Actin cytoskeletal modulation of pressure-induced depolarization and Ca\(^{2+}\) influx in cerebral arteries

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Gokina, Natalia I., and George Osol. Actin cytoskeletal modulation of pressure-induced depolarization and Ca\(^{2+}\) influx in cerebral arteries. Am J Physiol Heart Circ Physiol 282: H1410–H1420, 2002. First published December 16, 2001; 10.1152/ajpheart.00441.2001.—The objective of this study was to examine the role of the actin cytoskeleton in the development of pressure-induced membrane depolarization and Ca\(^{2+}\) influx underlying myogenic constriction in cerebral arteries. Elevating intraluminal pressure from 10 to 60 mm Hg induced membrane depolarization, increased intracellular cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{c}) and elicited myogenic constriction in both intact and denuded rat posterior cerebral arteries. Pretreatment with cytochalasin D (5 \text{ mM}) or latrunculin A (3 \text{ mM}) abolished constriction but enhanced the [Ca\(^{2+}\)]\text{c}; similarly, acute application of cytochalasin D to vessels with tone, or in the presence of 60 \text{ mM K}\(^{+}\), elicited relaxation accompanied by an increase in [Ca\(^{2+}\)]\text{c}. The effects of cytochalasin D were inhibited by nifedipine (3 \text{ mM}), demonstrating that actin cytoskeletal disruption augments Ca\(^{2+}\) influx through voltage-sensitive L-type Ca\(^{2+}\) channels. Finally, pressure-induced depolarization was enhanced in the presence of cytochalasin D, further substantiating a role for the actin cytoskeleton in the modulation of ion channel function. Together, these results implicate vascular smooth muscle actin cytoskeletal dynamics in the control of cerebral artery diameter through their influence on membrane potential as well as via a direct effect on L-type Ca\(^{2+}\) channels.

myogenic tone; wall tension; cytochalasin D; latrunculin A

PRESSURE-INDUCED MYOGENIC constriction represents a fundamental property of resistance-size arteries and arterioles and contributes to autoregulation of regional blood flow and maintenance of capillary hydrostatic pressure. It is also an essential component of basal vascular tone, which allows blood vessels to constrict or dilate in response to vasoactive mediators, and to elevations or reductions in transmural pressure, respectively. The myogenic response occurs independently of the endothelium and perivascular nerves and is therefore an inherent property of vascular smooth muscle (VSM) cells (10, 12, 13, 24, 33, 40, 45).

Numerous studies (10, 12, 16, 18, 26, 33, 40, 48) have demonstrated the importance of membrane depolarization, with resultant Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels and an increase in intracellular free Ca\(^{2+}\), as the principal mechanism by which pressure or stretch elicits smooth muscle contraction. In cerebrovascular smooth muscle, L-type Ca\(^{2+}\) channels are themselves sensitive to mechanical load (stretch), an observation that adds further complexity to the mechanisms underlying Ca\(^{2+}\) elevation induced by intraluminal pressure (29). Some studies (12, 14, 25, 33) have suggested that, in addition to enhanced Ca\(^{2+}\) influx, increased smooth muscle Ca\(^{2+}\) sensitivity may also contribute to the development of myogenic tone.

Despite considerable progress in our understanding of the mechanisms underlying myogenic tone and reactivity, the major questions of which cellular structures within the vascular wall detect the mechanical load imposed by transmural pressure and of how this signal is transduced into the myogenic response, remain unresolved. In a variety of nonmuscle cell types, the complex of extracellular matrix, transmembrane integrins, and intracellular cytoskeletal structures has been implicated in mechanotransduction (3, 8, 22, 23, 39). Hence, the detection of mechanical deformation, presumably the initial step in the signal transduction pathway, may occur within the cell membrane, cytoskeleton, or both.

Two models have been proposed to explain how cells can sense a mechanical load (3). According to the inside-out signaling model, the cytoskeleton primarily detects mechanical deformation and transfers tension to the mechanosensory integrin-dependent system in the cell membrane. This, in turn, triggers a cascade of biochemical events that subsequently evoke the appropriate cellular response. It is also possible that membrane-associated integrin molecules play a key role in detecting the mechanical load and transmit the mechanical stimulus from the cell membrane to the interior of the cell, which responds by structural reorganization and/or polymerization of one or more cytoskeletal components, an elevation in the intracellular cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{i}) or other signaling molecules (the outside-in model) (3, 8, 11, 13, 47, 49).

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Thus far, very little is known about the specific role of the cytoskeleton in VSM mechanoreception and mechanotransduction. Together with intermediate filaments and microtubules, actin represents a well-defined and complex cytoskeletal structure in smooth muscle cells (42). A role for actin polymerization in response to the contractile activation of tracheal smooth muscle has been proposed by Mehta and Gunst (30). A study (6) from our laboratory showed that disruption of the actin filamentous system with cytochalasin B resulted in accelerated forced dilatation of cerebral arteries, suggesting a similar role for the actin cytoskeleton in pressure-induced vasoconstriction. On the basis of the latter study, we hypothesized that the actin cytoskeleton might be important in linking the cellular deformation induced by transmural pressure to myogenic vasoconstriction. The actin cytoskeleton of VSM is physically coupled to or part of the contractile machinery (42) and, therefore, any manipulation of actin cytoskeletal structure and dynamics could also influence constriction. Hence, the objective of this study was to examine the effects of actin cytoskeletal disruption on pressure-induced membrane depolarization and Ca$^{2+}$ influx, the aforementioned events that are fundamental to the mechanism underlying the development of myogenic constriction. We used two structurally unrelated compounds, cytochalasin D and latrunculin A, which disrupt actin filamentous structure by binding to filamentous actin (F-actin) and monomeric actin (G-actin), respectively. Despite their different mechanisms of action, both compounds disrupt and disorganize the actin cytoskeletal structure within living cells (2, 19, 35, 43).

**METHODS**

*Animals.* Adult (16–24 wk old) male Wistar-Kyoto rats ($n = 72$) were anesthetized by an intraperitoneal injection of methohexitol sodium (Brevital; 50 mg/kg) and killed by decapitation. The brain was removed and immersed in a dissection medium composed of 140 mM KCl, 20 mM NaCl, 5 mM a-Tris, 5 mM MgCl$_2$, 1.2 mM CaCl$_2$, 14 mM NaHCO$_3$, 11.0 mM glucose, and 0.83 mM MgSO$_4$ equilibrated with a mixture of 95% O$_2$-5% CO$_2$, pH 7.4. Potassium solution (60 mM) was prepared by substituting equimolar amounts of NaCl with CaCl$_2$. For the fura 2 calibration procedure, we used a solution composed of 140 mM KCl, 20 mM NaCl, 5 mM HEPES, 5 mM EGTA, 1 mM MgCl$_2$, 5 μM nigericin, and 10 μM ionomycin, pH 7.15.

All chemicals were purchased from Sigma (St. Louis, MO) with the exception of cytochalasin D, latrunculin A, and ionomycin, which were obtained from Calbiochem (La Jolla, CA). Fura 2-AM, pluronic acid, and nigericin were purchased from Molecular Probes (Eugene, OR). Fura 2-AM was dissolved in DMSO as a 1 mM stock solution and frozen in 2-μl aliquots until used. Wortmannin, cytochalasin D, and latrunculin A were prepared as 10 mM stock solutions in DMSO. The maximal final concentration of DMSO in the bath solution was 0.05%. Nifedipine was solubilized in ethanol to yield a stock solution of 10 mM. 2,3-Butanediene monoxime (BDM) was dissolved directly in PSS just before use. Ionomycin and nigericin were prepared as 10 mM stock solutions in methanol.

**Calculations and statistical analysis.** Arterial wall [Ca$^{2+}$], was calculated using the following equation (17): [Ca$^{2+}$] = $K_d(R - R_{	ext{min}})/(R_{	ext{max}} - R)$, where $R$ is the experimentally measured ratio (340:380 nm) of fluorescence intensities, $R_{	ext{min}}$ is the ratio in the absence of [Ca$^{2+}$], $R_{	ext{max}}$ is the ratio at Ca$^{2+}$-saturated fura 2 conditions, and $K_d$ is the dissociation constant of fura 2. $β$ is a ratio of the fluorescence intensities at 380-nm excitation wavelength at $R_{	ext{min}}$ and $R_{	ext{max}}$, $R_{	ext{min}}$, $R_{	ext{max}}$, and $β$ were determined by an in situ calibration procedure using arteries treated with nigericin (5 μM) and (95% O$_2$-5% CO$_2$) at 37°C. Fura 2 fluorescence was measured using a photomultiplier system (IonOptix) in which background-corrected ratios of the 510-nm emission from arteries alternately excited at 340 and 380 nm were obtained at a sampling rate of 5 Hz. Arterial diameter was simultaneously recorded using the SoftEdge Acquisition Subsystem (IonOptix). All experimental protocols were started after a 30-min equilibration period at 10 mmHg.

In a separate set of experiments, the endothelium was removed by infusing an air bubble into the arterial lumen for 1–2 min, followed by gentle and brief (5 s) perfusion with regular PSS. The effectiveness of this denudation procedure was confirmed by the absence of a dilatory response to the application of acetylcholine, as described previously (16). Papaverine (100 μM) was applied in the end of each experiment to calculate maximal arterial diameter.

**Measurements of membrane potential.** For measurements of membrane potential we used glass microelectrodes filled with 0.5 M KCl having tip resistances of 110–150 MΩ; an Ag-AgCl pellet was used as an indifferent electrode. Microelectrodes were made from capillary tubing filled with 1 M KCl. The excised posterior cerebral artery, including its branches, was removed and carefully dissected free from surrounding connective tissues under a stereodissection microscope. All experiments were conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Vermont.

**Measurement of smooth muscle Ca$^{2+}$ in pressurized arteries.** To obtain simultaneous measurements of arterial diameter and [Ca$^{2+}$] in VSM cells, arteries were loaded with fura 2, a Ca$^{2+}$-sensitive fluorescent dye. Fura 2-acetoxymethyl ester (AM), 2 μl of 1 mM stock dissolved in dimethyl sulfoxide (DMSO), was premixed with an equal volume of a 25% solution of pluronic acid in DMSO and then diluted in PSS to yield a final concentration of 5 μM. The excised posterior cerebral artery, including its branches, was incubated in the fura 2-AM-PSS loading solution at room temperature in the dark for 60 min. Fura 2-loaded arteries were then washed with PSS and kept refrigerated until use. Each arterial segment was cannulated, mounted in an arteriograph and continuously superfused at 3 ml/min with oxygenated PSS
ionomycin (10 μM) as previously described (26). These values were then pooled and used to convert the ratio values into [Ca$^{2+}$]. $K_d$ was 282 nM, as determined by using in situ titration of Ca$^{2+}$ in fura 2-loaded posterior cerebral arteries (26). Arterial diameter, pressure, ratio, and membrane potential values were recorded using an IonWizard data-acquisition program and imported into SigmaPlot and SigmaStat programs for graphic 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. A paired or unpaired Student’s $t$-test or ANOVA were used to determine the significance of differences between sets of data, with $P < 0.05$ considered significant.

RESULTS

Cytochalasin D and latrunculin A inhibit pressure-induced constriction but augment associated elevation of [Ca$^{2+}$]. To explore the role of the actin cytoskeleton in mechanotransduction in small cerebral arteries, we studied the effects of actin cytoskeletal disruption with cytochalasin D and latrunculin A on pressure-induced elevations in smooth muscle [Ca$^{2+}$], and constriction. In control experiments, after a 30-min equilibration period at 10 mmHg, intraluminal pressure was increased to 60 mmHg. The initial distention of the artery was followed by myogenic constriction (24 ± 3% of maximal diameter) and elevation of [Ca$^{2+}$] from 88 ± 8 to 237 ± 10 nM. After stabilization of the myogenic response (10–15 min), pressure was returned to 10 mmHg. A second pressure step from 10 to 60 mmHg was applied 15–20 min later. This time was necessary for the restoration of both arterial diameter and [Ca$^{2+}$] level (86 ± 9 nM) to the control values. The extent of constriction and [Ca$^{2+}$] elevation in response to the second pressure step were not different from the control values: 25 ± 3% and 234 ± 12 nM, respectively ($P > 0.05; n = 14$).

In a second series of experiments, after the [Ca$^{2+}$] response and constriction induced by first pressure step were recorded, arteries were exposed to 5 μM cytochalasin D for 30 min at 10 mmHg. A subsequent increase in intraluminal pressure resulted in an elevation of [Ca$^{2+}$] that was significantly higher (345 ± 26 nM) than in controls (222 ± 13 nM, $P < 0.05; n = 14$), although pressure-induced constriction was almost completely inhibited (3 ± 1 vs. 24 ± 9% of maximal diameter, $P < 0.05$; Fig. 1). In 7 out of 14 arteries, we tested diameter changes and [Ca$^{2+}$] responses induced by a second pressure step in the presence of cytochalasin D (after ~60 min of cytochalasin D application).

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Fig. 1. Effects of cytochalasin D (Cyt-D) on pressure-induced elevation of intracellular cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]) smooth muscle in arterial wall and constriction of rat cerebral arteries. A: representative traces showing an increase in arterial wall [Ca$^{2+}$], and a decrease in arterial diameter in response to an elevation of intraluminal pressure from 10 to 60 mmHg before (control) and after 30 min of treatment with cytochalasin D (5 μM). B and C: bar graphs summarizing the effects of cytochalasin D on pressure-induced elevation in [Ca$^{2+}$], and associated vasoconstriction. *$P < 0.05$, significant difference. $d_{max}$, Maximal diameter.
This resulted in an elevation of \([\text{Ca}^{2+}]_i\) to 322 ± 32 nM, a value significantly greater than the arterial response before treatment with cytochalasin D \((219 \pm 23 \text{nM})\) \((P < 0.05)\), and a complete abolition of myogenic tone \((1 \pm 0.3\%)\).

Similar results were obtained when arteries were pretreated for 30 min with 3 μM latrunculin A, an inhibitor of actin polymerization. As with cytochalasin D, the pressure-induced \([\text{Ca}^{2+}]_i\) response was significantly augmented \((303 \pm 17 \text{nM})\) compared with the values obtained from the same arteries before treatment with latrunculin A \((233 \pm 13 \text{nM}; P < 0.05)\).

Myogenic vasoconstriction in response to pressure elevation was significantly reduced from 24 ± 2% to 10 ± 1% \((n = 15, P < 0.05; \text{Fig. 2})\). A second pressure step in the presence of latrunculin A \((3 \mu M)\) that was significantly increased \((318 \pm 18 \text{nM})\) compared with the response of the same artery before application of latrunculin A \((241 \pm 15 \text{nM}; P < 0.05)\). The associated myogenic constriction was further reduced to 5 ± 2%.

We also studied the effects of acute application of cytochalasin D and latrunculin A on pressure-induced myogenic tone and \([\text{Ca}^{2+}]_i\) responses. As shown in Fig. 3A, after stabilization of diameter and \([\text{Ca}^{2+}]_i\) levels in response to pressure elevation from 10 to 60 mmHg, application of 5 μM cytochalasin D resulted in immediate relaxation and an additional increase in smooth muscle \([\text{Ca}^{2+}]_i\). Constriction was significantly decreased from 24 ± 4 to 7 ± 1% \((P < 0.05)\), and \([\text{Ca}^{2+}]_i\) was elevated from 219 ± 15 to 288 ± 24 nM \((P < 0.05; n = 6, \text{Fig. 3B})\). Similar results were obtained in the presence of latrunculin A \((3 \mu M)\). Application of this compound was followed by a reduction in myogenic tone from 25 ± 4 to 10 ± 3% and an elevation in \([\text{Ca}^{2+}]_i\) from 202 ± 14 to 269 ± 19 nM \((both\ P < 0.05; n = 7, \text{Fig. 3C})\).

In four other arteries, acute application of 0.05% DMSO (used as a solvent for cytochalasin D and latrunculin A) resulted in no effect on pressure-induced myogenic constriction \((19 \pm 4\ vs. 19 \pm 3\%\ in\ control)\) and \([\text{Ca}^{2+}]_i\) \((211 \pm 14\ vs. 213 \pm 15\ \text{nM}\ in\ control)\). Subsequent addition of 5 μM cytochalasin D decreased the tone to 4 ± 1% and significantly elevated \([\text{Ca}^{2+}]_i\) to 342 ± 51 nM \((P < 0.05)\).

The effects of cytochalasin D at a higher concentration \((10 \mu M)\) and extended time of application \((to\ 1\ \text{h})\) were studied next. Under these conditions, elevation of pressure resulted in a significantly enhanced \([\text{Ca}^{2+}]_i\),
pressure-induced constriction (4 ± 2 vs. 28 ± 6% in control; \(P < 0.05\)), but significantly potentiated the associated elevation in \([\text{Ca}^{2+}]_i\) (295 ± 20 vs. 222 ± 19 nM in control; \(P < 0.05; n = 4\)). On the basis of these data, we conclude that the cytochalasin-enhanced elevation in \([\text{Ca}^{2+}]_i\), and inhibition of myogenic tone are due to specific disruption of the actin cytoskeleton in VSM cells.

Cytochalasin D-induced augmentation in \([\text{Ca}^{2+}]_i\), in response to pressure elevation is not due to increased wall tension. As already shown, both cytochalasin D and latrunculin A abolished constriction in response to elevated intraluminal pressure. This effect was associated with a substantial (~125% of control) increase in wall tension due to arterial dilation. To test whether the increased wall tension was itself responsible for the enhancement in pressure-induced \([\text{Ca}^{2+}]_i\), a different group of arteries was treated with either of two inhibitors of myosin light chain kinase, BDM (5 mM) and wortmannin (5 μM). These compounds have been shown to attenuate force generation in VSM cells and were therefore likely to inhibit myogenic constriction as well (4, 34).

In five arteries, elevation of pressure from 10 to 60 mmHg resulted in \([\text{Ca}^{2+}]_i\) increasing from 88 ± 15 to 252 ± 15 nM and myogenic constriction. Pretreatment of arteries with BDM for 20 min completely abolished the myogenic response to a pressure step, but also partially decreased the pressure-induced \([\text{Ca}^{2+}]_i\); elevation from 252 ± 15 to 167 ± 25 nM (\(P < 0.05\)). When cytochalasin D was applied to the same arteries treated with BDM, however, a subsequent increase in pressure produced similar arterial distention (arterial diameter 194 ± 10 vs. 195 ± 10 μm in control; \(P > 0.05\)) but significantly augmented the \([\text{Ca}^{2+}]_i\), response (to 277 ± 23 nM; Fig. 4A).

In another series of experiments, wortmannin (5 μM) was applied to the vessels with myogenic tone (at 60 mmHg). This resulted in virtually complete relaxation with a reduction in tone from 22 ± 1 to 3 ± 1% (\(P < 0.05\)). As seen in Fig. 4B, despite the increase in wall tension secondary to relaxation, \([\text{Ca}^{2+}]_i\) level was not significantly altered, averaging 237 ± 20 nM before and 243 ± 20 nM after wortmannin application (\(P > 0.05, n = 6\)).

Collectively, our data indicate that increased arterial wall tension due to dilation of pressurized arteries by cytochalasin D or latrunculin A is not responsible for the enhancement in smooth muscle \([\text{Ca}^{2+}]_i\). We therefore suggest that changes in actin cytoskeletal dynamics and/or organization modulate pressure-induced \([\text{Ca}^{2+}]_i\), signaling mechanisms in VSM cells of cerebral arteries.

Actin cytoskeletal disruption augments pressure-induced \([\text{Ca}^{2+}]_i\) influx through L-type channels. A role for the actin cytoskeleton in regulating \([\text{Ca}^{2+}]_i\) signaling mechanisms was implicated in a number of different cell types, and effects on both \([\text{Ca}^{2+}]_i\) influx and \([\text{Ca}^{2+}]_i\) release from intracellular stores have been demonstrated in earlier studies (23). The next series of experiments were designed to elucidate the mechanism of

**Fig. 3. Effects of acute application of cytochalasin D and latrunculin A on arterial wall \([\text{Ca}^{2+}]_i\), and diameter of pressurized rat posterior cerebral arteries.**

(A) Original traces showing an increase in arterial wall \([\text{Ca}^{2+}]_i\), and the appearance of myogenic constriction in response to an elevation of intraluminal pressure from 10 to 60 mmHg. Application of cytochalasin D (5 μM) resulted in an additional increase in \([\text{Ca}^{2+}]_i\), and dilation of the artery. (B and C) Bar graphs summarizing the effects of cytochalasin D and latrunculin A on arterial wall \([\text{Ca}^{2+}]_i\), and pressure-induced constriction. *\(P < 0.05\), significant difference.
additional \([\text{Ca}^{2+}]\) elevation induced in pressurized cerebral arteries by actin cytoskeletal disruption. We first tested the effects of nifedipine (3 \(\mu\)M), a dihydropyridine inhibitor of L-type \(\text{Ca}^{2+}\) channels, on enhanced pressure-induced \([\text{Ca}^{2+}]\) responses in arteries treated with cytochalasin D. As shown in Fig. 5, the application of nifedipine resulted in an immediate decrease in \([\text{Ca}^{2+}]\), with no significant effect on arterial diameter (because the vessel was already relaxed due to cytochalasin D). A second pressure step in the presence of both cytochalasin D and nifedipine resulted only in arterial distention with minimal elevation in smooth muscle \([\text{Ca}^{2+}]\).

Cytochalasin D induces additional \([\text{Ca}^{2+}]\) elevation in pressurized arteries depolarized with high-potassium solution. It is well documented that pressure-induced membrane depolarization stimulates the \(\text{Ca}^{2+}\) influx in small cerebral arteries (18, 26, 48). Therefore, both a direct potentiating effect on voltage-gated \(\text{Ca}^{2+}\) channels, as well as indirect augmentation of \(\text{Ca}^{2+}\) influx due to enhanced pressure-induced depolarization, should be considered as potential mechanisms for the observed effects of cytochalasin D. To test the first possibility, we studied the effects of cytoskeletal disruption in arteries depolarized with high-K\(^+\) solution, to prevent any changes in the membrane potential of VSM cells. As shown in Fig. 6A, high-K\(^+\) solution raised the \([\text{Ca}^{2+}]\) to 390 ± 38 nM and constricted arteries pressurized to 60 mmHg. Application of cytochalasin D (10 \(\mu\)M) resulted in an additional increase in \([\text{Ca}^{2+}]\), to 462 ± 40 nM \((P < 0.05; n = 7)\) and almost complete arterial relaxation (11 ± 1% of high-K\(^+\)-induced constriction). When wortmannin (5 \(\mu\)M) was used instead of cytochalasin D in conjunction with 60 mM K\(^+\), significant arterial relaxation was observed.

### Fig. 4. Effects of 2,3-butanedione monoxime (BDM) and wortmannin on pressure-induced changes in arterial wall \([\text{Ca}^{2+}]\), and diameter of rat posterior cerebral arteries. A: original traces demonstrating an increase in \([\text{Ca}^{2+}]\), and constriction in response to an elevation of intraluminal pressure from 10 to 60 mmHg during exposure of artery to BDM (5 mM) or combination of BDM (5 mM) and cytochalasin D (5 \(\mu\)M). B: application of wortmannin (5 \(\mu\)M) did not alter the level of \([\text{Ca}^{2+}]\), but dilated the pressurized artery.

### Fig. 5. Effects of nifedipine (3 \(\mu\)M) on pressure-induced elevation of arterial wall \([\text{Ca}^{2+}]\), and diameter of cerebral arteries pretreated with cytochalasin D. A: elevation of intraluminal pressure in the presence of cytochalasin D resulted in an increase in \([\text{Ca}^{2+}]\), that was not associated with arterial constriction. Application of nifedipine was followed by an abrupt decrease in \([\text{Ca}^{2+}]\). B: original traces demonstrating an inhibition of \([\text{Ca}^{2+}]\), changes in response to elevation of intraluminal pressure in the same artery treated with nifedipine and cytochalasin D. C and D: summary of the effects of cytochalasin D and nifedipine on pressure-induced elevation of \([\text{Ca}^{2+}]\), and constriction of posterior cerebral arteries. \(^* P < 0.05\), significant difference.
without any change in the level of [Ca\(^{2+}\)](i) (392 ± 32 nM before and 389 ± 30 nM after, n = 6, Fig. 6B). In four other arteries, nifedipine was added once high-K\(^+\)-induced constrictions and [Ca\(^{2+}\)](i) increases had stabilized; this was followed by an abolition of constriction and a decrease in [Ca\(^{2+}\)](i) from 384 ± 31 to 94 ± 11 nM. Subsequent application of cytochalasin D has no effect on arterial diameter or on [Ca\(^{2+}\)](i) (98 ± 13 nM). These experimental results favor a direct effect (i.e., not mediated by membrane depolarization) of the actin cytoskeleton on voltage-dependent Ca\(^{2+}\) channels.

**Cytochalasin D depolarizes smooth muscle cells in pressurized cerebral arteries.** In separate experiments, we measured the changes in membrane potential and arterial diameter of pressurized posterior cerebral arteries before and after treatment with cytochalasin D. Resting membrane potential in VSM cells of arteries at 10 mmHg averaged −67 ± 1 mV (n = 15). An elevation of pressure from 10 to 60 mmHg resulted in membrane depolarization of ~30 mV (to −38 ± 1 mV; n = 10; P < 0.05). This depolarization was associated with the development of myogenic tone (31 ± 3%). Application of 5 μM cytochalasin D resulted in an additional depolarization of 5 ± 1 mV (n = 6), and the appearance of membrane potential oscillations that were associated with a complete arterial dilation (Fig. 7).

**DISCUSSION**

The results of the present study demonstrate that the actin cytoskeleton has a modulatory but not a sensory role in the process of transducing the stimulus of intraluminal pressure into VSM contraction. This conclusion is based on our observation that the disruption of actin cytoskeletal structure did not attenuate but augmented two vital components of the pressure-induced VSM cellular response: membrane depolarization and elevation in [Ca\(^{2+}\)](i), fundamental events that precede the development of myogenic tone in small cerebral arteries (11–13, 16, 18, 26, 33, 40, 48).

To disrupt the actin cytoskeleton in VSM cells, we used two structurally unrelated compounds with essentially different mechanisms of action. Cytochalasins are membrane-permeable fungal metabolites that have long served as unique tools for studying the functional role of the actin cytoskeleton in cell motility, growth, and development (9, 19, 23, 27, 30, 32, 35, 37, 43, 44). These compounds are known to bind to the rapidly growing end of actin filaments, thereby inhibiting the association and dissociation reactions at those ends and preventing actin polymerization (9, 43). In contrast, the latrunculins are a novel class of compounds that induce highly specific sequestration of monomeric actin (G-actin). Their net effect is to inhibit polymerization of actin and to promote actin depolymerization (2, 43). Despite these different mechanisms of action, both cytochalasins and latrunculins disorganize and disassemble filamentous actin structures in nonmuscle as well as smooth muscle cells (2, 19, 35, 43).

In the experiments described herein, cytochalasin D and latrunculin A profoundly inhibited the contraction induced by intraluminal pressure or high-K\(^+\) solution, indicating the loss of VSM force-generating capability. These observations are consistent with the findings of others showing that disruption of the supramolecular organization of the actin cytoskeleton with cytochalasins resulted in an inhibition of smooth muscle contraction (30, 32, 38, 44). The latter does not result from an attenuation of associated Ca\(^{2+}\) influx, however, as either no change (32, 38) or an increase (this study) in...
[Ca\textsuperscript{2+}], occurs. Cytochalasins bind to the barbed end of actin filaments and compete with actin-binding proteins that provide attachment of actin filaments to the membrane structures (9, 43). Considering this mechanism of action, the inhibitory effect of cytochalasins on contraction can be attributed to the detachment of actin filaments from the membrane, thereby compromising force development in VSM. However, in our study, this effect is probably not responsible for attenuation of VSM force generation because, in contrast to cytochalasin D, latrunculin A (which binds directly to G-actin but does not interfere with F-actin or sites of its attachment to plasma membrane) also inhibits myogenic tone (2, 43).

We interpret the fact that latrunculin A attenuates the pressure-induced vasoconstriction as evidence that cerebrovascular smooth muscle possesses a considerable pool of G-actin, which is in dynamic equilibrium with F-actin. Similar to the observations made in other cell types, it is likely that sequestration of G-actin by latrunculin A shifts the dynamic equilibrium promoting F-actin depolymerization, diminishing amount of F-actin and thereby decreasing force production in VSM (2, 35). Therefore, these experiments suggest that alteration of actin cytoskeletal dynamics might be an important new mechanism regulating myogenic tone of cerebral arteries.

Our results demonstrate that actin cytoskeletal disruption inhibits myogenic tone but not pressure-induced depolarization or Ca\textsuperscript{2+} influx. Taking into consideration that membrane depolarization and Ca\textsuperscript{2+} influx preceded the development of myogenic tone, these data do not support the hypothesis that the actin cytoskeleton acts as a primary system for the detection of a mechanical load (i.e., as a mechanosensor). A role for the two other components of the cytoskeleton, intermediate filaments and microtubules, in mechanotransduction cannot be ruled out. However, in vimentin-deficient mice, elevation of intraluminal pressure resulted in the development of myogenic tone in small mesenteric arteries, where vimentin is a major component of the smooth muscle intermediate filamentous system (20). In a different study, depolymerization of microtubules resulted in Ca\textsuperscript{2+}-independent constriction of isolated cremaster arterioles and a preservation of myogenic responses over a wide range of intralumi-
nal pressures (36). Together, these observations do not support a role for the VSM cytoskeletal network in the detection of pressure-induced mechanical deformation in small arteries and therefore argue against the involvement of the actin cytoskeleton in the inside-out signaling model of mechanotransduction.

On the other hand, the results of this study do support a modulatory role for the actin cytoskeleton in smooth muscle Ca$^{2+}$ handling, as both acute application and pretreatment of arteries with cytochalasin D or latrunculin A resulted in a significant enhancement of pressure-induced [Ca$^{2+}$]; elevation in cerebrovascular smooth muscle. The cytochalasin D-induced effect was also observed in denuded vessels, eliminating a possible causal role of factors released from vascular endothelium due to cytoskeletal disruption.

We utilized several approaches in an effort to better understand the mechanisms responsible for the potentiation of pressure-induced [Ca$^{2+}$]; increases by cytochalasin D or latrunculin A in small cerebral arteries. Disruption of the actin filamentous system resulted in almost complete dilation of pressurized arteries and, consequently, a significant increase in arterial wall tension. Arterial wall tension is considered to be one of the potential mechanisms for triggering the pressure-induced myogenic responses in small arteries (12, 33, 40) and could therefore be responsible for the additional Ca$^{2+}$ influx associated with cytochalasin D or latrunculin A-induced dilation. However, this does not seem likely because cytochalasin D produced an increase in [Ca$^{2+}$]; in arteries that were already completely dilated with BDM. In addition, inhibition of myosin light chain kinase with wortmannin (5 μM) resulted in a complete arterial dilation with no significant changes in pressure- or high-K$^+$-induced [Ca$^{2+}$]; responses. Collectively, these data do not support a role for wall tension as the major cause for the additional increases in [Ca$^{2+}$]; induced by actin cytoskeletal disruption in our study.

A link between cytoskeletal structure and intracellular levels of Ca$^{2+}$ has been suggested in other studies, and a number of underlying mechanisms have been proposed (23, 27, 28, 35, 44, 49). The actin cytoskeleton may itself be part of the intracellular Ca$^{2+}$ store, and actin depolymerization or treadmilling might result in cytoplasmic Ca$^{2+}$ elevation (28). This mechanism probably is not responsible for the cytochalasin D effect in our experiments because there was no change in [Ca$^{2+}$]; in response to cytochalasin D application in the presence of nifedipine in arteries depolarized with high K$^+$. Therefore, an augmentation of the pressure-induced [Ca$^{2+}$]; response by cytochalasin D or latrunculin A may be the result of the following: 1) a direct modulatory effect on the function of L-type channels, 2) augmentation of pressure-induced membrane depolarization with a subsequent effect on voltage-dependent Ca$^{2+}$, channels, or 3) both. The finding that cytochalasin D further increased the Ca$^{2+}$ level in pressurized arteries depolarized with high-K$^+$ solution, suggests that disruption of F-actin eliminates its inhibitory effect on Ca$^{2+}$ channels, resulting in an enhanced Ca$^{2+}$ influx.

Application of cytochalasin D also resulted in an additional depolarization of VSM cells in the wall of pressurized (60 mmHg) cerebral arteries (Fig. 7), indicating that the actin cytoskeleton can modulate the activity of ion channels different from L-type Ca$^{2+}$ channels. A role of the actin cytoskeleton in the regulation of ion channel activity has been demonstrated in a number of cellular types, and both channel inhibition and activation have been reported (1, 5, 15, 31, 37, 41, 49). Disruption of the actin cytoskeleton with cytochalasins resulted in significant potentiation of Cl$^-$ channel activity responsible for volume regulation in different cell types (1, 5, 21, 41) and an induction of Na$^+$ channel activity, but an inhibition of K$^+$ channel currents in A6 epithelial cells (5). In some studies (21, 23, 41), stabilization of the actin cytoskeleton with phalloidin, which prevents depolymerization, abolished the channel effect of cytochalasins. Chaetoglobosin C, a cytochalasin that does not depolymerize actin, failed to affect ion channel activity (41). These observations argue in favor of a specific actin cytoskeleton-mediated effect of cytochalasins on ion channels. Mechanically gated ion channels are difficult to activate in a whole cell configuration, but actin cytoskeletal disruption resulted in the acceleration and considerable enhancement of currents through mechanosensitive channels (27, 46, 49). These observations suggest that intact cortical cytoskeleton normally protects plasma membrane from undergoing significant increases in tension during mechanical deformation.

There is limited information on the role of the actin cytoskeleton in the regulation of ion channel function in smooth muscle cells. The effect of actin cytoskeletal disruption on the function of L-type Ca$^{2+}$ channels has been studied in smooth muscle cells isolated from the ileum and in cultured cells from the aorta; either no effect (50 μM cytochalasin B) (32) or inhibition (100 μM cytochalasin D) (31) has been reported. Differences between these findings and our observations in this study may be explained in part by the fact that voltage-dependent Ca$^{2+}$ channels in cerebral artery VSM cells are sensitive to mechanical load, and this mechanism might contribute to the pressure-induced elevation in intracellular Ca$^{2+}$ (29). Disruption of the actin cytoskeleton might increase the mechanosensitivity of these Ca$^{2+}$ channels that, if present, cannot be observed on the single cell level.

There is also evidence that the actin cytoskeleton inhibits the activity of K$^+$ channels in a smooth muscle cell line (DDT; MP-2), and that depolymerization of actin with cytochalasin B disinhbits K$^+$ channel activity, resulting in membrane hyperpolarization (15). However, it seems that this mechanism cannot explain our findings because actin filament disruption was clearly associated with depolarization rather than hyperpolarization of smooth muscle in small cerebral arteries (Fig. 7).

A recent study (48) showed that activation of nonselective cation channels is involved in the pressure-
induced VSM depolarization of rat posterior cerebral arteries. Therefore, additional depolarization induced by cytochalasin D in pressurized vessels might result from the potentiation of the activity of these channels by actin cytoskeletal disruption. Both inhibition of K⁺ channels and activation of nonselective cation channels are potential mechanisms for explaining the cytochalasin D-induced depolarization observed in this study.

Mechanisms mediating actin cytoskeletal regulatory effects on ion channels are not well understood. Negative regulation of Ca²⁺ influx through mechanosensitive cation channels by actin filaments has been shown in fibroblasts, an effect that was mediated by integrin-induced actin polymerization and enhanced ABP-280 protein-dependent cross-linking of actin filaments (27, 49). Cipolla et al. (7) recently showed that a similar mechanism might operate in small cerebral arteries where an elevation of intraluminal pressure resulted in actin polymerization evidenced by a substantial decrease in VSM cell G-actin content. Furthermore, our present data suggest that pressure-induced actin filament reorganization might not only be important in determining actin-myosin interactions and force production, but may also serve to modulate Ca²⁺ influx into VSM cells. Whether this effect is a purely mechanical, i.e., effected by actin anchoring or cross-linking, or mediated by diffusible second messenger molecules such as tyrosine and RhoA kinases, protein kinase C, or cAMP-dependent protein kinases, has yet to be resolved.

In conclusion, our data demonstrate an important role for actin cytoskeletal dynamics in the regulation of pressure-induced Ca²⁺ influx, membrane depolarization, and myogenic tone in pressurized small cerebral arteries. Modulation of actin structural organization in VSM cells under physiological and pathophysiological conditions might therefore be an additional and novel mechanism for controlling cerebrovascular resistance and blood flow.

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