Functional Roles of the Rho/Rho Kinase Pathway and Protein Kinase C in the Regulation of Cerebrovascular Constriction Mediated by Hemoglobin

Relevance to Subarachnoid Hemorrhage and Vasospasm

Grant Wickman, Christopher Lan, Bozena Vollrath

Abstract—Although there is evidence that the Rho/Rho kinase pathway and protein kinase C (PKC) are involved in the development of cerebral vasospasm, the mechanism by which subarachnoid hemorrhage (SAH) activates these pathways is unclear. A large body of evidence points to oxyhemoglobin (OxyHb) as a major causative component of blood clot responsible for vasospasm. Therefore, the present studies were conducted to explore whether the Rho/Rho kinase and PKC may be involved in a sustained vasoconstriction induced by OxyHb in cerebral arteries. OxyHb evoked sustained vasoconstriction in the endothelium-denuded rabbit basilar arteries, which was reversed by the selective inhibitors of Rho kinase, Y-27632, and HA-1077, with the IC50 values of 0.26±0.02 and 0.74±0.1 μmol/L, respectively. In quiescent cerebrovascular smooth muscle (CVSM) cells, OxyHb induced Rho translocation, as assessed by immunoblotting, with a time course, which paralleled the contractile action of OxyHb. Rho translocation was also observed in intact arteries stimulated with OxyHb for 24 hours (219%) and 48 hours (160%). The increase in Rho translocation was fully inhibited by GGTI-297, an inhibitor of Rho prenylation. OxyHb also caused significant translocation of both PKα and PKε (P<0.01), which was maximal at the time corresponding to maximal tension developed in response to OxyHb. Ro-32-0432, an inhibitor of PKC, attenuated vasoconstriction mediated by OxyHb in basilar artery. These results show, for the first time, that OxyHb-mediated signaling in CVSM utilizes the Rho/Rho kinase and PKC-based mechanisms.

Key Words: hemoglobin ■ vasospasm ■ smooth muscle ■ Rho kinase ■ protein kinase C

Cerebral vasospasm is a serious complication of subarachnoid hemorrhage that frequently arises from rupture of a cerebral aneurysm, but the pathogenesis of this condition is still far from clear.1 A large body of evidence points to oxyhemoglobin (OxyHb), the ferrous form of hemoglobin, released from lysed erythrocytes, as a putative mediator of vasospasm.2 OxyHb causes prolonged contraction of isolated cerebral arteries,3 and intracisternal injections of this agent result in cerebral vasospasm.4 Furthermore, the presence of OxyHb in the cerebrospinal fluid (CSF) of patients after SAH and the extent of hemorrhage are correlated with the distribution, severity, and time course of vasospasm.2,5 However, the molecular mechanisms by which this compound evokes sustained vasoconstriction are still not clear. There is evidence that vasospasm starts as smooth muscle contraction, which is sustained for a long period of time.6,7 The initial phase of contraction is mediated by a rise in intracellular calcium and results in calmodulin-dependent activation of myosin light chain kinase (MLCK).8 Phosphorylation of myosin light chain (MLC) by MLCK leads to interaction between actin and phosphorylated MLC, activation of myosin ATPase, and subsequently to smooth muscle contraction.8,9 The sustained phase of vascular contraction is thought to involve the Ca2+ sensitization mechanisms,9 and it is presumably this phase that initiates clinical vasospasm.10 The major mechanism of Ca2+ sensitization of contraction is through inhibition of the smooth muscle myosin light chain phosphatase (MLCP), resulting in increased MLC phosphorylation and smooth muscle contraction at a constant intracellular calcium level.9,11 One of the hypotheses proposed for Ca2+ sensitization in vascular smooth muscle involves PKC, a group of enzymes that are broadly engaged in vital cellular functions, including smooth muscle contraction.12 The concept of a role for PKC in cerebral vasospasm is supported by the observations that phorbol esters, potent activators of PKC, induce angiographic vasospasm,13 and that the PKC inhibitors improve the outcome in experimental models of vasospasm.14 Whether PKC activation may initiate vasospasm is unclear, but an important step may be the phosphorylation of CPI-17 (PKC-potentiated phosphatase inhibitory protein of...
by guest on October 16, 2017

17 kDa), an inhibitor protein involved in the regulation of MLCP.12,15,16 Phosphorylation of CPI-17 by PKC inhibits MLCP activity and subsequently maintains the vasoconstriction by sustained phosphorylation of MLC.16 Although there is considerable evidence in support of a role for PKC, it is now recognized that the monomeric G protein Rho and its downstream target Rho-kinase can participate in sustained vasoconstriction by phosphorylating and inhibiting myosin binding subunit of MLCP.9,17 Rho kinase has been proposed to play a role in a variety of vascular smooth muscle disorders including hypertension, coronary and cerebral vasospasm.10,18,19 There is recent evidence that phosphorylation of MLCP increases after experimental hemorrhage and that this increase is associated with concomitant increase in Rho kinase activity.10 Furthermore, HA-1077, the selective inhibitor of Rho kinase, attenuates cerebral vasospasm in experimental animals and humans.19,20 Together, these observations imply that Rho/Rho kinase pathway plays a role in the pathogenesis of cerebral vasospasm. However, to date, there is no information available regarding the mechanism by which SAH activates the Rho/Rho kinase or PKC pathways. Moreover, it remains unexplored whether Rho/Rho kinase pathway plays a role in the OxyHb-mediated cerebral vasoconstriction. Therefore, the goal of the present investigation was to determine the possible involvement of PKC and Rho/Rho kinase in the sustained vasoconstriction mediated by OxyHb in cerebral arteries.

Materials and Methods

Tissue Preparation

All procedures were performed according to protocols approved by the University of Alberta Animal Care Committee. New Zealand rabbits were killed by an intravenous overdose of pentobarbital, and the basilar artery was quickly removed to a dissection dish filled with oxygenated Krebs-Henseleit buffer (KHB). Each artery was cut into sections about 3 mm in length and attached to a force transducer (Grass FT.03) connected to a Grass Polygraph model 7D. Ring preparations were equilibrated for 1 hour under a resting tension of 10 mN. This value was chosen on the basis of prior experiments to permit OxyHb-induced sustained contraction, causing a concentration-dependent relaxation to the basal force with the IC_{50} values of 0.26±0.02 and 0.74±0.135 μmol/L, respectively (Figures 1A through 1D). Y-27632 administered at a single concentration of 1 μmol/L rapidly reversed contraction, thus demonstrating that contractile activity of OxyHb could be abolished by inhibition of Rho kinase (Figure 1B).

Inhibition of Rho Kinase Activity Reverses Cerebral Vasospasm Induced by OxyHb

The concentration of OxyHb in the blood clot after subarachnoid hemorrhage is in the high micromolar range,2,4 thus, a concentration of 10 μmol/L OxyHb was used in our experiments. This treatment caused a slowly developing sustained contraction of the endothelium-denuded ring preparations of rabbit basilar arteries, which reached a plateau at about 20 minutes and was maintained for several hours. To assess the involvement of Rho kinase in the sustained vasoconstriction induced by OxyHb, we examined the effects of two selective inhibitors of Rho kinase, Y-27632 and HA-1077. To mimic the situation in vasospasm, in which therapeutic agents are usually administered after vasospasm had developed, cumulative concentrations of Y-27632 and HA-1077 were administered to preparations, in which a tonic response to OxyHb had developed. Both Y-27632 and HA-1077, potently inhibited OxyHb-induced sustained contraction, causing a concentration-dependent relaxation to the basal force with the IC_{50} values of 0.26±0.02 and 0.74±0.135 μmol/L, respectively (Figures 1A through 1D). Y-27632 administered at a single concentration of 1 μmol/L rapidly reversed contraction, thus demonstrating that contractile activity of OxyHb could be abolished by inhibition of Rho kinase (Figure 1B).

OxyHb Stimulates Membrane Targeting of Rho

Recruitment of Rho to the plasma membrane is crucial for activation of Rho GTPase activity and subsequent activation of Rho kinase. We investigated, therefore, in quiescent CVSM cells, the distribution of Rho mediated by OxyHb (10 μmol/L). In these experiments, cultured CVSM cells were

Cerebrovascular Smooth Muscle Cell Culture

Cells were prepared as previously published.21,22 Briefly, basilar arteries were isolated under sterile conditions and placed in a Petri dish containing Dulbecco’s modified Eagle medium (DMEM). The vessels were cut into segments and the endothelium was removed mechanically. The explants were transferred to culture flasks containing Dulbecco’s modified Eagle medium (DMEM). The dish containing Dulbecco’s modified Eagle medium (DMEM) was modified with oxygenated Krebs-Henseleit buffer (KHB). Each artery was cut into sections about 3 mm in length and attached to a force transducer (Grass FT.03) connected to a Grass Polygraph model 7D. Ring preparations were equilibrated for 1 hour under a resting tension of 10 mN. This value was chosen on the basis of prior experiments to permit OxyHb-induced sustained contraction, causing a concentration-dependent relaxation to the basal force with the IC_{50} values of 0.26±0.02 and 0.74±0.135 μmol/L, respectively (Figures 1A through 1D). Y-27632 administered at a single concentration of 1 μmol/L rapidly reversed contraction, thus demonstrating that contractile activity of OxyHb could be abolished by inhibition of Rho kinase (Figure 1B).

Immunoblotting

PKC isozyme expression and translocation were measured by immunoblot analysis with isofrom-specific antibodies using rat brain (PKCs α, β, γ, δ, ε, λ) and Jurkat cell (PKCθ) lyases as positive controls for antibody specificity (BD Transduction Laboratories). Rho expression was examined using a mouse monoclonal anti-Rho antibody (Santa Cruz Biotechnology). Total cellular proteins were obtained by homogenization of basilar arteries or cultured cells in sample buffer containing the following: (in mmol/L) Tris-HCl (pH 7.5) 50, EDTA 5, EGTA 5, sodium orthovanadate 1; and (in μg/mL) phenylmethylsulphonyl fluoride 50, leupeptin 10, aprotinin 10, and pepstatin 10. Protein-matched samples were electrophoresed by SDS-PAGE, transferred to nitrocellulose membranes, and blocked with nonfat milk. Equal loading was confirmed by inspection of the membranes after reversible Ponceau staining. Blots were then incubated with a primary antibody followed by HRP-labeled goat anti-mouse secondary antibody. The bands were detected on film using an ECL perfection 636 scanner with Epson Twain scanning software. The density of bands was measured using Sigma-Gel software (Jandel Corp).

In the experiments in which Rho and PKC isozyme translocation was measured, the cytosolic and particulate fractions of total cellular contents were separated by centrifugation as described previously.23

Solutions and Materials

KHB contained (in mmol/L) 120 NaCl, 5.9 KCl, 2.5 CaCl_2, 1.2 MgCl_2, 25 NaHCO_3, 1.2 NaH_2PO_4, and 11 dextrose at pH 7.4 when bubbled with 95% O_2+5%CO_2.

The following drugs were used: hemoglobin (Sigma), Ro-32-0432, HA-1077, and GGTI-297 (Calbiochem). Hemoglobin obtained commercially was converted before use to OxyHb (about 96% of OxyHb) as previously described.21 Y-27632 was generously provided by the Welfide Corporation (Osaka, Japan). All other reagents were of analytical grade.

Statistics

All values are expressed as mean±SE. The results were analyzed using ANOVA. Differences were considered significant when values were P<0.05.

Results

OxyHb Stimulates Membrane Targeting of Rho

Recruitment of Rho to the plasma membrane is crucial for activation of Rho GTPase activity and subsequent activation of Rho kinase. We investigated, therefore, in quiescent CVSM cells, the distribution of Rho mediated by OxyHb (10 μmol/L). In these experiments, cultured CVSM cells were
exposed to OxyHb for 10, 30, 60 minutes, and 3 hours, and then subjected to SDS-PAGE followed by Western blotting using the monoclonal anti-Rho antibody. Proteins were visualized using a goat anti-mouse antibody conjugated to HRP and a chemiluminescence detection system. Top, Representative Western blot illustrating Rho translocation induced with OxyHb (lines 3 to 10). Position of Rho is indicated on the right and that of molecular weight (M.W.) on the left of the figure. Quantification of intensities of Rho bands was performed by scanning densitometry. Bars represent the time course of Rho translocation expressed as a percentage of respective controls. Results are expressed as mean±SE for 11 experiments. C indicates cytosol; M, membrane fraction. **P<0.01; ***P<0.001.

Figure 2. Time course of Rho translocation stimulated by OxyHb in cultured cerebrovascular smooth muscle (CVSM) cells. Quiescent CVSM cells were stimulated with OxyHb (10 μmol/L) for 10, 30, 60 minutes, and 3 hours. Cytosolic and membrane lysates were resolved by SDS-PAGE and immunoblotted with the monoclonal anti-Rho antibody. Proteins were visualized using a goat anti-mouse antibody conjugated to HRP and a chemiluminescence detection system. Top, Representative Western blot illustrating Rho translocation induced with OxyHb (lines 3 to 10). Position of Rho is indicated on the right and that of molecular weight (M.W.) on the left of the figure. Quantification of intensities of Rho bands was performed by scanning densitometry. Bars represent the time course of Rho translocation expressed as a percentage of respective controls. Results are expressed as mean±SE for 11 experiments. C indicates cytosol; M, membrane fraction. **P<0.01; ***P<0.001.

Figure 3. Rho translocation stimulated by OxyHb in intact basilar artery preparations. Cultured preparations were stimulated with OxyHb (10 μmol/L) for 24 and 48 hours. Cytosolic and membrane lysates were resolved by SDS-PAGE and immunoblotted with the Rho antibody as described in Figure 2. Top, Representative Western blot illustrating Rho translocation. Quantification of intensities of Rho bands was performed by scanning densitometry. Bars represent the Rho translocation expressed as a percentage of respective controls. Results are expressed as mean±SE for 4 experiments. C indicates cytosol; M, membrane fraction. **P<0.01.
PKC Isoform Expression in Cerebrovascular Smooth Muscle in the Absence of Stimuli

Because PKC represents a large family of related isoenzymes, we performed analysis of the PKC isoform expression in basilar artery homogenates using Western blotting techniques with specific antibodies against individual PKC isoforms. Positive controls for all the antibodies were performed with either rat brain lysate (PKCs α, β, γ, δ, ε, and λ) or Jurkat cell lysate (PKCθ). These studies have demonstrated that rabbit basilar artery expresses both the Ca\(^{2+}\)-dependent PKC isoforms (PKC\(\alpha\) and PKC\(\beta\)) as well as the Ca\(^{2+}\)-independent PKC enzymes (PKC\(\epsilon\), PKC\(\theta\), and PKC\(\lambda\)) (Figure 6). In these studies, PKC\(\alpha\) and PKC\(\epsilon\), previously shown to be universally

Figure 4. Effect of GGTL-297 on Rho translocation stimulated by OxyHb in CVSM cells. Quiescent cells were pretreated with GGTL-297 (200 nmol/L) for 24 hours and further stimulated with OxyHb (10 μmol/L) for 60 minutes. Cytosolic and membrane lysates were resolved by SDS-PAGE and immunoblotted with the anti-Rho antibody as described in Figure 2. Top, Representative Western blot showing Rho translocation in the presence and absence of GGTL-297. Results are expressed as mean±SE from 3 experiments. C indicates cytosol; M, membrane fraction. *P<0.05 compared with control; ###P<0.001 compared with OxyHb.

Figure 5. Effects of Ro-32-0432 on contraction evoked by OxyHb and phorbol-myristate acetate (PMA) in the rabbit basilar artery rings. Representative traces showing the action of Ro-32-0432 on contraction induced by OxyHb (A) and PMA (B). Ro-32-0432 was administered to the preparations after maximum response to the vasoconstrictors had developed as indicated by arrows. Concentrations of Ro-32-0432 correspond to the values (2×IC\(_{50}\)) for inhibition of PKC\(\alpha\) and PKC\(\beta\) (18 and 56 nmol/L) and PKC\(\epsilon\) (360 nmol/L) isoforms. C, Bar graph of the effects of Ro-32 to 0432 on vasoconstriction induced by OxyHb and PMA. Results are expressed as a percentage of force, which occurred in the maximal sustained responses induced either by OxyHb (10 μmol/L) or PMA (160 nmol/L) and are presented as mean±SE; n=6 to 8 preparations from 3 experiments. *P<0.05; **P<0.01 compared with controls.

that markedly reduced contraction to OxyHb, indicating that PKC was not activated by Rho kinase (results not shown).

**Contractile Action of OxyHb Involves PKC Activation**

To determine if PKC may play a role in sustained vasoconstriction induced by OxyHb, we examined the effect of a bisindolylmaleimide inhibitor of PKC, Ro-32-0432, which exhibits several fold greater selectivity for the classical PKC isoforms over PKC\(\epsilon\). As shown in Figure 5, Ro-32-0432 administered at the concentrations twice as high as the IC\(_{50}\) values for the PKC (18, 56, and 360 nmol/L for PKC\(\alpha\), PKC\(\beta\), and PKC\(\epsilon\), respectively), produced a concentration-dependent attenuation of sustained contraction evoked by 10 μmol/L OxyHb, which was 85±5%, 57±13% (P<0.05), and 10±6% (P<0.01) of control, respectively. Ro-32-0432 at 360 nmol/L was found to significantly block contractions evoked by OxyHb, thus indicating that PKC\(\epsilon\) may play a major role in the OxyHb-mediated sustained vasoconstriction. Representative experiments are shown in Figure 5A, and the results from multiple experiments are shown in Figure 5C. To further examine whether PKC is involved in the sustained phase of contraction, we assessed the effect of Ro-32-0432 on contraction induced by phorbol myristate acetate (PMA) (160 nmol/L), a potent PKC activator. Ro-32-0432 (360 nmol/L) produced significant (P<0.01) relaxation of the PMA-induced contraction, suggesting that PKC\(\epsilon\) was the predominant isoform involved in the effects of PMA (Figure 5B).

The contractile action of PMA was unaltered by either Y-27632 or HA-1077, applied at the range of concentrations
expressed in tissues, were indeed expressed in basilar artery walls. In contrast, PKCγ and PKCδ were undetectable.

**Temporal Profile of PKC Isoforms Translocation Induced by OxyHb in Cerebrovascular Smooth Muscle Cells**

Because activated classical and novel PKCs translocate to the plasma membrane, a process which is a hallmark of activation, the levels of these enzymes were determined in both cytosolic and membrane fractions of CVSM cells. In these experiments, Western blot analyses were performed using CVSM cells exposed to OxyHb for 10 or 60 minutes. As can be seen in Figures 7 and 8, OxyHb induced the membrane association of both PKCα and PKCε compared with untreated controls. Densitometric analysis has shown that stimulation with OxyHb for 10 or 60 minutes resulted in 114±17% and 173±18% (P<0.01) increase in the amount of membrane-associated PKCα, respectively (Figure 7). Similar increases were observed in membrane-bound PKCε isoform (122±9%; P<0.05 and 141±14%; P=0.01, after 10 and 60 minutes of stimulation, respectively) (Figure 8). There were corresponding decreases in the cytosolic fractions after 60 minutes of stimulation (87±4% and 86±8% of control for PKCα and PKCε, respectively), thus confirming that stimulation with OxyHb resulted in spatial translocation of these PKC isoforms.

Discussion

Although a variety of compounds released from subarachnoid blood clot produce vasoconstriction, considerable evidence has implicated OxyHb as a major causative factor responsible for the development of cerebral vasospasm.2,4,5 The results presented here demonstrate, for the first time, that (1) the Rho/Rho kinase pathway is involved in OxyHb-mediated sustained cerebral vasoconstriction, (2) the selective inhibitors of Rho kinase, Y-27632 and HA-1077, reverse sustained vasoconstriction triggered by OxyHb, and (3) PKC isoenzymes, PKCα and PKCε, contribute to sustained vasoconstriction, as assessed by the vasodilatory action of a selective inhibitor of these enzymes and by the PKCs translocation.

The involvement of Rho in the contractile effects of OxyHb is documented in the present studies by the finding that the time course of Rho translocation in CVSM cells closely matches the time course of sustained vasoconstriction induced by OxyHb in cerebral arteries. Further support to this notion is provided by the ability of OxyHb to stimulate chronic translocation of Rho in intact arteries. The Rho translocation, an indicator of this protein activation, has been previously exploited in order to determine activation of Rho in response to agonists acting on G-protein–coupled receptors (GPCR) and was correlated with MLCP inhibition and Ca2+ sensitization of contractile force.26 The present study is the first to show that, although the OxyHb-mediated effects

![Figure 6](Image) Western blot analysis of the PKC isoform expression in the rabbit basilar artery lysates. Five isoforms are shown, representing the 3 main subclasses of PKC enzymes. Protein-matched samples were resolved by SDS-PAGE and immunoblotted with the PKC isoform-specific antibodies as described in Materials and Methods. Rat brain (PKCs α, β, γ, δ, ε, λ) or Jurkat cell (PKC θ) lysates were utilized as positive controls (C+) for antibody specificity. Immunoreactive bands were visualized using a goat anti-mouse IgG and a chemiluminescence detection system. Numbers on the left of the representative experiment is presented for each PKC group.

![Figure 7](Image) Spatial distribution of PKCα, mediated by OxyHb in CVSM cells. Quiescent cells were stimulated with OxyHb (10 μmol/L) for 10 minutes (A) or 60 minutes (B). Proteins from cytosolic and membrane fractions were resolved by SDS-PAGE and immunoblotted with the PKCα-specific antibody. Rat brain lysate was utilized as a positive control (C+) for antibody specificity. Top, Representative Western blots illustrating PKCα translocation induced with OxyHb (lines 3 to 4). Position of PKCα is indicated on the right and that of molecular weight (M.W.) on the left of the figures. Quantification of intensities of PKCα bands was performed by densitometry (arbitrary units). Bars represent the PKC translocation expressed as a percentage of respective controls. Results are expressed as mean±SE from 6 experiments. C indicates cytosolic; M, membrane fraction. **P<0.01 compared with control.
are most likely independent of GPCRs, this compound does stimulate Rho translocation and hence its activation in CVSM cells. Further evidence that Rho is involved in the effects of OxyHb is provided by the observations that Rho translocation mediated by OxyHb could be prevented by GGTI-297, a selective inhibitor of geranylgeranyl-transferase, an enzyme that catalyzes prenylation of Rho proteins. The Rho prenylation, which involves the attachment of geranylgeraniol, an isoprenoid intermediate of the cholesterol biosynthesis pathway, is a key step in the activation of Rho proteins.2,24,27 Inhibition of prenylation by geranylgeranyl transferase inhibitors prevents Rho translocation and hence Ca\(^{2+}\) sensitization mediated by this protein. Thus, our observation that OxyHb-mediated Rho translocation is sensitive to a selective inhibitor of geranylgeranyl transferase, supports the concept that OxyHb-induced sustained vasoconstriction may involve Rho prenylation and activation, a process associated with Ca\(^{2+}\) sensitization of contractile force.

It is now established that an important downstream target of Rho is Rho kinase, which phosphorylates the 130 kDa myosin binding subunit (MBS) of MLCP thereby inhibiting phosphatase activity and promoting an increase in MLCP phosphorylation and smooth muscle contraction.1,2,24 To assess the involvement of Rho kinase in OxyHb-mediated sustained vasoconstriction, we used two selective inhibitors of this kinase, Y-27632 and HA-1077 (fasudil). Both Y-27632 and HA-1077 have been shown to attenuate experimental vasospasm.10,21 Administration of Y-27632 or HA-1077 reversed the OxyHb-induced sustained cerebrovascualar constriction with the IC\(_{50}\) values of 0.26±0.02 and 0.74±0.13 \(\mu\)mol/L, respectively. These IC\(_{50}\) values are similar to previously reported values for agonist-induced contraction and to the \(K_c\) values reported using purified Rho kinase (0.14 and 0.4 \(\mu\)mol/L for Y-27632 and HA-1077, respectively).24 These observations and the high degree of selectivity of both inhibitors, indicate that the kinase involved in the contractile action of OxyHb is indeed Rho kinase. It is important to stress, that the concentration of OxyHb used in our studies to stimulate Rho translocation, as well as initiate sustained cerebral vasoconstriction, is in the range of concentrations of this agent detected in the CSF of patients after SAH.2,24 Therefore, the present study suggests that OxyHb-dependent activation of the Rho/Rho-kinase pathway in cerebrovascular smooth muscle is a step in the signaling cascade underlying the development of cerebrovascular spasm. It has been demonstrated in a variety of blood vessels that Rho kinase inhibitors decrease the MLCP inhibition and subsequent elevation of MLC phosphorylation, mediated by receptor stimulation or the activation of G-proteins, but have no effect on the transient or sustained increase in intracellular calcium.1,24,29 These observations suggest that sustained elevation of calcium and MLCK activation are not sufficient to cause sustained contraction, unless MLCP is simultaneously inhibited. It has previously been shown that, although OxyHb-stimulated contraction is not mediated by G-protein–coupled receptors, this compound can trigger the sustained elevation of intracellular calcium.7,29,30 It therefore seems likely that prolonged elevation of intracellular calcium mediated by OxyHb in CVSM is not sufficient to produce sustained contraction, and that MLCP inhibition is also required. This contention is supported by the observations that, although the intracellular Ca\(^{2+}\) level is elevated in cerebral vasospasm, the vasoconstriction is mediated through the inhibition of MLCP and the Y-27632-sensitive MLC phosphorylation.10

The mechanism by which OxyHb activates the Rho/Rho kinase pathway is unclear. OxyHb was shown to activate a number of signaling processes, including free-radical generation, elevation of intracellular Ca\(^{2+}\), activation of tyrosine kinases and mitogen-activated protein kinases, compatible with a prolonged vascular response.13,22,29,31 Whether free radical generation, or other actions mediated by OxyHb, may play a role in the activation of Rho/Rho-kinase pathway is presently unknown and it remains to be elucidated.

The involvement of PKC in the OxyHb-mediated vasoconstriction is suggested in the present study based on findings with Ro-32-0432, a selective inhibitor of PKC isoforms.24,25,32 Ro-32-0432, at the concentration previously reported to inhibit novel PKC isoforms, nearly completely inhibited the sustained contraction, indicating that this group of PKC enzymes may be involved in the late phase of the OxyHb-induced response. The same concentrations of Ro-32-0432 that reversed the sustained responses to OxyHb also reversed the PMA-dependent vasoconstriction, implying that this agent activated novel PKC isoenzymes. The concentrations of Ro-32-0432 reported to inhibit the classical PKC isoforms were less effective, suggesting that classical PKCs may not be essential for sustained contraction.
PKC activation has been associated with the translocation of PKC isoforms to the plasma membrane. This event is required for the functional PKC enzymes. The translocation of both PKCα and PKCε was observed in CVSM cells at the time corresponding to the maximal sustained contraction, pointing toward possible involvement of these isoforms in OxyHb–mediated contraction. Thus, the effects of OxyHb were quite selective, being restricted to only two isoenzymes of PKC expressed in the rabbit basilar artery. Together, these findings imply that regulation of cerebrovascular constriction by OxyHb involves selective activation of PKCε and, to a lesser extent, PKCα. How PKC may initiate vasoconstriction mediated by OxyHb is unclear, but an important step may be the phosphorylation of CPI-17, a potent repressor protein in the regulation of MLCP. It has been shown that agonists and phorbol esters evoke Ca²⁺ sensitization of smooth muscle contraction through the PKC-dependent phosphorylation of CPI-17, which increases MLC phosphorylation indirectly through the inhibition of MLCP. Furthermore, inhibitors of PKC reduce contraction and CPI-17 phosphorylation caused by GPCR agonists, indicating that CPI-17 functions downstream of PKC. Although there is strong evidence to support a PKC role in Ca²⁺ sensitization, the involvement of PKC has been questioned on the basis of findings that PKC inhibitors are not always effective in attenuation of Ca²⁺ sensitization caused by agonists or GTPγS. However, most of the evidence against a role for PKC is derived from permeabilized preparations from which PKC and CPI-17 may be lost.

The mechanism by which OxyHb stimulates PKC activity is unknown, but may involve the kinase oxidation mediated by the free radicals generated in the process of OxyHb oxidation to methemoglobin. This suggestion is supported by the observations indicating that PKC oxidation may be dependent on the presence of Fe²⁺, a reaction that yields the constitutively active enzyme, due to selective oxidation of the regulatory domain.

The ability of both Y-27632 and Ro-32-0432 to reverse vasoconstriction induced by OxyHb suggests that activation of Rho kinase and PKC may be functionally linked. Indeed, a direct interrelationship between PKC and Rho has been recently proposed by Hippenstiel et al. However, our observations of vasoconstriction induced by PMA was unaffected by Y-27632, together with the previous study of the effects of PMA on the vasodilatation caused by Y-27632, argue against this suggestion. Further studies are required to clarify the potential relationship between Rho and PKC.

Although the precise molecular events leading to activation of the Rho A/Rho kinase pathway and PKC by OxyHb and the relationship between these pathways await further elucidation, the present studies demonstrate, for the first time, that both the Rho/Rho-kinase and PKC pathways contribute to the sustained cerebral vasoconstriction mediated by OxyHb.

In conclusion, results from these studies indicate that OxyHb activates the Rho/Rho kinase pathway in intact CVSM, a phenomenon that has not been previously demonstrated. We further demonstrate that the Rho kinase inhibitors, Y-27632 and HA-1077, completely reverse sustained cerebral vasoconstriction mediated by OxyHb. Our results showing that OxyHb–mediated contraction is dependent on both activation of the Rho/Rho kinase pathway and PKC provide new clues to the understanding of the functions of OxyHb and suggest that these signaling events could play a role in the development of cerebral vasospasm.

Acknowledgments

These studies were supported by a grant-in-aid from the Heart and Stroke Foundation of Canada (to B.V.). We are grateful to the Pharmaceutical Research Division, Wefeld Corporation (Osaka, Japan) for the generous gift of Y-27632. The authors would like to thank Andrew Wlosokowicz and Debarsi Das for their excellent assistance during this study.

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_Circ Res._ 2003;92:809-816; originally published online March 13, 2003;
doi: 10.1161/01.RES.0000066663.12256.B2

_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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