Citation for published version (APA):
Shaifta, Y., Snetkov, V. A., Prieto-Lloret, J., Smirnov, S. V., Knock, G., Aaronson, P., & Ward, J. (2015). Sphingosylphosphorylcholine potentiates vasoreactivity and voltage-gated Ca\textsuperscript{2+} entry via NOX1 and reactive oxygen species. Cardiovascular Research, 106, 121–130. https://doi.org/10.1093/cvr/cvv029
Sphingosylphosphorylcholine potentiates vasoreactivity and voltage-gated Ca\(^{2+}\) entry via NOX1 and reactive oxygen species

Yasin Shaifta\(^{1\dagger}\), Vladimir A. Snetkov\(^{1\dagger}\), Jesus Prieto-Lloret\(^1\), Greg A. Knock\(^1\), Sergey V. Smirnov\(^2\), Philip I. Aaronson\(^1\), and Jeremy P.T. Ward\(^1*\)

\(^1\)Division of Asthma, Allergy, and Lung Biology, King’s College London, 5th Floor Tower Wing, Guy’s Campus, London SE1 9RT, UK; and \(^2\)Department of Pharmacy and Pharmacology, University of Bath, Bath, UK

Received 7 August 2014; revised 29 January 2015; accepted 30 January 2015; online publish-ahead-of-print 6 February 2015

Time for primary review: 39 days

Aims

Sphingosylphosphorylcholine (SPC) elicits vasoconstriction at micromolar concentrations. At lower concentrations (≤ 1 \(\mu\)mol/L), however, it does not constrict intrapulmonary arteries (IPAs), but strongly potentiates vasoreactivity. Our aim was to determine whether this also occurs in a systemic artery and to delineate the signalling pathway.

Methods and results

Rat mesenteric arteries and IPAs mounted on a myograph were challenged with ≈ 25 mmol/L \([\text{K}^+]\) to induce a small vasoconstriction. SPC (1 \(\mu\)mol/L) dramatically potentiated this constriction in all arteries by ≈ 400%. The potentiation was greatly suppressed or abolished by inhibition of phospholipase C (PLC; U73122), PKC\(\gamma\) (inhibitory peptide), Src (PP2), and NADPH oxidase (VAS2870), and also by Tempol (superoxide scavenger), but not by inhibition of Rho kinase (Y27632). Potentiation was lost in mesenteric arteries from p47phox \(-/-\) mice. The intracellular superoxide generator LY83583 mimicked the effect of SPC. SPC elevated reactive oxygen species (ROS) in vascular smooth muscle cells, and this was blocked by PP2, VAS2870, and siRNA knockdown of PKC\(\gamma\). SPC (1 \(\mu\)mol/L) significantly reduced the EC\(_{50}\) for U46619-induced vasoconstriction, an action ablated by Tempol. In patch-clamped mesenteric artery cells, SPC (200 nmol/L) enhanced Ba\(^{2+}\) current through L-type Ca\(^{2+}\) channels, an action abolished by Tempol but mimicked by LY83583.

Conclusion

Our results suggest that low concentrations of SPC activate a PLC-coupled and NOX1-mediated increase in ROS, with consequent enhancement of voltage-gated Ca\(^{2+}\) entry and thus vasoreactivity. We speculate that this pathway is not specific for SPC, but may also contribute to vasorestriction elicited by other G-protein coupled receptor and PLC-coupled agonists.

Keywords

NADPH oxidase • Vascular smooth muscle • L-type Ca\(^{2+}\) channels • Protein kinase C epsilon • Reactive oxygen species

1. Introduction

Sphingosylphosphorylcholine (SPC) is derived from membrane sphingomyelin and is present in the plasma in submicromolar-free concentrations and as a major component of low- and high-density lipoproteins; it is also released from activated platelets.\(^1\)–\(^3\) Sphingolipids including SPC and sphingosine-1-phosphate (S1P) have been associated with cardiovascular disease, but whereas S1P has been extensively investigated and its receptors cloned, no specific receptor for SPC has been positively identified and there are no selective pharmacological antagonists. The actions of SPC are, however, stereospecific and dependent on phospholipase C (PLC), suggesting that they are mediated via a G-protein coupled receptor.\(^1\)–\(^3\)–\(^4\)

SPC induces vasoconstriction in isolated arteries via activation of Ca\(^{2+}\) entry and Rho kinase-mediated Ca\(^{2+}\)-sensitization, with an EC\(_{50}\) of ≈ 12 \(\mu\)mol/L.\(^5\)–\(^9\) However, plasma concentrations are at least
10-fold less than this, raising questions concerning the physiological relevance of vasoconstriction induced by SPC. Conversely, we have demonstrated in rat intrapulmonary arteries (IPAs) that although low concentrations (1 μmol/L or less) of SPC do not on their own elicit vasoconstriction, cause depolarization, elevate \([Ca^{2+}]_{i}\), or activate Rho kinase, they strongly potentiate vasoactivity by enhancing \(Ca^{2+}\) entry induced by other stimuli.\(^{10}\) Although this effect was also stereospecific and dependent on PLC,\(^{10}\) it therefore clearly differs from the mechanisms previously shown to underlie vasoconstriction induced by higher concentrations of SPC. This implies an additional and hitherto unrecognized high affinity signalling pathway that could be of physiological relevance.

SPC has been reported to induce generation of reactive oxygen species (ROS) in a variety of non-muscle cell types, probably via NADPH oxidase (NOX),\(^{11–14}\) and ROS have been shown to enhance \(Ca^{2+}\) entry through L-type voltage-gated \(Ca^{2+}\) channels in vascular smooth muscle and cardiac myocytes.\(^{15–18}\) ROS have also been implicated in signalling pathways initiated by other PLC-coupled vasoconstrictor agonists, including angiotensin II and endothelin.\(^{16,17,19}\) This led us to hypothesize that a NOX/ROS-mediated pathway might underlie the SPC-induced enhancement of IPA vasoactivity. As the pulmonary vasculature exhibits some unique responses to changes in redox state and ROS,\(^{20,21}\) we focused here on mesenteric artery (MA) to determine whether our previous observations were specific to the pulmonary vasculature. We show that low, subcontractile concentrations of SPC potentiate vasoactivity of both MA and IPA through the same pathway, which involves PLC- and PKCa-dependent activation of NOX1, increased production of ROS, and consequent enhancement of \(Ca^{2+}\) entry via L-type channels.

2. Methods

2.1 Animals and tissues

The study conforms with UK Home Office regulations and Directive 2010/63/EU of the European Parliament. Adult male Wistar rats were killed by a lethal overdose of pentobarbital (i.p.). The lungs, mesentery, and in some cases sections of small renal or main femoral artery were excised and placed in cold physiological saline solution (PSS; in mmol/L: 118 NaCl, 24 NaHCO\(_3\), 1 mg/mL, 0.44 NaH\(_2\)PO\(_4\), 4 KCl, 5.5 glucose, and 1.8 CaCl\(_2\)). Male mice (6–8 weeks old) lacking genes for \(p97^{\text{plus}}\)\(^{22}\) (NOX2), \(p47^{\text{phox}}\)\(^{23}\) (background for both C57BL/6) or PKCa\(^{10}\) (background 129/5V)\(^{24}\) or matched wild-type (WT) were killed by a Home Office approved method, the mesentery removed and placed in cold PSS.

Small IPA, MA, renal artery (200–500 μm i.d.) or femoral artery segments (1–2 mm i.d.) were dissected free of connective tissue, mounted on a micrograph (Danish Myo Technology, Denmark), and bathed in PSS gassed with 5% CO\(_2\), balance air (pH 7.4) at 37 °C. Vessels were stretched to equivalent transmural pressures of \( \pm 25 \) (IPA) or \( \pm 90 \) (MA and renal), and pre-conditioned by repeated exposure to 80 mmol/L K\(^+\) PSS (KPSS, equimolar substitution for NaCl) as previously described.\(^ {10,20}\) Experiments were performed after \( \pm 30 \) min to allow for stabilization. Tension was recorded using Acquisition Engine software (Cairn Research Ltd, Faversham, UK).

2.2 Cell culture, siRNA design, and cell transfection

Pulmonary artery smooth muscle cells (PASMCs) were dispersed from IPA using collagenase (type XI, 2 mg/mL) and papain (1 mg/mL), and cultured in DMEM containing 10% FCS as previously described.\(^ {10}\) PASMCs from passages 3–4 were growth-arrested in serum-free medium for 24 h before use; each cell line was verified as smooth muscle by immunostaining for smooth muscle α-actin, calponin, and desmin (Sigma-Aldrich, Poole, UK). siRNAs were designed as described previously.\(^ {25}\) The 19-nucleotide target sequences (PKCa·siRNA; position 883–901, GenBank accession no. BC076505; PKCa·siRNA: position 2079–2097, GenBank accession no. AY642593) were synthesized into 64–65 mer oligonucleotides with BamH1/Hindlll overhangs (Sigma-Aldrich) and cloned into the expression vector pSilencer 3.0-H1 (Life Technologies Ltd, Paisley, UK). All clones were purified using an EndoFree Plasmid Maxi Kit (Qiagen Ltd, Crawley, UK) and sequenced (Geneservice Ltd, Cambridge, UK). PASMCs were transfected using the Basic Nucleofector\(^ {TM}\) Kit for primary mammalian smooth muscle cells and a nucleofector device (Nucleofector\(^ {TM}\) Technology, Lonza, Slough, UK); after 72 h cells were serum starved for 24 h prior to use. Transfection efficiency was \( \geq 80\% \), as determined using pmaxGFP (green fluorescent protein-expressing vector) provided in the kit and confirmed by fluorescence microscopy. Efficiency and selectivity of knockdown was confirmed by western blot.

2.3 Estimation of ROS

As a qualitative, real-time estimation in intact arteries maintained under identical conditions to contraction studies, MAs were mounted on a confocal wire myograph (Danish Myo Technology) and pre-conditioned as above. Following incubation with 10 μmol/L carboxy 2,7′-dichlorofluorescin–diaacetate (C-DCFH/DA) for 45 min at 37 °C, excess dye was washed off and tissue fluorescence of oxidized C-DCF (excitation 490 nm and emission 530 nm) recorded every 30 s using an inverted microscope (Zeiss UK Ltd) and microfluorimeter (Cairn Research Ltd). After a stable baseline was established (-30 min), SPC was added to the bath.

ROS generation in cultured cells was estimated using lucigenin-enhanced luminescence. PASMCs (passage 4) were cultured to confluence in 24-well plates and growth-arrested for 24 h. Medium was then replaced with gassed PSS at 37 °C containing 5 μmol/L lucigenin and 100 μmol/L NADPH, to which SPC and pharmacological inhibitors were added. Luminescence was measured at 37 °C using a Hitex Chameleon plate reader (Hitex, Finland).

2.4 Electrophysiology

Freshly isolated MA smooth muscle cells (MASCs) were obtained from third- to fourth-order MA by enzymatic dispersion, and recordings of whole-cell currents performed with patch clamp as described previously.\(^ {26}\) Ba\(^{2+}\) was used as a charge carrier to record currents (\(I_{\text{Ba}}\)) through voltage-gated L-type \(Ca^{2+}\) channels, with an extracellular solution containing (mmol/L): 10 BaCl\(_2\), 130 NaCl, 5 CsCl, 1 MgCl\(_2\), 5 HEPES, 5 glucose, pH 7.35 and a pipette solution containing (mmol/L): 120 Cs methansulfonate, 20 CsCl, 2 MgATP, 0.5 NaGTP, 0.3 MgCl\(_2\) and 5 HEPES, pH 7.2. Cells were equilibrated with pipette solution for 3 min after whole-cell access before recording the control (time 0) current–voltage (\(I–V\)) relationship using 120 ms voltage steps between -80 and +80 mV; holding potential was -70 mV. Cells were then incubated for 5 min with either 200 mmol/L SPC, 1 μmol/L LY83853, or in the absence of drug (time control), prior to recording of the test \(I–V\) relationship. The effect of Tempol (3 mmol/L) was studied on cells preincubated for 2 min before addition of 200 mmol/L SPC.

2.5 Calculations and statistical analysis

Tension was normalized to the response to KPSS, or for potentiation experiments to the control response prior to addition of SPC. Results are expressed as means ± SEM. Concentration–response curves were fitted to individual experiments using a Hill equation to provide \(EC_{50}\) and fitted maximum (\(Y_{\text{max}}\) Sigmaplot 12, Systat Software Inc., CA, USA); for analysis \(EC_{50}\) was expressed as \(pD_{2}\) (\(-\log EC_{50}\)). Statistical analysis was performed using ANOVA with a Holm-Sidak post hoc unless otherwise stated (Sigmaplot, Systat Software Inc.). Statistical significance was deemed if \(P < 0.05\).
2.6 Reagents
U73122, Go6983, Go6976, PP2, and rottlerin were obtained from Calbiochem, UK; C-DCFH/DA from Invitrogen, UK, and all other reagents including PKCs translocation inhibitor peptide from Sigma-Aldrich.

3. Results

3.1 Potentiation of vasoconstriction by subcontractional concentrations of SPC
As previously reported for rat IPA,10 1 μmol/L SPC alone had no effect on tension in rat or mouse MA (e.g. Figures 1A and 2A), or rat renal or femoral artery.

Rat small MAs were challenged with sequential 5 min applications of PSS containing ~25 mmol/L [K+] to cause a small depolarization-induced rise in tension of 5.8 ± 0.6% (n = 31) of that induced by KPSS, as previously described.10 SPC (1 μmol/L) was added to the bath, and the procedure repeated at 15 min intervals in the continued presence of SPC. SPC strongly potentiated the subsequent response to depolarization, and this gradually increased with time (Figure 1A and B), such that at 30 min (second challenge post-SPC) force was increased to 512 ± 40% of control (n = 31; P < 0.001; Figure 1C). Baseline tension measured immediately before each depolarizing challenge was unchanged from control (Figure 1B), consistent with the lack of effect of SPC alone, and in the absence of SPC the response to repeated depolarization was also unchanged over 45 min (Figure 1A). SPC (1 μmol/L) caused the same degree of potentiation at 30 min in IPA (to 505 ± 39% control, n = 47, P < 0.001) and small renal arteries (510 ± 107% control, n = 9, P < 0.01) as MA, but had a smaller effect in large femoral arteries (163 ± 23%, n = 12, P < 0.05).

SPC also potentiated agonist-induced constriction in MA. Preincubation with SPC (1 μmol/L) caused a substantial leftward shift in the PGF2α cumulative concentration–response curve, reducing the EC50 from ~20 to ~7 μmol/L (pD2: control: 4.78 ± 0.30, n = 5; SPC: 5.29 ± 0.14, n = 6; P < 0.05). We previously reported the same for IPA.10

3.2 Signalling pathways involved in SPC-mediated potentiation
Arteries were incubated with pharmacological inhibitors for 15 min, and two control depolarizations were performed before 1 μmol/L SPC was added as above. Potentiation at 30 min (second challenge post-SPC) was greatly suppressed or abolished by U73122 (PLC inhibitor, 10 μmol/L), Go6983 (broad-spectrum PKC inhibitor, 3 μmol/L), and rottlerin (putative PKCδ inhibitor, 1 μmol/L, though see below), but not by Go6976 (inhibitor of conventional, but not novel PKC, isoforms, 3 μmol/L) or Y27362 (Rho kinase inhibitor, 3 μmol/L; Figure 1C). The SPC-induced potentiation of depolarization-induced contraction in MA thus exhibited the same pharmacological profile as we previously reported for IPA,10 suggesting the same underlying mechanism.

Based on the differential effects of Go6976 and broad-spectrum PKC inhibitors, rottlerin, and PKCδ translocation studies, we previously suggested that SPC-induced potentiation of IPA vasoactivity involved the novel PKCδ isoform.10 However, concerns about the specificity of
rottlerin\textsuperscript{27} led us to examine this further; indeed, we found no difference between MA from C57BL/6 and 129/SV WT mice, so the data were pooled. (A) Typical tension recordings from MA of WT, PKC\textsuperscript{d/2}, gp91\textsuperscript{phox/2}, and p47\textsuperscript{phox/2} mice for 5 min challenges with PSS containing 25 mmol/L [K\textsuperscript{+}] in the presence of 1 mmol/L SPC. (B) Mean data from WT (n = 11, 7 mice), PKC\textsuperscript{d/2} (n = 4, 4 mice), gp91\textsuperscript{phox/2} (n = 6, 4 mice), and p47\textsuperscript{phox/2} mice (n = 11, 7 mice; challenge 2). Bars = SEM. \textsuperscript{††}P < 0.001 vs. control; two-way ANOVA, Holm-Sidak post hoc.

3.3 Role of NOX and ROS

Both PKC\textsubscript{e} and Src kinase are known to activate NOX\textsuperscript{30–32} and Src has been implicated in the actions of SPC in coronary artery.\textsuperscript{31} PP2

\textbf{Figure 2} SPC-induced potentiation in MA of mice. We found no difference between MA from C57BL/6 and 129/SV WT mice, so the data were pooled. (A) Typical tension recordings from MA of WT, PKC\textsuperscript{d/2}, gp91\textsuperscript{phox/2}, and p47\textsuperscript{phox/2} mice for 5 min challenges with PSS containing 25 mmol/L [K\textsuperscript{+}] in the presence of 1 mmol/L SPC. (B) Mean data from WT (n = 11, 7 mice), PKC\textsuperscript{d/2} (n = 4, 4 mice), gp91\textsuperscript{phox/2} (n = 6, 4 mice), and p47\textsuperscript{phox/2} mice (n = 11, 7 mice; challenge 2). Bars = SEM. \textsuperscript{††}P < 0.001 vs. control; two-way ANOVA, Holm-Sidak post hoc.

\textbf{Figure 3} Effects of inhibitors on SPC-induced potentiation in MA and IPA. (A) SPC-induced potentiation (challenge 2) of MA for SPC alone (n = 31, 24 rats), and in the presence of PKC\textsubscript{e} peptide inhibitor (n = 6), PP2 (Src inhibitor, n = 7), Tempol (n = 11, 8 rats), and VAS2870 (NOX inhibitor, n = 4); 24 rats. (B) SPC-induced potentiation (challenge 2) of IPA for SPC alone (n = 47, 30 rats), and in the presence of PKC\textsubscript{e} peptide inhibitor (n = 8), PP2 (n = 8), Tempol (n = 11), and VAS2870 (n = 4); 20 rats. (C) Typical trace showing reversal of SPC-induced potentiation in a MA by addition of Tempol (3 mmol/L). Bars = SEM. \textsuperscript{***}P < 0.001 vs. SPC alone; two-way ANOVA, Holm-Sidak post hoc.
(Src inhibitor, 10 µmol/L), VAS2870 (a novel selective inhibitor of NOX, 10 µmol/L), and Tempol (membrane-permeable catalytic superoxide scavenger, 3 mmol/L) all strongly suppressed SPC-induced potentiation of depolarization-induced contraction in both rat MA and IPA (Figure 3A and B). Similarly, application of Tempol on top of an established constriction reversed SPC-induced potentiation by 95 ± 12% (P < 0.01; n = 4) in MA (Figure 3C).

To determine the NOX isoform, we used the same protocol but with MA from mice lacking gp91phox (NOX2) or p47phox, the organizer subunit for NOX2 and NOX1. While potentiation was the same in MA from WT and gp91phox−/− mice, it was absent in mice lacking p47phox (Figure 2A). This strongly suggests that activation of NOX1 and consequent generation of ROS are essential for the potentiating actions of SPC.

We examined whether ROS were also responsible for SPC-induced potentiation of agonist-induced contraction. We utilized U46619, as unlike PGF2α, it only activates TP receptors; experiments were performed in the presence of 100 µmol/L L-NAME to mitigate against any complicating effects of nitric oxide. U46619 concentration–response curves are repeatable; three were performed on each MA: control, following incubation with SPC (1 µmol/L), and SPC plus Tempol (3 mmol/L). A separate set of time-matched experiments were performed with Tempol alone. SPC caused a leftward shift in the U46619 concentration–response relationship (P < 0.001), which was ablated in the presence of Tempol such that the relationship was shifted significantly to the right of control (P < 0.05; Figure 4A). There was no difference between the effects of SPC plus Tempol and Tempol alone.

To determine the mechanism by which SPC potentiates U46619-induced contraction, we performed similar experiments using nifedipine (3 µmol/L; L-type channel blocker). Nifedipine suppressed the response to U46619 (Figure 4B), and in its presence SPC was without any effect. This suggests that SPC potentiates U46619-induced contraction by enhancing voltage-gated Ca2+ entry.

As Tempol suppressed the effects of subcontractile concentrations of SPC, we examined whether it would also suppress the effects of higher concentrations of SPC, which do elicit vasoconstriction. Indeed, Tempol suppressed SPC-induced contraction in MA by ~75% (P < 0.001; Figure 4C). Conversely, nifedipine only reduced the response to SPC by ~50%, as expected because SPC at more than ~5 µmol/L also activates Rho kinase-mediated Ca2+ sensitization.13–15 These results imply that SPC-induced contraction in MA is largely mediated via ROS.

### 3.4 Does SPC increase ROS generation?

As a qualitative measure of ROS production we examined the effect of SPC on C-DCFH oxidation in intact, unstimulated MA mounted on a myograph. SPC increased the rate of C-DCFH oxidation in a concentration-dependent manner, under identical conditions to those used for contraction studies (Figure 5A).

---

**Figure 4** U46619 and SPC-induced contraction, and effects of Tempol and nifedipine. (A) Cumulative concentration–response curves for U46619: control (filled circle), SPC (1 µmol/L; open circle), SPC + Tempol (open square); 12 MA (6 rats). Tempol alone (filled square); n = 8 MA (4 rats). Mean pD2: control: 7.11 ± 0.02; SPC: 7.53 ± 0.03, P < 0.001 vs. control; SPC + Tempol: 6.99 ± 0.02, P < 0.001 vs. SPC, P < 0.02 vs. control; RM ANOVA, Holm-Sidak post hoc. pD2 for Tempol alone: 6.90 ± 0.08, NS vs. Tempol + SPC; P < 0.05 vs. Control; one-way ANOVA, Holm-Sidak post hoc. Bars = SEM. (B) Similar to A, but in the presence of nifedipine (3 µmol/L; open square), and SPC + nifedipine (filled square); 15 MA (9 rats). Control: pD2: 7.04 ± 0.06, Vmax: 144 ± 13% KPSS, nifedipine: pD2: 6.64 ± 0.05, P < 0.001 vs. control, Vmax: 43 ± 5% KPSS, P < 0.001 vs. control; SPC + nifedipine: pD2: 6.67 ± 0.03, NS vs. nifedipine alone, Vmax: 53 ± 6% KPSS, NS vs. nifedipine alone; RM ANOVA, Holm-Sidak post hoc. (C) Similar cumulative concentration–response curves for SPC in MA (filled circles); 16 MA (10 rats), with Tempol (filled squares); 8 MA (5 rats), or nifedipine (open squares); 10 MA (6 rats). SPC: pD2: 5.08 ± 0.04, Vmax: 16.8 ± 1.3% KPSS, SPC + Tempol: pD2: 4.84 ± 0.06, P < 0.02 vs. control, Vmax: 42.4 ± 1.2% KPSS, P < 0.001 vs. control; SPC + nifedipine: pD2: 5.10 ± 0.06, NS vs. control, Vmax: 8.25 ± 1.1% KPSS, P < 0.002 vs. control, P < 0.05 vs. Tempol; one-way ANOVA, Holm-Sidak post hoc. Bars = SEM (not shown if smaller than symbol).
Addition of SPC (1 μmol/L) caused a ∼40% increase in lucigenin-enhanced luminescence in unstimulated cultured PASMCs (n = 11, P < 0.001), which was effectively abolished by preincubation with Gö6983 (3 μmol/L), PP2 (10 μmol/L), and VAS2870 (10 μmol/L) such that there was no significant elevation above basal luminescence (Figure 5B); none of these agents alone had any effect on basal luminescence. SPC (10 μmol/L) had a significantly stronger effect (∼80% increase, n = 12, P < 0.001, 10 vs. 1 μmol/L SPC). However, while VAS2870 still strongly suppressed the response to 10 μmol/L SPC, Gö6983 and PP2 were only partially effective at this concentration (Figure 5B).

To confirm a role for PKCα upstream of ROS generation, and to positively identify the PKC isoform, we transfected cells with siRNA against PKCα or PKCe, or scrambled siRNA. PKCe-siRNA reduced protein expression to 19.7 ± 2.6% of control, and strongly suppressed the increase in lucigenin luminescence induced by 1 and 10 μmol/L SPC (n = 5, P < 0.001 for both). PKCα-siRNA reduced protein expression to 20.5 ± 1.3%, but had no effect on SPC-induced luminescence (n = 4; Figure 5C). Neither siRNA had a significant influence on basal luminescence.

3.5 Do exogenous ROS mimic the effects of SPC?

The above results suggest that SPC-induced potentiation of vasoreactivity is mediated via a PLC-, PKCe- and Src-dependent activation of NOX1, and consequent generation of ROS. We therefore examined whether exogenous ROS could mimic the effects of SPC using LY83583, a membrane-permeable quinolinequinone that acts within the cell to generate intracellular superoxide.20,36 At 1 μmol/L, LY83583 does not itself alter vascular tension20,36 (and see Figure 6A), but like SPC it substantially enhanced the response to depolarization with ∼25 mmol/L [K+] in both MA and IPA (Figure 6A and B; P < 0.001). In contrast to SPC, however, the effects were not significantly inhibited by Gö6983, PP2, or VAS2870 (Figure 6B), consistent with ROS being downstream of PKCe- and Src-mediated activation of NOX1.

3.6 SPC potentiation of voltage-gated Ca2+ channels

We examined the effects of SPC on voltage-gated Ca2+ currents using whole-cell patch-clamp and Ba2+ as a charge carrier, in freshly isolated myocytes from rat MA (Figure 7). We utilized 200 μmol/L SPC because 1 μmol/L caused rapid loss of attachment. To account for current rundown, comparisons were made between different cells 5 min after addition of SPC or solvent (PSS). SPC (200 μmol/L) increased peak current at 5 min to 176 ± 17% (n = 7; P < 0.01) of that in control cells, and this enhancement was abolished in the presence of 3 mmol/L Tempol (90 ± 13%, n = 6; P < 0.01 vs. SPC alone, NS vs. control; Figure 7). As predicted, the intracellular ROS generator LY83583 (1 μmol/L) had a similar effect to that of SPC (145 ± 12%, n = 6; P < 0.05).

4. Discussion

We previously reported that concentrations of SPC ≤1 μmol/L, insufficient to elicit elevation of [Ca2+]i, or vasoconstriction alone, nevertheless strongly potentiate depolarization- and agonist-induced constriction of small IPA by enhancing Ca2+ entry.10 The precise mechanism, however, remained unclear, and it was unknown whether this phenomenon was limited to pulmonary arteries, which have unique

Figure 5 Effect of SPC on ROS generation. (A) Mean of real-time recordings from intact MA from three rats showing increased oxidation of C-DCFH (fluorescence at 530 nm) following addition of SPC. Data normalized to initial fluorescence; bars = SEM, shown at 2 min intervals for clarity. (B) Increase in lucigenin luminescence in PASMCs cultured from eight rats following addition of 1 (n = 11) or 10 (n = 12) μmol/L SPC, and in the presence of Gö6983 (n = 6 and 7), PP2 (n = 9 and 11), and VAS2870 (n = 4 and 4). **P < 0.01, ##P < 0.001 vs. control (no SPC); ***P < 0.001 vs. 1 μmol/L SPC alone; ^P < 0.05, ##^P < 0.001 vs. 10 μmol/L SPC alone; one-way ANOVA, Holm-Sidak post hoc. Bars = SEM. (C) Increase in lucigenin luminescence following addition of 1 or 10 μmol/L SPC in PASMCs cultured from six rats transfected with scrambled siRNA (n = 12), PKCα siRNA (n = 4), or PKCe-siRNA (n = 5). **P < 0.01, ##P < 0.001 vs. control (no SPC); ***P < 0.001 vs. scrambled siRNA with 1 or 10 μmol/L SPC; one-way ANOVA, Holm-Sidak post hoc. Bars = SEM.
responses to hypoxia and some other stimuli. The key findings of the present study are that such concentrations of SPC also strongly potentiate vasoreactivity of small MAs and renal arteries, and that for both MA and IPA this is mediated via an increase in ROS generated by NOX1 and enhancement of Ca\(^{2+}\) entry through L-type channels.

SPC (1 \(\mu\)mol/L) enhanced constriction induced by mild depolarization with \(~25\) mmol/L \([K^+]_o\) by the same extent (\(~400\%) at 30 min in small IPA, MA, and renal arteries, but to a lesser degree in a large femoral artery (\(~60\%). The latter may reflect the suggestion that SPC plays a greater role in distal compared with large proximal arteries. The relatively slow onset, with a maximum effect around 50–60 min (Figure 1B), was similar in all artery types and to vasoconstriction induced by high (e.g. 10 \(\mu\)mol/L) concentrations of SPC. While this could reflect accumulation of SPC in the membrane or of a secondary mediator, it is noted that there was no increase in basal tension over at least 45 min (Figure 1B). As we previously reported for IPA, pretreatment with 1 \(\mu\)mol/L SPC also potentiated the response of MA to PGF\(_{2\alpha}\) and U46619, demonstrated here by a large shift to the left of the concentration–response curve (Section 3.1 and Figure 4A).
The differential effects of G6983 and G6976 imply a novel isoform of PKC, which like conventional PKCs are activated by PLC-derived diacylglycerol; conventional PKCs have been previously shown to play no role in the action of SPC.28 While we originally proposed PKCδ, we show here that SPC-induced potentiation of constriction was unaltered in MA from mice lacking PKCδ11–19 and PKCε is known to activate NOX in cardiac31 and pulmonary artery myocytes.40 Both PKC and Src phosphorylate the p47phox organizer subunit of NOX1 and NOX2, essential for translocation of cytosolic subunits and activation of the oxidase complex.32 Interestingly, Src and PKCε are themselves redox-sensitive, giving rise to the possibility of positive feedback-mediated amplification.40,41 Consistent with a central role for NOX-generated ROS, SPC-induced potentiation of depolarization-induced constriction was strongly suppressed in both MA and IPA by the novel NOX inhibitor VAS2870.33 S Src inhibitor PP2, and superoxide scavenger Tempol (Figure 3). Tempol also reversed the potentiation by SPC of U46619-induced vasocostruction (Figure 4A). Moreover, the potentiating effect of SPC was abolished in MA from p47phox−/−, but not gp91phox−/−, mice (Figure 2); this strongly implicates NOX1 as the relevant isoform, as only NOX1 and NOX2 require p47phox.

Consistent with the above, 1 μmol/L SPC increased ROS generation both in intact MA and in cultured vascular smooth muscle cells, an action effectively abolished in the latter by G6983, PP2, and VAS2870, and also following siRNA knockdown of PKCε (but not PKCδ; Figure 5). Furthermore, subcontractional concentrations of the intracellular superoxide generator LY83583 mimicked the actions of SPC (Figure 6), and we have previously shown that such concentrations also enhance agonist-induced constriction in MA and IPA.20 Inhibition of PKC or Src did not affect LY83583-induced potentiation (Figure 6), suggesting that in these circumstances PKCe and Src play a primarily upstream role to generation of ROS. These results strongly suggest that the effects of SPC reported here are mediated by increased generation of NOX1-derived ROS.

Concerning the mechanism by which low concentrations of SPC enhance vascular reactivity, our current and previous10 results exclude any role for a Rho kinase- (or PKC) mediated increase in Ca2+ sensitivity, although at higher concentrations both SPC and ROS do activate Rho kinase.19,20,39 However, voltage-dependent Ca2+ entry induced by depolarization or agonist was increased.10 While this might occur if SPC induced some depolarization itself, and NOX-derived ROS have been reported to inhibit K+ channels in pulmonary artery,40,42 SPC did not suppress K+ currents in IPA, and indeed still potentiated the response in maximally depolarized arteries.10 Moreover, we have reported that intracellularly generated ROS cause a negative shift in the activation of Kv current in both MA and IPA, which would tend to have a hyperpolarizing effect.20 ROS and redox state are known to affect the function of L-type Ca2+ channels, the α1C subunit of which contains multiple redox-sensitive cysteine residues,15 and NOX-derived ROS are reported to enhance L-type Ca2+ channel currents in both cardiac and vascular smooth muscle.16–19 Consistent with this, we found that 200 nmol/L SPC potentiated L-type Ca2+ channel current (Ba2+ as a charge carrier) in MA smooth muscle cells, and this was prevented by Tempol and mimicked by the superoxide generator LY83583 (Figure 7).

Our results are consistent with a model where subcontractile concentrations of SPC activate NOX1 through a PLC, PKCε, and Src-dependent mechanism, and the consequent increased generation of ROS enhances Ca2+ entry through L-type channels, when these are activated by other means. The pathway is apparently identical in MA and IPA. This mechanism may not be limited to SPC, as a similar pathway, albeit at concentrations sufficient to cause constriction alone, has been proposed for the archetypical NOX activator angiotensin II in cerebral arteries,16 ET-1 in cardiac myocytes,15 and U46619 in pulmonary artery.17,42 Notably, Tempol caused a shift to the right of the control U46619 concentration–response curve in MA (Figure 4A). While these reports may differ in terms of specific isoforms of PKC and NOX, and in pulmonary artery the possible involvement of Kv channels, the underlying signalling pathway is very similar. Note, however, that all these studies utilized agonist concentrations that in themselves cause constriction and activation of parallel Ca2+ mobilization and other pathways, which might underlie reported differences.

Considering the above, we briefly examined whether ROS and NOX signalling was important for constriction elicited by higher concentrations of SPC, which has been attributed to activation of voltage-dependent and -independent Ca2+ entry and Rho kinase-mediated Ca2+ sensitization.16–19 Notably, all of these have been reported to be activated by ROS.32,41 We found that Tempol suppressed SPC-induced constriction by >80% in MA (Figure 4B), and that 10 μmol/L SPC doubled the rate of ROS generation compared with 1 μmol/L (Figure 5B). While this implies that ROS form a key signalling component for SPC at any concentration, the fact that G6983, PP2, and knockdown of PKCe only partially inhibited the elevation of ROX elicited by 10 μmol/L SPC suggests that an additional pathway may be activated by concentrations >1 μmol/L, as previously suggested for other cell types.43

5. Conclusion

We demonstrate here that low concentrations of SPC, insufficient to elicit vasoinconstriction alone, strongly potentiate vasoreactivity via PLC, PKCε, and Src-dependent activation of NOX1, increased generation of ROS, and consequent enhancement of Ca2+ entry through L-type channels. SPC has been implicated in cardiovascular disease, though the majority of in vitro studies utilized concentrations probably well in excess of physiological levels.1–3 The concentrations used here and previously (≤1 μmol/L)10 are closer to those reported in plasma,44 although physiologically relevant concentrations at the cell surface are difficult to determine because of autocrine and paracrine production, and extensive binding to albumin, HDLs, and LDLs.1,4,12 Nevertheless, our results suggest that physiological or pathophysiologic concentrations of SPC could greatly increase vascular reactivity to other stimuli. In addition, we can speculate from the data shown in Figure 4B that many of the vascular actions of SPC might be mediated
Agonist activation of NOX1 enhances vasoreactivity

via increased ROS. In this respect, there are similarities between SPC and angiotensin II, which share many downstream pathways.1,45

The question arises as to why SPC and low concentrations of ROS should have similar effects in MA and IPA, whereas higher concentrations of exogenous ROS are reported to constrict pulmonary arteries.20,21 It is probable that this relates to compartmentalization of SPC-induced ROS signalling, synonymous to that for Ca2+. Notably, angiotensin II causes highly localized sub-plasmalemmal generation of ROS and co-localized L-type channel activity in cerebral artery, suggesting clustering of receptors, NOX, and L-type channels in a micro-regional domain.16 We speculate that a similar situation exists for SPC and an as yet unidentified high affinity SPC receptor. Higher concentrations of exogenous ROS would, however, have more promiscuous effects, for example relaxing MA (but not IPA) by opening K+ channels,20 and constricting IPA (but not MA) in part by mobilizing ryanodine-sensitive Ca2+ stores.21,30

In summary, we present evidence for a novel pathway by which physiological concentrations of SPC strongly potentiate vasoreactivity, involving PLC, NOX1, and ROS-mediated enhancement of voltage-gated Ca2+ entry. Similarities with other studies suggest that this could potentially be common to other GqPCR and PLC-coupled agonists, with significant implications for vascular regulation and disease.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

p47phox−/−, gp91phox−/−, and PKCβ2−/−, as well as matched WT mice, were kindly donated by Professors Ajay Shah and Qingbo Xu, King’s College London.

Conflict of interest: none declared.

Funding

This work was supported by the Wellcome Trust (grant #087776). Funding to pay the Open Access publication charges for this article was provided by the Wellcome Trust.

References

1. Nixon GF, Mathieson FA, Hunter I. The multi-functional role of sphingosylphosphorylcholine. Prog Lipid Res 2008;47:62–75.
2. Kurokawa T, Yumina Y, Fujisawa H, Shirao S, Kashwagi S, Sato M, Kishi H, Miwa S, Mogami K, Kato S, Akimura T, Soma M, Ogawara K, Ogawa A, Kobayashi S, Suzuki M. Elevated concentrations of sphingosylphosphorylcholine in cerebrospinal fluid after subarachnoid hemorrhage: a possible role as a spasmogen. Clin Neurosci 2009;16:1064–1068.
3. Michel MC, Mulders AC, Jongsma M, Alewijnse AE, Peters SL. Vascular effects of sphingomyelin metabolites. Prog Lipid Res 2009;48:732–738.
4. Knock GA, Snetkov VA, Smirnov SV, Kjaer A, Aaronson PI, Ward JP. Superoxide differentially controls pulmonary and systemic L-type calcium channels by reactive oxygen species. Circ Res 2010;107:1002–1010.
5. Chakrabarti S, Chowdhury A, Das P, Shaikh S, Roy S, Chakrabarti T. Role of protein kinase C in NADPH oxidase derived O2(−)-mediated regulation of KV-LVOCC axis under U46619 induced increase in [Ca2+]i in pulmonary smooth muscle cells. Arch Biochem Biophys 2009;487:123–130.
6. Sonpal Y, Ho J, Kaur R, Jeppesen E, Chen J, Singh JH, Singh S. Notably, angiotensin II causes highly localized sub-plasmalemmal binding to sphingosylphosphorylcholine in Jurkat T cells. Prostaglandins Other Lipid Mediat 2007;84:174–183.
7. Thomas GD, Snetkov VA, Patel R, Leach RM, Aaronson PI, Ward JP. Sphingosylphosphorylcholine-induced vasoconstriction of pulmonary artery: activation of non-store-operated Ca2+ entry. Cardiovasc Res 2005;68:56–64.
8. Neteok VA, Thomas GD, Teague B, Leach RM, Shafta Y, Knock GA, Aaronson PI, Ward JP. Low concentrations of sphingosylphosphorylcholine enhance pulmonary artery vasoreactivity: the role of protein kinase C, delta and Ca2+ entry. Hypertension 2008;51:239–245.
9. Jean ES, Lee MJ, Sung SM, Kim JH. Sphingosylphosphorylcholine induces apoptosis of endothelial cells through reactive oxygen species-mediated activation of ERK. J Cell Biochem 2007;100:1536–1547.
10. Han M, Kim YL, Sackett SJ, Kim K, Kim HL, Jo YJ, Ha NC, Im DS. Effect of direct albumin binding to sphingosylphosphorylcholine in Jurkat T cells. Prostaglandins Other Lipid Mediat 2007;84:174–183.
11. Choi H, Kim S, Kim HJ, Kim KM, Lee CH, Shin JH, Noah M. Sphingosylphosphorylcholine down-regulates flgillin gene transcription through NOX5-based NADPH oxidase and cyclooxygenase-2 in human keratinocytes. Biochem Pharmacol 2010;80:95–103.
12. Jeon ES, Kang YJ, Hye In HS, Kim HS, Ryu SH, Kim YK, Kim JH. Sphingosylphosphorylcholine generates reactive oxygen species through calcium-, protein kinase Cdelta- and phospholipase D-dependent pathways. Cell Signal 2005;17:777–787.
13. Hool LC, Corry B. Redox control of calcium channels: from mechanisms to therapeutic opportunities. Antioxid Redox Signal 2009;7:409–435.
14. Amberg GC, Siipola R. Low concentrations of sphingosylphosphorylcholine by reactive oxygen species. Circ Res 2010;107:1002–1010.
15. Hool LC, Corry B. Redox control of calcium channels: from mechanisms to therapeutic opportunities. Antioxid Redox Signal 2009;7:409–435.
16. Amberg GC, Siipola R. Low concentrations of sphingosylphosphorylcholine by reactive oxygen species. Circ Res 2010;107:1002–1010.
17. Chakrabarti S, Chowdhury A, Das P, Shaikh S, Roy S, Chakrabarti T. Role of protein kinase C in NADPH oxidase derived O2(−)-mediated regulation of KV-LVOCC axis under U46619 induced increase in [Ca2+]i in pulmonary smooth muscle cells. Arch Biochem Biophys 2009;487:123–130.
18. Song YH, Cho H, Ryu SY, Yoon YJ, Park SH, Noh CI, Lee SH, Ho WK. L-type Ca2+ channel facilitation mediated by H(2)O(2)-induced activation of CaMKII in rat ventricular myocytes. J Mol Cell Cardio 2010;48:773–800.
19. Zeng Q, Zhou Q, Yao F, O'Rourke ST, Sun C. Endothelin-1 regulates cardiac L-type calcium channels via NAD(P)H oxidase-derived superoxide. J Pharmacol Exp Ther 2008;326:732–738.
20. Snetkov VA, Smirnov SV, Kjaer A, Aaronson PI, Ward JP. Knock GA. Superoxide differentially controls pulmonary and systemic vascular tone through multiple signalling pathways. Cardiovasc Res 2011;89:214–224.
21. Sylvester JT, Shimoda LA, Aaronson PI, Ward JP. Hypoxic pulmonary vasorelaxation. Physiol Rev 2012;92:569–602.
22. Bendall JK, Cave AC, Heymes C, Gall N, Shah AM. Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. Circulation 2002;105:293–296.
23. Landmesser U, Cai H, Dilakov S, McCallin J, Lhwang JG, Hunt HM, Harrison DG. Role of p47(phox) in vascular oxidative stress and hypertension caused by angiotensin II. Hypertension 2002;40:511–515.
24. Bai X, Margariti A, Hu Y, Sato Y, Zeng L, Ivecis A, Habi O, Mason JC, Wang X, Xu Q. Protein kinase C(delta) deficiency accelerates neointimal lesions of mouse injured artery involving delayed reendothelialization and vasoohibin-1 accumulation. Arterioscler Thromb Vasc Biol 2010;30:2467–2474.
25. Knock GA, Snetkov VA, Shafta Y, Dmarski S, Ward JP. Aaronson PI. Role of src family kinases in hypoxic vasoconstriction of rat pulmonary artery. Cardiovasc Res 2008;80:453–460.
26. Finch AL, Gordenko DV, Yuill KH, Smirnov SV. Cellular localization of mitochondrial NOX5 contributes to K+ channel-mediated regulation of cellular excitability in pulmonary but not mesenteric circulation. Am J Phys Lung Cell Mol Physiol 2009;296:L347–L360.
27. Soltoff SP, Rottlerin: an inappropriate and ineffective inhibitor of PKCdelta. Trends Pharmacol Sci 2007;28:453–458.
28. Kim YS, Sang HJ, Park SY, Min YS, Im BO, Ko SK, Whang WK, Sohn UJ. The signaling mechanism of the sphingosylphosphorylcholine-induced contraction in cat esophageal smooth muscle cells. Arch Pharm Res 2007;30:1608–1618.
29. Way KJ, Chou E, King GL. Identification of PKC-isofrom-specific biological actions using pharmacological approaches. Trends Pharmacol Sci 2000;21:181–187.
30. Wang YX, Zheng YM. ROS-dependent signaling mechanisms for hypoxic Ca2+ responses in pulmonary artery myocytes. Antioxid Redox Signal 2010;12:611–623.
31. White CN, Figtree GA, King GL. Identification of PKC-isoform-specific biological actions using pharmacological approaches. Trends Pharmacol Sci 2000;21:181–187.
37. Aaronson PI, Robertson TP, Knock GA, Becker S, Lewis TH, Snetkov V, Ward JP. Hypoxic pulmonary vasoconstriction: mechanisms and controversies. J Physiol 2006; 570:53–58.
38. Hedemann J, Fetscher C, Michel MC. Comparison of noradrenaline and lysosphingolipid-induced vasoconstriction in mouse and rat small mesenteric arteries. Auton Autacoid Pharmacol 2004; 24:77–85.
39. Choi SK, Ahn DS, Lee TH. Comparison of contractile mechanisms of sphingosylphosphorylcholine and sphingosine-1-phosphate in rabbit coronary artery. Cardiovasc Res 2009; 82:243–252.
40. Rathore R, Zheng YM, Niu CF, Korde A, Ho YS, Wang YX. Hypoxia activates NADPH oxidase to increase [ROS] and [Ca\(^{2+}\)] through the mitochondrial ROS-PKCepsilon signaling axis in pulmonary artery smooth muscle cells. Free Radic Biol Med 2008; 45:1223–1231.
41. Knock GA, Ward JP. Redox regulation of protein kinases as a modulator of vascular function. Antioxid Redox Signal 2011; 15:1531–1547.
42. Cogolludo A, Frazziano G, Cobeno L, Moreno L, Lodi F, Villanor E, Tamargo J, Perez-Vizziano F. Role of reactive oxygen species in K\(_c\) channel inhibition and vasoconstriction induced by TP receptor activation in rat pulmonary arteries. Ann N Y Acad Sci 2006; 1091:41–51.
43. Ignatov A, Lintzel J, Hermans-Borgmeyer I, Krenkamp HJ, Joost P, Thomsen S, Mether A, Schaller HC. Role of the G-protein-coupled receptor GPR12 as high-affinity receptor for sphingosylphosphorylcholine and its expression and function in brain development. J Neurosci 2003; 23:907–914.
44. Lilom K, Sun G, Binder M, Virag T, Nusser N, Baker DL, Wang DA, Fabian MJ, Brandsen B, Bender K, Eckel A, Malik KU, Miller DD, Desiderio DM, Tigg D, Pott L. Sphingosylphosphocholine is a naturally occurring lipid mediator in blood plasma: a possible role in regulating cardiac function via sphingolipid receptors. Biochem J 2001; 355:189–197.
45. Nguyen Dinh Cat A, Montezano AC, Burger D, Touyz RM. Angiotensin II, NADPH oxidase, and redox signaling in the vasculature. Antioxid Redox Signal 2013; 19:1100–1120.