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## LOW CONCENTRATIONS OF SPHINGOSYLPHOSPHORYLCHOLINE ENHANCE PULMONARY ARTERY VASOREACTIVITY: ROLE OF PKC $\delta$ AND Ca $^{2+}$ ENTRY

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### Abstract

Sphingosylphosphorylcholine (SPC) is a powerful vasoconstrictor, but *in vitro* its EC<sub>50</sub> is ~100-fold more than plasma concentrations. We examined whether sub-contractile concentrations of SPC (1  $\mu$ mol/l) modulated vasoreactivity of rat intrapulmonary arteries (IPA), using myography and measurement of intracellular [Ca $^{2+}$ ]<sub>i</sub> ([Ca $^{2+}$ ]<sub>i</sub>). SPC (1  $\mu$ mol/l) had no effect on force or [Ca $^{2+}$ ]<sub>i</sub> on its own, but dramatically potentiated constrictions induced by ~25 mmol/l [K $^{+}$ ], such that at 40 min force and [Ca $^{2+}$ ]<sub>i</sub> (Fura PE3 340/380 ratio) were increased by 429  $\pm$  96% and 134  $\pm$  26% respectively. The potentiation was stereospecific, apparent at concentrations >100 nmol/l SPC, and independent of the endothelium, 2-APB-sensitive Ca $^{2+}$  entry, and Rho kinase. It was abolished by the PLC inhibitor U73122, the broad spectrum PKC inhibitor Ro 31-8220 and the PKC $\delta$  inhibitor rottlerin, but not by Gö6976, which is ineffective against PKC $\delta$ . The potentiation could be attributed to enhancement of Ca $^{2+}$  entry. SPC also potentiated the responses to PGF<sub>2 $\alpha$</sub>  and U436619, which activate a 2-APB sensitive non-selective cation channel (NSCC) in IPA. In this case potentiation was partially inhibited by diltiazem, but abolished by 2-APB, Ro31-8220 and rottlerin. 1  $\mu$ mol/l SPC caused translocation of PKC $\delta$  to the perinuclear region and cytoskeleton of cultured IPA smooth muscle cells. We present the novel finding that low, sub-contractile concentrations of SPC potentiate Ca $^{2+}$  entry in IPA through both voltage-dependent and independent pathways, via a receptor-dependent mechanism involving PKC $\delta$ . This has implications for the physiological role of SPC, especially in cardiovascular disease where SPC is reported to be elevated.

### Keywords

Pulmonary artery; Lysosphingolipids; Sphingosylphosphorylcholine; L-type Ca $^{2+}$  channels; Protein kinase C $\delta$

### Introduction

The lysosphingolipid sphingosylphosphorylcholine (SPC) is an important cardiovascular mediator derived from sphingomyelin and carried by plasma lipoproteins<sup>1,2</sup>. It has been associated with multifarious processes including proliferation, angiogenesis, inflammation and vasoconstriction<sup>1-7</sup>, and elevated SPC concentrations may contribute to cardiovascular

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disease<sup>2</sup>, including hypercholesterolemia and atherosclerosis<sup>3,8</sup>, cerebral and coronary vasospasm<sup>6,7</sup> and hypertension<sup>9</sup>. In systemic arteries SPC-induced vasoconstriction is reported to be mediated via pertussis toxin (PTX) sensitive G<sub>i</sub>-proteins, IP<sub>3</sub>-induced Ca<sup>2+</sup> release, Rho kinase (RhoK)-mediated Ca<sup>2+</sup> sensitization, and Ca<sup>2+</sup> entry via voltage-dependent L-type channels<sup>1,2,10</sup>, although in some arteries Ca<sup>2+</sup> sensitization may be the major mechanism<sup>6,7</sup>. In contrast, we recently reported that in rat small intrapulmonary arteries (IPA) the SPC-induced elevation of [Ca<sup>2+</sup>]<sub>i</sub> is primarily due to activation of a voltage-independent Ca<sup>2+</sup> entry pathway, that is insensitive to PTX and Ca<sup>2+</sup> release from stores<sup>4</sup>.

A conceptual problem regarding any physiological role for SPC is that the EC<sub>50</sub> for most of its effects *in vitro* is in the range of 7-18 μmol/l<sup>1,2,4-6</sup>, whereas plasma concentrations may be as low as 50 nmol/l, though in serum this rises to 130 nmol/l suggesting release from activated platelets<sup>11</sup>. It has been argued that lysophospholipids act in a paracrine or autocrine fashion, with higher local concentrations than in plasma, especially at sites of thrombus formation, atherosclerosis and inflammation<sup>1,2,6,7</sup>; tissue concentrations of ~10 μmol/l SPC are reported for certain types of inflammation<sup>12</sup>. Studies on SPC are hindered by lack of specific antagonists, and its receptors remain unidentified. SPC is a low affinity ligand for sphingosine-1-phosphate (S1P) receptors, but this cannot account for the majority of its actions. Although GPR4, OGR1 and G2A have been proposed as SPC receptors, recent evidence suggests this family responds to protons and not SPC<sup>13,14</sup>.

We considered whether concentrations of SPC insufficient to exert direct vasoactive effects might potentiate vasoconstriction induced by other means, as demonstrated for some other agonists in IPA<sup>15</sup>, and examined the effects of sub-contractile concentrations of SPC (1 μmol/l) on depolarization- and agonist-induced vasoconstriction of rat IPA. We report the novel and potentially important finding that these low concentrations of SPC substantially potentiate IPA vasoreactivity via a PKCδ-dependent enhancement of both voltage-dependent and independent Ca<sup>2+</sup> entry, and that this mechanism differs from that underlying vasoconstriction induced by higher concentrations of SPC.

## Materials and Methods

Male Wistar rats (200-300g) were killed by cervical dislocation; the investigation 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). Small IPA (3<sup>rd</sup>-4<sup>th</sup> branch; 150-450 μm i.d.) were mounted in a myograph (Danish MyoTechnology, Denmark) containing physiological salt solution (PSS) gassed with 95% air / 5% CO<sub>2</sub> (pH 7.4) at 37°C, as previously described<sup>16</sup>. Endothelial denudation was achieved by rubbing the lumen, and confirmed by loss of relaxation to acetylcholine. Pulmonary artery smooth muscle cells (PASMCs) were dispersed from similar sized IPA using collagenase (type XI, 2mg/ml) and papain (1mg/ml)<sup>16</sup>, and used immediately for electrophysiology or cultured in DMEM containing 10% FCS. PASMCs from passages 3-4 were grown on 13mm coverslips and growth-arrested in serum-free medium for 24 hrs before use; each cell line was verified as smooth muscle by immunostaining for smooth muscle α-actin, calponin and desmin (Santa Cruz Biotechnology, CA).

### Estimation of [Ca<sup>2+</sup>]<sub>i</sub>

IPA were incubated for 1 hour at 37°C in PSS with 4 μmol/l Fura PE-3/AM followed by washing with PSS. The myograph was mounted on an inverted microscope and microfluorimeter (Cairn Ltd., Faversham, U.K.). Force was recorded simultaneously with the ratio of emission intensities at >510 nm from excitation wavelengths of 340 and 380 nm (F<sub>340/380</sub>).

## Electrophysiology

Freshly dispersed PASMCs were studied at ~20°C using whole cell patch clamp (Axopatch-200c, Axon Instruments Inc., CA). The bath was continuously perfused with HEPES-buffered PSS containing 2 mmol/l tetraethylammonium (TEA) to block BK<sub>Ca</sub> current. The pipette solution contained (mmol/l): KCl 140; MgCl<sub>2</sub> 2; EGTA 5; HEPES 10; MgATP 2.0, Li<sub>2</sub>GTP 0.2; pH adjusted to 7.2 with KOH. Current-voltage relationships were obtained using a voltage ramp protocol, with a holding potential of -60 mV and a 0.5 s ramp from -90 to +100mV every 5 s.

## α-toxin permeabilization of IPA

Isometric force was recorded in α-toxin-permeabilized arteries, as described previously<sup>4</sup>. IPA were mounted as above, but incubated at 26°C and permeabilized with 60 μg/ml α-toxin at pCa 6.5. IPA were relaxed with solution containing 10 mmol/l EGTA before sub-maximal vasoconstriction was induced by increasing [Ca<sup>2+</sup>] to 200 nmol/l (pCa 6.7) by adjusting the K<sub>2</sub>EGTA/CaEGTA ratio.

## PKCδ translocation

Cultured PASMCs were exposed to 1 μM SPC for 10 min, before fixation with 4% paraformaldehyde and 4% PEG6000, and permeabilization with 0.1% Triton. Cells were stained with anti-PKCδ antibody (Santa Cruz Biotechnology) overnight at 4C, followed by Alexa 488 labelled secondary antibody (goat anti-rabbit IG, Invitrogen) for 2 hrs at room temperature. Coverslips were viewed using a CARVII confocal imager (BD Biosciences) and Zeiss Axovert microscope. Specificity of anti-PKCδ was confirmed by Western blot; staining was negative with secondary antibody alone.

## Solutions and drugs

Physiological salt solution (PSS) contained (mmol/l): 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. In experiments where [K<sup>+</sup>] was altered, osmolarity was maintained by equimolar substitution of K<sup>+</sup> for Na<sup>+</sup>. HEPES-buffered PSS contained (mmol/l) NaCl 130; KCl 4; CaCl<sub>2</sub> 2.0, MgCl<sub>2</sub> 2; glucose 5; HEPES 10, buffered to pH 7.4 with NaOH. Chemicals were obtained from Calbiochem (Notts., UK), except 2-APB (2-aminoethoxydiphenylborane; Tocris Cookson Ltd., Bristol, UK), and *D-erythro*-SPC and *L-threo*-SPC (Avanti Lipids Inc., AL). Unless otherwise stated, diastereomeric SPC was used in experiments. Lysosphingolipids were dissolved in 2:1 chloroform:methanol for storage at -80°C. Before use, solvent was evaporated and the residue dissolved in PSS.

## Calculations and statistical analysis

Changes in force and [Ca<sup>2+</sup>]<sub>i</sub> (F<sub>340/380</sub>) were normalized to the response to a 2 min exposure to KPSS<sub>4,17</sub>, and expressed as %KPSS. Values for EC<sub>50</sub> (IC<sub>50</sub>), and maximum response were derived from fitting to the Hill equation (Sigmaplot, Systat Software Inc., CA). Results are shown as mean ± S.E.M., and compared using paired or unpaired Student's *t* test, or ANOVA with a Holm-Sidak *post hoc* as appropriate (SigmaStat, Systat Software Inc., CA).

## Results

As we previously reported for diastereomeric SPC<sub>4</sub>, *D-erythro*-SPC induced vasoconstriction of IPA only at concentrations >1 μmol/l, with a similar Hill coefficient (2.4 ± 0.9), maximum vasoconstriction (88 ± 6% KPSS), and EC<sub>50</sub> (7.7 ± 0.2 μmol/l; n=3). *L-threo*-SPC only elicited constriction at 30 μmol/l (11 ± 4% KPSS; n=3).

### Sub-contraction concentrations of SPC and depolarization-induced IPA vasoconstriction

IPA were challenged with sequential 5 min applications of PSS containing 21–25 mmol/l  $[K^+]$  to give a rise in tension ~15% of that induced by KPSS. SPC (1  $\mu\text{mol/l}$ ) was added to the bath, and the procedure repeated at 20 min intervals in the continued presence of SPC. Alone, 1  $\mu\text{mol/l}$  SPC had no effect on tension and caused only a very small, transient (<3 min) elevation in  $[Ca^{2+}]_i$  ( $1.9 \pm 0.7\%$  KPSS,  $n=13$ ). However, it subsequently caused a dramatic potentiation of the response to depolarization that gradually increased to a plateau at ~40–60 min (Fig. 1), such that at 20 min force was increased to  $294 \pm 47\%$  and  $[Ca^{2+}]_i$  ( $F_{340/380}$ ) to  $198 \pm 14\%$  ( $n=7$ ,  $p<0.01$ ), and at 40 min to  $529 \pm 96\%$  and  $234 \pm 26\%$  respectively ( $n=6$ ,  $p<0.001$ ). In the absence of SPC the response to repeated depolarization was unchanged over the entire experimental period ( $95 \pm 10\%$  of control at 80 min,  $n=4$ ). The SPC-induced potentiation of force was apparent at concentrations above 100 nmol/l, concentration dependent and stereospecific (Fig. 2A).

We examined the effects of SPC on the concentration-response relationship to  $[K^+]$  (Fig. 2B). Pre-incubation with 1  $\mu\text{mol/l}$  SPC for 40 min shifted this relationship to the left ( $EC_{50}$ : control:  $36 \pm 2$  mmol/l  $[K^+]$ ; SPC:  $31 \pm 1$  mmol/l  $[K^+]$ ;  $n=9$ ,  $p<0.001$ ). The maximum response was increased (control:  $109 \pm 3\%$  KPSS; SPC:  $120 \pm 4\%$  KPSS;  $p<0.001$ ), indicating enhancement of voltage-gated  $Ca^{2+}$  entry and/or increased  $Ca^{2+}$  sensitivity rather than depolarization. 1  $\mu\text{mol/l}$  SPC had no effect on  $K_V$  current amplitude in myocytes from IPA, with an unchanged zero current potential (control:  $-24 \pm 1$  mV; SPC:  $-22 \pm 1$  mV;  $n=5$ ). Direct examination of voltage-activated  $Ca^{2+}$  current ( $I_{Cav}$ ) proved impossible, as this current is small and highly variable in IPA and ran down over the period required (>30min).

### Mechanism of SPC-induced potentiation of vasoconstriction

Using the sequential  $K^+$  challenge protocol shown in Fig. 1, we examined the effects of endothelial denudation, 2-APB (75  $\mu\text{mol/l}$ ), cyclopiazonic acid (CPA, SERCA inhibitor, 10  $\mu\text{mol/l}$ ), Y27632 (RhoK inhibitor, 3  $\mu\text{mol/l}$ ), U73122 (PLC inhibitor, 10  $\mu\text{mol/l}$ ), and three PKC inhibitors: Ro31-8220 (broad spectrum, 3  $\mu\text{mol/l}$ ), Gö6976 (conventional and some novel isozymes, but not PKC $\delta$ , 3  $\mu\text{mol/l}$ ), and rottlerin (PKC $\delta$ , 1  $\mu\text{mol/l}$ ). IPA were pre-incubated with inhibitor for 20 min, then challenged four times at 20 min intervals with PSS containing ~24 mmol/l  $[K^+]$ , with SPC (1  $\mu\text{mol/l}$ ) applied during the final two challenges. Tension developed during these last two challenges was compared with the paired controls, i.e. following preincubation with inhibitor but before addition of SPC.

SPC-induced potentiation of tension was unaffected by removal of the endothelium, 2-APB, CPA, Y27632 or Gö6976, but was substantially reduced or abolished by U73122, Ro31-8220, and rottlerin (Fig. 3). This suggests the mechanism of potentiation involves PLC and PKC $\delta$ , but not endothelium-derived mediators, store release or 2-APB-sensitive  $Ca^{2+}$  entry, RhoK-mediated  $Ca^{2+}$  sensitization, or other PKC isozymes.

Quantification of changes in  $[Ca^{2+}]_i$  in the presence of PKC inhibitors was complicated by their fluorescence, but in 3 experiments we were able to show that Ro 31-8220 abolished both the SPC-induced potentiation of tension and the rise in  $[Ca^{2+}]_i$  (tension:  $99 \pm 15\%$  control;  $[Ca^{2+}]_i$  ( $F_{340/380}$ ):  $104 \pm 20\%$  control) (Fig. 4A & B). PdBu (100 nmol/l), an activator of conventional and novel PKC isozymes, also potentiated the depolarization-induced rise in  $[Ca^{2+}]_i$  ( $147 \pm 11\%$  control,  $n=3$ ,  $p<0.05$ ; Fig. 4C).

1  $\mu\text{mol/l}$  SPC had no detectable effect on tension ( $n=4$ ) in  $\alpha$ -toxin permeabilized IPA constricted to ~30% of maximum response with a pCa of 6.7, obviating any contribution from PKC-mediated  $Ca^{2+}$  sensitization.

### Effect of 1 $\mu\text{mol/l}$ SPC on agonist-induced IPA vasoconstriction

SPC (1  $\mu\text{mol/l}$ ) caused a significant left-ward shift of the  $\text{PGF}_{2\alpha}$  concentration-response relationship (Fig. 5A).  $\text{PGF}_{2\alpha}$ -induced vasoconstriction of IPA is mediated via TP receptors and associated with  $\text{Ca}^{2+}$  entry via a 2-APB-sensitive voltage-independent pathway and diltiazem-sensitive L-type channels<sup>17</sup>. As expected, diltiazem (10  $\mu\text{mol/l}$ ) partially suppressed the response to  $\text{PGF}_{2\alpha}$ , but only reduced and did not abolish the SPC-induced left-ward shift of the  $\text{PGF}_{2\alpha}$  concentration-response relationship (Fig. 5B). Consistent with this, and using the sequential challenge protocol shown in Fig. 1 but with  $\text{PGF}_{2\alpha}$ , SPC (1  $\mu\text{mol/l}$ ) in the presence of diltiazem potentiated 3 $\mu\text{mol/l}$   $\text{PGF}_{2\alpha}$ -induced constriction to  $341 \pm 125\%$  ( $n=3$ ) at 40 min, and U436619 (20 nmol/l)-induced tension and elevation in  $[\text{Ca}^{2+}]_i$  (tension:  $563 \pm 159\%$ ;  $[\text{Ca}^{2+}]_i$  ( $F_{340/380}$ ):  $212 \pm 57\%$ ;  $n=3$ ; Fig. 4D), indicating that SPC also potentiates voltage-independent  $\text{Ca}^{2+}$  entry.

Repeated challenges with  $\text{PGF}_{2\alpha}$  showed less consistency than for  $\text{K}^+$ , so in order to characterise in more detail the SPC-mediated potentiation of the response to  $\text{PGF}_{2\alpha}$ , we applied SPC during a stable established  $\text{PGF}_{2\alpha}$ -induced constriction equivalent to  $\sim 25\%$  KPSS, with potentiation measured at 10 min (Fig. 6A). Due to the slowly developing nature of the SPC-induced potentiation, its magnitude using this shortened protocol was smaller than for the repeated challenge protocol, but was still highly significant ( $140 \pm 6.2\%$  control,  $n=12$ ,  $p<0.001$ ). For comparison, SPC-induced potentiation of  $\text{K}^+$ -induced constriction was  $165 \pm 26\%$  ( $n=3$ ) at 10 min using this protocol.

Using the above protocol we investigated the effects of diltiazem (10  $\mu\text{mol/l}$ ), 2-APB (75  $\mu\text{mol/l}$ ), Y27632 (3  $\mu\text{mol/l}$ ), Ro31-8220 (3  $\mu\text{mol/l}$ ), rottlerin (1  $\mu\text{mol/l}$ ) and CPA (10  $\mu\text{mol/l}$ ) on the potentiation induced by 1 mol/l SPC. IPA were preincubated with inhibitors for 20 min;  $\text{PGF}_{2\alpha}$  was then applied to induce a constriction of  $\sim 25\%$  KPSS (3-10  $\mu\text{mol/l}$   $\text{PGF}_{2\alpha}$ ). Fig. 6B shows the effects of these inhibitors on SPC-induced potentiation. Consistent with the data shown in Fig. 3 for depolarization-induced constriction, the potentiation was unaffected by Y27632 but substantially reduced or abolished by Ro31-8220 and rottlerin, and consistent with figs. 4D and 5B diltiazem significantly reduced the potentiation by  $\sim 50\%$ , whereas 2-APB abolished it. Following constriction with U-46619 (20 nmol/l) in the presence of diltiazem, SPC (1  $\mu\text{mol/l}$ ) caused a further rise in both tension ( $148 \pm 10\%$  control,  $n=6$ ,  $p<0.01$ ) and  $[\text{Ca}^{2+}]_i$  ( $184 \pm 16\%$ ,  $p<0.01$ ), again confirming that SPC potentiates voltage-independent  $\text{Ca}^{2+}$  entry.

### Effect of 1 $\mu\text{mol/l}$ SPC on PKC $\delta$ translocation in cultured PSMCs

The data obtained in the presence of the three PKC inhibitors strongly suggested a role for PKC $\delta$ ; we therefore examined the effect of 1  $\mu\text{mol/l}$  SPC on PKC $\delta$  sub-cellular localization in PSMCs cultured from IPA. In untreated cells PKC $\delta$  staining was strongest within the nucleus, with diffuse staining throughout the cytoplasm (Fig. 7). In SPC treated cells staining in the nucleus was reduced, with translocation of PKC $\delta$  to the perinuclear region, cytoskeleton and possibly membrane.

## Discussion

Our key finding is that concentrations of SPC insufficient to cause vasoconstriction or elevation of  $[\text{Ca}^{2+}]_i$  in IPA can nevertheless substantially enhance vasoconstriction elicited by other means. Such concentrations (300-1000 nmol/l) are  $>10$  fold smaller than the  $\text{EC}_{50}$  reported for SPC-induced vasoconstriction *per se*, or indeed most other actions of SPC ( $\sim 12$   $\mu\text{mol/l}$ )<sup>1,2,4-6</sup>, and are consequently much closer to those reported for plasma<sup>11</sup>. Notably, SPC was stereospecific for the effects elicited by both high and low concentrations, implying that both are receptor mediated (Fig. 2A).

The signalling pathways underlying potentiation of IPA vasoreactivity by  $1 \mu\text{mol/l}$  SPC differ from those underlying vasoconstriction to higher concentrations. We have shown that the latter is mediated by increased RhoK-mediated  $\text{Ca}^{2+}$  sensitivity and activation of 2-APB-sensitive, voltage-independent  $\text{Ca}^{2+}$  entry pathway, but is independent of  $\text{Ca}^{2+}$  release from stores and PLC4. In contrast, the potentiation of depolarization-induced constriction by  $1 \mu\text{mol/l}$  SPC was unaffected by 2-APB or inhibition of RhoK, but was strongly suppressed by inhibition of PLC and PKC (Fig. 3).

The related sphingolipid S1P inhibits  $\text{K}_V$  channels and depolarises cerebral arteries by via a PKC-dependent mechanism<sup>18</sup>. However, although the  $[\text{K}^+]/\text{force}$  relationship was shifted to the left by  $1 \mu\text{mol/l}$  SPC, the maximum response was also enhanced (Fig. 2B), and there was no effect on  $\text{K}_V$  currents in IPA smooth muscle cells. This suggests that neither  $\text{K}_V$  channel inhibition nor depolarization can account for the potentiation of vasoreactivity. Both RhoK and PKC modulate  $\text{Ca}^{2+}$  sensitivity in pulmonary artery<sup>19</sup>, but as inhibition of RhoK was without effect and  $1 \mu\text{mol/l}$  SPC did not alter  $\text{Ca}^{2+}$  sensitivity of  $\alpha$ -toxin permeabilized IPA, this suggests the potentiation is mediated entirely via the observed enhancement of the elevation of  $[\text{Ca}^{2+}]_i$  (Figs. 1 and 4).

PKC enhances  $\text{Ca}^{2+}$  entry via L-type channels in smooth muscle<sup>20</sup> by increasing channel availability and prolonging open time<sup>21,22</sup>, and there is some evidence for differential effects of conventional and novel PKC isozymes<sup>20</sup>. Ro31-8220 has equivalent potencies against conventional ( $\alpha$ ,  $\beta_I$ ,  $\beta_{II}$ ,  $\gamma$ ), novel ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ,  $\mu$ ) and atypical ( $\iota$ ,  $\lambda$ ) PKC isozymes ( $\text{IC}_{50} < 1 \mu\text{mol/l}$ ), whereas Gö6976 has selectivity for conventional isozymes ( $\text{IC}_{50} < 1 \mu\text{mol/l}$ ), but is relatively ineffective against PKC $\delta$  ( $\text{IC}_{50} \sim 30 \mu\text{mol/l}$ ). Rottlerin has a 10-fold greater potency for PKC $\delta$  ( $\text{IC}_{50} \sim 1\text{-}6 \mu\text{mol/l}$ ) than for PKC  $\alpha$ ,  $\beta$  and  $\gamma$ , and  $\sim 50$ -fold for  $\epsilon$ ,  $\theta$  and  $\zeta$ <sup>19,23</sup>. Ro31-8220 and rottlerin abolished the SPC-induced potentiation of vasoreactivity whereas Gö6976 had no effect, suggesting that PKC $\delta$  is a critical component of the signal transduction pathway. Accordingly,  $1 \mu\text{mol/l}$  SPC induced PKC $\delta$  translocation in PSMCs from the nucleus to the perinuclear region, cytoskeleton and possibly membrane (Fig. 7); activation of PKC $\delta$  has previously been associated with a similar sub-cellular translocation in vascular smooth muscle and cardiomyocytes<sup>24,25</sup>.

The potentiation of  $\text{PGF}_{2\alpha}$ -induced vasoconstriction was inhibited by diltiazem by only 50%, and unlike that for depolarization-induced vasoconstriction was abolished by 2-APB (Fig. 6B). This implies that SPC also potentiates voltage-independent  $\text{Ca}^{2+}$  entry, and this was found to be the case (see Fig. 4D). This is also PKC $\delta$ -dependent as the potentiation was abolished by Ro31-8220 and rottlerin (Fig. 6B). The complete block by 2-APB and partial block by diltiazem is consistent with a model whereby TP receptor agonists activate NSCC, resulting in both  $\text{Ca}^{2+}$  and  $\text{Na}^+$  entry, with the latter causing depolarization and additional  $\text{Ca}^{2+}$  entry via L-type channels (see Fig. 5 and 17).

Consistent with our results, other studies have shown that SPC activates PKC $\delta$  and PLC in pancreatic islet endothelial cells<sup>26</sup>, and PKC $\delta$  has been reported to mediate the hypoxia-induced enhancement of  $\text{Ca}^{2+}$  entry via L-type channels in carotid body glomus cells<sup>27</sup>. Moreover, SPC has also been reported to potentiate 2-APB-sensitive  $\text{Ca}^{2+}$  entry in thyroid FRO cells via a PKC- and PLC-dependent mechanism, independent of intracellular  $\text{Ca}^{2+}$  release or any increase in inositol phosphates<sup>28</sup>. SPC-induced vasoconstriction in systemic arteries is in part mediated by PLC/ $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release<sup>1,10</sup>, though this is not the case for IPA<sup>4</sup>; this reflects our previous finding that activation of PLC/ $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release does not couple to vasoconstriction in IPA<sup>17</sup>. Considering the above, we speculate that the potentiation of IPA vasoreactivity elicited by SPC is mediated via a GPCR linked to PLC, with subsequent activation of PKC $\delta$  by DAG and PKC $\delta$ -dependent phosphorylation of L-type channels and a 2-APB-sensitive NSCC.

In summary, we report the novel and potentially important finding that sub-contractile concentrations (  $1 \mu\text{mol/l}$ ) of SPC cause significant potentiation of IPA vasoreactivity via a PKC $\delta$ -mediated enhancement of Ca $^{2+}$  entry via both L-type channels and a voltage-independent, 2-APB-sensitive NSCC. This pathway involves different mechanisms from those previously shown to underlie vasoconstriction induced by higher concentrations of SPC in IPA4 and other vascular beds see<sup>1,2,10</sup>, possibly implying the existence of more than one GPCR for SPC.

## Perspectives

Our results have considerable implications for the pathophysiological role of SPC. Whilst lysosphingolipids may act in a paracrine or autocrine fashion, with localised concentrations considerably higher than in plasma especially at sites of thrombus formation, atherosclerosis and inflammation<sup>1,2,6,7</sup>, the discrepancy between plasma SPC concentrations<sup>11</sup> and those required for most of its reported effects is still in the order of 100 fold. The phenomenon we describe here however was apparent at 300 nmol/l, and could conceivably be active in healthy tissues, and certainly if SPC increases in cardiovascular disease as suggested<sup>2,9</sup>. This could be of particular importance in the lung, as pulmonary vascular disease is commonly associated with inflammation and microemboli, factors that would be expected to increase SPC. However the phenomenon does not seem to be limited to IPA, as in preliminary experiments we have found that it also occurs in a range of small systemic arteries. As SPC potentiates the response to TP agonists and is released from activated platelets<sup>11</sup>, an intriguing speculation is that this phenomenon could also contribute to the initial vasoconstrictor response associated with haemostasis and platelet-derived thromboxane A $_2$ .

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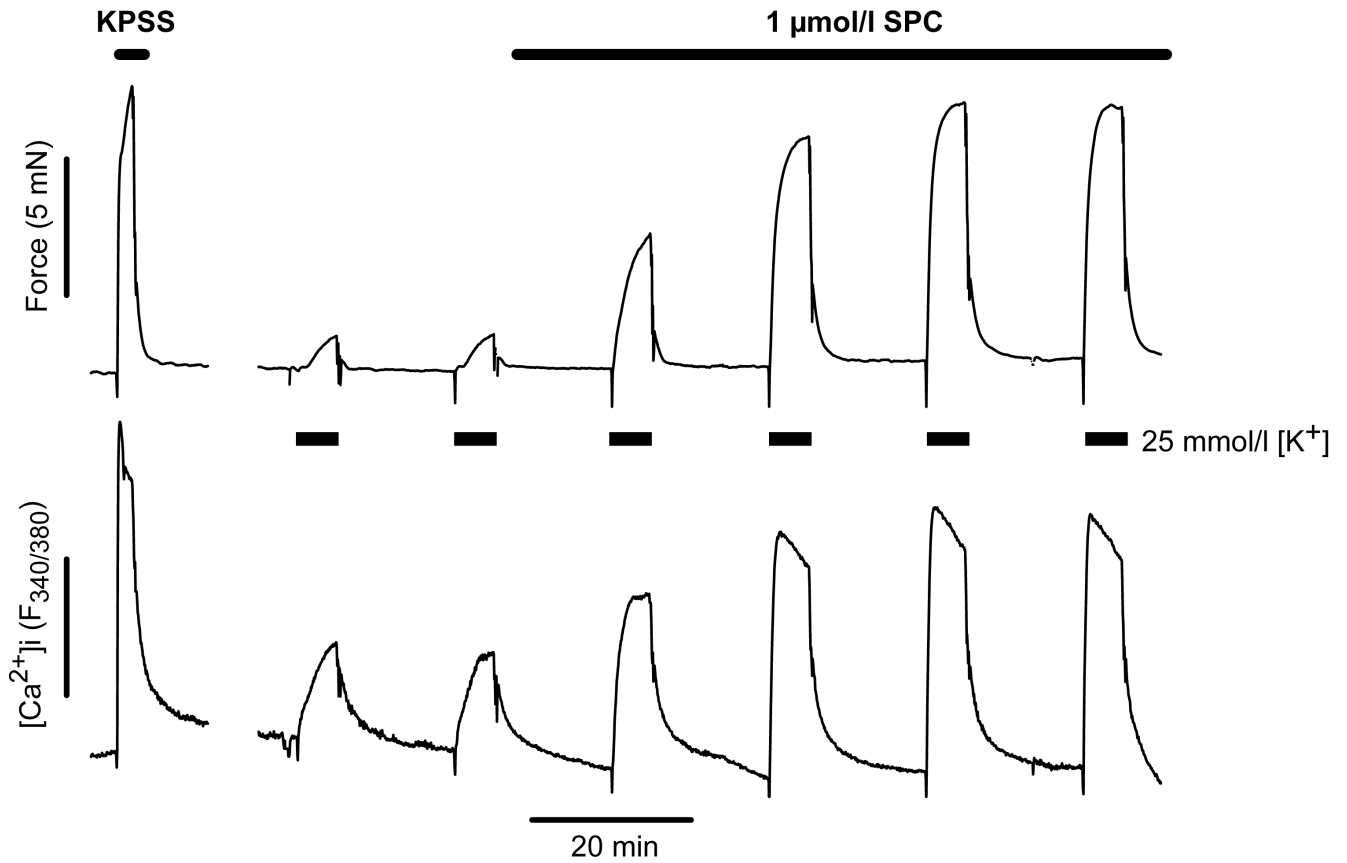
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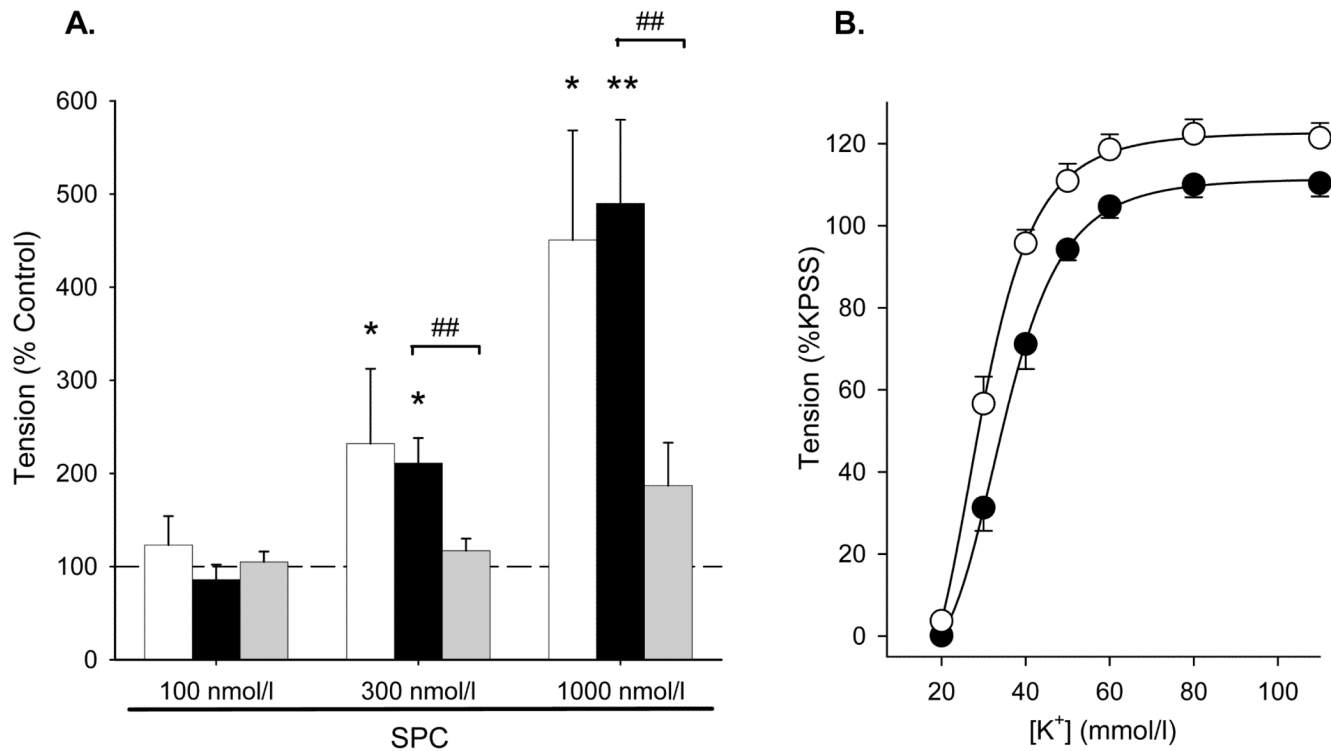


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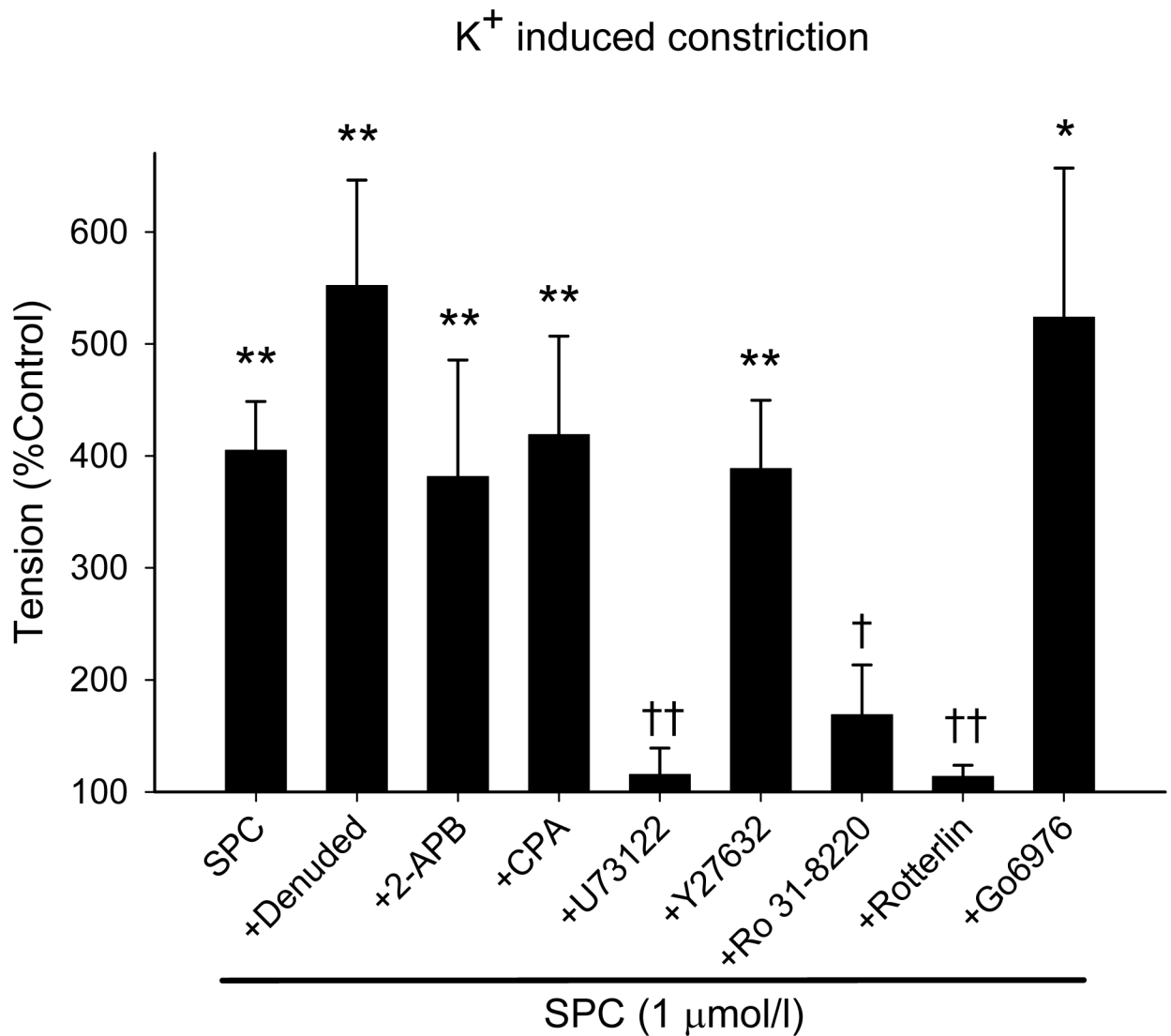
**Figure 1.**

A typical experimental trace of simultaneous force and [Ca<sup>2+</sup>]<sub>i</sub> (expressed as F<sub>340/380</sub>) recordings in a small IPA, illustrating the sequential challenge protocol with PSS containing 25 mmol/l [K<sup>+</sup>]. Note that 1 μmol/l SPC had no effect on force or [Ca<sup>2+</sup>]<sub>i</sub> on its own, but caused a substantial, slowly developing potentiation of the constriction elicited by 25 mmol/l [K<sup>+</sup>]. The response to depolarization with KPSS is shown for comparison.



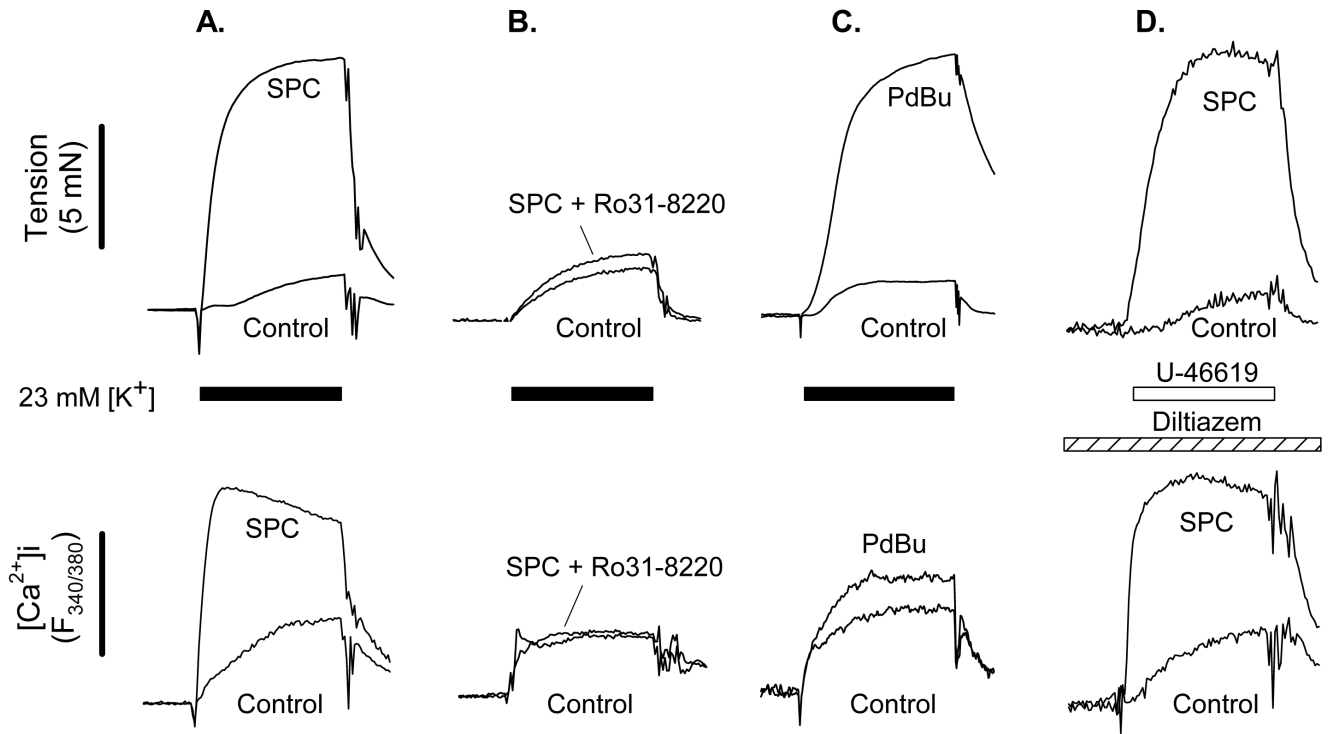
**Figure 2.**

A. Concentration-dependence of SPC-induced potentiation at 40 min of constriction elicited by ~25mmol/l [K<sup>+</sup>], showing stereospecificity. Diastereomeric SPC (open bars); *D-erythro*-SPC (black bars); *L-threo*-SPC (grey bars). Bars are mean ± S.E.M, n=4-5. \* = p<0.05, \*\* = p<0.01 versus control (100%); ## = p<0.01 *D-erythro*-SPC versus *L-threo*-SPC. B. Cumulative concentration-response relationship for K<sup>+</sup> (equimolar substitution for Na<sup>+</sup>) in IPA (filled circles; EC<sub>50</sub>: 36 ± 2 mmol/l; maximum: 109 ± 3% KPSS; n=9), and following preincubation with 1 μmol/l SPC (open circles; EC<sub>50</sub>: 31 ± 1 mmol/l, p<0.001; maximum: 120 ± 4% KPSS, p<0.001; n=9). Symbols are mean ± S.E.M.



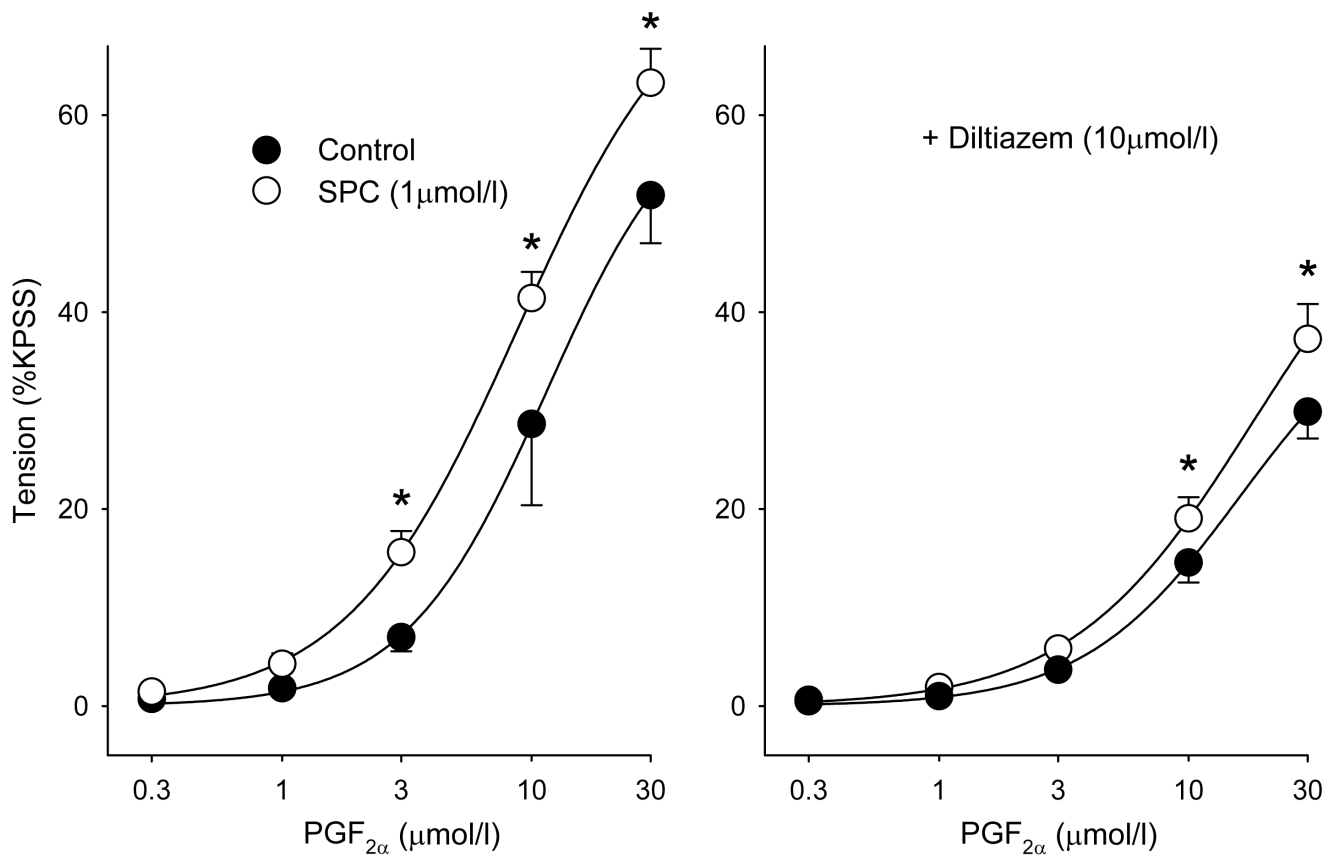
**Figure 3.**

Potential of depolarization ( $\sim 25$ mmol/l  $[K^+]$ )-induced vasoconstriction of IPA by 1  $\mu$ mol/l SPC measured at 40 min, and following removal of the endothelium or preincubation with inhibitors. Data were compared with their respective paired controls (100%), i.e. following preincubation with inhibitor but before addition of SPC. Bars are mean  $\pm$  S.E.M.,  $n = 4-31$ . \* =  $p < 0.05$ , \*\* =  $p < 0.01$  versus control; † =  $p < 0.05$ , †† =  $p < 0.01$  versus SPC.

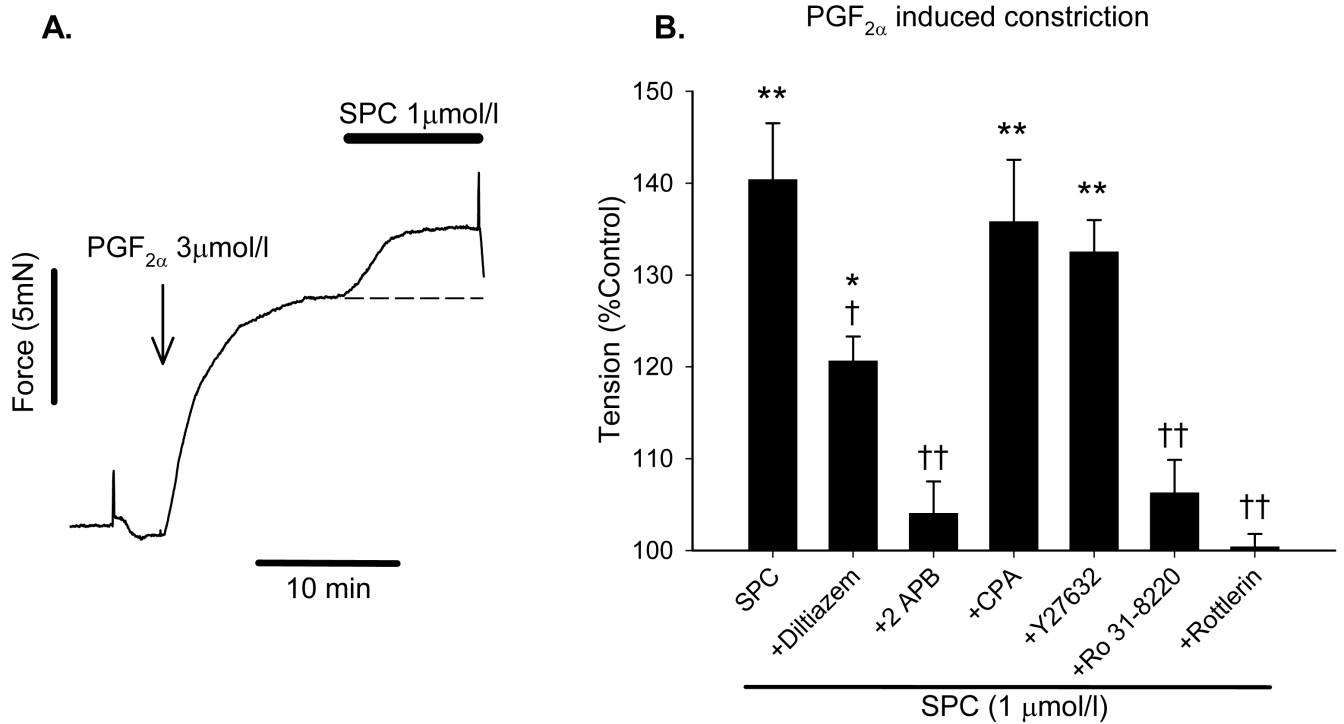


**Figure 4.**

Representative experimental traces of simultaneous force and intracellular  $[Ca^{2+}]_i$  (expressed as  $F_{340/380}$ ) recordings, showing the control response superimposed on the response in the same IPA following 40 min incubation with 1  $\mu\text{mol/l}$  SPC (panels A, B & D) or 100 nmol/l PdBu (panel C). In panels A, B & C constriction was elicited by PSS containing 23 mmol/l  $[K^+]$ , and in panel D by the TP agonist U-46619 (20 nmol/l) in the presence of diltiazem (10  $\mu\text{mol/l}$ ).

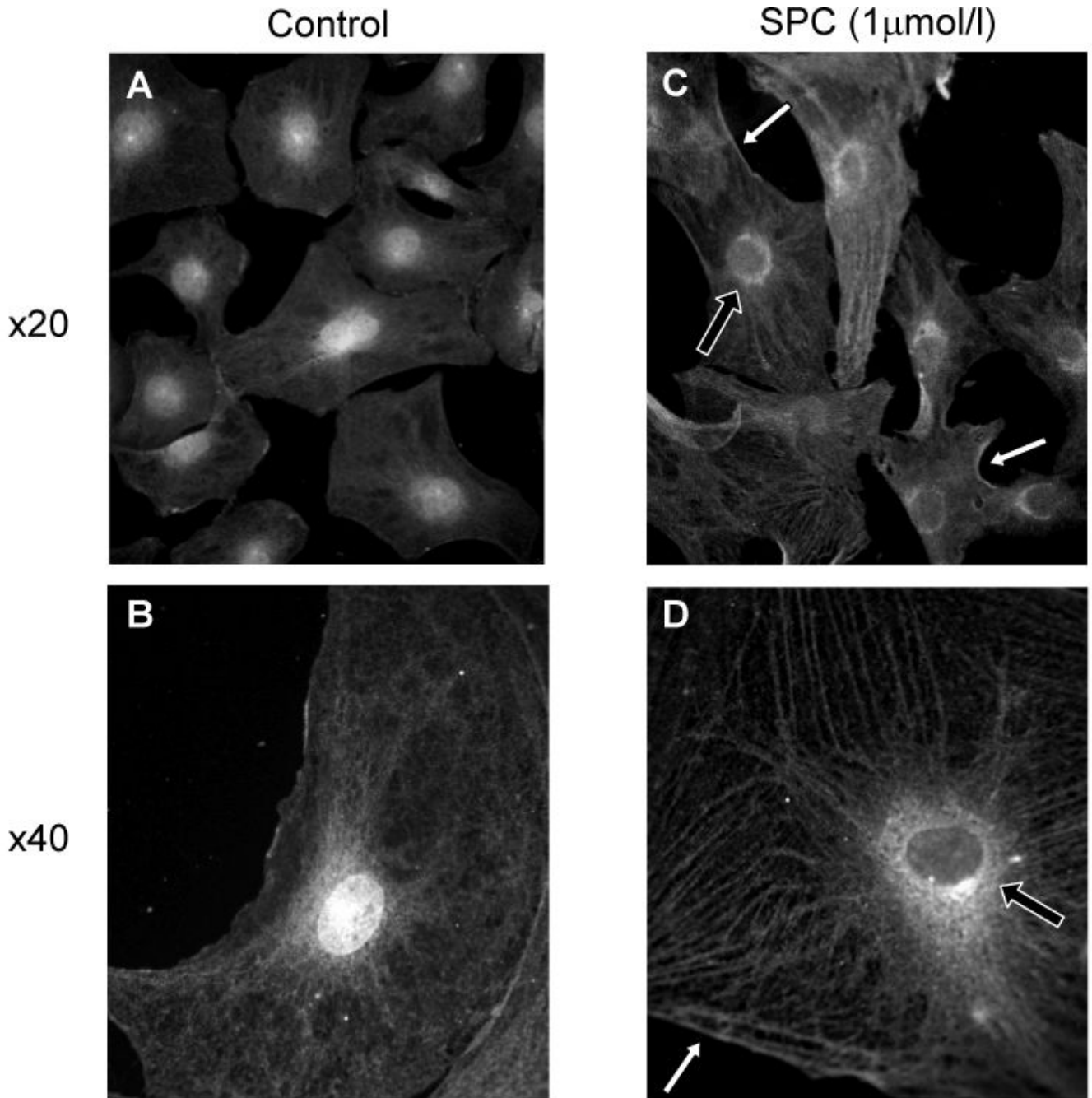


**Figure 5.** Cumulative concentration-response relationships for PGF<sub>2α</sub> in IPA. The left hand panel shows the effect of preincubation with 1 μmol/l SPC (Control, filled circles; SPC, open circles); the right hand panel shows the response in the presence of 10 μmol/l diltiazem. In both cases control and experimental curves were significantly different (-diltiazem:  $p < 0.01$ ,  $n = 4$ ; +diltiazem:  $p < 0.05$ ,  $n = 5$ ; 2 way ANOVA); symbols are mean  $\pm$  S.E.M., \* =  $p < 0.01$  (Holm-Sidak *post hoc*).



**Figure 6.**

A: Representative trace illustrating the second, shorter protocol where SPC is added on top of an established stable vasoconstriction induced by PGF<sub>2α</sub>. SPC was added once developed force reached a plateau, and caused a further increase in force; in the absence of SPC tension was stable for at least 10 min, illustrated by the dashed line. B: Increase in PGF<sub>2α</sub>-induced force caused by 1 μmol/l SPC measured after 10 min using the protocol shown in A., and following preincubation with inhibitors; 100% represents tension in the presence of inhibitors but before addition of SPC. Bars are mean ± S.E.M., n = 4-12. \* = p<0.05, \*\* = p<0.01 versus control; † = p<0.05, †† = p<0.01 versus SPC.



**Figure 7.** SPC induced translocation of PKC $\delta$  immunoreactivity in PASMCs from the nucleus to the perinuclear regions (e.g. black arrows) and cytoskeleton, with some evidence for translocation to the membrane (e.g. white arrows). A & B: control; C & D: treatment with 1  $\mu$ mol/l SPC for 10 min. B & D are at higher magnification (oil immersion objective) to show detail. Figures typical for 6-10 coverslips from each of 6 cell lines derived from different animals. >90% cells demonstrated translocation.