Effects of Rho kinase inhibition on cerebral artery myogenic tone and reactivity

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Several recent studies have implicated the RhoA-Rho kinase pathway in arterial myogenic behavior. The goal of this study was to determine the effects of Rho kinase inhibition (Y-27632) on cerebral artery calcium and diameter responses as a function of transmural pressure. Excised segments of rat posterior cerebral arteries (100–200 μm) were cannulated and pressurized in an arteriograph at 37°C. Increasing pressure from 10 to 60 mmHg triggered an elevation of cytosolic calcium concentration ([Ca\(^{2+}\)]\(_i\)) from 113 ± 9 to 199 ± 12 nM and development of myogenic tone. Further elevation of pressure to 120 mmHg induced only a minor additional increase in [Ca\(^{2+}\)]\(_i\) and constriction. Y-27632 (0.3–10 μM) inhibited myogenic tone in a concentration-dependent manner at 60 and 120 mmHg with comparable efficacy; conversely, sensitivity was decreased at 120 vs. 60 mmHg (50% inhibitory concentration: 2.5 ± 0.3 vs. 1.4 ± 0.1 μM; P < 0.05). Dilatation was accompanied by further increases in [Ca\(^{2+}\)]\(_i\) and an enhancement of Ca\(^{2+}\) oscillatory activity. Y-27632 also effectively dilated the vessels permeabilized with α-toxin in a concentration-dependent manner. However, dilator effects of Y-27632 at low concentrations were larger at 60 vs. 100 mmHg. In summary, the results support a significant role for RhoA-Rho kinase pathway in cerebral artery mechanotransduction of pressure into sustained vasoconstriction (myogenic tone and reactivity) via mechanisms that augment smooth muscle calcium sensitivity. Potential downstream events may involve inhibition of myosin phosphatase and/or stimulation of actin polymerization, both of which are associated with increased smooth muscle force production.

Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) due to Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. This response occurs secondary to pressure-induced depolarization of VSM and can be abolished by inhibitors of voltage-dependent Ca\(^{2+}\) channels (5, 11, 12, 19, 24, 29, 30, 33).

In a previous study (30), our laboratory found that the largest increment in membrane depolarization and subsequent elevation of [Ca\(^{2+}\)]\(_i\) occurs during the development of tone, most often between 40 and 60 mmHg; further elevation in pressure elicits little or no additional membrane depolarization and is associated with very modest [Ca\(^{2+}\)]\(_i\) elevation despite the significantly enhanced force development by VSM that is required to withstand high levels of intraluminal pressure.

This response, termed myogenic reactivity, can also be demonstrated in arteries depolarized with high K\(^+\) solution, indicating that mechanisms other than membrane depolarization might be involved (20). In particular, increased force in the absence of increased cytosolic [Ca\(^{2+}\)]\(_i\) suggests that pressure can augment VSM calcium sensitivity. This phenomenon is well documented in VSM and involves several mechanisms, e.g., the RhoA-Rho kinase pathway whose activation inhibits myosin phosphatase activity and stimulates actin polymerization (27, 31, 36). Several recent publications have demonstrated the importance of this pathway in the initiation and maintenance of pressure-induced myogenic tone in some vascular beds (16, 26, 32, 37, 39).

The purpose of this study was to examine the role of RhoA-Rho kinase signaling pathway in cerebral artery mechanotransduction. To accomplish this, we examined the effects of a specific inhibitor of Rho kinase (Y-27632) on isolated intact and endothelium-denuded rat cerebral arteries to determine whether 1) Y-27632 was able to inhibit pressure-induced myogenic tone by a direct action on VSM (as opposed to endothelium), 2) its vasodilatory action was related to a reduction in [Ca\(^{2+}\)]\(_i\), 3) sensitivity to Y-27632 was modulated by pressure, 4) a vasodilatory effect of Y-27632 could be preserved in the absence of normal membrane ionic gradients and under conditions in which changes in [Ca\(^{2+}\)]\(_i\), however subtle, could not be invoked. To accomplish the latter, we utilized Staphylococcus aureus α-toxin to induce VSM permeabilization. The results support a significant role for RhoA-Rho kinase pathway in cerebral artery mechanotransduction of pressure into sustained vasoconstriction (myogenic tone and reactivity) via mechanisms that augment VSM calcium sensitivity.

PRESURE-INDUCED VASOCONSTRICTION, often called myogenic tone, is an important determinant of peripheral resistance and contributes to the autoregulation of blood flow in the cerebral, coronary, and renal circulations. Myogenic tone occurs in the absence of endothelial or neural input and is an inherent property of vascular smooth muscle (VSM) (3, 5, 18, 29, 33). Although the mechanisms that underlie arterial myogenic behavior have been studied intensively for many years, the cellular structures and pathways involved in the mechanotransduction of pressure or stretch into sustained vasoconstriction are still not well understood.

We and others have shown that the development of myogenic tone is associated with a substantial elevation in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) due to Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. This response occurs secondary to pressure-induced depolarization of VSM and can be abolished by inhibitors of voltage-dependent Ca\(^{2+}\) channels (5, 11, 12, 19, 24, 29, 30, 33).

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METHODS

Animals. All experiments were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals (National Institutes of Health publication 85-23, 1985), and the experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Adult (16–24 wk old) male Wistar-Kyoto rats (n = 32) were anesthetized by an intraperitoneal injection of methohexital sodium (50 mg/kg; Brevital) and killed by decapitation. The brain was removed and immersed in a dissection dish filled with physiological salt solution (PSS; composition presented in Solutions and drugs). The entire posterior cerebral artery was carefully dissected free from surrounding connective tissues under a stereo dissection microscope.

Experimental protocol 1: effect of Y-27632 on myogenic tone. Arterial segments were cannulated in an arteriograph, placed on the stage of the inverted microscope, and pressurized to 10 mmHg. After a 1-h equilibration period at 37°C, intraluminal pressure was elevated from 10 mmHg to either 60 or 120 mmHg. After the development and stabilization of myogenic vasoconstriction (10–15 min), increasing concentrations of Y-27632 were added cumulatively to the superfusate. Each new concentration was applied after stabilization of the diameter in response to the previous application (typically after 10–15 min). A cocktail containing a mixture of papaverine (100 μM, a phosphodiesterase inhibitor) and diltiazem (10 μM, a calcium channel blocker) was applied at the end of each experiment to obtain the maximally relaxed arterial diameter (Dmax). Lumen diameter was continuously monitored using an arterial diameter analyzing system (Living Systems Instrumentation) or the SoftEdge Acquisition Subsystem (IonOptix).

In a separate set of experiments, the endothelium was removed by infusing air into the arterial lumen for 2–3 min, followed by gentle and brief (5 s) perfusion with regular PSS before pressurization of the artery. The effectiveness of this denudation procedure was confirmed by the absence of a dilatory response to acetylcholine.

Experimental protocol 2: measurement of smooth muscle Ca2+ in pressurized arteries. To obtain simultaneous measurements of arterial diameter and [Ca2+]i in VSM cells, arterial smooth muscle cells were first loaded with fura-2, a Ca2+-sensitive fluorescent dye. Fura 2-AM (1 mM stock dissolved in anhydrous DMSO) was premixed with an relaxing solution (for composition of the relaxing solution, see below) and continuously monitored using an arterial diameter analyzing system (Living Systems Instrumentation) or the SoftEdge Acquisition Subsystem (IonOptix).

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Experimental protocol 3: effects of Y-27632 on arteries permeabilized with S. aureus α-toxin. Arterial segments (0.5–1.0 mm in length) were transferred to an arteriograph filled with Ca2+-free relaxing solution (for composition of the relaxing solution, see below), cannulated, and pressurized to 60 mmHg. An arteriograph containing a cannulated arterial segment was placed on the stage of an inverted microscope equipped with a video camera. Permeabilization was carried out in relaxing solution by the addition of S. aureus α-toxin (800 U/ml) for 20 min at room temperature, as previously described (13). Arterial segments were then rinsed two to three times with relaxing solution to remove any remaining α-toxin from the bath solution. Vessels were allowed to equilibrate for 30 min at 37°C. Constrictor responses to increasing concentrations of Ca2+ were obtained in a cumulative fashion by applying each concentration of Ca2+ (pCa, −log [Ca2+], was 6.75–6.0) until arterial diameter was reduced by 30–40%, usually 5–10 min at each concentration. Once constriction was sufficient and stable, Y-27632 was applied in increasing concentrations (typically 2–3 concentrations were tested in any 1 vessel). At the end of an experiment, arteries were exposed to Ca2+-free relaxing solution to obtain the fully relaxed diameter.

In a separate set of experiments, after permeabilization of the arteries with α-toxin, pressure was elevated to 100 mmHg. Vessels were then preconstricted with Ca2+ to reduce diameter by 30–40%, and Y-27632 was tested in increasing concentrations, as described above. A slightly lower level of intraluminal pressure than for intact vessels was used since α-toxin-permeabilized vessels pressurized to 100 mmHg demonstrated more stable constrictor responses to Ca2+ elevation than at 120 mmHg.

Solutions and drugs. PSS contained (in mM) 119 NaCl, 4.7 KCl, 24.0 NaHCO3, 1.2 KH2PO4, 1.6 CaCl2, 1.2 MgSO4, 0.023 EDTA, and 11.0 glucose, pH 7.4. For the fura 2 calibration procedure, we used a solution of the following composition: 140 mM KCl, 20 mM NaCl, 5 mM HEPES, 5 mM EGTA, 1 mM MgCl2, 5 μM nigericin, and 10 μM ionomycin, pH 7.1.

Relaxing Ca2+-free solution contained the following (in mM): 63.6 potassium methanesulfonate, 2.0 MgCl2, 4.5 MgATP, 2.0 EGTA, 10.0 phosphocreatine, and 30.0 piperoxane-N,N’-bis(2-ethanesulfonic acid); pH was adjusted to 7.1 with 10.0 N KOH. The composition of the activating solution was similar to that of the relaxing solution, except that it contained 10.0 mM EGTA. The amount of CaCl2 needed to yield the desired free ion concentration was calculated by a computer program that solves a set of simultaneous equations describing the multiple equilibria of ions in solution (1).

Both relaxing and activating solutions contained 1.0 μM carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone, a mitochondrial blocker, and 1.0 μM leupeptin, a protease inhibitor. Ionic strength was kept constant at 200 mM by adjusting the concentration of potassium methanesulfonate accordingly. GTP (10 μM) was added to all activation solutions to preserve the function of RhoA-Rho kinase pathway, as described elsewhere (7).

All chemicals were purchased from Sigma (St. Louis, MO) with the exception of ionomycin, nigericin, GTP, and S. aureus α-toxin, which were obtained from Calbiochem (La Jolla, CA). Fura 2-AM and phosphoric acid were purchased from Molecular Probes. Fura 2-AM was dissolved in dehydrated DMSO as a 10 mM stock solution and stored at −20°C.

Calculations and statistical analysis. Smooth muscle [Ca2+]i was calculated using the following equation (14): [Ca2+]i = Keβ(R − Rmin)/(Rmax − R), where Ke is the dissociation constant of fura 2, R is experimentally measured ratio (340/380 nm) of fluorescence intensities, Rmin is a ratio in the absence of [Ca2+]i, and Rmax is a ratio at Ca2+-saturated fura 2 conditions. β is a ratio of the fluorescence intensities at 380-nm excitation wavelength at Rmin and Rmax. Rmin and β were determined by an in situ calibration procedure using arteries treated with nigericin (5 μM) and ionomycin (10 μM), as previously described (11). These values were then pooled and used to convert the ratio values into a [Ca2+]i.

The Ka was 282 nM, as determined by using in situ titration of Ca2+ in fura 2-loaded posterior cerebral arteries (19). Arterial diameter, pressure, and ratio values were recorded using an Ion Wizard data acquisition program and...
imported into Sigma Plot and Sigma Stat programs for graphical representation, calculations, and statistical analysis. Data are expressed as means ± SE, where each n is the number of arterial segments studied. Only one vessel per animal was used for a particular protocol. Paired or unpaired Student’s t-tests and one- or two-way repeated-measures ANOVA, followed by a multiple comparisons test (Holm-Sidak method), were used to determine the significance of differences, with P < 0.05 considered significant.

RESULTS

Inhibition of Rho kinase with Y-27632 results in endothelium-independent attenuation of myogenic tone. To explore the role of the RhoA-Rho kinase pathway in mechanotransduction in small cerebral arteries, we studied the effects of Y-27632, a potent inhibitor of Rho kinase, on pressure-induced vasoconstriction. In the first set of experiments, after an equilibration period at 10 mmHg, intraluminal pressure was increased to 60 mmHg. The initial distention of the artery was followed by development of myogenic constriction. In the first set of experiments, the role of the RhoA-Rho kinase pathway in mechanotransduction in small cerebral arteries, we studied the effects of Y-27632, a potent inhibitor of Rho kinase, on pressure-induced vasoconstriction. In the first set of experiments, the addition of Y-27632 in increasing concentrations resulted in dose-dependent vasodilation of all arteries tested (Fig. 1, A and B). The maximal concentration of Y-27632 used (10 μM) induced nearly maximal dilatation of the arteries that averaged 88 ± 3% of D_max. Washout of Y-27632 was followed by a slow restoration of pressure-induced constriction (shown in Fig. 1A).

There is evidence that the RhoA-Rho kinase pathway can also be involved in the regulation of endothelial NO production, raising the possibility that the observed vasodilation in response to Y-27632 could be due to an effect of Y-27632 on endothelial cells, with subsequent modulation of smooth muscle function (25, 38). To test this possibility, we performed additional experiments using endothelium-denuded arteries (see METHODS).

As in intact vessels, elevation of intraluminal pressure from 10 to 60 mmHg was followed by sustained vasoconstriction of 38 ± 3% of D_max (n = 8). Application of ACh (3 μM) did not elicit dilation in denuded arteries. Y-27632 produced a dose-dependent vasodilation of endothelium-denuded vessels that was similar to that described for intact cerebral arteries, indicating that Y-27632 has a direct effect on VSM cells within the arterial wall (Fig. 1B).

Y-27632-induced inhibition of myogenic tone is associated with an increase in smooth muscle [Ca^{2+}]. It is well documented that elevation of intraluminal pressure in cerebral arteries results in smooth muscle depolarization with a subsequent elevation of cytosolic [Ca^{2+}], (12, 15, 19, 24, 30). Recent studies indicate that Rho kinase inhibition can reduce [Ca^{2+}] and, subsequently, decrease force production in different types of smooth muscle (9, 17, 23, 34). Therefore, our next experiments were aimed at evaluating the effects of Y-27632 on pressure-induced [Ca^{2+}] and diameter responses of cerebral arteries.

Figure 2A demonstrates the effect of increasing concentrations of Y-27632 on [Ca^{2+}], and lumen diameter of cerebral arteries pressurized at 60 mmHg. Y-27632 induced concentration-dependent dilation of the vessel that was associated with a significant increase in total cytosolic [Ca^{2+}]. At a concentration of 3 μM, Y-27632 inhibited myogenic tone by 64 ± 5% but significantly elevated [Ca^{2+}], from 199 ± 12 to 224 ± 11 nM (P < 0.05; n = 5) (Fig. 2, A and B).

As shown in Fig. 3, A and B, the overall increase in [Ca^{2+}], was primarily due to a significant augmentation of VSM...
[Ca$^{2+}$]$_i$ oscillations, which increased in amplitude from 25 ± 3 to 100 ± 15 nM in 3 μM Y-27632 (n = 5).

We also studied the effect Y-27632 on diameter and [Ca$^{2+}$]$_i$ responses of cerebral arteries to pressure elevation from 60 to 120 mmHg (myogenic reactivity). As evident from Fig. 4A, elevating pressure from 10 to 60 mmHg resulted in a significant increase in [Ca$^{2+}$]$_i$ and the development of vasoconstriction (myogenic tone). Increasing intraluminal pressure further to 120 mmHg induced an additional rise in [Ca$^{2+}$]$_i$ and transient constriction. Intraluminal pressure was then decreased to 60 mmHg, and Y-27632 was applied at a concentration of 3 μM. Figure 4B illustrates the response of the same artery to pressure challenge from 60 to 120 mmHg in the presence of Y-27632 (3 μM). A subsequent increase in intraluminal pressure resulted in an elevation of [Ca$^{2+}$]$_i$ that was significantly higher (335 ± 48 nM) than in controls (246 ± 41 nM).

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**Fig. 2.** A: original traces demonstrating changes in smooth muscle cytosolic calcium concentration ([Ca$^{2+}$]$_i$) and arterial diameter as a function of stepwise elevation of intraluminal pressure from 10 to 60 mmHg and in response to application of Y-27632. B: bar graph summarizing the effect of Y-27632 on smooth muscle [Ca$^{2+}$]$_i$ of cerebral arteries pressurized to 60 mmHg. Different letters indicate significant differences (P < 0.05) between treatment means (1-way repeated-measures ANOVA). Lumen diameters of arteries at 10 mmHg were 109 ± 7 μm; maximal diameters at 60 mmHg were 164 ± 10 μm (n = 5).

**Fig. 3.** A: Y-27632-induced potentiation of smooth muscle [Ca$^{2+}$]$_i$ oscillations is associated with arterial dilation. B: bar graph summarizing the effect of Y-27632 on [Ca$^{2+}$] oscillatory activity. *Significantly different from controls at P < 0.05 (1-way repeated-measures ANOVA). Lumen diameters of arteries at 10 mmHg were 109 ± 7 μm; maximal diameters at 60 mmHg were 164 ± 10 μm (n = 5).
nM; \( P < 0.05; n = 4 \), although pressure-induced constriction was almost completely inhibited \((7 \pm 1 \ vs. \ 35 \pm 5\%; \ P < 0.05) \). The bar graphs in Fig. 4 summarize the effect of Y-27632 on the pressure-induced myogenic response (Fig. 4C) and associated elevation in \([\text{Ca}^{2+}]_i\) (Fig. 4D).

Intraluminal pressure elevation decreases the sensitivity of cerebral arteries to Y-27632. The results presented above demonstrate that pressure-induced vasoconstriction is abolished by Y-27632, thereby implicating RhoA-Rho kinase activation in the development and maintenance of myogenic tone in cerebral arteries. The next series of experiments was designed to elucidate whether the contribution of the RhoA-Rho kinase pathway to myogenic tone is dependent on the level of intraluminal pressure. To test this possibility, we measured the vasodilator effect of Y-27632 in cerebral arteries pressurized to 120 (100) mmHg and compared it with Y-27632-induced vasodilation of arteries at 60 mmHg in intact pressurized arteries and in vessels permeabilized with bacterial \( \alpha \)-toxin.

Intact arteries. In these experiments, intraluminal pressure was elevated from 10 to 120 mmHg. Once myogenic tone developed and stabilized \((32 \pm 2\% \ of \ D_{\text{max}}; n = 8) \), exposure of arteries to increasing concentrations of Y-27632 resulted in a dose-dependent vasodilation (Fig. 5A). At the maximal concentration used \((10 \ \mu M) \), inhibition of myogenic tone was \( 81 \pm 3\% \). The magnitude of this response (efficacy) did not differ from that observed earlier at 60 mmHg \((P > 0.05) \). On the other hand, as shown in Fig. 5, B and C, arteries pressurized to 120 mmHg were significantly less sensitive to the dilator effects of Y-27632 than those at 60 mmHg. The average concentration of Y-27632 required to inhibit 50% of myogenic tone in vessels pressurized to 120 mmHg \((2.5 \pm 0.3 \ \mu M) \) was significantly higher than the corresponding value for vessels at 60 mmHg \((1.4 \pm 0.1 \ \mu M; \ P < 0.05; \text{Fig. } 5C) \).

Arteries permeabilized with \( S. \ aureus \alpha \)-toxin. We also tested the effects of Y-27632 in arteries permeabilized with \( \alpha \)-toxin and pressurized to two different levels of pressure: 60 and 100 mmHg. After the permeabilization protocol was completed, arteries were preconstricted with a \( \text{Ca}^{2+} \)-containing solution by 30–40% to match the level of the constriction present in intact arterial segments. As shown in Fig. 6, A and B, Y-27632 effectively dilated arteries at both levels of pressure in a concentration-dependent manner. However, the dilator effects of 0.1 and 0.3 \( \mu M \) Y-27632 were larger at 60 vs. 100 mmHg. At higher concentrations (1 and 3 \( \mu M \)), Y-27632 was equally potent in arteries pressurized at both levels of pressure (Fig. 6C).

DISCUSSION

The main findings of the present study, carried out in isolated, pressurized cerebral vessels, are that 1) inhibition of Rho kinase with Y-27632 attenuates both myogenic tone and reactivity and is associated with an increase in smooth muscle cytosolic \([\text{Ca}^{2+}]_i\), and \([\text{Ca}^{2+}]_i\), oscillations; 2) Rho kinase inhibition is also effective in abolishing \( \text{Ca}^{2+} \)-induced constriction of vessels permeabilized with \( \alpha \)-toxin in a concentration-dependent manner, further substantiating an effect that is independent of mechanisms that regulate cytosolic \([\text{Ca}^{2+}]_i\), or membrane potential; and 3) the effects of Y-27632 in both intact and \( \alpha \)-toxin permeabilized arteries are modulated by intraluminal pressure such that higher concentrations of the inhibitor are required to produce similar vasodilation at 120 vs. 60 mmHg. Together, these observations implicate the RhoA-Rho kinase pathway in cerebral artery mechanotransduction (myogenic behavior) and suggest that activation of the RhoA-Rho kinase pathway is enhanced by transmural pressure.
RhoA-Rho kinase pathway in the regulation of cerebrovascular smooth muscle contractility. It is well established that pressure-induced myogenic tone is an important determinant of cerebral resistance and blood flow autoregulation (18). The present study demonstrates, for the first time, that cerebral artery responsiveness to pressure can be nearly eliminated by a potent inhibitor of Rho kinase (Y-27632) in a concentration-dependent manner. Y-27632 is a relatively specific inhibitor of Rho kinase and has been widely used to probe the functional role of RhoA-Rho kinase pathway in different types of smooth muscles. Its potency for inhibiting Rho kinase is 100 times greater than that for conventional protein kinase C (PKC) isoforms (36). In femoral artery smooth muscle, relatively low concentrations of Y-27632 were recently shown to inhibit PKC-δ, a calcium-insensitive isoform of PKC (6). In our previous study, we demonstrated that constriction of small cerebral arteries in response to activation of PKC requires elevated cytosolic Ca\(^{2+}\) and is therefore mediated by Ca\(^{2+}\).

**Fig. 5.** A: experimental traces demonstrating the concentration-dependent effects of Y-27632 on the diameter of cerebral arteries pressurized to 120 mmHg. B: concentration-response curves showing differences in sensitivity to the inhibitory effects of Y-27632 on myogenic tone in arteries pressurized to 60 vs. 120 mmHg. *Significantly different at $P < 0.05$ (2-way repeated-measures ANOVA). Maximal lumen diameters of arteries pressurized to 60 and 120 mmHg were 170 ± 5 (n = 11) and 185 ± 7 μm (n = 8), respectively. Lumen diameters of the arteries at 10 mmHg were 107 ± 4 and 113 ± 3 μm, respectively. C: bar graph showing the concentrations of Y-27632 required to inhibit 50% of myogenic tone (IC\(_{50}\)) in vessels pressurized to 60 vs. 120 mmHg. *Significantly different at $P < 0.05$ (unpaired Student’s t-test).

**Fig. 6.** A and B: experimental traces illustrating the effects of Y-27632 on α-toxin-permeabilized arteries preconstricted with Ca\(^{2+}\) at 2 different levels of transmural pressure. C: bar graphs showing the pressure-dependence of arterial sensitivity to Y-27632. *Significantly different at $P < 0.05$ (2-way repeated-measures ANOVA). Initial diameters of arteries pressurized to 60 and 100 mmHg were 185 ± 5 (n = 11) and 184 ± 7 μm (n = 9), respectively.
sensitive isofoms of PKC (11). Moreover, PKC-α has been shown to mediate myogenic reactivity in basilar artery (39). Based on these considerations, it appears likely that the major part of the vasodilator effect of Y-27632 in the present study results from Rho kinase inhibition, although other nonspecific actions cannot be completely ruled out.

The potency of the inhibitory effect of Y-27632 on pressure-induced vasoconstriction of cerebral vessels was similar to that previously shown for mesenteric arteries and renal afferent arterioles but considerably higher than in renal efferent arterioles and tail arteries (26, 32, 37). The distinct effect of Y-27632 on pressure-induced myogenic constriction may be attributable to a different role of the RhoA-Rho kinase pathway in the mechanotransduction of control of SMC contraction in different vascular beds, or even in different parts of the same vascular bed (e.g., the renal vasculature (26)). The role of Rho kinase in the control of pressure-induced myogenic tone of skeletal muscle resistance arteries was recently confirmed by using vessels overexpressing dominant negative mutants of RhoA or Rho kinase. As in the experiments with use of Rho kinase inhibitors, these arteries did not constrict in response to pressure elevation (2).

In the present study, the dilatory effects of Y-27632 were completely preserved after endothelial denudation, thereby eliminating the possibility that endothelium-derived signals are responsible for this effect, and indicating that smooth muscle cells of cerebral arteries are the major target of Y-27632, a widely used Rho kinase inhibitor. Our present observations, together with recently published data from other investigators (2, 26, 32, 37), support an important role for Rho kinase in the control of resistance artery myogenic tone.

The mechanisms by which activation of the RhoA-Rho kinase pathway increases smooth muscle cell contractility are not well understood and could be attributable to 1) its modulation of ion channel activity that results in an elevation in cytosolic [Ca^{2+}], 2) an increased Ca^{2+} sensitivity of contractile process, or 3) other mechanisms such as enhanced actin polymerization. Our laboratory previously documented a stimulatory effect of transmural pressure on cerebral artery actin polymerization and hypothesized that this process may facilitate force development by providing additional sites for actin-myosin interaction (4). The findings in this study dovetail with the earlier observation and suggest that actin polymerization in response to pressure elevation may be effected via RhoA activation. Although this hypothesis awaits confirmation by direct measurement of active vs. total RhoA (which was beyond the scope of this study), the linkage between RhoA and actin polymerization has been made in a number of studies using various tissues, including smooth muscle (27, 28, 31, 35).

**RhoA-Rho kinase pathway and regulation of cytosolic calcium**

Recent publications have also demonstrated that the RhoA-Rho kinase pathway is involved in the regulation of smooth muscle ion channel function with a subsequent modulation of membrane potential, Ca^{2+} influx, and cytosolic [Ca^{2+}] (9, 17, 22, 34). These newly established mechanisms may be responsible for the vasodilatory effects of Rho kinase inhibition on VSM and could operate in addition to its modulation of Ca^{2+} sensitivity or actin filament assembly. Earlier studies from our and other laboratories have shown that pressure-induced cerebral artery constriction is associated with membrane depolarization and an elevation of [Ca^{2+}]_i (12, 15, 19, 24, 30). Simultaneous measurements of smooth muscle [Ca^{2+}]_i and diameter in the present study revealed that Y-27632-induced inhibition of pressure-induced myogenic tone occurs without a decrease in [Ca^{2+}]_i (Fig. 2). Moreover, Y-27632-induced attenuation of myogenic tone in cerebral vessels was associated with a significant increase in smooth muscle [Ca^{2+}]_i.

Closer examination of the calcium data reveals that the increase in average cytosolic [Ca^{2+}]_i is mostly due to the augmentation of [Ca^{2+}]_i oscillations secondary to Y-27632 exposure. As shown in Fig. 3, Y-27632 increased the amplitude of cerebral artery VSM Ca^{2+} oscillatory activity in a concentration-dependent manner. The oscillatory pattern appearing to be one of transient and generally symmetrical increases in [Ca^{2+}]_i, superimposed on the existing baseline, with a frequency on the order of 0.5 Hz. In our laboratory’s previous study using pressurized cerebral arteries (30), our laboratory demonstrated that pressure elevation results in membrane depolarization and generation of fast oscillations in smooth muscle membrane potential of variable amplitude. In human cerebral arteries, both spontaneous oscillations in membrane potential and associated rhythmic contractions were abolished by nifedipine, implicating L-type Ca^{2+} channels in generation of the oscillatory activity (10). This electrical activity most probably is the major underlying mechanism for [Ca^{2+}]_i oscillations observed in the present study.

The cellular basis for augmentation of [Ca^{2+}]_i oscillations in the present study is not known and may involve several mechanisms. Modulation of smooth muscle membrane potential can profoundly affect the generation of Ca^{2+}-dependent membrane oscillations. It has been recently demonstrated that Rho kinase can regulate ion channel function in smooth muscle (9, 22, 34). Inhibition of Rho kinase with Y-27632 in the present study can therefore potentially affect the function of ion channels responsible for pressure-induced membrane depolarization with a subsequent modulation of membrane potential and amplitude of [Ca^{2+}]_i oscillations.

It has been shown that voltage-gated Ca^{2+} channels of cerebrovascular myocytes are stretch sensitive (21). Therefore, another possibility for the increased amplitude of [Ca^{2+}]_i oscillations is an augmented activity of Ca^{2+}-permeable channels due to increased wall tension (stretch) following Y-27632-induced vasodilation. Because this response was observed secondary to Rho kinase inhibition with Y-27632, an alternative interpretation is that Rho kinase activation suppresses the activity of Ca^{2+}-permeable channels and that its inhibition results in an increase in Ca^{2+} channel activity with a subsequent enhancement of oscillations in cytosolic [Ca^{2+}]. Either way, the relatively large oscillations in [Ca^{2+}]_i (100 nM in the presence of 10 μM Y-27632) were not associated with any significant constriction and were, in fact, accompanied by a loss of myogenic tone (dilation). These data underscore the importance of mechanisms other than [Ca^{2+}]; elevation in VSM force production during the development of myogenic tone and implicate the RhoA-Rho kinase pathway in this process.

Additional supporting evidence for the involvement of the Rho kinase in regulating smooth muscle force production was obtained in α-toxin-permeabilized vessels. Under these experimental conditions, the intracellular concentration of smooth muscle Ca^{2+} can be tightly controlled by adding the desirable...
amount of Ca\(^{2+}\) to the bath solution. This method also eliminates the possibility of changes in cytosolic [Ca\(^{2+}\)], due to α-toxin-induced hyperpermeability of the VSM cell membrane to small molecules such as Ca\(^{2+}\) ions (8). The effectiveness of low concentrations (0.3–1 μM) of Y-27632 in dilating α-toxin-permeabilized vessels under constant levels of cytosolic Ca\(^{2+}\) confirms that the actions of Rho kinase in the control of cerebral artery smooth muscle contractility can extend beyond membrane events and regulation of cytosolic Ca\(^{2+}\).

**Pressure-dependence of Y-27632-induced vasodilation.** To further clarify the role of the RhoA-Rho kinase pathway in mediating the conversion of a mechanical stimulus (intraluminal pressure) into cellular response (vasoconstriction), we determined the potency of Y-27632 in inhibiting the myogenic tone at two different levels of intraluminal pressure: 60 and 120 mmHg. If pressure enhances the activity of Rho kinase, it seems reasonable that a higher concentration of the inhibitor might be required to produce a comparable effect at 120 vs. 60 mmHg. The results indicate that, although the efficacy of the inhibitor is comparable at both pressures, there is a significant reduction in Y-27632 sensitivity at 120 vs. 60 mmHg, as evidenced by the difference in the concentration of Y-27632 required to inhibit 50% of myogenic tone values at the two pressures (Fig. 5C). In addition to the difference in concentration, it deserves note that, at comparable diameters, wall tension at 120 mmHg is twice that at 60 mmHg. Hence, the circumferential force attempting to distend the vessel is much greater. This fact, combined with the observation that vessels were able to maintain tone at 120 mmHg in concentrations of Y-27632 that were effective in relaxing vessels at 60 mmHg, further substantiates the likelihood of a significant stimulatory effect of transmural pressure on VSM RhoA activation. Confirmation of this hypothesis awaits direct measurement of active vs. total RhoA as a function of pressure, as does the understanding of changes secondary to RhoA activation (e.g., the creation of additional actin filaments and/or enhanced calcium sensitivity due to the inhibition of myosin phosphatase) responsible for the increased VSM force production.

**GRANTS**

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**REFERENCES**


