Sphingosylphosphorylcholine Is a Novel Messenger for Rho-Kinase–Mediated Ca\(^{2+}\) Sensitization in the Bovine Cerebral Artery

Unimportant Role for Protein Kinase C

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Abstract—Although recent investigations have suggested that a Rho-kinase–mediated Ca\(^{2+}\) sensitization of vascular smooth muscle contraction plays a critical role in the pathogenesis of cerebral and coronary vasospasm, the upstream of this signal transduction has not been elucidated. In addition, the involvement of protein kinase C (PKC) may also be related to cerebral vasospasm. We recently reported that sphingosylphosphorylcholine (SPC), a sphingolipid, induces Rho-kinase–mediated Ca\(^{2+}\) sensitization in pig coronary arteries. The purpose of this present study was to examine the possible mediation of SPC in Ca\(^{2+}\) sensitization of the bovine middle cerebral artery (MCA) and the relation to signal transduction pathways mediated by Rho-kinase and PKC. In intact MCA, SPC induced a concentration-dependent (EC\(_{50}=3.0\) \(\mu\)mol/L) contraction, without [Ca\(^{2+}\)] elevation. In membrane-permeabilized MCA, SPC induced Ca\(^{2+}\) sensitization even in the absence of added GTP, which is required for activation of G-proteins coupled to membrane receptors. The SPC-induced Ca\(^{2+}\) sensitization was blocked by a Rho-kinase inhibitor (Y-27632) and a dominant-negative Rho-kinase, but not by a pseudosubstrate peptide for conventional PKC, which abolished the Ca\(^{2+}\)-independent contraction induced by phorbol ester. In contrast, phorbol ester–induced Ca\(^{2+}\) sensitization was resistant to a Rho-kinase inhibitor and a dominant-negative Rho-kinase. In primary cultured vascular smooth muscle cells, SPC induced the translocation of cytosolic Rho-kinase to the cell membrane. We propose that SPC is a novel messenger for Rho-kinase–mediated Ca\(^{2+}\) sensitization of cerebral arterial smooth muscle and, therefore, may play a pivotal role in the pathogenesis of abnormal contraction of the cerebral artery such as vasospasm. The SPC/Rho-kinase pathway functions independently of the PKC pathway. (Circ Res. 2002;91:112-119.)

Key Words: vasospasm ■ sphingolipid ■ protein kinase C ■ Rho-kinase ■ membrane permeabilization

Cerebral vasospasm, a sustained abnormal contraction of the cerebral artery, is one of the major causes of lethal complications in patients with subarachnoid hemorrhage (SAH). The mechanisms responsible have been under intense investigation but remain poorly understood. The primary determinant of smooth muscle contraction is phosphorylation of 20-kDa myosin light chain (MLC), which is regulated by not only the Ca\(^{2+}\)/calmodulin (CaM)-dependent MLC kinase (MLCK)–mediated pathway but also a Ca\(^{2+}\)-independent mechanism (=Ca\(^{2+}\) sensitization). Excitatory agonists, including spasmodogenic agonists, initially increase intracellular Ca\(^{2+}\) levels leading to activation of MLCK. This action is followed immediately by a decrease in MLC phosphatase activity and an increase in MLC phosphorylation, which induces Ca\(^{2+}\) sensitization, the result of which is an abnormally enhanced contraction of vascular smooth muscle (VSM).

Because pharmacological interventions with Ca\(^{2+}\) channel blockers in experimental models\(^{4,5}\) and clinical trials\(^{6,7}\) have showed no significant reduction in the incidence of cerebral vasospasm, the Ca\(^{2+}\) sensitization mechanism, which is resistant to Ca\(^{2+}\) channel blockers, is currently considered to play a more important role in the pathogenesis of cerebral vasospasm. In addition, multiple second messengers/signaling pathways, including the Rho A/Rho-kinase,\(^{8-10}\) protein kinase C (PKC),\(^{11,12}\) and arachidonic acid pathways,\(^{13}\) have been linked to Ca\(^{2+}\) sensitization mechanisms.

Sato et al\(^{14}\) reported that activation of Rho-kinase was observed during the vasospasm induced by SAH. It was also reported that HA1077 (fasudil), an inhibitor of Rho-
kinase,\textsuperscript{10,12} prevents the development of cerebral vasospasm in two-hemorrhage canine models\textsuperscript{16} and in humans.\textsuperscript{17} Taken together, these observations strongly suggest that a Rho-kinase pathway plays an important role in the pathogenesis of cerebral vasospasm.

The upstream mediator of the Rho-kinase pathway causing Ca\textsuperscript{2+} sensitization of cerebral arteries has remained to be determined. Because translocation and membrane binding is required for activation of Rho-kinase,\textsuperscript{18} we hypothesized that a component of cell membrane or its metabolites may be present upstream of the Rho-kinase pathway. After extensive screening of molecules, we recently found that sphingosylphosphorylcholine (SPC) induces a sustained and concentration-dependent contraction in coronary arteries and that this contraction was abolished by a Rho-kinase inhibitor (Y-27632).\textsuperscript{19} SPC is a sphingolipid generated by N-deacylation of sphingomyelin, one of the most abundant lipids in the cell membrane, and was found to be a critical molecule in apoptosis, cell proliferation, and endothelial NO production.\textsuperscript{20–22} The slowly developing and sustained nature of the SPC-induced contraction mediated by Rho-kinase\textsuperscript{19} suggests a possible role for this mechanism in the abnormal, prolonged vasoconstriction which occurs in cerebral vasospasm. However, the role of this novel signal transducing molecule in cerebral arterial contractions has not been identified.

In the present study, we tested the hypothesis that SPC is a potential signal mediator for Ca\textsuperscript{2+} sensitization of cerebral arterial smooth muscle contraction as an upstream initiator of the Rho-kinase pathway. Because PKCs may play a role in Ca\textsuperscript{2+} sensitization,\textsuperscript{23} interrelationships between a PKC-mediated pathway and a newly discovered pathway mediated by Rho-kinase and SPC were also examined.

For these purposes, we directly evaluated Ca\textsuperscript{2+} sensitization of middle cerebral artery (MCA) contraction and its signal transduction mechanisms, using simultaneous measurement of \([\text{Ca}^{2+}]_{i}\) and force and the membrane permeabilization in combination with cytotoxic application of a pseudosubstrate peptide for PKCs and a dominant-negative form of Rho-kinase.

**Materials and Methods**

Ca\textsuperscript{2+} sensitization of the bovine MCA, which we obtained from a local slaughterhouse\textsuperscript{24} (Ube Shokumiku Center, Ube, Japan), was examined using simultaneous measurement of \([\text{Ca}^{2+}]_{i}\), and force in intact MCA loaded with fura-2,\textsuperscript{19} and membrane permeabilization with \(\alpha\)-toxin\textsuperscript{25} and \(\beta\)-escin.\textsuperscript{26} The roles of Rho-kinase and PKCs were assessed using a dominant-negative form of Rho-kinase, GST-RB/PH(TT)\textsuperscript{27} and a PKC\textsubscript{19-31} pseudosubstrate inhibitor,\textsuperscript{28} respectively, which were added to the cytosol of \(\beta\)-escin–permeabilized MCAs. The expression and translocation of Rho-kinase were investigated using Western blots in bovine MCA and rat aorta and confocal immunofluorescence image processing analysis in vascular smooth muscle cells (VSMCs) in primary culture, respectively.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

**Results**

**SPC-Induced Ca\textsuperscript{2+}-Independent Contraction in Intact VSM**

In the simultaneous measurement of \([\text{Ca}^{2+}]_{i}\), and force, SPC (30 \(\mu\text{mol/L}\)) induced a gradual increase in tension, which reached a maximum plateau level (105.8±33.6\%, \(n=4\)) within 30 minutes, with no change in the \([\text{Ca}^{2+}]_{i}\) (0.0±0.0\%, \(n=4\); Figure 1A). This Ca\textsuperscript{2+}-independent contraction continued for at least 2 hours and did not decrease even after replacing the buffer with normal PSS (data not shown).

The contraction induced by SPC (30 \(\mu\text{mol/L}\)) was reduced from 95.3±14.4\% (\(n=12\)) to 0.8±1.6\% by Y-27632 (2 \(\mu\text{mol/L}\)) (\(n=4\); \(P<0.01\); Figure 1), thereby indicating complete inhibition of the SPC-induced contraction by Y-27632. In contrast, Y-27632 (2 \(\mu\text{mol/L}\)) had no apparent effect on the 118 mmol/L \(K^{+}\)-PSS–induced \([\text{Ca}^{2+}]_{i}\)-elevation and contraction, which is widely regarded as a typical Ca\textsuperscript{2+}-dependent contraction (\(n=8\); \(P>0.05\); Figure 1A). This finding, taken together with the contractile response to a quick release during SPC-induced contraction, indicates that the irreversible contraction is physiological (see online data supplement for details).

The SPC-induced contraction was concentration-dependent with an \(EC_{50}\) of 3.0 \(\mu\text{mol/L}\) (Figure 1B). The maximum response was obtained at 30 \(\mu\text{mol/L}\) (95.3±14.4\%, \(n=12\); Figure 1B).
Figure 2. Differential requirement of GTP for the contractile response induced by SPC and U-46619 at constant Ca\(^{2+}\) (pCa 6.3, buffered with 10 mmol/L EGTA) in the 6-toxin-permeabilized bovine MCA. Sequential experiments were done using the same vascular strip. U-46619 (30 nmol/L) plus GTP (10 \(\mu\)mol/L) was applied in the presence of GDP\(\alpha\)S (1 mmol/L), and subsequently, GTP (10 \(\mu\)mol/L) was applied in the presence of U-46619 (30 nmol/L). Then, SPC (30 \(\mu\)mol/L) was applied in the absence of GTP to the same strip of VSM. GTP alone (without an agonist for a receptor) had no apparent contractile effect on the force.

SPC-Induced Ca\(^{2+}\) Sensitization in Membrane-Permeabilized MCA

Using 6-toxin-permeabilized MCA, the SPC-induced Ca\(^{2+}\) sensitization and the involvement of G protein were examined (Figure 2). Because molecules with a molecular mass of less than 1000 can penetrate the membrane in 6-toxin-permeabilized strips,\(^{25}\) [Ca\(^{2+}\)]\(_i\), can be controlled at a constant value with a highly buffering effect of 10 mmol/L EGTA. In constant [Ca\(^{2+}\)]\(_i\) (pCa 6.3), a G protein–coupled receptor agonist, U-46619 (30 nmol/L), required the presence of GTP to induce contraction and this was abolished by GDP\(\alpha\)S (1 mmol/L), thus indicating an essential role of G protein activation in the U-46619–induced contraction. In contrast, in the same strip, 30 \(\mu\)mol/L SPC induced Ca\(^{2+}\) sensitization at pCa 6.3, even in the absence of added GTP (Figure 2). GTP was also not required for the SPC-induced Ca\(^{2+}\) sensitization in –escin–permeabilized MCA (Figure 3). Neither GDP\(\alpha\)S nor GTP modified the Ca\(^{2+}\) sensitization induced by SPC.

These results indicate involvement of the G protein–independent pathway in the Ca\(^{2+}\) sensitization induced by SPC, although direct measurements of nucleotides and their kinetics in the permeabilized MCA are required for confirmation.

Effects of PKC\(\alpha\) 19-31, Y-27632, and GST-RB/PH (TT) on SPC-Induced Ca\(^{2+}\) Sensitization in -escin–Permeabilized MCA

To investigate the signal transduction mechanism(s) involved in the SPC-induced Ca\(^{2+}\) sensitization, we used MCA strips permeabilized with –escin. Because permeabilization with –escin allows higher molecular mass compounds (up to 150 kDa) to penetrate the cell membrane, large molecules such as recombinant proteins can be introduced into the cytosol of the –escin–permeabilized smooth muscle.\(^{26,29}\) At constant [Ca\(^{2+}\)]\(_i\), (pCa 6.3), 30 \(\mu\)mol/L SPC elicited a significant additional 64.4\% increase in the force (=Ca\(^{2+}\) sensitization; n = 8; \(P<0.01\); Figures 3A and 5B) even in the absence of GTP, which is required for activation of G proteins coupled to membrane receptors. This 30 \(\mu\)mol/L SPC-induced Ca\(^{2+}\)-independent contraction was abolished by a Rho-kinase inhibitor (2 \(\mu\)mol/L Y-27632) (\(P<0.05\); Figures 3C and 5B) and by a dominant-negative form of Rho-kinase (0.5 \(\mu\)g/mL GST-RB/PH (TT)) (\(P<0.05\); Figures 3D and 5B), whereas a PKC pseudosubstrate, PKC\(\alpha\) 19-31, had no effect on SPC-induced Ca\(^{2+}\) sensitization (Figures 3B and 5B). In contrast, the force at pCa 6.3 (=Ca\(^{2+}\)-induced contraction) was not affected by 2 \(\mu\)mol/L Y-27632 or by 0.5 \(\mu\)g/mL GST-RB/PH (TT) (Figure 5A). These results strongly suggest the requirement of Rho-kinase, but not of G proteins and PKCs, for SPC-induced Ca\(^{2+}\) sensitization.

Effects of PKC\(\alpha\) 19-31, Y-27632, and GST-RB/PH (TT) on Phorbol 12,13-Dibutyrate (PDBu)–Induced Ca\(^{2+}\) Sensitization in -escin–Permeabilized MCA

In –escin–permeabilized smooth muscle and at constant [Ca\(^{2+}\)]\(_i\) (pCa 6.3), 0.03 \(\mu\)mol/L PDBu elicited a significant additional 84.8\% increase in the force (=Ca\(^{2+}\) sensiti-
zation; n=3; P<0.01; Figures 4A and 5C). A pseudosubstrate peptide for conventional and novel PKCs (50 μmol/L PKCa 19-31) abolished the Ca2+ sensitization induced by 0.03 μmol/L PDBu (n=4; P<0.05; Figures 4B and 5C) without affecting the force at pCa 6.3 (Ca2+-induced contraction) (P>0.05, n=4; Figure 5A). In contrast, the 0.03 μmol/L PDBu-induced Ca2+ sensitization of force was not affected by preincubation with 2 μmol/L Y-27632 and 0.5 μg/mL GST-RB/PH (TT) (Figures 4C, 4D, and 5C), both of which abolished the SPC-induced Ca2+ sensitization presumably mediated by Rho-kinase. These results strongly suggest that Rho-kinase is not required for the Ca2+ sensitization mediated by phorbol ester–sensitive PKCs, which are selectively inhibited by their pseudosubstrate peptide, PKCa 19-31.

**Immunoblot Analysis and Subcellular Localization of Rho-Kinase**

Because translocation of Rho-kinase from the cytosol to the surface membrane plays an important role in activation of the enzyme, we asked if SPC would also induce translocation of Rho-kinase in VSMCs. As shown in Figure 6A, the antibody against Rho-kinase (ROKα) recognized a single protein with an approximate molecular mass of 180 kDa, as determined by immunoblotting bovine MCA and rat aorta, and thereby indicating specificity of the antibody for Rho-kinase. Using the same antibody to immuno localize ROKα, the ROKα label intensities in the cytosol (C) and surface membrane (M) were digitized, and the ratio of the membrane signal to the cytosol signal (M/C) was calculated (Figures 6B and 6C). In the control cells (before SPC stimulation), homogeneous distribution of Rho-kinase in the cytosol, but not in the nucleus, was observed. Treatment of rat aortic VSMCs in primary culture with SPC (30 μmol/L, 30 minutes) led to translocation of cytosolic ROKα to the free edge of the cells and thereby induced a marked increase in M/C (P<0.01; Figures 6B and 6C). The M/C increased gradually with a time course compatible with slow Ca2+ sensitization induced by SPC: 1.45±0.28 (n=12) at 20 minutes and 2.08±0.41 (n=9) at 30 minutes. Gong et al also noted translocation of Rho-kinase to the membrane in portal vein smooth muscle corresponding to Ca2+ sensitization. The nucleus was negatively stained. No specific fluorescence was observed when only the secondary antibody (without an anti-ROKα antibody) was used for immunostaining. In addition, increase in the M/C ratio induced by SPC was reduced by preincubation (30 minutes) with Y-27632.

![Figure 4. Effects of PKCa 19-31, Y-27632, and GST-RB/PH (TT) on PDBu-induced Ca2+ sensitization of β-escin-permeabilized bovine MCA at constant Ca2+ (pCa 6.3, buffered with 10 mmol/L EGTA). PDBu (0.03 μmol/L) was applied in the absence (A) and presence of several blockers (B through D): 50 μmol/L PKCa 19-31 (B), 2 μmol/L Y-27632 (C), and 0.5 μg/mL GST-RB/PH (TT) (D). At the end of each experiment, a maximal contraction induced by pCa 4.5 was observed for normalization in the data analysis (see Figure 5).](Image)

![Figure 5. Summary of experiments shown in Figures 3 and 4. A, Effects of PKCa 19-31 (50 μmol/L), Y-27632 (2 μmol/L), and GST-RB/PH (TT) (0.5 μg/mL) on the force level at pCa 6.3 (Control) (n=4 to 9). B, Effects of PKCa 19-31 (100 μmol/L), Y-27632 (2 μmol/L), and GST-RB/PH (TT) (0.5 μg/mL) on the Ca2+-independent contraction induced by 0.03 μmol/L PDBu. Control indicates the force level at pCa 6.3 (n=5 to 7). C, Effects of PKCa 19-31 (50 μmol/L), Y-27632 (2 μmol/L), and GST-RB/PH (TT) (0.5 μg/mL) on the Ca2+-independent contraction induced by 0.03 μmol/L PDBu. Control indicates the force level at pCa 6.3 (n=3 to 4). Tension levels were expressed as a percentage, assigning the values in normal relaxing (pCa>8, nominally zero Ca2+ with 10 mmol/L EGTA) and activating (pCa 4.5, buffered with 10 mmol/L EGTA) solutions to be 0% and 100%, respectively. Data are expressed as the mean±SD. *P<0.05.](Image)
Effects of PKC Inhibitors on the SPC-Induced Contraction in Intact VSM

The effect of chelerythrine chloride, a nonselective PKC inhibitor, and bisindolylmaleimide 1, which is a relatively selective inhibitor of conventional PKC (cPKC) and novel PKC (nPKC) isoforms, on the PDBu-induced contraction were examined using intact VSM strips. PDBu developed a concentration-dependent contraction, ranging from 0.0001 to 1\textsuperscript{H}mol/L, and the maximum response was obtained at 0.03\textsuperscript{H}mol/L (150.8\textsuperscript{H}21.3\%, n=10, data not shown). Chelerythrine chloride (10\textsuperscript{H}mol/L) antagonized the PDBu-induced contraction, with a rightward shift of the concentration-response curve (data not shown), and the maximal tension was inhibited from 150.8\textsuperscript{H}21.3\% (n=10) to 35.6\textsuperscript{H}12.1\% (n=5; \(P<0.01\); Figure 7D). Bisindolylmaleimide 1 (1\textsuperscript{H}mol/L) also inhibited the PDBu-induced contraction more potently than did chelerythrine chloride, and the maximal tension was inhibited from 150.8\textsuperscript{H}21.3\% (n=10) to 5.5\textsuperscript{H}4.0\% (n=5; \(P<0.01\); Figure 7D).

The SPC (30\textsuperscript{H}mol/L)-induced contraction was markedly inhibited by a nonselective PKC inhibitor, chelerythrine chloride (10\textsuperscript{H}mol/L) (Figure 7), and was blocked only slightly by a relatively selective blocker of cPKC and nPKC, bisindolylmaleimide 1 (1\textsuperscript{H}mol/L) (Figure 7). Bisindolylmaleimide 1 (1\textsuperscript{H}mol/L) also inhibited the 118 mmol/L K\textsuperscript{+} depolarization contraction from 100\% to 85.85\textsuperscript{H}6.66\% (n=6; \(P<0.01\); Figure 7D).

Figure 6. Immunoblot detection of Rho-kinase and translocation of Rho-kinase in VSMCs in primary cultures of the rat aorta. A, Lane a, Positive control (RSV-3T3 lysate, prepared from a mouse fibroblast cell line). Lanes b through d, Homogenates of the bovine MCA smooth muscles (10 \(\mu\)g of protein) (b), bovine brain (9 \(\mu\)g of protein) (c), and VSMCs in primary culture of the rat aorta (10 \(\mu\)g of protein) (d), respectively. Note that Rho-kinase was recognized to be a single protein with an approximate molecular mass of 180 kDa. B, Rat VSMCs (Control; B-1) were stimulated with SPC at 30 \(\mu\)mol/L for 30 minutes at 37\textdegree C (B-2). Cells were fixed with paraformaldehyde and stained with anti-ROK\(\alpha\) antibody. In B-2, the SPC-induced contraction of cultured VSMCs results in the cell shape change and irregular outline of the cells. C, Changes in the ratio of the membrane signal to the cytosol signal (M/C). ROK\(\alpha\) label intensities were digitized along the white line (a direction was indicated by an arrow). The ratio of the intensities of the surface membrane (M) to the intensities of the cytosol (C), M/C, was calculated (n=9 to 15). Data are expressed as the mean\(\pm\)SD. *\(P<0.01\).

Figure 7. Effects of PKC inhibitors on contractions induced by PDBu (0.03 \(\mu\)mol/L) or SPC (30 \(\mu\)mol/L) in the intact bovine MCA. A, Control contraction induced by 30 \(\mu\)mol/L SPC in the absence of the inhibitors. B and C, 10 \(\mu\)mol/L chelerythrine chloride (B) and 1 \(\mu\)mol/L bisindolylmaleimide 1 (C) were applied 15 minutes before the subsequent application of SPC. D, Summary of the experiments investigating the effects of PKC inhibitors on the PDBu-induced contraction (n=5, left) and on the contractions induced by SPC or 118 mmol/L K\textsuperscript{+} depolarization (n=4 to 12, right). Force was expressed as a percentage, assuming the values in normal (5.9 mmol/L K\textsuperscript{+}) and 118 mmol/L K\textsuperscript{+}-PSS to be 0\% and 100\%, respectively. Data are expressed as the mean\(\pm\)SD. *\(P<0.01\); **\(P<0.05\).
Figure 7D), a finding compatible with the reported nonspecific effect of these PKC inhibitors on the Ca²⁺-induced contraction. This nonspecific effect may be due to the inhibition of a kinase downstream of Rho-kinase.

**Discussion**

The major findings of this study are as follows: (1) SPC, a novel mediator of the Rho-kinase–induced Ca²⁺ sensitization of the cerebral artery, induces a sustained and last-longing Ca²⁺-independent contraction (with no [Ca²⁺], elevation), which is characteristic of vasospasm in the cerebral artery; (2) SPC may not require activation of G proteins to induce Ca²⁺ sensitization and, therefore, may act intracellularly, but not extracellularly, as agonists for G protein–coupled receptors; (3) phorbol ester–sensitive PKCs play only a minor role in SPC-induced Rho-kinase–mediated Ca²⁺ sensitization, and Rho-kinase apparently does not contribute to the Ca²⁺ sensitization induced by phorbol ester–sensitive PKCs (cPKC and nPKC); and (4) translocation of Rho-kinase from the cytosol to the cell membrane may play a role in SPC-induced Ca²⁺ sensitization mechanisms.

In the present study, Ca²⁺ sensitization of the MCA contraction was evaluated directly using 2 techniques: (1) simultaneous measurement of [Ca²⁺], and force in intact MCA loaded with fura-2 and (2) membrane permeabilization with β-escin or α-toxin. In intact MCA, SPC induced a large and sustained contraction without affecting the [Ca²⁺], level (Figure 1), thereby indicating that the SPC–induced contraction does not require activation of the Ca²⁺/CaM-dependent MLCK pathway. This Ca²⁺–independence was confirmed by findings that 30 μmol/L SPC elicited a significant additional increase in force at constant [Ca²⁺] (pCa 6.3, buffered with 10 mmol/L EGTA) in MCA permeabilized with β-escin or α-toxin (Figures 2, 3A, and 5B). Using a Rho-kinase inhibitor (Y-27632) and a dominant-negative Rho-kinase, we found that the SPC–induced contraction of the cerebral artery in the absence of a change in [Ca²⁺], is mediated by Rho-kinase (Figures 1 and 3). This also supports the Ca²⁺-sensitizing effect of SPC, because we earlier found that cytosolic application of constitutively active Rho-kinase induces maximal contraction even with complete removal of cytosolic Ca²⁺ with 10 mmol/L EGTA in membrane-permeabilized VSM, which clearly indicates that activation of Rho-kinase induces a Ca²⁺–independent contraction of VSM. We earlier reported the involvement of Rho-kinase in the SPC–induced Ca²⁺ sensitization in the pig coronary artery, although we used only a Rho-kinase inhibitor and not a dominant-negative Rho-kinase, and PKC involvement was not investigated in that study.

Although Y-27632 is widely used as a relatively specific inhibitor of Rho-kinase, the possibility that the observed inhibitory effect of Y-27632 on the SPC–induced Ca²⁺ sensitization (Figures 1 and 3) may be due to inhibition of other unknown protein kinases sensitive to Y-27632, but not of Rho-kinase, needs to be ruled out. Therefore, in the present study, in order to specifically inhibit Rho-kinase, we examined the effect of a dominant-negative Rho-kinase, GST-RB/PH (TT), on the SPC–induced contraction in the MCA permeabilized with β-escin, which allows for large molecules (up to 150 kDa) to enter the cytosol of VSM. SPC (30 μmol/L)–induced Ca²⁺ sensitization was abolished by GST-RB/PH (TT) (0.5 μg/mL), thus providing direct evidence that SPC–induced Ca²⁺ sensitization is mediated by Rho-kinase (Figures 3D and 5B). Such specific effects of GST-RB/PH (TT) on Rho-kinase–mediated Ca²⁺ sensitization were supported by findings that GST-RB/PH (TT) (up to 1 μg/mL) had no effect on Ca²⁺ (pCa 6.3)–induced contraction (Figure 3D) and on the Ca²⁺ sensitization induced by the PKC activator, phorbol ester (Figures 4D and 5C), in membrane-permeabilized vascular strips.

It was reported that sphingolipids, including SPC, are ligands for G protein–coupled membrane receptors, AGR 163 and ovarian cancer G protein–coupled receptor 1 (OGR1). Activation of these sphingolipid receptors induces Ca²⁺ mobilization. However, the present study showed that SPC did induce contraction of bovine MCA with no change in the [Ca²⁺], (Figure 1). In addition, we also found that SPC–induced Ca²⁺ sensitization was not affected by GTP and GDPβS, which respectively activates and inactivates G proteins coupled to membrane receptors (including AGR16 and OGR1). Furthermore, the potency of SPC for OGR1 (Kₐ = 33.3 nmol/L) is far different from that noted in the present study (EC₅₀ = 3.0 μmol/L). Taken together, all of these findings strongly support the notion of intracellular actions of SPC as an intracellular messenger. Indeed, exogenously applied SPC is rapidly taken up by intact cells, therefore, the contractile effect of SPC on intact muscle may not necessarily be mediated through the action of SPC on these membrane receptors. It seems highly likely that SPC may act intracellularly, not extracellularly (as agonist for these G protein–coupled membrane receptors). We propose that SPC may be a novel mediator for Ca²⁺ sensitization of cerebral VSM contraction, with a possible signal transduction by Rho-kinase. However, it is also possible that the contractile effect of SPC may be mediated through the action of SPC on yet to be identified membrane receptors not coupled to G protein.

In the present study, the PKCα 19-31 pseudosubstrate had no effect on the Ca²⁺ (pCa 6.3)–induced contraction of β-escin–permeabilized MCA (Figure 5A). In this study, the SPC (30 μmol/L)–induced Ca²⁺ sensitization was not blocked by PKCα 19-31 (100 μmol/L), whereas the PDBu (0.03 μmol/L)–induced Ca²⁺ sensitization was abolished by PKCα 19-31 (50 μmol/L) (Figures 3, 4, and 5), thereby indicating a highly specific effect of PKCα 19-31 on PDBu-induced Ca²⁺ sensitization. In contrast, the SPC (30 μmol/L)–induced Ca²⁺ sensitization was blocked by preincubation with either Y-27632 (2 μmol/L) or GST-RB/PH (TT) (0.5 μg/mL), whereas the PDBu-induced Ca²⁺ sensitization was not affected (Figures 3, 4, and 5). These results suggest that the Ca²⁺ sensitization mediated by phorbol ester–sensitive PKCs plays only a minor role in SPC–induced Rho-kinase–mediated Ca²⁺ sensitization (Figure 8). The notion that PKCs may play a role in Ca²⁺ sensitization was based mainly on the finding that excitatory agonists producing diacyl glycerol (DAG) and exogenous phorbol esters, both of which activate PKCs, also cause Ca²⁺ sensitization. However, the physiological roles of PKCs in Ca²⁺ sensitization have recently been
Rho-kinase-sensitive PKCs (cPKC and nPKC) play only minor roles in the agonists- and G protein-mediated Ca\(^{2+}\) sensitization. Y-27632 and GST-RB/PH (TT) inhibit the activation of cPKC and/or nPKC, which in turn induces Ca\(^{2+}\) sensitization mediated by SPC. Phorbol 12,13-dibutyrate (PDBu) activates cPKC and/or nPKC, which in turn induces Ca\(^{2+}\) sensitization by SPC. PKC \(\alpha\) 19-31 inhibits the activation of cPKC and/or nPKC and thereby abolishes the Ca\(^{2+}\) sensitization induced by PDBu. PKC \(\alpha\) 19-31 has no apparent effect on the SPC-induced Ca\(^{2+}\) sensitization, and Y-27632 and GST-RB/PH (TT) do not affect the PDBu-induced Ca\(^{2+}\) sensitization. Taken together, our results indicate complete separation of the phorbol ester-sensitive PKC (cPKC and nPKC) pathway and the SPC/Rho-kinase pathway in the bovine cerebral artery VSM.

Complete separation of the Ca\(^{2+}\)-sensitizing pathways mediated by SPC/Rho-kinase and phorbol ester–sensitive PKCs was clearly demonstrated by the cytotoxic application of a pseudosubstrate peptide of PKC and a dominant-negative Rho-kinase to the membrane-permeabilized MCA (Figures 3 through 5 and 8). Although membrane permeabilized techniques are useful tools for investigations of Ca\(^{2+}\) sensitization and its signal transduction, membrane permeabilization is not a physiological condition. Therefore, we also examined this point using intact muscles, although use of less specific pharmacological agents was needed. In the present study, a selectively inhibitory of cPKC and nPKC isoforms, bisindolylmaleimide 1 (1 \(\mu\)mol/L), abolished the PDBu-induced contraction, whereas at the same concentration, it had a minimal inhibitory effect on the SPC-induced contraction (Figure 7). In intact bovine MCA, chelerythrine chloride, a nonspecific PKC inhibitor, antagonized both PDBu- and SPC-induced contractions (Figure 7). The differential effects of bisindolylmaleimide 1 and chelerythrine chloride are compatible with our finding that cPKC and nPKC, which is blocked by the PKC 19-31 pseudosubstrate and is more sensitive to bisindolylmaleimide 1, may not contribute to the Ca\(^{2+}\) sensitization induced by SPC and Rho-kinase. However, our results do not rule out the possible involvement of aPKC, which is much less sensitive to bisindolylmaleimide 1 than is chelerythrine chloride, in Ca\(^{2+}\) sensitization mediated by a SPC/Rho-kinase pathway. This notion is also supported by data that aPKC, but not cPKC and nPKC, may play an important role in the Ca\(^{2+}\) sensitization induced by agonists.

SPC is produced by N-deacylation of sphingomyelin, one of the most abundant lipids in cell membrane. In addition, it is likely, but not proven, that membrane lipids, including SPC, are released into the perivascular subarachnoid space in the process of degradation of RBC, WBC, and platelets, as induced by SAH. If SAH induces increasing amounts of SPC into the cerebrospinal fluid, long term narrowing of the cerebral artery can occur through the vasospasm induced by an SPC/Rho-kinase pathway. We propose that SPC is a novel mediator for the pathological Ca\(^{2+}\)-independent contraction of the cerebral artery (=cerebral vasospasm) as mediated by Rho-kinase, but not by conventional and novel PKCs. A very recent in vivo study demonstrated that Rho-kinase, but not PKC, activity contributes to cerebral vascular tone. Therefore, taken together with this finding, our results in the present study support the new concept that the Ca\(^{2+}\) sensitization induced by Rho-kinase, but not by PKC, may play a major role also in the maintenance of cerebral artery tone under normal conditions. To examine the physiological roles of SPC, measurements of SPC concentrations in human cerebrospinal fluid are currently under investigation.


Sphingosylphosphorylcholine Is a Novel Messenger for Rho-Kinase–Mediated \( \text{Ca}^{2+} \) Sensitization in the Bovine Cerebral Artery: Unimportant Role for Protein Kinase C

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