$ ,  $*P < 0.01$ ,  $n = 7$  arteries, compared with time controls,  $n = 7$  arteries].



**Figure 3** Effects of PGF<sub>2α</sub> on *src* family kinases tyr-416 auto-phosphorylation and tyrosine phosphorylation of multiple proteins in intra-pulmonary artery. (A) PGF<sub>2α</sub>-enhanced phospho-*src* (tyr-416) immunoreactivity at 60 and 54 kDa in a time-dependent manner (20 μM, \**P* < 0.05, *n* = 10–12 rats). (B) PGF<sub>2α</sub> also enhanced phospho-tyrosine immunoreactivity at multiple protein bands (20 μM, \**P* < 0.01, *n* = 10–15 rats). This was inhibited by PP2 (30 μM, †*P* < 0.05 vs. PGF<sub>2α</sub>, *n* = 10–15 rats) but not by PP3 (30 μM, *n* = 12 rats). Data are expressed as the ratio of phospho-tyrosine/β-actin immunoreactivity.

PGF-stimulated increase in phosphorylation peaked at 10 min. This time point was therefore used in subsequent phosphorylation experiments.

### 3.5 MYPT-1 and MLC<sub>20</sub> phosphorylation are sensitive to inhibition of *src* family kinases and rho-kinase

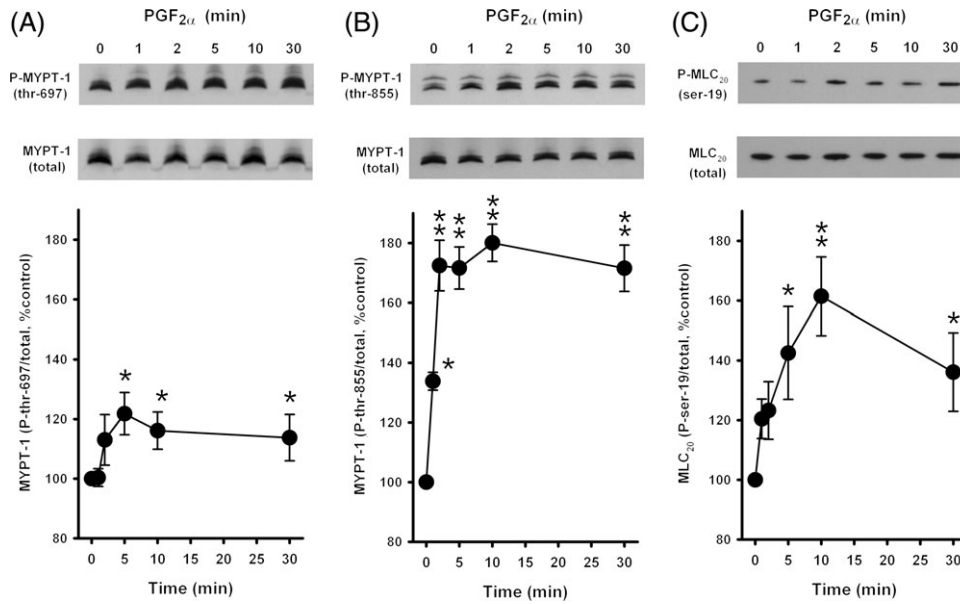
Basal phosphorylation of MYPT-1 at thr-697 and thr-855 and MLC<sub>20</sub> at ser-19 was not significantly affected by 30 μM SU6656, but was reduced by the rho-kinase inhibitor Y27632 (10 μM) (Figure 5A–C). Y27632 was much more effective against MYPT-1 phosphorylation at thr-855 than at thr-697. After 10 min exposure to PGF<sub>2α</sub>, phosphorylation of MYPT-1 at thr-697 and thr-855 and MLC<sub>20</sub> at ser-19 was enhanced (Figure 5D–F). This PGF<sub>2α</sub>-induced enhancement of MYPT-1 phosphorylation at thr-855 and MLC<sub>20</sub> phosphorylation at ser-19 was nearly abolished by 30 μM SU6656 (Figure 5E and F). Y27632 also greatly inhibited the phosphorylation of these two sites. The effects of the two inhibitors when used in combination were however not additive (Figure 5D–F).

### 3.6 Interaction between *src* family kinases and rho-kinase during PGF<sub>2α</sub>-mediated Ca<sup>2+</sup>-sensitization

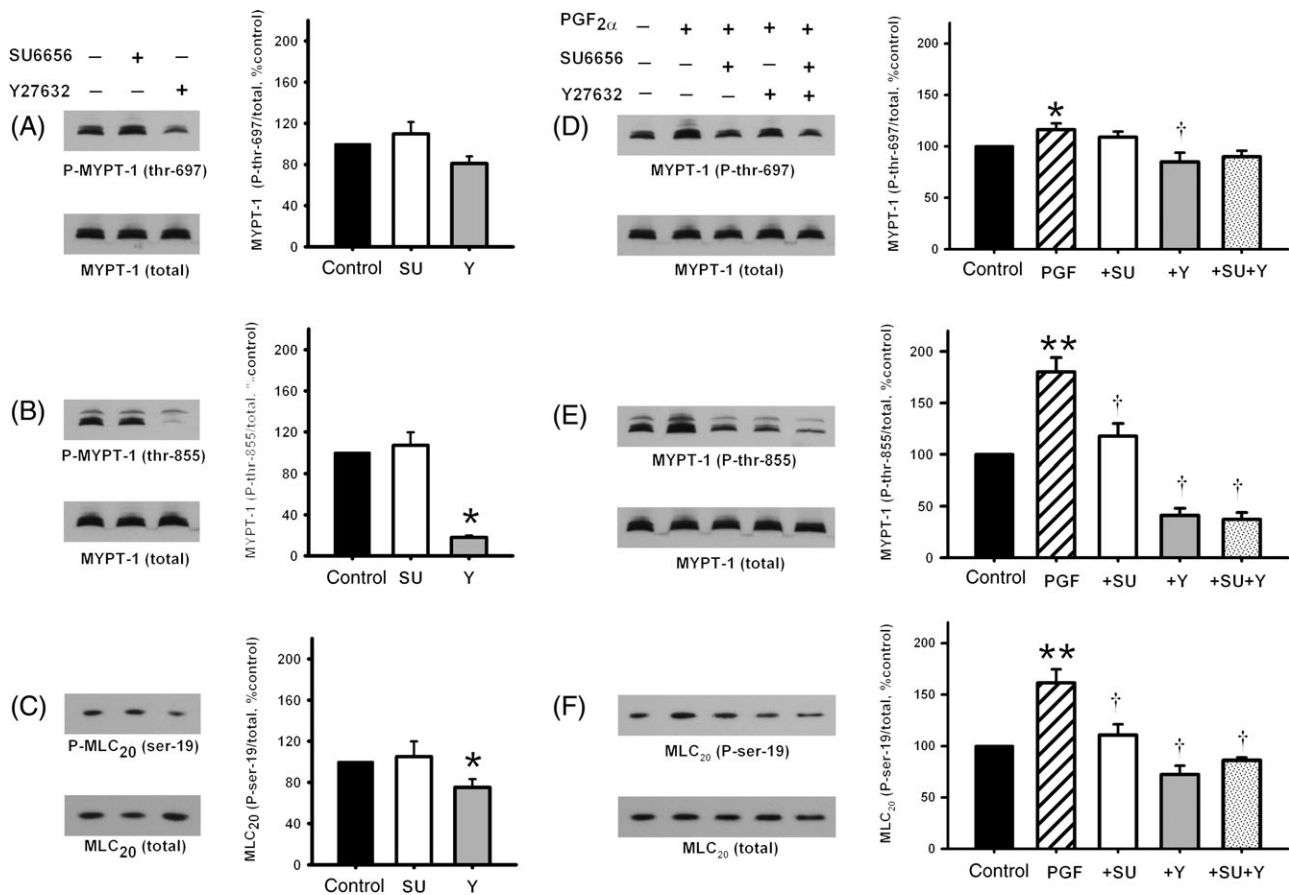
The results shown in Figure 5 suggest that *src*FK activation may be part of the same pathway as PGF<sub>2α</sub>-induced

rho-kinase-mediated phosphorylation of MYPT-1. This was corroborated by further contraction experiments in α-toxin-permeabilized IPA. As expected, 30 μM SU6656 did not have any significant effect on pCa 6.9-induced contraction in the absence of PGF<sub>2α</sub> (Figure 6A). PP2, PP3, and 2-bromopalmitate were similarly without effect (PP2 and PP3: 30 μM, *n* = 8, 2-BP: 100 μM, *n* = 5, not shown). Y27632, however, did relax this contraction, indicative of significant basal rho-kinase activity in the absence of agonist (Figure 6A). Y27632 also greatly relaxed the PGF<sub>2α</sub>-induced contraction, but this relaxation was not significantly greater when SU6656 was applied simultaneously with Y27632 (Figure 6B). The PKC-δ selective blocker rottlerin was completely without effect on PGF<sub>2α</sub> contraction (see Supplementary material online, Figure S<sub>v</sub>), eliminating the possibility that our results were influenced by inhibition of PKC-δ by Y27632, as shown previously by Wilson *et al.*<sup>22</sup>

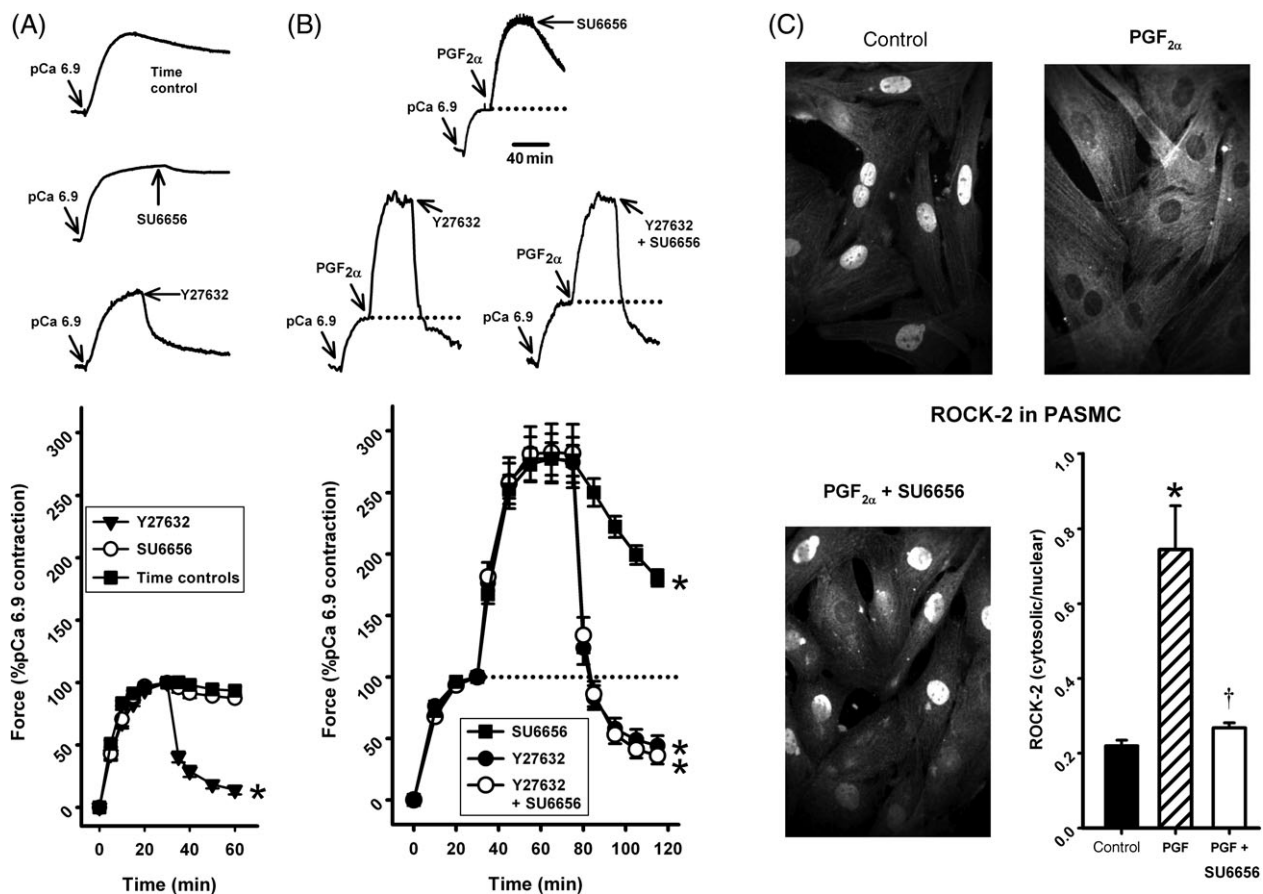
To examine more closely the interaction between *src*FK and rho-kinase during stimulation with PGF<sub>2α</sub>, we examined in PASCs the subcellular translocation of rho-kinase, a process that is required for its activation and therefore precedes phosphorylation of MYPT-1.<sup>5</sup> In control cells, the majority of rho-kinase (ROCK-2, the isoform activated by RhoA) antibody staining was localized to the nucleus; with some cytoplasmic staining, but upon stimulation with PGF<sub>2α</sub>, most of this translocated to the cytoskeleton. This movement was almost completely reversed by



**Figure 4** Effects of  $\text{PGF}_{2\alpha}$  on phosphorylation of MYPT-1 (120–130 kDa) and  $\text{MLC}_{20}$  (18 kDa) in intra-pulmonary artery. Phosphorylation was enhanced on MYPT-1 at thr-697 [(A) \* $P < 0.05$ ,  $n = 6-8$  rats] and thr-855 [(B) \* $P < 0.05$ ; \*\* $P < 0.01$ ,  $n = 6-10$  rats] and on  $\text{MLC}_{20}$  at ser-19 [(C) \* $P < 0.05$ ; \*\* $P < 0.01$ ,  $n = 6-11$  rats].



**Figure 5** Effects of inhibition of *src* family kinases and rho-kinase on MYPT-1 (120–130 kDa) and  $\text{MLC}_{20}$  (18 kDa) phosphorylation. [(A)–(C)] In the absence of  $\text{PGF}_{2\alpha}$ , phosphorylation of all three sites was not affected by SU6656 (30  $\mu\text{M}$ ,  $n = 7$  rats), but variably inhibited by Y27632 (10  $\mu\text{M}$ , \* $P < 0.05$ ,  $n = 8$  rats). [(D)–(F)] In the presence of  $\text{PGF}_{2\alpha}$ , phosphorylation of all three sites was enhanced (\* $P < 0.05$ ; \*\* $P < 0.01$ ,  $n = 8-11$  rats), and this enhancement was inhibited by SU6656 (30  $\mu\text{M}$ , MYPT-1 at thr-855 and  $\text{MLC}_{20}$  at ser-19, † $P < 0.01$ ,  $n = 8$  rats), Y27632 (10  $\mu\text{M}$ , † $P < 0.01$ ,  $n = 9$  rats), and the two drugs in combination († $P < 0.01$ ,  $n = 8$  rats). No additive effect of SU6656 and Y27632 was apparent.



**Figure 6** Interaction between *src* family kinases and rho-kinase activity in PGF<sub>2α</sub>-stimulated intra-pulmonary artery and pulmonary artery smooth muscle cells. (A) In intra-pulmonary artery, in the absence of PGF<sub>2α</sub>, the pCa<sub>6.9</sub> contraction was inhibited by Y27632 (10 μM, \**P* < 0.001, *n* = 8, compared with time controls, *n* = 8 arteries) but not by SU6656 (30 μM, *n* = 8 arteries). (B) Y27632 also inhibited contraction in the presence of PGF<sub>2α</sub> (10 μM, 84 ± 2%, \**P* < 0.001, *n* = 8 arteries) and this relaxation was unaltered by the additional presence of SU6656 (30 μM, 87 ± 2%, \**P* < 0.001, *n* = 8 arteries). The effect of SU6656 alone (from Figure 2) is included as a comparison. (C) In pulmonary artery smooth muscle cells, PGF<sub>2α</sub> triggered translocation of rho-kinase (ROCK-2) immunoreactivity from the nucleus to the cytoskeleton (20 μM, \**P* < 0.05, *n* = 6 determinations) and this was prevented by SU6656 (30 μM; \**P* < 0.05, *n* = 6 determinations).

pre-treatment with SU6656 (Figure 6C). In the absence of PGF<sub>2α</sub>, SU6656 was without effect (not shown). No ROCK-2 was detected in plasma membrane.

#### 4. Discussion

The results of this study clearly demonstrate the involvement of *src*FKs in agonist-induced contraction of the pulmonary artery. Most previous works have relied on the use of non-selective tyrosine kinase inhibitors such as genistein and tyrphostins, which do not distinguish between receptor and non-receptor tyrosine kinases, and have focused on the role of these kinases in Ca<sup>2+</sup> homeostasis (reviewed in Ward *et al.*<sup>15</sup>). The few that have addressed the question of Ca<sup>2+</sup>-sensitization have drawn opposing conclusions. For example, in α-toxin-permeabilized rat mesenteric arteries, ET-1-induced contractions were inhibited by tyrphostin-A23,<sup>14</sup> whereas in β-escin-permeabilized guinea-pig mesenteric arteries, direct Ca<sup>2+</sup>-mediated contraction was unaffected by tyrphostin.<sup>11</sup> This discrepancy may be explained by our current findings, since we showed that the *src*FK-selective inhibitors SU6656<sup>23</sup> and PP2<sup>24</sup> both selectively inhibited PGF<sub>2α</sub>-induced but not direct Ca<sup>2+</sup>-induced contraction in α-toxin-permeabilized rat pulmonary artery and inhibited PGF<sub>2α</sub>-mediated but not basal MLC<sub>20</sub> phosphorylation at

ser-19, suggesting the involvement of *src*FK only during stimulation of G-protein-coupled receptors.

MYPT-1, the regulatory subunit of myosin phosphatase, is an important mediator of changes in contractile force independent of a change in [Ca<sup>2+</sup>]<sub>i</sub> (Ca<sup>2+</sup>-sensitization).<sup>2</sup> As has been previously shown for PGF<sub>2α</sub> and other agonists in other vascular preparations,<sup>22,25,26</sup> we observed that PGF<sub>2α</sub> enhanced MYPT-1 phosphorylation at two sites, thr-697 and thr-855 (the rat homologues of thr-696 and thr-850 in humans), but it was principally phosphorylation at thr-855 which was most sensitive to rho-kinase inhibition by Y27632. Although both threonine residues were previously shown to be targets of rho-kinase,<sup>3,4</sup> our results agree with several other reports that thr-855 or its equivalent in other vascular preparations is the principal target of rho-kinase and that thr-697 is phosphorylated basally by another as-yet-unspecified serine/threonine kinase.<sup>22,25,26</sup> As with phosphorylation of MLC<sub>20</sub>, the enhancement of MYPT-1 phosphorylation at thr-855 by PGF<sub>2α</sub> was inhibited by SU6656 although basal phosphorylation was unaffected. Interestingly, Y27632 nearly abolished the pCa 6.9-induced contractions in IPA as well as the basal MYPT-1 phosphorylation at thr-855 and significantly inhibited MLC<sub>20</sub> phosphorylation, suggesting that there is considerable basal rho-kinase activity in the rat pulmonary artery. In any case, it is stimulated further by the addition of



agonist. A similar observation was made in U46619-stimulated rat caudal artery.<sup>22</sup>

The *src* family members most likely to be required for the responses described in this study are the highly expressed *src* and *fyn*, which have both been previously implicated in smooth muscle contraction,<sup>7,16</sup> and possibly *yes*, which is also highly expressed. Unfortunately, existing kinase inhibitors cannot easily distinguish between *src*, *fyn*, or *yes*. However, these kinases do differ in the way that they are post-translationally modified; *src* is dual-myristoylated, whereas both *fyn* and *yes* are also palmitoylated.<sup>8</sup> Previously, using inhibitors of fatty acid acylation and palmitoylation, Nakao *et al.*<sup>16</sup> suggested that sphingosylphosphorylcholine-induced Ca<sup>2+</sup>-sensitization in porcine coronary artery was mediated specifically by *fyn*, not *src*. We, however, found that the non-selective acylation inhibitor 2-bromopalmitate, but not the selective palmitoylation inhibitor EPA,<sup>21</sup> inhibited PGF<sub>2 $\alpha$</sub> -mediated Ca<sup>2+</sup>-sensitization. Accordingly, but bearing in mind that other important membrane-signalling proteins are also myristoylated and palmitoylated,<sup>27</sup> we may tentatively suggest that it is specifically *src* and not *fyn* or *yes* which is the *src*FK involved in PGF<sub>2 $\alpha$</sub> -mediated Ca<sup>2+</sup>-sensitization in rat IPA. The additional contractions caused by 2-bromopalmitate and EPA are presumably due to further actions of these drugs, independent of PGF<sub>2 $\alpha$</sub>  stimulation or *src*FK activity.

The question arises as to whether *src*FK activation in response to PGF<sub>2 $\alpha$</sub>  is upstream of rho/rho-kinase or enhances Ca<sup>2+</sup>-sensitivity through a separate pathway. The actions of SU6656 and Y27632 were not additive with respect to both inhibition of PGF<sub>2 $\alpha$</sub> -induced MYPT-1 and MLC<sub>20</sub> phosphorylation and inhibition of the associated PGF<sub>2 $\alpha$</sub> -induced contraction, suggesting that *src*FK and rho-kinase may be part of the same pathway. In order to test this hypothesis, we examined translocation of rho-kinase in PASMC, a process that is important for the activation of the kinase.<sup>5</sup> In control cells, rho-kinase staining was concentrated in the nucleus, as has been shown previously,<sup>28</sup> but upon PGF<sub>2 $\alpha$</sub>  treatment, it translocated to the cytoskeleton. Importantly, this movement was reversed by pre-treatment with SU6656, indicating that the movement of rho-kinase in response to stimulation by PGF<sub>2 $\alpha$</sub>  was likely to be mediated by *src*FK. In contrast to the study of Nakao *et al.*,<sup>16</sup> we found no evidence for the presence of rho-kinase at the plasma membrane, either in control or in stimulated cells.

PGF<sub>2 $\alpha$</sub>  mediates Ca<sup>2+</sup>-sensitization via the TP receptor,<sup>29</sup> which is coupled to G $\alpha_{q/11}$ .<sup>30</sup> How this coupling results in activation of *src*FK and/or rho/rho-kinase is unclear. For full kinase activity, *src*FK require auto-phosphorylation at tyr-416<sup>10</sup> and we showed that this is enhanced by PGF<sub>2 $\alpha$</sub> , indicating that *src*FK activity was increased. *Src* activation often goes hand in hand with focal adhesion kinase (FAK) which is activated by *src*.<sup>31,32</sup> FAK may be the ~120 kDa protein whose tyrosine phosphorylation in the current study was enhanced by PGF<sub>2 $\alpha$</sub>  and inhibited by PP2. A well-established connection between G-protein-linked receptors such as the TP prostenoid receptor (which is activated by PGF<sub>2 $\alpha$</sub> ) and mitogenic signalling involves *src*-dependent transactivation of the EGF-receptor tyrosine kinase.<sup>33</sup> This mechanism also involves G $\alpha_i$ , which is known to activate *src* by direct coupling.<sup>34</sup> Studies with the tyrosine phosphatase inhibitor vanadate have suggested that RhoA

translocation is stimulated by as-yet-unspecified tyrosine phosphorylation.<sup>35</sup> Furthermore, a pathway for tyrosine kinase-dependent modulation of rho activation has been suggested: active rho requires GTP, and it acquires this from rho-specific RhoGEF.<sup>6</sup> Two RhoGEFs in native smooth muscle that may enhance RhoA activation by G $\alpha_q$  or G $\alpha_{12/13}$  are leukaemia-associated RhoGEF (LARG) and PDZ-RhoGEF,<sup>26,36</sup> and LARG is indeed activated by tyrosine phosphorylation.<sup>37</sup>

In conclusion, we have shown for the first time that agonist-mediated, rho-kinase-dependent Ca<sup>2+</sup>-sensitization but not basal rho-kinase activity in rat pulmonary artery requires *src*FK activity. This has implications for the regulation of rho and rho-kinase-dependent vascular tone and vascular remodelling in health and disease. The precise mechanism(s) by which G-protein-linked receptors couple to *src*FK and subsequent rho-kinase activation remain to be determined.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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