

## REVIEW ARTICLE

# The role of actin filament dynamics in the myogenic response of cerebral resistance arteries

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The myogenic response has a critical role in regulation of blood flow to the brain. Increased intraluminal pressure elicits vasoconstriction, whereas decreased intraluminal pressure induces vasodilatation, thereby maintaining flow constant over the normal physiologic blood pressure range. Improved understanding of the molecular mechanisms underlying the myogenic response is crucial to identify deficiencies with pathologic consequences, such as cerebral vasospasm, hypertension, and stroke, and to identify potential therapeutic targets. Three mechanisms have been suggested to be involved in the myogenic response: (1) membrane depolarization, which induces  $\text{Ca}^{2+}$  entry, activation of myosin light chain kinase, phosphorylation of the myosin regulatory light chains ( $\text{LC}_{20}$ ), increased actomyosin MgATPase activity, cross-bridge cycling, and vasoconstriction; (2) activation of the RhoA/Rho-associated kinase (ROCK) pathway, leading to inhibition of myosin light chain phosphatase by phosphorylation of MYPT1, the myosin targeting regulatory subunit of the phosphatase, and increased  $\text{LC}_{20}$  phosphorylation; and (3) activation of the ROCK and protein kinase C pathways, leading to actin polymerization and the formation of enhanced connections between the actin cytoskeleton, plasma membrane, and extracellular matrix to augment force transmission. This review describes these three mechanisms, emphasizing recent developments regarding the importance of dynamic actin polymerization in the myogenic response of the cerebral vasculature.

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## INTRODUCTION—THE MYOGENIC RESPONSE

Cognition and brain integrity are critically dependent on appropriate control of blood flow within the cerebral circulation, with insufficient flow leading to ischemia and excessive flow provoking small-vessel rupture and blood–brain barrier disruption. Cerebral blood flow is controlled through the interplay of several physiologic factors that regulate the contractile state of vascular smooth muscle cells within the walls of cerebral arteries and arterioles. These factors include intravascular pressure and circulating or locally released vasoactive molecules.<sup>1,2</sup>

Bayliss<sup>3</sup> made the seminal observation that small arteries are sensitive to intravascular pressure, with vasoconstriction occurring in response to an increase in pressure and vasodilatation in response to a decrease in pressure. This mechanism, known as the *myogenic response*,<sup>2</sup> maintains blood flow constant during variations in systemic pressure, i.e., flow autoregulation.<sup>4</sup> The sensitivity of resistance vessels (< ~200  $\mu\text{m}$  internal diameter) to pressure can be traced to cellular mechanisms inherent to the smooth muscle cells, i.e., *intrinsic* myogenic mechanisms.<sup>2</sup> The modulatory actions of *extrinsic* vasoactive molecules are superimposed on these *intrinsic* mechanisms to match blood flow to metabolic demand under dynamic physiologic conditions,<sup>2</sup> such as regional neuronal activity.<sup>1</sup>

Vascular smooth muscle cells of cerebral resistance vessels, therefore, respond to an increase in intravascular pressure and

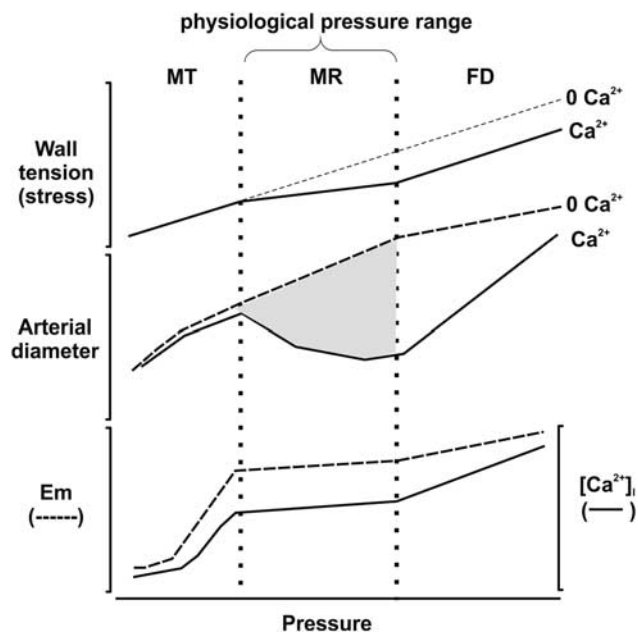
increased wall tension by contracting, with diameter progressively decreasing with pressure elevation beyond a threshold level (Figure 1).<sup>5,6</sup> The intrinsic development of myogenic tone permits flow autoregulation by reducing flow with pressure elevation, and permitting dilatation and increased flow with pressure reduction. Each resistance vessel has a different operating range of pressure,<sup>2,7</sup> e.g., rat middle cerebral arteries exhibit myogenic constriction between ~60 and 130 mm Hg,<sup>6,8,9</sup> but penetrating arterioles are active between ~20 and 140 mm Hg.<sup>10</sup> Outside the autoregulatory range, diameter (Figure 1) and flow (Figure 2) increase proportionally with pressure in the presence of physiologic extracellular-free  $\text{Ca}^{2+}$  concentrations. High pressure overwhelms the capacity of the smooth muscle cells to generate increased force, leading to forced dilatation and unregulated flow augmentation with further pressure increase (Figures 1 and 2).<sup>2,11</sup>

The cerebral myogenic response is independent of, but modulated by extrinsic factors from the endothelium (including nitric oxide, prostacyclin, electrical coupling, endothelin-1, and thromboxane  $\text{A}_2$ ), perivascular nerves (e.g., nitric oxide, calcitonin gene-related peptide, and serotonin), the blood (e.g., angiotensin II), or released locally from astrocytes (e.g., prostaglandins and  $\text{K}^+$  ions) surrounding smaller arterioles (i.e., neurovascular coupling).<sup>2,7</sup> *In vivo* measurement of the myogenic response is complicated by these extrinsic factors, but the experimental

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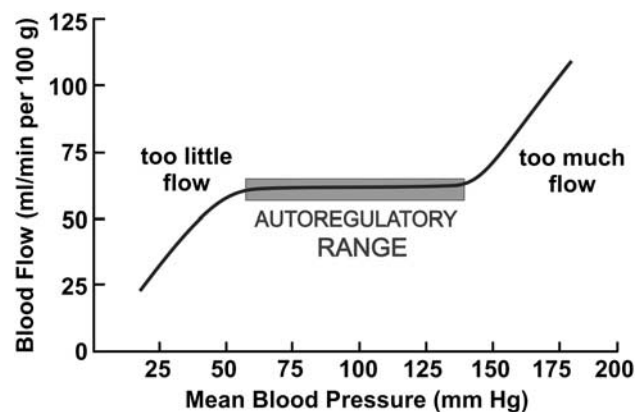


**Figure 1.** The relationship between wall tension, arterial diameter, membrane potential or  $[Ca^{2+}]_i$ , and intraluminal pressure in rat cerebral arteries. Three phases of the diameter response to increased intraluminal pressure have been identified. As pressure is increased toward the normal physiologic range, myogenic tone (MT) begins to develop. Myogenic reactivity (MR) is observed within the physiologic pressure range, beyond which forced dilatation (FD) occurs as myogenic vasoconstriction fails to counteract the high intraluminal pressure. The myogenic response within the physiologic pressure range is shown in gray shading and is the difference between the diameter versus pressure curves in the absence and presence of extracellular  $Ca^{2+}$ . Circumferential wall tension or stress is thought to be the stimulus for the myogenic response. Importantly, membrane potential (Em) and  $[Ca^{2+}]_i$  increase as pressure is increased to 40 to 60 mm Hg, but exhibit modest changes over the physiologic pressure range. Redrawn from Figure 8 of Osol *et al* (2002).<sup>11</sup>

approach of *in vitro* pressure myography facilitates analysis of the molecular basis of the myogenic response and its modulation by individual factors in isolation.<sup>2,7</sup>

Inappropriate myogenic control of cerebral blood flow is observed in animal models of hypertension, stroke, and diabetes, and is prognostic of neurologic deterioration and poor outcome in patients with these conditions.<sup>12–17</sup> Current strategies to optimize cerebral perfusion pressure and improve flow autoregulation are crude (e.g., volume expansion, dopamine, or epinephrine to increase systemic pressure) and not appropriate in some clinical situations.<sup>16</sup> Progress in the treatment of dysfunctional cerebral autoregulation requires elucidation of the molecular basis of the myogenic response and the defects responsible for abnormal myogenic control of cerebral blood flow.

Much of our knowledge of the cellular mechanisms of excitation–contraction coupling (the process whereby a stimulus, e.g., a contractile agonist or an increase in intraluminal pressure, induces contraction) and its regulation in vascular smooth muscle has come from studies of large conduit arteries. This is due principally to the size of these vessels, which provide adequate amounts of tissue for biochemical analysis. Since resistance arteries of  $<200\ \mu\text{m}$  internal diameter are primarily responsible for blood flow regulation,<sup>18</sup> it is essential that the molecular mechanisms that regulate contraction and relaxation of such vessels will be studied directly. Recent advances, particularly improvements in the sensitivity of detection and quantification of



**Figure 2.** The relationship between blood flow and perfusion pressure in rat middle and posterior cerebral arteries. The autoregulatory region extends from  $\sim 60$  to 130 mm Hg and is indicated by the shading. The presence of a myogenic response maintains flow constant over a wide range of pressure. Outside the autoregulatory range, there is too little flow at  $<60$  mm Hg and, at high pressure, force generation by vascular smooth muscle cells is insufficient to counteract the distending force of intracellular pressure, resulting in forced dilatation and too much flow.

protein phosphorylation levels,<sup>8,9,19</sup> have enabled direct analysis of signal transduction pathways involved in the control of contraction and relaxation of smaller vessels, including the myogenic response of cerebral and other resistance arteries.

### THE MECHANOSENSOR

The mechanosensor responds to an increase in intraluminal pressure and transduces this stimulus to vasoconstriction. Candidate mechanosensors include the integrins and G protein-coupled receptors. Integrins are transmembrane glycoproteins that link the extracellular matrix, via interaction with proteins such as collagen, laminin, and fibronectin,<sup>20,21</sup> to the cytoskeleton, via interaction with focal adhesion proteins, and thereby transduce mechanical force across the plasma membrane. Evidence has been provided in support of a role for integrins in the myogenic response,<sup>22</sup> which was found to be suppressed by integrin-specific peptides and blocking antibodies.<sup>20,21,23</sup> Smooth muscle integrins are located in membrane-associated dense plaques, structural analogs of focal adhesions of cultured cells.<sup>24</sup> Integrin stimulation (clustering) induces tyrosine phosphorylation and activation of focal adhesion kinase (FAK) and Src family tyrosine kinases, and increased tyrosine phosphorylation is associated with the myogenic response.<sup>25,26</sup> Furthermore, the myogenic response of cerebral arteries is sensitive to tyrosine kinase inhibition.<sup>27</sup> There are likely several important substrates of these activated tyrosine kinases, but only some have been identified. Candidates, based on agonist- and depolarization-induced tyrosine phosphorylation in airway and vascular smooth muscles, include the cytoskeletal proteins paxillin<sup>28–30</sup> and talin,<sup>31</sup> the adapter protein p130 Crk-associated substrate (CAS)<sup>32</sup> and p42/44 mitogen-activated protein kinases (MAPKs).<sup>33</sup> Stimulation of  $\alpha_5\beta_1$  integrin has been implicated in activation of the L-type  $Ca^{2+}$  channel in vascular myocytes via a tyrosine phosphorylation cascade involving FAK, Src, and various focal adhesion proteins, such as paxillin and vinculin.<sup>34</sup>

$G_{q/11}$ -coupled receptors (specifically  $AT_1$  receptors) have also been proposed as mechanosensors in the myogenic response, with mechanically activated receptors adopting an active conformation that enables G protein coupling, activation of phospholipase C, opening of TRPC channels, and membrane depolarization.<sup>35</sup>

## THE ROLE OF $Ca^{2+}$ IN THE MYOGENIC RESPONSE

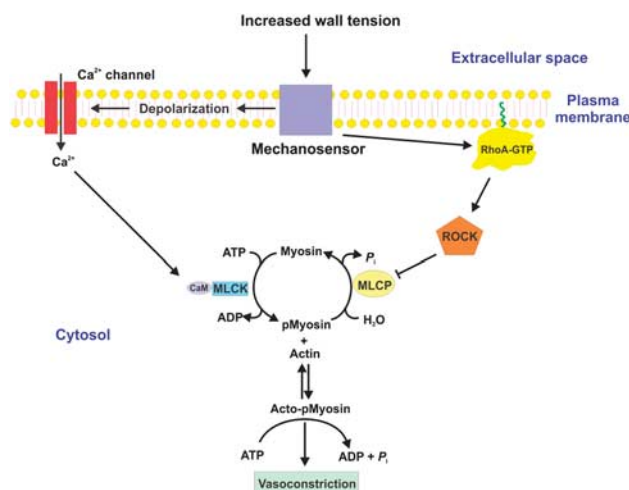
The primary mechanism for the activation of vascular smooth muscle contraction involves an increase in cytosolic-free  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) in response to a variety of contractile stimuli, including smooth muscle cell membrane depolarization elicited by neurotransmitters released from nerve terminals, occupancy of a range of smooth muscle plasma membrane G protein-coupled receptors by ligands transported in the bloodstream or released from neighboring cells (e.g., endothelial cells, parenchymal cells, fibroblasts, and adipocytes), and an increase in intraluminal pressure.<sup>2,36,37</sup> This increase in  $[Ca^{2+}]_i$  results from  $Ca^{2+}$  entry from the extracellular space and  $Ca^{2+}$  release from intracellular stores, principally the sarcoplasmic reticulum.

Increased arterial wall tension because of an increase in intraluminal pressure is detected by the mechanosensor, which evokes membrane potential depolarization via activation of nonselective cation channels, leading to opening of voltage-gated  $Ca^{2+}$  channels in the sarcolemma.<sup>2,6</sup>  $Ca^{2+}$  then enters the cell and decreases its electrochemical gradient (Figure 3).  $Ca^{2+}$  binds to calmodulin and the  $(Ca^{2+})_4$ -calmodulin complex activates myosin light chain kinase (MLCK).<sup>38,39</sup> MLCK phosphorylates a unique substrate, the two 20-kDa regulatory light chain subunits (LC<sub>20</sub>) of myosin II.<sup>40</sup> This phosphotransferase reaction, whereby a phosphoryl group is covalently attached to Ser-19 of LC<sub>20</sub>, is necessary and sufficient for activation of the MgATPase activity of actomyosin, cross-bridge cycling and contraction of demembranated smooth muscle.<sup>41</sup>

It was initially considered that the myogenic response is due exclusively to increased MLCK activity.<sup>6</sup> However, pressure elevation also evokes asynchronous  $Ca^{2+}$  waves within individual vascular smooth muscle cells because of release of  $Ca^{2+}$  from the sarcoplasmic reticulum,<sup>42,43</sup> similarly to the  $Ca^{2+}$  waves observed during agonist-induced contraction.<sup>44</sup> Thus, myogenic constriction is suppressed by membrane hyperpolarization, block of voltage-gated  $Ca^{2+}$  channels, removal of extracellular  $Ca^{2+}$ , or application of ML-7 (which inhibits MLCK activity).<sup>2,6,43,45,46</sup> The extent of myogenic depolarization must be precisely controlled to obtain an appropriate increase in  $[Ca^{2+}]_i$ . This control is provided by negative-feedback activation of large-conductance  $Ca^{2+}$ -activated  $K^+$  channels<sup>2,47,48</sup> and voltage-gated  $K^+$  channels.<sup>46,48–52</sup>

## $Ca^{2+}$ SENSITIZATION VIA INHIBITION OF MYOSIN LIGHT CHAIN PHOSPHATASE

Knot and Nelson<sup>6</sup> showed that an increase in intravascular pressure in rat cerebral arteries from 60 to 100 mmHg depolarized the membrane potential by 8 mV and increased  $[Ca^{2+}]_i$  by  $\sim 55$  nmol/L. Pressure-induced depolarization, leading to activation of voltage-gated  $Ca^{2+}$  channels, entry of extracellular  $Ca^{2+}$  and  $Ca^{2+}$ -dependent activation of MLCK, is clearly necessary for myogenic vasoconstriction.<sup>6,42</sup> However, these changes are not sufficient to explain the phenomenon.<sup>2,7,11,53–56</sup> On the basis of the three phases described in Figure 1, which are characterized by distinct pressure-dependent changes in  $[Ca^{2+}]_i$  that do not parallel arterial diameter, Osol *et al*<sup>11</sup> suggested the existence of additional regulatory mechanisms in the myogenic response. One such mechanism involves the inhibition of myosin light chain phosphatase (MLCP) via Rho-associated kinase (ROCK) or protein kinase C (PKC), leading to an increase in LC<sub>20</sub> phosphorylation and vasoconstriction without a change in  $[Ca^{2+}]_i$ . This process is generally referred to as  $Ca^{2+}$  sensitization (Figure 3) and was first described for agonist-induced smooth muscle contraction.<sup>57–59</sup> The ability of inhibitors of ROCK and PKC, or suppression of RhoA or ROCK with dominant-negative mutants, to block myogenic constriction increased the possibility that  $Ca^{2+}$  sensitization may also be involved in the myogenic response.<sup>2,11,14,54–56,60–62</sup>



**Figure 3.** Pressure-induced vasoconstriction via increased  $[Ca^{2+}]_i$  and via  $Ca^{2+}$  sensitization mediated by inhibition of myosin light chain phosphatase (MLCP). The mechanosensor detects an increase in arterial wall tension, which leads to membrane depolarization, opening of voltage-gated  $Ca^{2+}$  channels, and influx of  $Ca^{2+}$  from the extracellular space. Extracellular-free  $[Ca^{2+}]$  is  $\sim 2.5$  mM and cytoplasmic-free  $[Ca^{2+}]$  at rest is  $\sim 120$  to  $150$  nmol/L. After stimulation, the free  $[Ca^{2+}]$  in the cytoplasm rises to  $\sim 300$  to  $500$  nmol/L. This increase in  $[Ca^{2+}]_i$  saturates the four  $Ca^{2+}$ -binding sites of calmodulin (CaM), leading to activation of  $Ca^{2+}$ /CaM-dependent myosin light chain kinase (MLCK). Activated MLCK phosphorylates the two 20-kDa light chain subunits of myosin II at Ser-19, which activates cross-bridge cycling and vasoconstriction, driven by the energy derived from the hydrolysis of ATP within the head regions of phosphorylated myosin bound to actin filaments. Detection of an increase in wall tension by the mechanosensor also leads to activation of the small GTPase RhoA. The mechanism of activation of RhoA likely involves activation of a guanine nucleotide exchange factor, which catalyzes GDP-GTP exchange on RhoA. This induces dissociation of a guanine nucleotide dissociation inhibitor and a conformational change involving exposure of a hydrophobic geranylgeranyl moiety on RhoA. RhoA-GTP then translocates from the cytosol to the plasma membrane where it attaches to the membrane via the geranylgeranyl moiety (indicated in green) and activates Rho-associated kinase (ROCK). Activated ROCK phosphorylates the myosin targeting subunit of MLCP, MYPT1, at Thr-855 (and perhaps Thr-697), resulting in inhibition of phosphatase activity, increased phosphorylation of LC<sub>20</sub> at Ser-19 by MLCK, and enhanced contraction, without an additional change in  $[Ca^{2+}]_i$ . ROCK can also phosphorylate CPI-17 at Thr-18, which thereby becomes a potent inhibitor of MLCP; however, this does not occur during the myogenic response of rat cerebral arteries. Protein kinase C (PKC) can also phosphorylate CPI-17 at Thr-38 but, since this does not occur during the myogenic response of rat cerebral arteries, this mechanism cannot explain the role of PKC in the myogenic response.

Myosin light chain phosphatase is a type 1 protein serine/threonine phosphatase. The native enzyme is a trimer composed of a catalytic subunit (PP1c $\delta$ ), a regulatory MYPT1 subunit (myosin phosphatase targeting subunit)<sup>63</sup> and a 20- to 21-kDa subunit that is not required for activity or regulation.<sup>64</sup> ROCK phosphorylates two sites in MYPT1 (Thr-697 and Thr-855 in rat sequence numbering), both of which result in reduced phosphatase activity.<sup>65,66</sup> ROCK<sup>67</sup> and PKC<sup>68</sup> phosphorylate CPI-17, a 17-kDa cytosolic protein that becomes a potent inhibitor of MLCP when phosphorylated at Thr-38.<sup>69</sup> In its phosphorylated state, CPI-17 binds to the catalytic subunit of MLCP and inhibits the activity of the holoenzyme. The ROCK-catalyzed phosphorylation of MYPT1 and CPI-17 thereby shifts the MLCK:MLCP activity ratio in favor of the kinase, resulting in an increase in LC<sub>20</sub> phosphorylation at Ser-19 and contraction without a change in  $[Ca^{2+}]_i$ .

While MLCP exhibits constitutive activity, it is subject to complex regulation, both inhibition and activation.<sup>64</sup> We will focus on mechanisms of inhibition of MLCP activity since they are largely responsible for  $\text{Ca}^{2+}$  sensitization, the mechanism whereby contraction occurs without a change in  $[\text{Ca}^{2+}]$ .<sup>57</sup> A variety of ligands interact with G protein-coupled receptors that are linked to members of the  $\text{G}_{12/13}$  family of heterotrimeric G proteins. Ligand occupancy of the receptor activates guanine nucleotide exchange factors such as LARG, PDZ-RhoGEF, and p115RhoGEF,<sup>57,70</sup> which in turn activate the small GTPase RhoA by catalyzing GDP-GTP exchange.<sup>57,71</sup> RhoA-GTP activates ROCK, which phosphorylates the regulatory subunit of MLCP, leading to inhibition of phosphatase activity.<sup>72</sup> Studies with PKC inhibitors have suggested a role for this kinase in the myogenic response of resistance arteries, including cerebral arteries,<sup>14,54,73,74</sup> but the underlying mechanism remains unclear. For example, the PKC inhibitors GF109203X (a general PKC inhibitor) and Gö6976 (a selective inhibitor of the  $\text{Ca}^{2+}$ -dependent PKC isoforms) suppressed the myogenic response of rat middle cerebral arteries.<sup>8</sup> This could have been explained by inhibition of pressure-induced phosphorylation of CPI-17 at Thr-38, the PKC site;<sup>69</sup> however, CPI-17 was not phosphorylated at any pressure examined (10, 60, or 100 mm Hg). Furthermore, while GF109203X induced vasodilatation at 100 mm Hg, there was no change in phosphorylation of MYPT1 at either of the two phosphatase inhibitory sites (Thr-697 and Thr-855) or in phosphorylation of  $\text{LC}_{20}$ . We concluded that PKC-dependent  $\text{Ca}^{2+}$  sensitization via phosphorylation of CPI-17 and MYPT1, which would lead to MLCP inhibition, does not contribute to the myogenic response. We can also conclude from these experiments that the PKC inhibitors do not block the myogenic response by inhibiting ROCK.

Integrins have been widely implicated in activation of the Rho family of small GTPases.<sup>75</sup> Since ROCK is a prominent downstream target of RhoA,  $\text{Ca}^{2+}$  sensitization via the RhoA/ROCK pathway attracted a great deal of interest in the context of the myogenic response. Attenuation of this pathway by inhibitors of ROCK, or dominant-negative mutants of RhoA or ROCK, suppressed the myogenic response.<sup>14,54,55,61,62,76</sup> Direct demonstration that increased intraluminal pressure induced phosphorylation of MYPT1 supported an important role for  $\text{Ca}^{2+}$  sensitization in the myogenic response of cerebral resistance arteries.<sup>8</sup> Myogenic vasoconstriction, which was markedly inhibited by pretreatment with two structurally distinct ROCK inhibitors, Y27632 and H1152, correlated with an increase in phosphorylation of MYPT1 at Thr-855 and of  $\text{LC}_{20}$  at Ser-19. Importantly, these increases in phosphorylation were observed when intraluminal pressure was increased from 60 to 100 mm Hg, i.e., within the physiologic range. Interestingly, no changes in phosphorylation of MYPT1 at Thr-697, or of CPI-17 at Thr-38, were detected. MYPT1 exhibited basal phosphorylation (at 10 mm Hg intraluminal pressure) at both Thr-697 and Thr-855, suggesting that MLCP is partially inhibited under these conditions, whereas no phosphorylation of CPI-17 was detected at any pressure.<sup>8</sup>

$\text{Ca}^{2+}$  sensitization is also involved in the contractile response of cerebral resistance arteries to serotonin in the presence of physiologic levels of intraluminal pressurization. Vasoconstriction induced by serotonin at 10 mm Hg was observed to correlate with an increase in phosphorylation of  $\text{LC}_{20}$ (Ser-19), but with no evidence of a change in MYPT1 phosphorylation.<sup>9</sup> In contrast, a combination of increased intraluminal pressure (to 60 mm Hg) and serotonin elicited further increases in vasoconstriction, MYPT1(Thr-855) and  $\text{LC}_{20}$ (Ser-19) phosphorylation, and an increase in MYPT1(Thr-697) phosphorylation, all of which were attenuated by ROCK inhibition.<sup>9</sup> Similar effects were observed in response to a supra-physiologic pressure increase (to 140 mm Hg). Once again, no phosphorylation of CPI-17 was detected under any conditions.<sup>9</sup> These data imply that the cellular mechanism(s) used by extrinsic

vasoconstrictor factors to evoke contraction are influenced by the presence of myogenic activation. In the example cited above, serotonin-evoked contraction was due exclusively to MLCK activation in the absence of intraluminal pressurization. However, in the presence of myogenic depolarization and a preexisting activation of MLCK at 60 mm Hg, subsequent vasoconstriction in response to serotonin required that an alternative mechanism of MLCP inhibition be used to increase the level of  $\text{LC}_{20}$ (Ser-19) phosphorylation and force generation. A major goal for future research must be to understand the molecular basis of the integration of extrinsic vasoconstrictor and dilator signaling with the cellular mechanisms that are responsible for the myogenic response.

## ACTIN CYTOSKELETAL DYNAMICS

Accumulating evidence indicates that dynamic cytoskeletal reorganization influences force generation in smooth muscle.<sup>24</sup> Actin filaments of the contractile apparatus are anchored to the cytoplasmic tails of integrins by a complex of adhesion proteins, and to each other at cytosolic dense bodies. Integrins and focal adhesion complexes are connected to, and reinforced by the cortical actin cytoskeleton. Together, these elements distribute and transmit force generated by cross-bridge cycling at the level of the contractile apparatus (myofilaments) over the cell membrane and to the extracellular matrix. Dynamic reorganization of the actin cytoskeleton is postulated to strengthen these interactions and enhance force transmission. The importance of actin polymerization in smooth muscle contraction was originally suggested by the effects of inhibitors of actin polymerization on the contractile responses of a variety of smooth muscle tissues. Thus, treatment of airway,<sup>77,78</sup> vascular,<sup>28,29,79–83</sup> gastrointestinal,<sup>84</sup> and uterine<sup>81</sup> smooth muscles with latrunculins (which sequester G-actin monomers), or cytochalasins (which cap the fast-growing, barbed end of actin filaments), markedly attenuate contractility. Furthermore, blockade of specific steps in the activation of actin polymerization inhibits contraction in response to a variety of stimuli. For example, dynamic reorganization in agonist-evoked smooth muscle contraction,<sup>24</sup> and possibly the myogenic response,<sup>11,80,85,86</sup> have been attributed to ROCK, PKC, and/or mechanostimulation of FAK.<sup>87–89</sup> The requirement of an intact cytoskeleton for myogenic constriction is suggested by the use of cytochalasins (which induce depolymerization) and jasplakinolide (which enhances polymerization).<sup>80,85,86</sup> Alternatively, latrunculin B is thought to disrupt dynamic reorganization of the actin cytoskeleton in the myogenic response by binding G-actin.<sup>80,85,90</sup> The effects on smooth muscle contractility described above were not attributable to disruption of the contractile apparatus itself (i.e., the myofilaments), suggesting that formation of new actin filaments is essential for normal contractile function.

A large body of evidence supports the conclusion that smooth muscle contraction is accompanied by an increase in F-actin and a decrease in G-actin content.<sup>78,80,86,91–93</sup> Quantification of F- and G-actin levels at rest and after stimulation with acetylcholine (canine trachea) or norepinephrine (carotid artery) indicated that a small proportion of cellular actin (~10% to 12%) undergoes polymerization, the F-actin content increasing from ~70% to 80% at rest to ~80% to 92% after stimulation.<sup>32,89,92,94</sup> This is consistent with the existence of two pools of cellular actin: one ('contractile actin') involved in the contractile machinery, associated with tropomyosin and stabilized in the filamentous form, and the other ('cytoskeletal actin') localized to the cell cortex, not associated with tropomyosin and undergoing reversible polymerization–depolymerization.<sup>95</sup> Contractile and cytoskeletal actins may involve distinct isoforms,<sup>96,97</sup> but this is controversial.<sup>92,98</sup>

### Actin Dynamics in the Myogenic Response

As discussed earlier, the myogenic response requires MLCK activation via increased  $[Ca^{2+}]_i$  and MLCP inhibition via MYPT1 phosphorylation at Thr-855, leading to an increase in LC<sub>20</sub> phosphorylation.<sup>37</sup> Several lines of evidence suggest, however, that the myogenic response of rat cerebral arteries cannot be fully explained by increased MLCK and decreased MLCP activities: (1) depletion of RhoA in rat cerebral arteries in organ culture abolished the contractile response to an increase in intraluminal pressure from 15 to 80 mmHg without affecting LC<sub>20</sub> phosphorylation, i.e., without a change in the MLCK:MLCP activity ratio;<sup>88</sup> (2) microcystin treatment (MLCP inhibition) induced robust LC<sub>20</sub> phosphorylation in RhoA-depleted arteries, but failed to elicit a contractile response;<sup>88</sup> (3) PKC inhibition abolished the myogenic response without a change in phosphorylation of LC<sub>20</sub>, CPI-17, or MYPT1;<sup>8</sup> (4) serotonin-induced vasoconstriction at 10 and 60 mmHg was almost completely abolished by inhibition of ROCK or PKC, but the level of LC<sub>20</sub> phosphorylation declined only to the level observed in control tissues at 60 mmHg, at which pressure a significant myogenic response was observed;<sup>8,9</sup> (5) serotonin increased LC<sub>20</sub> phosphorylation by ~25% at 10 mmHg and ~10% at 60 mmHg, but the vasoconstriction at 60 mmHg was significantly greater;<sup>9</sup> and (6) under three different experimental conditions (an increase in intraluminal pressure from 80 to 120 mmHg, application of serotonin at 80 mmHg, or pressurization to 80 mmHg in the presence of serotonin), pressure- or agonist-induced vasoconstriction was observed without an increase in LC<sub>20</sub> phosphorylation above the preexisting level of ~0.5 mol P<sub>i</sub>/mol LC<sub>20</sub> (El-Yazbi *et al*, submitted). These observations suggested that the myogenic response of cerebral vessels involves a third mechanism, in addition to MLCK activation and MLCP inhibition. Based on the evidence provided above, a likely candidate for this third mechanism involves dynamic regulation of the actin cytoskeleton.

In summary: (1) *de novo* formation of actin filaments is required for force development; (2) actin polymerization and myosin phosphorylation are independent events (but see Chen *et al*<sup>99</sup> for evidence of actin polymerization in response to LC<sub>20</sub> phosphorylation); and (3) both actin filament formation and myosin phosphorylation are required for smooth muscle contraction.

We recently investigated pressure-induced actin polymerization as the potential third mechanism involved in the myogenic response of rat middle cerebral arteries (El-Yazbi *et al*, submitted). Latrunculin B treatment had no effect on resting diameter (at 10 mmHg intraluminal pressure) or on the magnitude of serotonin-induced constriction at 10 mmHg, but induced dilatation to the passive diameter observed in the absence of extracellular Ca<sup>2+</sup> in vessels pressurized to 80 mmHg. This vasodilatation occurred without a change in LC<sub>20</sub> phosphorylation. Latrunculin B also completely dilated vessels precontracted with serotonin at 80 mmHg, and vessels constricted in response to an increase in pressure from 10 to 80 mmHg after serotonin pretreatment. These results are consistent with force generation during the myogenic response, and in response to serotonin in the presence of myogenic tone, being sensitive to sequestration of G-actin. Quantification of G-actin levels under different conditions supported a role for actin polymerization in the myogenic response. G-actin content progressively decreased as intraluminal pressure was increased from 10 to 80 to 120 mmHg. However, serotonin treatment at 10 mmHg was not associated with a significant change in G-actin content. Latrunculin B treatment at 80 mmHg, however, was accompanied by a fourfold increase in G-actin content. Finally, G-actin levels were significantly lower in the presence than in the absence of serotonin at 80 mmHg, and at 80 mmHg after pretreatment with serotonin, compared with 10 mmHg in the presence of serotonin. Therefore, in every case in which vasoconstriction

occurred in the absence of a change in LC<sub>20</sub> phosphorylation, G-actin content was reduced.

Our observation that cerebral arteries treated with latrunculin B at 120 mmHg, or at 80 mmHg in the presence of serotonin, dilated to the passive diameter rather than to the preexisting diameter at 80 mmHg or in the presence of serotonin at 10 mmHg, suggests that increased actin polymerization is not the only process involved in the reorganization of the cytoskeleton. Since LC<sub>20</sub> phosphorylation did not change, we can conclude that sequestration of G-actin by latrunculin B does not affect membrane potential, Ca<sup>2+</sup> influx, MLCK or ROCK activation, or MLCP inhibition. Complete loss of force would be predicted, however, if pressurization evoked a remodeling process that involves breaking of existing connections between the contractile machinery, the plasma membrane, and the extracellular matrix that are required for force distribution and transmission. Severing of existing connections within the cytoskeleton and between the actin cytoskeleton and the contractile machinery would prevent transmission of force generated by cross-bridge cycling to the sarcolemma and extracellular matrix, resulting in complete dilatation.  $\alpha$ -Actinin may have a key role in this regard: recruitment of  $\alpha$ -actinin to membrane-associated dense bodies in tracheal smooth muscle is required for force development but not for actin polymerization or LC<sub>20</sub> phosphorylation.<sup>100</sup>  $\alpha$ -Actinin crosslinks actin filaments and also binds to integrins,<sup>101</sup> which would strengthen the connections between the cytoskeleton and extracellular matrix, thereby enhancing the efficiency of the transmission of force generated by cross-bridge cycling.

Based on accumulating evidence of a change in F-actin: G-actin ratio with pressure elevation in resistance arteries, it is worthwhile to consider the potential physiologic significance of cytoskeletal reorganization in the myogenic response. A well-recognized, fundamental feature of smooth muscle contraction is that force is maintained during sustained contraction, although the level of LC<sub>20</sub>(Ser-19) phosphorylation and the rate of ATP hydrolysis by actomyosin ATPase (i.e., energy consumption) are substantially lower than during the phase of force generation after agonist or mechanical stimulation.<sup>102,103</sup> The molecular basis of this phenomenon of force maintenance at reduced levels of LC<sub>20</sub> phosphorylation and energy consumption, referred to as the 'latch state,' is not known. It has been attributed to: (1) dephosphorylated, slowly cycling cross-bridges,<sup>104</sup> (2) slower kinetics of myosin head interaction with actin when phosphorylated at a single *versus* both heads,<sup>105</sup> and more recently, (3) stimulated actin polymerization.<sup>30</sup> Consistent with the latter view, Jones *et al*<sup>106</sup> showed that F-actin stabilization with phalloidin before Ca<sup>2+</sup>-induced activation of Triton X-100-permeabilized airway smooth muscle was associated with an increased rate of ATP hydrolysis compared with untreated tissues during sustained contractions. The lower level of energy consumption associated with sustained contraction may arise because of a reduction in load on the contractile apparatus as a result of the dynamic reorganization of actin filaments. In the context of the myogenic response, reorganization and strengthening of the cortical cytoskeleton, as well as connections between the contractile apparatus and cytoskeleton at dense bodies may, therefore, optimize the efficiency of energy utilization during sustained contractions that are required to hold arteriolar diameter constant at maintained levels of physiologic intraluminal pressurization. Direct measurements of ATP hydrolysis in the presence and absence of cytoskeletal reorganization after changes in intraluminal pressure are required to substantiate this view.

### Cellular Signaling Mechanisms and Adapter/Effector Proteins Involved in Actin Cytoskeletal Dynamics

As yet, we know very little about the signal transduction pathways that mediate pressure-induced actin cytoskeletal rearrangement during the myogenic response in the cerebral vasculature. Work in

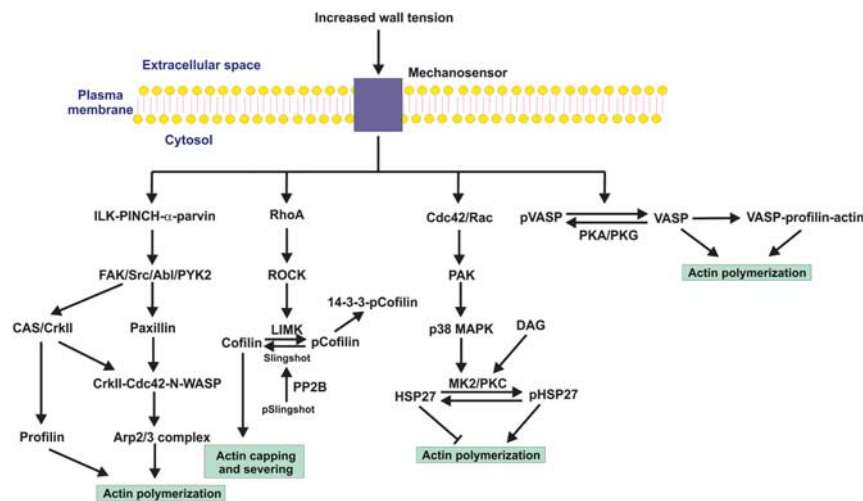
this area is being directed by previous studies with larger, agonist-activated smooth muscle tissues that lack myogenic tone. Based largely on the work of Gunst *et al*<sup>24</sup> with airway smooth muscle, newly formed actin filaments in the subplasmalemmal region are thought to connect to membrane-associated dense plaques, specifically the cytoplasmic domain of  $\beta$  integrins, via linker proteins such as talin and vinculin.  $\beta$  Integrins thereby form a physical continuum that connects the extracellular matrix to the contractile machinery to mediate mechanical force transmission. Several proteins are recruited to the dense plaques on stimulation, e.g., FAK, talin, paxillin, vinculin,<sup>93,107</sup>  $\alpha$ -actinin,<sup>100</sup> and the  $\text{Ca}^{2+}$ -dependent nonreceptor tyrosine kinase PYK2 (proline-rich tyrosine kinase 2).<sup>28</sup> An early event in the response of tracheal smooth muscle to acetylcholine is the recruitment to the membrane of a stable cytosolic trimeric complex<sup>108</sup> composed of the protein kinase ILK (integrin-linked kinase), which binds to the cytoplasmic domain of  $\beta$  integrins and also serves a scaffolding role,<sup>109</sup> PINCH (particularly interesting new cysteine-histidine-rich protein, an LIM (Lin 11, Isl-1, Mec-3) domain-only adapter protein)<sup>110</sup> and  $\alpha$ -parvin (an actin-binding protein).<sup>111</sup> Association of the complex with paxillin and vinculin initiates actin polymerization via the actin nucleation initiating factor N-WASP (neuronal Wiskott-Aldrich syndrome protein) pathway.<sup>108</sup>

**The Neuronal Wiskott-Aldrich Syndrome Protein Pathway.** Various pathways leading to N-WASP activation have been identified. For example, FAK is activated by autophosphorylation at Tyr-397 in response to integrin activation.<sup>112,113</sup> Paxillin tyrosine phosphorylation, which is catalyzed by activated FAK and other tyrosine kinases, has been observed in tracheal<sup>107,114</sup> and vascular

smooth muscles,<sup>28–30</sup> and phosphorylated paxillin activates N-WASP via the adapter protein Crkl and the small GTPase Cdc42 (Figure 4).<sup>91,94</sup>

An alternative pathway to activation of N-WASP involves the focal adhesion protein CAS,<sup>115,116</sup> which is phosphorylated by the tyrosine kinases Src or Abl in response to contractile stimulation.<sup>32</sup> Norepinephrine induced an increase in force, LC<sub>20</sub> phosphorylation, and F-actin: G-actin ratio in canine carotid artery.<sup>116</sup> Downregulation of CAS by antisense oligonucleotide treatment inhibited this norepinephrine-induced force response and the increase in F-actin: G-actin ratio, without affecting LC<sub>20</sub> phosphorylation.<sup>32</sup> Phosphorylation of CAS by Abl increased its binding to Crkl<sup>117</sup> and contractile stimulation increased the association of Crkl with N-WASP,<sup>91</sup> thereby exposing a binding site for the actin-related protein (Arp)2/3 complex to initiate actin polymerization and branching.<sup>91,92,94,117</sup> Silencing of Abl by short-hairpin RNA reduced CAS phosphorylation in resistance arteries on contractile stimulation.<sup>117</sup> Abl itself is activated by phosphorylation at Tyr-412 and this has been shown to occur in vascular smooth muscle cells in response to angiotensin II.<sup>118</sup> PYK2 may also phosphorylate CAS and paxillin.<sup>119,120</sup>

p130 Crk-associated substrate also lies upstream of the actin regulatory protein, profilin, which binds to actin monomers and can promote actin polymerization:<sup>121</sup> binding of the profilin-actin complex to N-WASP can deliver actin monomers to the Arp2/3 complex nucleation site or the barbed end of a growing actin filament.<sup>122</sup> Profilin-mediated actin polymerization has been implicated in the contractile response of canine carotid arteries to norepinephrine, referred to above, with the demonstration that latrunculin A pretreatment or downregulation of profilin expression by antisense oligonucleotide treatment inhibits



**Figure 4.** Potential pathways leading to actin polymerization in the myogenic response. Several signal transduction pathways have been implicated in the regulation of actin polymerization, based largely on studies of agonist stimulation of airway and vascular smooth muscles, but the possibility that one or more of them may have a role in the myogenic response deserves investigation. An early event after stimulation is the recruitment to the plasma membrane of a trimeric complex composed of integrin-linked kinase (ILK), PINCH (particularly interesting new cysteine-histidine-rich protein), and  $\alpha$ -parvin. A variety of tyrosine kinases are capable of phosphorylating paxillin, leading to the sequential activation of N-WASP (neuronal Wiskott-Aldrich syndrome protein) and the actin-related protein (Arp)2/3 complex, which activates actin polymerization. N-WASP can also be activated via p130 CAS (Crk-associated substrate) and the adapter protein, Crkl. In addition, the actin-binding protein profilin lies downstream of CAS and induces actin polymerization. In addition to their role in  $\text{Ca}^{2+}$  sensitization via myosin light chain phosphatase (MLCP) inhibition, RhoA and Rho-associated kinase (ROCK) may be involved in regulation of actin polymerization through the actin capping and severing protein cofilin. Lin 11, Isl-1, Mec-3 kinase (LIMK) is a substrate of ROCK which, when phosphorylated within its activation loop, phosphorylates cofilin at Ser-3. Phosphocofilin (pCofilin) loses the ability to cap and sever actin filaments, so activation of this pathway would stabilize preformed actin filaments in the subplasmalemmal domain. HSP27 is an actin capping protein that inhibits actin polymerization. This effect is alleviated by phosphorylation (pHSP27) by MK2, which lies downstream of p38 MAPK (mitogen-activated protein kinase) and the small GTPase-activated PAK (p21-activated protein kinase). Protein kinase C (PKC), activated by diacylglycerol (DAG), phosphorylates the same sites in HSP27 as does MK2. The vasodilator-stimulated phosphoprotein (VASP) is a PKA and PKG substrate, with phosphorylation (pVASP) preventing its interaction with actin and the profilin-actin complex. Dephosphorylation favors binding of VASP to G-actin and profilin-actin, which enhance actin polymerization. While the role of VASP has not been studied in relation to the myogenic response, the possibility that increased wall tension leads to dephosphorylation of VASP deserves attention.

norepinephrine-induced force development and the increase in F-actin: G-actin ratio, without affecting the increase in LC<sub>20</sub> phosphorylation. Finally, downregulation of CAS prevented the association of profilin with G-actin.<sup>32</sup> Future studies are needed to elucidate the importance and specific components of the N-WASP pathway(s) in the myogenic response of cerebral resistance arteries.

**The ROCK-LIMK-Cofilin Pathway.** In addition to regulating Ca<sup>2+</sup> sensitization via inhibition of MLCP, the small GTPase RhoA affects actin polymerization (Figure 4).<sup>87,123,124</sup> Support for a role for RhoA in the myogenic response came from the observation that RhoA translocates from the cytosol to the plasma membrane during the myogenic response of mesenteric arteries, and the myogenic response is blocked by treatment with C3 exoenzyme, which ADP-ribosylates and inactivates RhoA,<sup>125</sup> or by overexpression of dominant-negative RhoA.<sup>62</sup> Several studies suggest that ROCK is the important downstream target of activated RhoA leading to actin polymerization<sup>126,127</sup> and that ROCK has an important role in the myogenic response of cerebral, renal, and mesenteric vascular beds, as discussed earlier. Inhibition of ROCK activity also abolished UTP-induced actin polymerization and induced dilatation of vessels that were precontracted with UTP.<sup>90</sup>

The actin depolymerizing factor/cofilin family of proteins has been implicated in canine tracheal smooth muscle contraction.<sup>128</sup> Cofilin binds to and severs actin filaments, providing G-actin monomers and short, capped actin filaments with free barbed ends that provide nucleation sites for the formation of new actin filaments by the Arp2/3 complex. Cofilin is phosphorylated at Ser-3 by LIM kinase (LIMK), which is activated by phosphorylation within its activation loop by ROCK, among others.<sup>129</sup> Phosphorylation of cofilin abolished its actin binding and severing function.<sup>130,131</sup> Phosphocofilin binds to the intracellular scaffolding protein, 14-3-3, which sequesters cofilin and protects it from dephosphorylation.<sup>132</sup> The phosphatase(s) responsible for cofilin dephosphorylation in smooth muscle is unknown, but in other cell types two specific cofilin phosphatases have been identified: slingshot and chronophin.<sup>133,134</sup> Slingshot can be activated by dephosphorylation, and studies with cultured cells have implicated calcineurin (the Ca<sup>2+</sup>/calmodulin-dependent type 2B protein serine/threonine phosphatase, PP2B) as the responsible phosphatase.<sup>135</sup> Slingshot has also been shown to dephosphorylate and thereby inactivate LIMK.<sup>136</sup> Acetylcholine-induced contraction of canine tracheal smooth muscle correlates with dephosphorylation (activation) of cofilin, and expression of the dominant-negative phosphomimetic S3E mutant of cofilin in canine tracheal smooth muscle inhibited acetylcholine-induced cofilin dephosphorylation, actin polymerization and contraction.<sup>128</sup> Cofilin function appears to be restricted to a subcellular pool of actin that is distinct from the stable actin filaments involved in actomyosin complex formation and cross-bridge cycling. The latter pool is associated with tropomyosin, which inhibits cofilin binding, whereas the cortical actin pool involved in dynamic polymerization-depolymerization is free of tropomyosin and, therefore, accessible to cofilin.<sup>137,138</sup>

At first glance, it would appear that activation of the ROCK-LIMK-cofilin pathway has the opposite effect to what would be predicted, i.e., alleviation of cofilin's ability to cap and sever actin filaments would prevent the formation of new actin filaments that have been implicated in force development. However, the time course of events is likely to be critical here. Cofilin in resting vascular smooth muscle of the swine carotid artery is phosphorylated at Ser-3 and becomes dephosphorylated in response to K<sup>+</sup>-induced depolarization, which activates voltage-gated Ca<sup>2+</sup> channels to allow the entry of extracellular Ca<sup>2+</sup> and activation of contraction via LC<sub>20</sub> phosphorylation.<sup>30</sup> However, cofilin dephosphorylation is a slow process compared with force development: significant dephosphorylation of cofilin was not

detected until steady-state force had already been developed in response to K<sup>+</sup>. Removal of the depolarizing stimulus resulted in phosphorylation of cofilin. We suggest, therefore, that the ROCK-LIMK-cofilin pathway is constitutively active and cofilin remains phosphorylated and, therefore, may be inactive during the force development phase of the myogenic response. Actin polymerization during this rapid phase is then likely because of activation of the N-WASP pathway. Subsequently, when cofilin becomes dephosphorylated, its actin capping and severing activity is expressed to sustain dynamic actin polymerization during the force maintenance phase of the contractile response. Dephosphorylation of cofilin, therefore, may have a key role in cortical actin polymerization required for force maintenance rather than force development. However, stretch of the rat portal vein in organ culture increased cofilin phosphorylation and the F-actin: G-actin ratio.<sup>139</sup> Furthermore, different contractile agonists have been shown to either increase or have no effect on cofilin phosphorylation in pulmonary arterial ring preparations.<sup>140</sup>

**The HSP27 Pathway.** HSP27 is a well-known chaperone protein that provides protection against physical and chemical stresses. It has also been implicated, however, in regulation of the actin cytoskeleton.<sup>141</sup> The unphosphorylated protein inhibits actin polymerization by capping actin filaments, a property that is lost on phosphorylation.<sup>142</sup> HSP27 is phosphorylated<sup>143</sup> by MK2 (MAPK-activated protein kinase 2),<sup>144</sup> which itself is activated by phosphorylation by p38 MAPK that lies downstream of PAK (p21-activated kinase) and other kinases (Figure 4).<sup>145</sup> Agonist-induced translocation of HSP27 to membranes and the contractile apparatus has been shown in smooth muscle.<sup>93,146</sup> Norepinephrine stimulation of rat mesenteric resistance vessels results in the activation of p38 MAPK (leading to phosphorylation of HSP27) and Src (leading to phosphorylation of paxillin). HSP27 phosphorylation induces the translocation of HSP27 from the cytoskeleton to the cytosol, where it interacts with phosphorylated paxillin, thereby alleviating HSP27-mediated inhibition of actin polymerization and leading to increased F-actin content.<sup>93</sup> To date, there is no evidence implicating HSP27 phosphorylation in the myogenic response, but this pathway is worth investigating in this context. It is also of potentially great importance that PKC $\delta$  and, less effectively, PKC $\alpha$ , have been shown to phosphorylate the same sites in HSP27 as MK2,<sup>144</sup> given that PKC has been implicated in the myogenic response.<sup>8,9,14,54,73,74</sup> Direct evidence of PKC-mediated actin polymerization during the myogenic response has come from measurements of G-actin content in rat gracilis arteries. The myogenic response was inhibited by pretreatment with the PKC inhibitor GF109203X, without an effect on CPI-17 phosphorylation, and increased intraluminal pressure decreased G-actin content in a GF109203X-dependent manner (Moreno-Dominguez *et al*, submitted).

Another heat-shock protein, HSP20, has been implicated in cyclic nucleotide-dependent relaxation of vascular smooth muscle: HSP20 phosphorylated at Ser-16 by cAMP-dependent protein kinase (PKA) competes with phosphocofilin for binding to 14-3-3, leading to an increase in the amount of activated (dephosphorylated) cofilin.<sup>147</sup>

**The Vasodilator-stimulated Phosphoprotein Pathway.** Vasodilator-stimulated phosphoprotein (VASP) is a member of the Ena/VASP family of proteins that are involved in regulation of actin dynamics through actin filament elongation, and have been implicated in cancer cell invasion and metastasis, morphogenesis, axon guidance, endothelial barrier function<sup>148</sup> and, more recently, airway smooth muscle contraction.<sup>149</sup> It binds directly to G-actin to induce actin polymerization<sup>150</sup> and is localized to focal adhesions through interactions with proteins such as zyxin<sup>151</sup> and vinculin.<sup>152</sup> It also recruits profilin-actin heterodimers via its

central proline-rich domain.<sup>153</sup> It contains two principal cyclic nucleotide-dependent protein kinase phosphorylation sites: Ser-153 (predominant PKA site) and Ser-235 (predominant PKG (cGMP-dependent protein kinase) site) with minor phosphorylation of Thr-274 by both kinases.<sup>154</sup> Ser-153 is located within the proline-rich domain and has been implicated in targeting of VASP, whereas Ser-235 and Thr-274 lie within the actin-binding domain and are involved in the regulation of actin polymerization.<sup>155</sup> Phosphorylation inhibits VASP/G-actin interaction and prevents VASP-induced actin filament formation (Figure 4).<sup>156</sup>

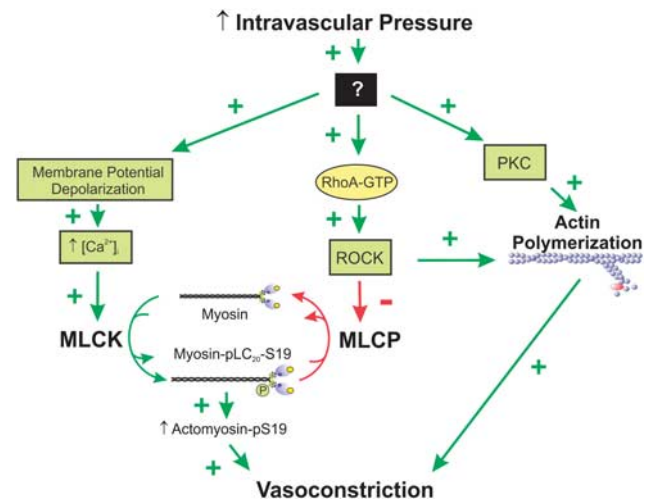
Vasodilator-stimulated phosphoprotein partially colocalizes with  $\alpha$ -actinin and vinculin in isolated aortic smooth muscle cells, consistent with its localization to adhesion plaques and dense bodies.<sup>83</sup> Phosphorylation of VASP decreases during phenylephrine stimulation, and treatment with cytochalasin D inhibits phenylephrine-induced actin polymerization in freshly isolated ferret aortic smooth muscle cells and force development in aortic rings.<sup>83</sup> However, no effect on the phenylephrine-induced increase in LC<sub>20</sub> phosphorylation was observed in the aortic ring preparations treated with cytochalasin D. Knockdown of VASP significantly reduces phenylephrine-induced force generation. Introduction into freshly isolated aortic smooth muscle cells of the expressed EVH1 domain of human EVL (another Ena/VASP family member), fused to the HIV-1 TAT sequence to achieve membrane permeability, inhibits phenylephrine-induced actin polymerization, whereas the corresponding F78S mutant (which should prevent interaction with vinculin and other target proteins)<sup>157</sup> has no effect. Finally, phenylephrine treatment induces modest, transient decreases in phosphorylation of VASP at all three PKA/PKG sites. The potential role of VASP and its dephosphorylation in dynamic actin cytoskeletal regulation in the cerebral circulation will be an important avenue for future exploration.

The relative importance of the pathways regulating actin polymerization is likely to vary according to cell type and mode of activation. It is, therefore, crucially important to identify the signal transduction pathways in diverse vascular beds that respond to specific stimuli. The myogenic response in rat cerebral arteries represents one important example.

## CONCLUDING REMARKS

Evidence has been presented indicative of the involvement of three signaling pathways in the myogenic response (Figure 5): (1) Ca<sup>2+</sup>-induced activation of contraction via MLCK-catalyzed phosphorylation of LC<sub>20</sub>; (2) Ca<sup>2+</sup> sensitization of contraction elicited by Rho-associated kinase-mediated phosphorylation and inhibition of MLCP; and (3) Ca<sup>2+</sup> sensitization of contraction elicited by dynamic regulation of the actin cytoskeleton. It is unclear whether a single or multiple mechanosensors are involved in activation of these three pathways. It is important to emphasize that Ca<sup>2+</sup> sensitization pathways are ineffective in the absence of Ca<sup>2+</sup>-dependent activation of MLCK. Ca<sup>2+</sup> channel blockers and removal of extracellular Ca<sup>2+</sup> are well known to abolish the myogenic response.<sup>43,48</sup> This does not mean, however, that MLCP inhibition and actin polymerization are not involved in the myogenic response since cross-bridge cycling absolutely requires LC<sub>20</sub> phosphorylation, which generally requires an increase in [Ca<sup>2+</sup>]<sub>i</sub>. It is important to note, therefore, that any treatment or manipulation that directly or indirectly affects the activity of voltage-gated Ca<sup>2+</sup> channels or Ca<sup>2+</sup> influx may confound interpretation of the involvement of MLCP inhibition and dynamic regulation of the actin cytoskeleton.

The cerebral myogenic response is key to three fundamental mechanisms: (1) it maintains cerebral blood flow constant during variations in systemic pressure (i.e., flow autoregulation);<sup>11</sup> (2) it establishes a regional blood flow reserve so that local flow may be



**Figure 5.** Three signaling pathways are implicated in the myogenic response: (1) Ca<sup>2+</sup>-induced activation of contraction via myosin light chain kinase (MLCK)-catalyzed phosphorylation of LC<sub>20</sub> in response to membrane depolarization; (2) Ca<sup>2+</sup> sensitization of contraction elicited by Rho-associated kinase (ROCK)-mediated phosphorylation of MYPT1 and inhibition of myosin light chain phosphatase (MLCP); and (3) Ca<sup>2+</sup> sensitization of contraction elicited by dynamic regulation of the actin cytoskeleton mediated by protein kinase C (PKC) and ROCK. The mechanisms whereby an increase in wall tension and intravascular pressure activate these signaling pathways remain to be identified, but likely involve integrins and G protein-coupled receptors as mechanosensors.

matched to metabolic demand by allowing vasodilatation from a partially constricted state,<sup>2</sup> and (3) it provides a critical shield that protects brain capillaries and the blood-brain barrier from damage by elevated intravascular pressure.<sup>2,158,159</sup> Inappropriate myogenic behavior impacts each of these. Enhanced myogenic constriction at low pressure and reduced force generation with increasing pressure are observed in several conditions that have an impact on neurologic function in humans, including hypertension, cerebral vasospasm, hemorrhagic and ischemic stroke, diabetes, and head trauma.<sup>1,12-17,158,160,161</sup> Impaired dilatation at low perfusion pressure in hypertension compromises blood flow reserve and increases the risk of ischemia. Conversely, impaired myogenic constriction leading to uncontrolled elevated levels of blood flow (i.e., 'breakthrough') in malignant hypertension, hemorrhagic stroke, type 2 diabetes, and trauma is associated with secondary damage owing to blood-brain barrier disruption, small vessel rupture (microbleeds), edema, and increased intracranial pressure. Notably, loss of the myogenic response may be a critical prelude to irreversible, 'true' vascular remodeling within the arterial wall involving hypertrophy and hyperplasia.<sup>161-163</sup> Only eutrophic remodeling of existing wall material around a smaller lumen is observed in small arteries that possess a myogenic response (conduit vessels lacking myogenic tone exhibit 'true' remodeling).<sup>163</sup> However, the loss of myogenic behavior and an inability of vessels to withstand the stress of increased pressure-evoked wall tension stimulate an adaptive, structural response to control blood flow.<sup>161-163</sup> Understanding the molecular defects that result in the loss of myogenic responsiveness will provide crucial information concerning the fundamental changes that contribute to this key event in the progression of vascular dysfunction.

There remain numerous gaps in our understanding of the signaling pathways involved in the myogenic response, their applicability to smooth muscle tissues in general, and whether they are involved in acute contractile responses and slower effects such as cell migration and proliferation. Essentially, actin



polymerization is regulated by proteins that control the availability of monomeric G-actin and of free barbed (plus) ends of short, nucleating actin filaments.<sup>24</sup> While relatively little is known regarding the identity of actin regulatory proteins in the context of smooth muscle contraction and the myogenic response, a number of candidate proteins have emerged from extensive knowledge of the regulation of actin polymerization–depolymerization in cell migration.<sup>164,165</sup> In the context of the cerebral circulation, we are embarking on a period of identification of the proteins involved in dynamic regulation of the actin cytoskeleton as an essential component of the myogenic response. This will lead to elucidation of defects responsible for pathologies of the cerebral circulation and identification of novel drug targets.

Finally, it is worth closing with a note about the concept of  $Ca^{2+}$  sensitization of smooth muscle contraction.  $Ca^{2+}$  sensitization can be defined as an increase in force without a change in  $[Ca^{2+}]_i$ . This can be achieved by activation of signaling pathways that lead to inhibition of MLCP activity and an increase in actin polymerization. It must be emphasized that activation of either of these mechanisms without a sufficiently high  $[Ca^{2+}]_i$  will not induce contraction since there must be a threshold level of  $LC_{20}$  phosphorylation to activate cross-bridge cycling.

#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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