Sphingosylphosphorylcholine induces Ca^{2+}-sensitization of vascular smooth muscle contraction: possible involvement of Rho-kinase

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Received 25 August 2000; accepted 5 September 2000

Edited by Guido Tettamanti

Abstract Sphingosylphosphorylcholine (SPC), a sphingolipid, concentration-dependently (1–50 μM) induced contraction and slight elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in smooth muscle of the pig coronary artery, the result being a marked increase in the force/$[Ca^{2+}]_i$ ratio. In α-toxin- or β-escin-permeabilized, but not Triton X-100-permeabilized, vascular strips, SPC induced contraction at constant $[Ca^{2+}]_i$ (pCa 6.3) in the absence of GTP, whereas a G-protein-coupled receptor agonist, histamine, required the presence of GTP to induce the contraction. The Rho-kinase blocker, Y-27632 (10 μM) abolished the SPC-induced Ca^{2+}-sensitization, without affecting the Ca^{2+}-induced contraction. These results suggest that SPC induces Ca^{2+}-sensitization of force in vascular smooth muscle, presumably through the activation of Rho-kinase (or a related kinase).

Key words: Sphingolipid; Cytosolic Ca^{2+} concentration; Ca^{2+} sensitivity; Smooth muscle; Coronary artery; Y-27632

1. Introduction

Sphingolipids are novel messengers for signal transduction in various cellular processes, including apoptosis and cell proliferation [1–3]. One of the sphingolipids, sphingosylphosphorylcholine (SPC) is generated by *N*-deacylation of sphingomyelin, which is one of the most abundant lipids in the cell membrane. In various cultured cells, SPC directly induces an increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [2,3], which might lead to cellular responses through Ca^{2+}-dependent enzymes. We recently found that SPC induces $[Ca^{2+}]_i$ elevation and NO production in endothelial cells in situ [4]. In smooth muscle cells, the contraction is primarily regulated by $[Ca^{2+}]_i$ via Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK). SPC was reported to decrease the length of smooth muscle cells isolated from the rectosigmoid of the rabbit [5]. These reports raised the possibility that SPC could induce the contraction of vascular smooth muscle. However, the effect of SPC on the contractile regulation of vascular smooth muscle is not well understood. In addition to the $[Ca^{2+}]_i$-dependent mechanism in smooth muscle, the modulation of Ca^{2+} sensitivity of the contractile apparatus was recently found to be the mechanism that can $[Ca^{2+}]_i$-independently regulate smooth muscle contraction [6,7]. We showed that Rho-kinase directly induces contraction through an increase in the phosphorylation of the myosin light chain, independent of the Ca^{2+}-calmodulin-dependent MLCK pathway [7,8]. A recent report shows that the Ca^{2+}-sensitization mediated by Rho-kinase contributes to blood pressure regulation in vivo and may participate in hypertension [9]. However, the activation mechanism of Rho-kinase mediation of the Ca^{2+}-sensitization remains to be determined [7], except for the possibility that RhoA [10–12] and arachidonic acid [13,14] may be involved in activation of this pathway. Therefore, increasing interest has been directed toward mechanisms involved in Ca^{2+}-sensitization in vascular smooth muscle.

In this study, using simultaneous measurement of $[Ca^{2+}]_i$ and force, and membrane permeabilization techniques, we demonstrate for the first time that SPC potentiates the Ca^{2+} sensitivity of the contractile apparatus in vascular smooth muscle, an activity abolished by the Rho-kinase inhibitor (Y-27632) [9] and observed even in the absence of GTP, which is required for activation of the receptor-coupled G-proteins. Our results are compatible with the notion that SPC may be a novel mediator for the Ca^{2+}-sensitization of vascular smooth muscle contraction, with possible mediation by Rho-kinase (or a related kinase), although the endogenous generation of SPC, associated with the Ca^{2+}-independent contraction of vascular smooth muscle, will need to be demonstrated.

2. Materials and methods

2.1. Materials

Y-27632 was a gift from Yoshitomi Pharmaceutical Industries (Osaka, Japan). Fura-2 AM (acetoxymethyl ester form of fura-2) and ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Dojindo Laboratories (Kumamoto, Japan); sphingosine (SP), phosphorylcholine (PC), sphingomyelinase, Triton X-100, PIPES, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and leupeptin were from Sigma (St. Louis, MO, USA); SPC was from Sigma or Biomol (Plymouth Meeting, PA, USA); β-escin was from Sigma or Katayama Chemical (Osaka, Japan); methanesulfonic acid, creatine phosphate disodium and hista-

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Abbreviations: SPC, sphingosylphosphorylcholine; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; MLCK, myosin light chain kinase; fura-2 AM, acetoxymethyl ester form of fura-2; SP, sphingosine; PC, phosphorylcholine; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; PSS, physiological salt solution; SPP, sphingosine-1-phosphate

0014-5793/00/$20.00
PII: S0014-5793(00)02046-9

mine dihydrochloride were from Wako (Osaka, Japan); *Staphylococcus aureus* α-toxin was from Research Biochemical International (Natick, MA, USA); C_2-ceraminde, sphingosine-1-phosphate (SPP) and calmodulin were from Biomol; ATP and GTP were from Boehringer Mannheim (Germany); ionomycin was from Calbiochem (Frankfurt, Germany); and other reagents used were from Katayama Chemical.

SPC was at first dissolved in 100% ethanol and subsequently the SPC/ethanol solution was dissolved in phosphate-buffered solution (PBS) containing 2 mg/ml bovine serum albumin to acquire a stock solution (2 mM), as described [4]. The SPC stock solution was stored at −80°C.

2.2. Simultaneous measurements of tension and $[Ca^{2+}]_i$ of arterial smooth muscle

The left circumflex coronary arteries (2–3 cm from the origin) of the pig were cut into strips (1×4×0.1 mm) without endothelium and adventitia and were loaded with $[Ca^{2+}]_i$ indicator dye, fura-2, as described [15–17]. In brief, strips were incubated for 4 h at 37°C in normal physiological salt solution (normal PSS in mM: NaCl, 123; KCl, 4.7; $NaHCO_3$, 15.5; KH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 1.25; and D-glucose, 11.5) gassed with a mixture of 5% CO_2 and 95% O_2 with the resulting pH of 7.4 and containing 12.5 μM fura-2 AM and 5% fetal bovine serum. The fura-2-loaded strips were mounted vertically in a quartz organ bath and the tension was measured using a force transducer (TB-612T, Nihon Koden, Japan). During 1 h of incubation in normal PSS, for purposes of equilibration, the strips were stimulated repeatedly with 118 mM K^+ PSS every 15 min, and the resting tension was adjusted to an optimal one (about 200 mg). To assess the $[Ca^{2+}]_i$ levels, the ratio (F_{340}/F_{380}) of fura-2 fluorescence intensities (at 500 nm) at alternating (400 Hz) excitation (340 and 380 nm) was monitored using a front-surface fura-2 fluorometer (CAM-230, Japan Spectroscopic Co., Japan), as described [15–17] but with a minor modification, in which randomly arranged optical fibers were used. In brief, strips were illuminated by guiding the excitation light from a xenon light source through quartz optic fibers randomly arranged in a circle (diameter 7 mm), and surface fluorescence of the strips was collected by glass optic fibers randomly arranged in the circle. Changes in tension and fluorescence ratio were expressed as a percentage, assuming the values at a steady state in normal (5.9 mM K^+) and 118 K^+ mM PSS to be 0 and 100%, respectively. The absolute values of $[Ca^{2+}]_i$ were determined in separate measurements, with the use of the method and the following equation described by Grynkiewicz et al. [18], with minor modifications [15,19]:

$$[Ca^{2+}]_i = K_d(R-R_{min})/(R_{max}-R)(S_{f2}/S_{b2})$$

where K_d is a dissociation constant and assumed to be 224 nM [18], R is F_{340}/F_{380}, R_{max} is obtained by the addition of 25 μM ionomycin in the presence of extracellular Ca^{2+} (1.25 mM Ca^{2+}) and R_{min} is obtained in the absence of extracellular Ca^{2+} (0 mM Ca^{2+}; 2 mM EGTA). S_{f2}/S_{b2} is the ratio of proportionality coefficients of free dye and Ca^{2+}-bound dye at one wavelength (380 nM) and is under the influence of various optical factors, including excitation intensity, path length and instrumental efficiency of collecting emitted photons [18,19]. The mean values of $[Ca^{2+}]_i$ at normal PSS (at rest; 0%) and 118 mM K^+-depolarization (100%) were 105.5 ± 3.8 nM (n = 10) and 718.2 ± 3.2 nM (n = 10), respectively. Because the calibration of $[Ca^{2+}]_i$ was made on the assumption that the fura-2 signal was homogeneous despite a multicellular preparation, the K_d value of fura-2 in the cytosol of vascular smooth muscle in the present experimental condition was 224 nM (a K_d for fura-2 in the buffer with normal ionic strength at pH 7.05 and 37°C) and S_{f2}/S_{b2} was 1; the $[Ca^{2+}]_i$ values obtained were an approximation of the true $[Ca^{2+}]_i$ value [19].

2.3. Membrane permeabilization of arterial smooth muscle

Small strips (150–200 μm×2 mm) of the left circumflex coronary artery smooth muscle of the pig were dissected out [16,17]. Isometric tension was measured using a force transducer (UL-2g, Minebea, Japan) in a well on a 'bubble' plate at 28°C [16,17,20,21]. The solution was changed by sliding the bubble plate to an adjacent well. When steady responses to high K^+ were observed, the strips were incubated for 10 min in normal relaxing solution (in mM: potassium methanesulfonate, 74.1; magnesium methanesulfonate, 2; MgATP, 4.5; EGTA, 1; creatine phosphate, 10; PIPES, 30) containing 1 μM FCCP as a mitochondria blocker and 1 μM leupeptin as a protease inhibitor.

α-Toxin permeabilization of the coronary artery smooth muscle strips was performed, as described [16,17]. In brief, the strips were incubated with 0.12 mg/ml *S. aureus* α-toxin for 45–50 min in activating solution (pCa 6.3), in which 10 mM EGTA was used, a specified amount of calcium methanesulfonate was added to give a desired concentration of free calcium ions, and the ionic strength was kept constant at 0.2 M by adjusting the concentration of potassium methanesulfonate.

For β-escin and Triton X-100 permeabilizations, the strips were incubated with 30 μM β-escin [20,21] and with 0.5% Triton X-100 [8], respectively, for 20–30 min in normal relaxing solution, as described. Calmodulin (1 μM) was added to the activating solution for β-escin- and Triton X-100-permeabilized strips.

The contractile effect of SPC on the tension was examined at pCa 6.3. The tension levels were expressed as a percentage, assigning the values in normal relaxing (pCa ≫ 8, nominally zero Ca^{2+} with 10 mM EGTA) and activating (pCa 4.5, buffered with 10 mM EGTA) solutions to be 0 and 100%, respectively. The value assigned to 100% response (obtained with pCa 4.5) was observed before the start of the experiments using α-toxin-permeabilized strips, and at the end of the experiments in β-escin- and Triton X-100-permeabilized strips. The values are expressed as the mean ± standard error. The Student's t-test was used to determine statistical significance, at $P < 0.05$.

3. Results

3.1. Effects of SPC on tension and $[Ca^{2+}]_i$ of arterial smooth muscle

SPC concentration-dependently (1–50 μM) induced sustained contraction and slight $[Ca^{2+}]_i$ elevation in smooth muscle of the porcine coronary artery (Fig. 1A,B). In Ca^{2+}-free PSS containing 2 mM EGTA instead of 1.25 mM $CaCl_2$ (complete removal of extracellular Ca^{2+}), SPC induced little or no elevation of $[Ca^{2+}]_i$ (data not shown), suggesting that SPC elevates $[Ca^{2+}]_i$ in vascular smooth muscle mainly by the influx of extracellular Ca^{2+}, but not by the release of intracellular Ca^{2+}. The extent of the contraction induced by 30 μM SPC was much larger than that expected from the extent of the $[Ca^{2+}]_i$ elevation, as compared with the contraction induced by 118 mM K^+-depolarization (Fig. 1A). The SPC effect on the vascular smooth muscle is specific, since SP and PC (up to 100 μM) (Fig. 1C,D), both metabolites of SPC, and 30 μM C_2-ceramide (data not shown) had no apparent effect on the force. In addition, the contractile effects of 30 μM SPP and 0.1 U/ml sphingomyelinase were only transient and much smaller (less than 20%) (data not shown) than that for 30 μM SPC (61.2 ± 9.5%, n = 4) (Fig. 1A,B). The specificity of the effect of SPC over other sphingolipids is also supported by the recent report that SPC selectively binds to orphan receptor OGR1 with high affinity and high specificity [22].

3.2. Effects of SPC on the relationship between tension and $[Ca^{2+}]_i$ of arterial smooth muscle

To examine the relationship between tension and $[Ca^{2+}]_i$, we simultaneously observed changes in tension and $[Ca^{2+}]_i$ when extracellular Ca^{2+} was added in a cumulative manner (from 0 to 1.25 mM) during 118 mM K^+-depolarization. The cumulative addition of extracellular Ca^{2+} induced a step-wise increase in tension and $[Ca^{2+}]_i$ (Fig. 2A). As shown in Fig. 2B, SPC concentration-dependently (1–50 μM) shifted the $[Ca^{2+}]_i$–tension curve to the left of the control curve obtained with high K^+-depolarization. These results indicate that SPC potentiates Ca^{2+} sensitivity of the contractile apparatus of vascular smooth muscle.

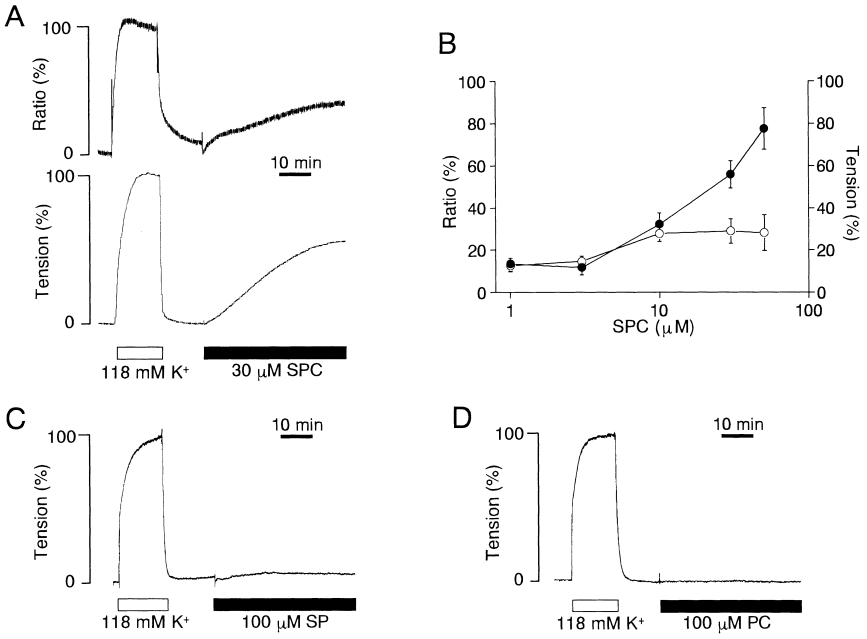

Fig. 1. Effects of SPC and its metabolites on $[Ca^{2+}]_i$ and tension in porcine coronary arterial strips. Resting levels and the plateau levels of the contraction induced by 118 mM K^+ were designated 0 and 100%, respectively. A: A representative recording showing the effect of 30 μM SPC on $[Ca^{2+}]_i$ and tension of the fura-2-loaded strips. B: Changes in $[Ca^{2+}]_i$ (open circle) and tension (closed circle) induced by various concentrations (in abscissa) of SPC. Data represent the means ($n=4$) of the values at 40 min after the application of SPC and are expressed as mean ± S.E. C and D: Representative recordings showing the effects of SP (C) and PC (D) on tension.

3.3. Effect of SPC on the tension of permeabilized arterial smooth muscle

To investigate the signal transduction pathway(s) involved in the SPC-induced Ca^{2+}-sensitization of the contractile apparatus, we used coronary arterial strips permeabilized with *S. aureus* α-toxin. Since the permeabilization with α-toxin allows molecules with a molecular mass of less than 1000 to penetrate the cell membrane [21,23], $[Ca^{2+}]_i$ can be controlled to a constant value in case of use of a high buffer, 10 mM EGTA. SPC (30 μM) enhanced (68.7 ± 2.9%, mean ± S.E., $n=4$) the contractile response to constant cytosolic Ca^{2+} (pCa 6.3) buffered with 10 mM EGTA (20.8 ± 1.1%, $n=4$; $P<0.01$) (Fig. 3A,B,D). This enhancement represents an increase in the Ca^{2+} sensitivity of the contractile apparatus.

A G-protein-coupled receptor agonist, histamine (10 μM), induced contraction at constant $[Ca^{2+}]_i$ (pCa 6.3) in the presence of 10 μM GTP but had little effect in the absence of GTP (Fig. 3A). In the same strip, 30 μM SPC induced Ca^{2+}-sensitization at pCa 6.3, even in the absence of GTP (Fig. 3A). These results are compatible with the involvement of the G-protein-independent pathway in Ca^{2+}-sensitization induced by SPC.

We reported that Rho-kinase induces smooth muscle contraction through an increase in the phosphorylation of myosin light chain, independently of the Ca^{2+}-calmodulin-dependent MLCK pathway [7,8]. Therefore, we examined the effect of Y-27632, a blocker of Rho-kinase [9], on the Ca^{2+}-sensitization induced by SPC. The Ca^{2+}-sensitization induced by SPC was partially inhibited by the subsequent addition of 10 μM Y27632 ($P<0.05$) (Fig. 3B,D). In contrast, the prior application of 10 μM Y27632 abolished the SPC-induced Ca^{2+}-sensitization (Fig. 3C,E). Y-27632 (up to 10 μM) itself had no effect on the contraction induced by pCa 6.3 (Fig. 3C,E), such being compatible with a previous observation that 10 μM Y-27632 specifically inhibits Rho-kinase, as compared to MLCK [9]. Similar results were obtained using smooth muscle strips permeabilized with β-escin (data not shown). In β-escin-permeabilized strips, higher molecular weight compounds (up to 150 kDa) can penetrate the membrane [21,24], more so than in α-toxin-permeabilized strips (< 1 kDa) [21,23], therefore Rho-kinase (> 150 kDa) should be retained in the cytosol of the permeabilized strips [7]. In contrast, 30 μM SPC had no effect on Triton X-100-permeabilized smooth muscle (data not shown). We earlier observed that most of the endogenous Rho-kinase in vascular smooth muscle strips leaked out of the cells after Triton X-100-permeabilization [7,8]. Taken together, these results suggest the involvement of Rho-kinase in the Ca^{2+}-sensitization induced by SPC.

4. Discussion

The novel finding of this study is that SPC induces not only a slight elevation of $[Ca^{2+}]_i$ but also the potentiation of Ca^{2+} sensitivity of the contractile apparatus, and induces smooth muscle contraction. In addition, we also suggest that the SPC-induced Ca^{2+}-sensitization was mediated by Rho-kinase (or a related kinase sensitive to Y27632). These results were obtained from experiments using a simultaneous measurement of $[Ca^{2+}]_i$ and force in intact smooth muscle and a measurement of Ca^{2+}-independent contraction in membrane-permeabilized smooth muscle.

We favor the view that SPC potentiates Ca^{2+} sensitivity of the contractile apparatus, for the following reasons: (1) SPC increased the force/$[Ca^{2+}]_i$ ratio and shifted the curve of the $[Ca^{2+}]_i$–force relationship to the left in intact vascular smooth muscle strips, as compared with the response to high K^+-depolarization (Figs. 1 and 2) and (2) SPC induced contrac-

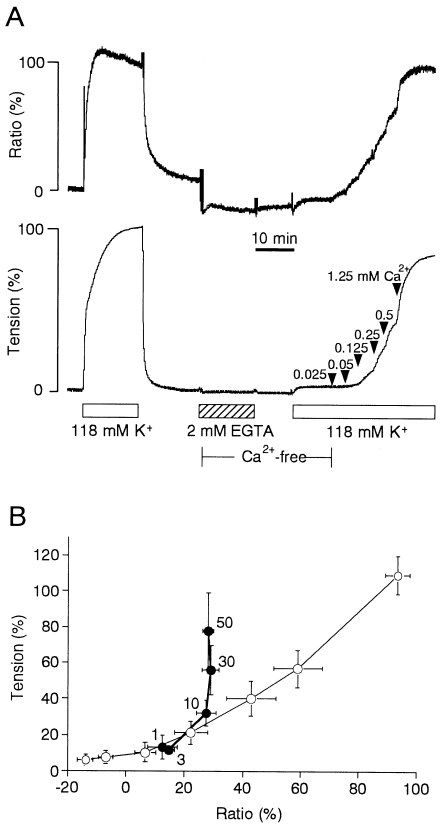

Fig. 2. A comparison of $[Ca^{2+}]_i$–tension relationships of contractions induced by SPC and by 118 mM K^+. A: A representative recording showing step-wise elevations of $[Ca^{2+}]_i$ and tension induced by cumulative applications of extracellular Ca^{2+} (0–1.25 mM) in the presence of 118 mM K^+. B: The $[Ca^{2+}]_i$–tension relationships of the contractions obtained by the experimental protocol shown in (A) (open circle, connected by a thin solid line) and of steady-state contractions (at 40 min) induced by the various concentrations (1, 3, 10, 30 and 50 μM, as indicated by the number shown in the figure) of SPC (closed circle, connected by a thick solid line). The data represent the means of four measurements with S.E.

tion at constant $[Ca^{2+}]_i$ in membrane-permeabilized strips (Fig. 3). It was also observed that SPC reduces the Ca^{2+} requirement for activating tissue transglutaminase [25].

The Rho-kinase inhibitor (10 μM Y-27632) abolished SPC-induced Ca^{2+}-sensitization without affecting the Ca^{2+}-induced contraction (Fig. 3), which suggests involvement of Rho-kinase in Ca^{2+}-sensitization induced by SPC. This notion is supported by findings in the present study that SPC can induce Ca^{2+}-sensitization in Rho-kinase-maintained vascular strips permeabilized with α-toxin or with β-escin, but not in the Triton X-100-permeabilized vascular strips in which Rho-kinase had leaked out of the cells [7,8] (Fig. 3). The mechanism(s) by which SPC activates Rho-kinase is unclear. Rho-kinase has a pleckstrin homology domain, a lipid-binding motif [13], which raises the possibility that some lipids may directly affect Rho-kinase and possibly regulate its kinase activity. It was recently reported that purified Rho-kinase is directly activated by arachidonic acid [13], a lipid messenger which was found to induce Ca^{2+}-sensitization of permeabilized smooth muscle [26]. This is also supported by the finding that the Ca^{2+}-sensitization induced by arachidonic acid was blocked by a Rho-kinase blocker, Y-27632 [14]. Therefore, one can hypothesize that SPC may also directly activate Rho-kinase. However, post- rather than pre-incubation with Y27632 more potently inhibited the arachidonic acid-induced Ca^{2+}-sensitization [14], whereas in the present study we obtained opposite results with the SPC-induced Ca^{2+}-sensitization: the pre-incubation with Y27632 showed a more potent inhibition of the SPC-induced Ca^{2+}-sensitization than did post-incubation (Fig. 3). Therefore, even if SPC directly activates Rho-kinase, it is also likely that the activation mechanism(s) of Rho-kinase may differ between SPC and arachidonic acid. In addition, arachidonic acid was reported to induce the Ca^{2+}-sensitization through a variety of other actions [7], including the direct inhibition of myosin phosphatase activity [26] and the activation of atypical kinase C [27].

It was reported that SPP strongly activates G-protein-coupled membrane receptors (EDG-1 and AGR16), which are expressed in various types of cells, including vascular smooth muscle cells [28,29]. It was also suggested that SPC may elevate $[Ca^{2+}]_i$ perhaps through activation of these membrane receptors [30,31], although SPC is a much less potent agonist for the membrane receptors than is SPP [30,31]. In the present study, we also found that SPC induced a slight elevation of $[Ca^{2+}]_i$ in intact vascular smooth muscle strips (Fig. 1). It may be that SPC-induced $[Ca^{2+}]_i$ elevation is mediated by the activation of membrane receptors. However, since exogenously applied SPC is rapidly taken up by intact cells [2,32], the contractile effect of SPC on the intact muscle may not necessarily be mediated through the action of SPC on these membrane receptors. Indeed, the contraction induced by SPC is much larger than that of SPP (data not shown). Furthermore, the present study showed that SPC stimulated the influx of extracellular Ca^{2+}, but not the release of intracellular Ca^{2+}, whereas it has been well documented that activation of EDG-1 and AGR16 stimulates both mechanisms of Ca^{2+} mobilization [30,31]. In contrast, our recent potency analysis of SPC and SPP suggested no important role for these membrane receptors in SPC-induced $[Ca^{2+}]_i$ elevation in endothelial cells in situ, while SPC stimulates both Ca^{2+} influx and Ca^{2+} release [4]. In addition, Fig. 3A suggests the involvement of a G-protein-independent pathway in Ca^{2+}-sensitization induced by SPC, whereas sphingolipid receptors, EDG-1 and AGR16, were reported to be coupled with G-protein. OGR1, expressed in several tissues including small intestine [33] and for which SPC was reported to be a high affinity ligand, is also coupled with G-protein and the potency of SPC for OGR1 (K_d = 33.3 nM) is far different from that in the present study [22]. Therefore, it is likely that SPC may act intracellularly, but not extracellularly (as agonist for these G-protein-coupled membrane receptors). Therefore, we propose that SPC may be a novel mediator for Ca^{2+}-sensitization of vascular smooth muscle contraction, with possible mediation by Rho-kinase (or a related kinase). On the other hand, it is also possible that the contractile effect of SPC may be mediated through the action of SPC on unknown membrane receptors, which are not coupled with G-protein and have a much lower affinity for SPC than currently known receptors, such as OGR1 and EDG. To confirm that SPC is a messenger in vascular smooth muscle, it remains to be determined if sphingomyelin generates SPC in vascular smooth muscle cells and what metabolites are formed from SPC, using radiolabeling procedures [34]; although ^{14}C-labeled SPC was the only labeled lipid detected by thin layer chromatography and there was no significant labeling of lysoPC, PC or sphingomyelin, even

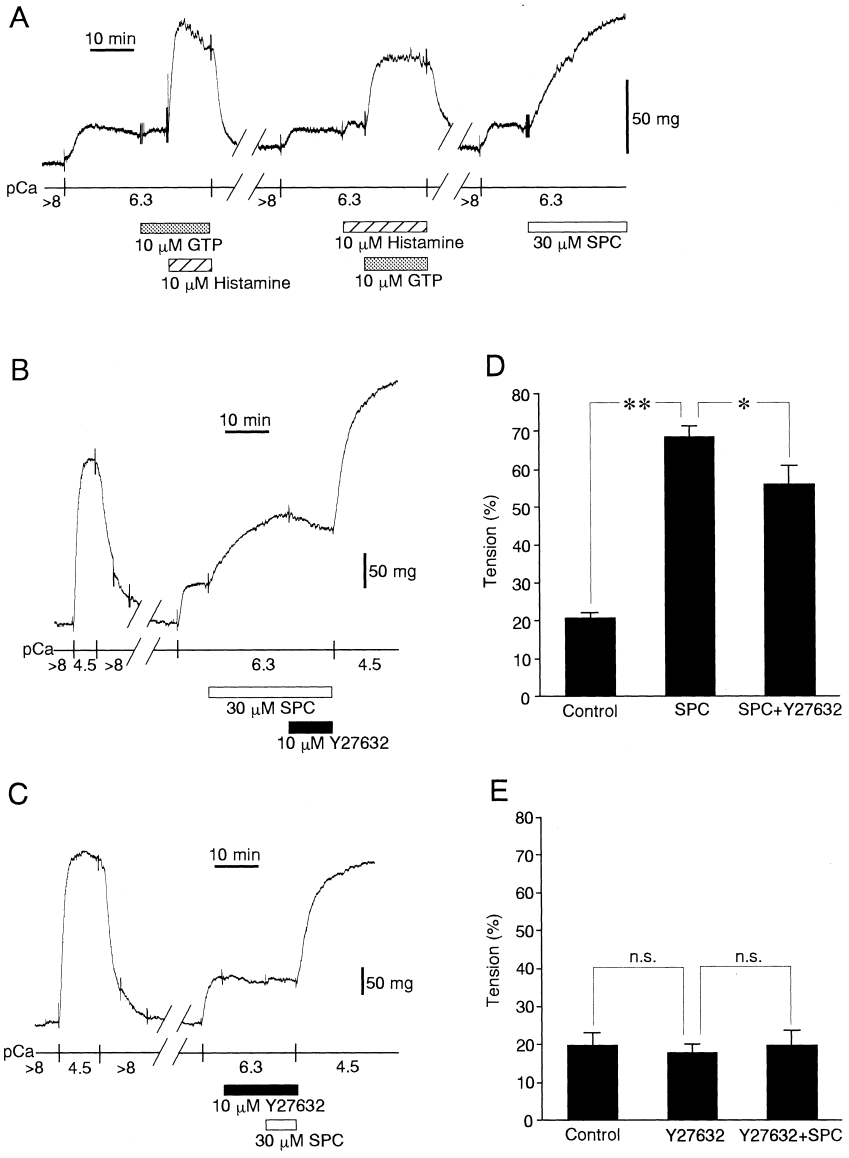

Fig. 3. Effects of SPC on tension of α-toxin-permeabilized coronary artery smooth muscle at constant Ca^{2+} (pCa 6.3, buffered with 10 mM EGTA). A: The differential requirement of GTP (10 μM) for the contractile responses induced by 30 μM SPC and by 10 μM histamine at constant Ca^{2+} (pCa 6.3) in the same vascular smooth muscle strip permeabilized with α-toxin. B: The inhibitory effect of 10 μM Y-27632 on the contraction induced by 30 μM SPC at pCa 6.3 in permeabilized smooth muscle. C: The lack of any contractile response of permeabilized smooth muscle in the presence of 10 μM Y-27632 to 30 μM SPC at pCa 6.3. D and E: Summary of the experiments carried out using protocols similar to those for (B) and (C), respectively. The tension levels were expressed as a percentage: the 100% contractions induced by activating solution (pCa 4.5) were observed before starting the experiments. The data represent the means of four experiments with S.E. $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., difference not significant.

at 1 h after ^{14}C-labeled SPC was applied to Swiss 3T3 fibroblasts [32].

SPC may play a role in disease states such as hypertension and vasospasm. It was reported that Ca^{2+}-sensitization mediated by Rho-kinase, which was suggested to be activated by SPC in the present study (Fig. 3), is involved in the pathogenesis of hypertension [9]. Y-27632, a specific inhibitor of Rho-kinase, controls hypertension in several hypertensive rat models [9]. Since Ca^{2+}-sensitization by SPC was also abolished by Y-27632, the biosynthesis of SPC in vascular smooth muscle may be closely related to hypertension.

Acknowledgements: We thank Dr. S. Narumiya (Kyoto University, Japan) and Mr. Y. Miura and Ms. A. Yoshimura (Yoshitomi Pharmaceutical Industries, Saitama, Japan) for generous gifts of Y-27632, R. Kimura for secretarial assistance and S. Miwa, K. Kuroki and N. Maeda for technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, and grants from the Mochida Memorial Foundation, Terumo Life Science Foundation, Suzuken Memorial Foundation, Ono Medical Research Foundation, Uehara Memorial Foundation, Japan Research Foundation for Clinical Pharmacology, Yamanouchi Foundation for Research on Metabolic Disorders and Japan Heart Foundation and Pfizer Pharmaceuticals for Research on Coronary Artery Disease.

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