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).\(^1\) 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),\(^2\) 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).\(^3\) Excitatory agonists, including spasmogenic 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,\(^4\) 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\(^5,6\) and clinical trials\(^7,8\) 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,\(^8,9\) protein kinase C (PKC),\(^10,11\) and arachidonic acid pathways,\(^12\) have been linked to Ca\(^{2+}\) sensitization mechanisms.

Sato et al\(^11\) 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, prevents the development of cerebral vasospasm in twohemorrhage canine models and in humans. 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\(^{2+}\) sensitization of cerebral arteries has remained to be determined. Because translocation and membrane binding is required for activation of Rho-kinase, 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). 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. The slowly developing and sustained nature of the SPC-induced contraction mediated by Rho-kinase 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\(^{2+}\) sensitization of cerebral arterial smooth muscle contraction as an upstream initiator of the Rho-kinase pathway. Because PKCs may play a role in Ca\(^{2+}\) sensitization, 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\(^{2+}\) sensitization of middle cerebral artery (MCA) contraction and its signal transduction mechanisms, using simultaneous measurement of [Ca\(^{2+}\)]\(_i\) and force, and membrane permeabilization in combination with cytosolic application of a pseudosubstrate peptide for PKCs and a dominant-negative form of Rho-kinase.

**Materials and Methods**

Ca\(^{2+}\) sensitization of the bovine MCA, which we obtained from a local slaughterhouse (Ube Shokumiku Center, Ube, Japan), was examined using simultaneous measurement of [Ca\(^{2+}\)]\(_i\), and force in intact MCA loaded with fura-2, and membrane permeabilization with α-toxin and β-escin. The roles of Rho-kinase and PKCs were assessed using a dominant-negative form of Rho-kinase, GST-RB/PH(TT) and a PKC19-31 pseudosubstrate inhibitor, respectively, which were added to the cytosol of β-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\(^{2+}\) Independent Contraction in Intact VSMC**

In the simultaneous measurement of [Ca\(^{2+}\)]\(_i\), and force, SPC (30 μmol/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 [Ca\(^{2+}\)]\(_i\) (0.0±0.0%, n=4; Figure 1A). This Ca\(^{2+}\)-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 μmol/L) was reduced from 95.3±14.4% (n=12) to 0.8±1.6% by Y-27632 (2 μmol/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 μmol/L) had no apparent effect on the 118 mmol/L K\(^+\)-PSS–induced Ca\(^{2+}\) elevation and contraction, which is widely regarded as a typical Ca\(^{2+}\)-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 μmol/L (Figure 1B). The maximum response was obtained at 30 μmol/L (95.3±14.4%, n=12; Figure 1B).
SPC-Induced Ca^{2+} Sensitization in Membrane-Permeabilized MCA With No Requirement for G Protein Activation

Using α-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 α-toxin–permeabilized strips,^{25} [Ca^{2+}], can be controlled at a constant value with a highly buffering effect of 10 mmol/L EGTA. In constant [Ca^{2+}], (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/βS (1 mmol/L), and subsequently, GTP (10 μmol/L) was applied in the presence of U-46619 (30 nmol/L). Then, SPC (30 μmol/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.

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

To investigate the signal transduction mechanism(s) involved in the SPC-induced Ca^{2+} sensitization, we used MCA strips permeabilized with β-escin. Because permeabilization with β-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 β-escin–permeabilized smooth muscle.^{26,29} At constant [Ca^{2+}], (pCa 6.3), 30 μmol/L SPC elicited a significant additional 64.4±7.3% 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 μmol/L SPC-induced Ca^{2+}-independent contraction was abolished by a Rho-kinase inhibitor (2 μmol/L Y-27632) (P<0.05; Figures 3C and 5B) and by a dominant-negative form of Rho-kinase (0.5 μg/ml GST-RB/PH (TT)) (P<0.05; Figures 3D and 5B), whereas a PKC pseudosubstrate, PKCα 19-31, had no effect on SPC-induced Ca^{2+} sensitization (Figures 3D and 5B). In contrast, the force at pCa 6.3 (=Ca^{2+}-induced contraction) was not affected by 2 μmol/L Y-27632 or by 0.5 μg/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 PKCα 19-31, Y-27632, and GST-RB/PH (TT) on Phorbol 12,13-Dibutyrate (PDBu)–Induced Ca^{2+} Sensitization in β-Escin–Permeabilized MCA

In β-escin–permeabilized smooth muscle and at constant [Ca^{2+}], (pCa 6.3), 0.03 μmol/L PDBu elicited a significant additional 84.8±3.4% increase in the force (=Ca^{2+} sensit-
abolished the SPC-induced Ca\(^{2+}\) sensitization (Figures 4C, 4D, and 5C), both of which against Rho-kinase (ROK)

Rho-kinase in VSMCs. As shown in Figure 6A, the antibody

PDBu (0.03 \(\mu\)mol/L) was applied in the absence (A) and presence of several blockers (B through D): 50 \(\mu\)mol/L PKCa 19-31 (B), 2 \(\mu\)mol/L Y-27632 (C), and 0.5 \(\mu\)g/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).

Effects of PKC

Summary of experiments shown in Figures 3 and 4. A, Effects of PKCa 19-31 (50 \(\mu\)mol/L), Y-27632 (2 \(\mu\)mol/L), and GST-RB/PH (TT) (0.5 \(\mu\)g/mL) on the force level at pCa 6.3 (Control) (n=4 to 9). B, Effects of PKCa 19-31 (100 \(\mu\)mol/L), Y-27632 (2 \(\mu\)mol/L), and GST-RB/PH (TT) (0.5 \(\mu\)g/mL) on the Ca\(^{2+}\)-independent contraction induced by 0.03 \(\mu\)mol/L PDBu. Control indicates the force level at pCa 6.3 (n=5 to 7). C, Effects of PKCa 19-31 (50 \(\mu\)mol/L), Y-27632 (2 \(\mu\)mol/L), and GST-RB/PH (TT) (0.5 \(\mu\)g/mL) on the Ca\(^{2+}\)-independent contraction induced by 0.03 \(\mu\)mol/L PDBu. Control indicates the force level at pCa 6.3 (n=5 to 7). Tension levels were expressed as a percentage, assigning the values in normal relaxing (pCa>8, nominally zero Ca\(^{2+}\) 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.

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,\(^{18}\) we asked if SPC would also induce translocation of Rho-kinase in VSMCs. As shown in Figure 6A, the antibody against Rho-kinase (ROK\(\alpha\)) 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 immunolocalize ROK\(\alpha\), the ROK\(\alpha\) 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 \(\mu\)mol/L, 30 minutes) led to translocation of cytosolic ROK\(\alpha\) 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 Ca\(^{2+}\) 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\(^{30}\) also noted translocation of Rho-kinase to the membrane in portal vein smooth muscle corresponding to Ca\(^{2+}\) sensitization. The nucleus was negatively stained. No specific fluorescence was observed when only the secondary antibody (without an anti-ROK\(\alpha\) 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.
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 1/1000 mol/L, and the maximum response was obtained at 0.03/1000 mol/L (150.8±21.3%, n=10, data not shown). Chelerythrine chloride (10 μmol/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±21.3% (n=10) to 35.6±4.0% (n=5; P<0.01; Figure 7D). Bisindolylmaleimide 1 (1 μmol/L) also inhibited the PDBu-induced contraction more potently than did chelerythrine chloride, and the maximal tension was inhibited from 150.8±21.3% (n=10) to 5.5±4.0% (n=5; P<0.01; Figure 7D).

The SPC (30 μmol/L)-induced contraction was markedly inhibited by a nonselective PKC inhibitor, chelerythrine chloride (10 μmol/L) (Figure 7), and was blocked only slightly by a relatively selective blocker of cPKC and nPKC, bisindolylmaleimide 1 (1 μmol/L) (Figure 7). Bisindolylmaleimide 1 (1 μmol/L) also inhibited the 118 mmol/L K⁺ depolarization contraction from 100% to 85.8±6.6% (n=10; Figure 7D).
findings that 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, AGR1633 and ovarian cancer G protein–coupled receptor 1 (OGR1).34 Activation of these sphingolipid receptors induces Ca²⁺ mobilization.33,34 However, the present study showed that SPC did induce contraction of bovine MCA with no change in [Ca²⁺]i (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 (Kᵢ=33.3 nmol/L) is far different from that noted in the present study (ECₑ₀=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 cells35; 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 PKCαs 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.12,36 However, the physiological roles of PKCs in Ca²⁺ sensitization have recently been
mediated Ca\(^{2+}\) sensitization, mediator for the pathological Ca\(^{2+}\) sensitization. Y-27632 and GST-RB/PH (TT) inhibit Rho-kinase to the membrane-permeabilized MCA (Figures 3–8). Although membrane permeabilized technology, Sports, and Culture, Japan. M. Ohara (Fukuoka) provided pharmacological agents was needed. In the present study, a nonselective PKC inhibitor, antagonized both PDBu-induced contraction (Figure 7). In intact bovine MCA, chelerythrine chloride, although use of less specific pharmacological agents was needed. In the present study, a nonselective PKC inhibitor, antagonized both PDBu-induced contraction (Figure 7). In intact bovine MCA, chelerythrine chloride, although use of less specific pharmacological agents was needed. In the present study, a nonselective PKC inhibitor, antagonized both PDBu-induced contraction (Figure 7). In intact bovine MCA, chelerythrine chloride, although use of less specific pharmacological agents was needed. In the present study, a nonselective PKC inhibitor, antagonized both PDBu-induced contraction (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.23

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.40 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.41 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.

Supporting data and expanded text can be found in the online data supplement available at http://www.circresaha.org.

Acknowledgments

Y-27632 was a generous gift from Welfide Corporation (Osaka, Japan). This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports, and Culture, Japan. M. Ohara (Fukuoka) provided language assistance.

References

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Circ Res. 2002;91:112-119; originally published online June 13, 2002; doi: 10.1161/01.RES.0000026057.13161.42

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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