Implications of \( \alpha_v \beta_3 \) Integrin Signaling in the Regulation of \( \text{Ca}^{2+} \) Waves and Myogenic Tone in Cerebral Arteries

Rania E. Mufti, Anil Zechariah, Maria Sancho, Neil Mazumdar, Suzanne E. Brett, Donald G. Welsh

Objective—The myogenic response is central to blood flow regulation in the brain. Its induction is tied to elevated cytosolic \( [\text{Ca}^{2+}] \), a response primarily driven by voltage-gated \( \text{Ca}^{2+} \) channels and secondarily by \( \text{Ca}^{2+} \) wave production. Although the signaling events leading to the former are well studied, those driving \( \text{Ca}^{2+} \) waves remain uncertain.

Approach and Results—We postulated that \( \alpha_v \beta_3 \) integrin signaling is integral to the generation of pressure-induced \( \text{Ca}^{2+} \) waves and cerebral arterial tone. This hypothesis was tested in rat cerebral arteries using the synergistic strengths of pressure myography, rapid \( \text{Ca}^{2+} \) imaging, and Western blot analysis. GRGDSP, a peptide that preferentially blocks \( \alpha_v \beta_3 \) integrin, attenuated myogenic tone, indicating the modest role for sarcoplasmic reticulum \( \text{Ca}^{2+} \) release in myogenic tone generation. The RGD peptide was subsequently shown to impair \( \text{Ca}^{2+} \) wave generation and myosin light chain 20 (MLC\(_{20}\)) phosphorylation, the latter of which was attributed to the modulation of MLC kinase and MLC phosphatase via MYPT1-T855 phosphorylation. Subsequent experiments revealed that elevated pressure enhanced phospholipase \( \text{C} \gamma_1 \) phosphorylation in an RGD-dependent manner and that phospholipase \( \text{C} \) inhibition attenuated \( \text{Ca}^{2+} \) wave generation. Direct inhibition of inositol 1, 4, 5-trisphosphate receptors also impaired \( \text{Ca}^{2+} \) wave generation, myogenic tone, and MLC\(_{20}\) phosphorylation, partly through the T-855 phosphorylation site of MYPT1.

Conclusions—Our investigation reveals a hitherto unknown role for \( \alpha_v \beta_3 \) integrin as a cerebral arterial pressure sensor. The membrane receptor facilitates \( \text{Ca}^{2+} \) wave generation through a signaling cascade, involving phospholipase \( \text{C} \gamma_1 \), inositol 1, 3, 4 trisphosphate production, and inositol 1, 4, 5-trisphosphate receptor activation. These discrete asynchronous \( \alpha_v \beta_3 \) events facilitate MLC\(_{20}\) phosphorylation and, in part, myogenic tone by influencing both MLC kinase and MLC phosphatase activity. (Arterioscler Thromb Vasc Biol. 2015;35:2571-2578. DOI: 10.1161/ATVBAHA.115.305619.)

Key Words: arterial pressure ■ \( \text{Ca}^{2+} \) waves ■ cerebral arteries ■ myography ■ phosphorylation

The cardiovascular system is composed of a muscular pump and a distribution network of arteries, veins, and capillaries, which attend to tissue metabolic demands. Within this integrated system, resistance arteries control the magnitude and distribution of tissue blood flow by responding to neurotransmitters, metabolites, and mechanical forces, such as intravascular pressure.\(^1-3\) The myogenic response refers to an artery’s ability to constrict to elevated pressure, a response of particular prominence in the brain.\(^4,5\) Past studies have shown that the myogenic response is transduced by changes in myosin light chain kinase (MLCK) and phosphatase (MLCP) activity, along with cytoskeletal organization.\(^6\) These downstream events are intimately, although not exclusively, linked to global \( [\text{Ca}^{2+}] \), an event primarily controlled by voltage-gated \( \text{Ca}^{2+} \) channels.\(^3,8\) Although extracellular \( \text{Ca}^{2+} \) influx is of key importance, recent work has revealed that pressure also mobilizes \( \text{Ca}^{2+} \) from the sarcoplasmic reticulum (SR) to modestly facilitate tone development.\(^10-12\) Smooth muscle \( \text{Ca}^{2+} \) waves are slow asynchronous events, which spread from end-to-end in a voltage insensitive manner.\(^10,13\) This internal release of \( \text{Ca}^{2+} \) from SR induces myosin light chain 20 (MLC\(_{20}\)) phosphorylation by: (1) activating MLCK and (2) inhibiting MLCP via phosphorylation of MYPT1-T855.\(^5\) Although \( \text{Ca}^{2+} \) waves have been linked to myogenic tone particular at pressures where vessels are more hyperpolarized,\(^10\) the signaling mechanisms driving these transient events, are yet to be fully elucidated.

Integrin receptors are membrane-bound glycoproteins that link the extracellular matrix to the actin cytoskeleton. Given their cellular positioning, they are often viewed as presumptive mechanotransducers; an interpretation consistent with integrin blockade (ie, \( \alpha_v \beta_3 \), \( \alpha_v \beta_3 \)) impairing pressure-induced constriction in skeletal muscle resistance arteries.\(^14\) As key members of focal adhesion complexes, integrins play an essential role in regulating other structural and signaling proteins. This includes focal adhesion kinase, an enzyme which when active, forms a high-affinity binding site with phospholipase \( \text{C} \gamma_1 \) (PLC\(_{\gamma_1}\)) inducing its phosphorylation.\(^15-19\) Activation of PLC\(_{\gamma_1}\) could facilitate inositol 1, 3, 4 trisphosphate (IP\(_3\))

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production and drive Ca\textsuperscript{2+} wave generation by opening its like named receptor (IP\textsubscript{R}).\textsuperscript{18,20,21} In this study of the cerebral circulation, we investigated whether the α\textsubscript{v}β\textsubscript{3} integrin facilitates Ca\textsuperscript{2+} wave generation and myogenic tone through a signaling cascade, involving PLCγ1 and IP\textsubscript{R}. To test this hypothesis, we used a methodological approach that synergistically combined pressure myography, rapid Ca\textsuperscript{2+} imaging, and Western blot analysis. We found that inhibiting α\textsubscript{v}β\textsubscript{3} integrin partially impaired myogenic tone, demonstrating the modest role of SR Ca\textsuperscript{2+} waves in the generation of myogenic tone. Consistent with the functional observations, elevated pressure was also shown to increase Ca\textsuperscript{2+} wave generation in rat cerebral arteries. These pressure-induced Ca\textsuperscript{2+} events were attenuated by α\textsubscript{v}β\textsubscript{3}-blocking peptides and facilitated MLC\textsubscript{20} phosphorylation by augmenting MLCK activity and inhibiting MLCP via the phosphorylation of MYPT1-T855. Further experiments revealed that: (1) elevated pressure augmented PLCγ1 phosphorylation in a α\textsubscript{v}β\textsubscript{3}-dependent manner and (2) inhibiting PLC or IP\textsubscript{R} diminished phosphorylation. Cumulatively, this investigation demonstrates that pressure-induced Ca\textsuperscript{2+} waves, in turn, enable MLC\textsubscript{20} phosphorylation and, in part, myogenic tone by regulating MLCK and MLCP activity.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Blocking αβ3 Integrin Attenuates Myogenic Tone

Experiments began by isolating cerebral arteries and using quantitative polymerase chain reaction to assess integrin mRNA expression. This analysis revealed the presence of 5α and 4β integrins, including those comprising αβ3 and αβ1 (Figure 1A and 1B in the online-only Data Supplement). Next, endothelial intact arteries were cannulated and rapidly pressurized from 15 to 80 mm Hg; diameter was subsequently assessed under control conditions and after treatment with peptides that preferentially block the αβ3 integrin. The GRGDSP peptide attenuated myogenic tone in a concentration-dependent manner, whereas its inactive analog GRADSP had no effect (Figure 1C and 1D in the online-only Data Supplement). Given that the myogenic response is intrinsic and partially driven by endothelial integrins may introduce a confounding influence, proceeding experiments were repeated in endothelial denuded arteries. Figure 1E and IF in the online-only Data Supplement shows that GRGDSP peptide attenuated myogenic tone in a concentration-dependent manner. The inactive analog GRADSP had no effect except at the highest concentration (210 μmol/L) where a notable constriction was observed. Antagonizing β3 integrin with a monoclonal antibody also attenuated pressure-induced responses (diameter at 15 mm Hg—diameter at 80 mm Hg) in cerebral arteries (control, −23.1±3.5 versus antibody treated, 11.99±2.54; p<0.05; n=5). Further control experiments implicated a role for the αβ3 integrin in the myogenic response as the EILDVSPT-blocking peptide diminished vasomotor tone (Figure II in the online-only Data Supplement). The inactive peptide analog had no significant effect, barring the highest concentration tested (210 μmol/L).

#### Blocking αβ3 Integrin Attenuates Ca\textsuperscript{2+} Waves and MLC\textsubscript{20} Phosphorylation

Having ascertained that a 70-μmol/L concentration of αβ3 integrin–blocking peptide moderates the myogenic response, we next sought to investigate its effects on Ca\textsuperscript{2+} signaling. As above, arteries were rapidly pressurized to 80 mm Hg and after 5 minutes, diameter was assessed under control conditions and after treatment with RGD/RAD peptide-diltiazem (30 μmol/L, L-type Ca\textsuperscript{2+} channel blocker) or ryanodine (10 μmol/L; SR depletion agent). Cerebral arteries treated with αβ3-blocking peptide (RGD) displayed attenuated myogenic tone (Figure 1A and 1B), whereas the inactive analog (RAD) had no effect (Figure 1C and 1D). Incubating RGD/RAD-treated vessels with diltiazem, further attenuated myogenic tone confirming that L-type Ca\textsuperscript{2+} channels remained active after αβ3 integrin blockade. In contrast, ryanodine did not attenuate myogenic tone in arteries pretreated with RGD peptide; it did, however, modestly attenuate tone in RAD-treated vessels (Figure 1E–1H). The latter observation aligns with previous myogenic experiments using ryanodine.\textsuperscript{10} Together, these observations indirectly suggest that αβ3 integrins contribute to SR store mobilization and Ca\textsuperscript{2+} wave generation, independent of L-type Ca\textsuperscript{2+} channel activity. The preceding analysis assumes that RGD peptides have few nonmyogenic effects.

Given the preceding novel but suggestive observations, we next used rapid Ca\textsuperscript{2+} imaging to directly examine whether αβ3 integrin was involved in the generation of pressure-induced Ca\textsuperscript{2+} waves. Cerebral arteries were loaded with Fluo-4 AM and Ca\textsuperscript{2+} waves monitored in the absence and presence of the αβ3 integrin–blocking peptide (RGD) or its inactive analog (RAD). Pressurization to 80 mm Hg increased the frequency and percentage of cells firing Ca\textsuperscript{2+} waves (Figure 2); past work has noted that these events are voltage insensitive and unaffected by diltiazem, an L-type Ca\textsuperscript{2+} channel blocker.\textsuperscript{10} Pressure-induced Ca\textsuperscript{2+} waves were markedly attenuated by the RGD but not by the RAD peptide. In a complimentary manner, the RGD but not RAD peptide diminished the ability
application of diltiazem but not ryanodine. The preceding perturbations did not influence MYPT1 T-697 phosphorylation (Figure 3C), a second regulatory site on this targeting subunit.

### Blocking αβ3 Integrin Impairs Downstream PLCγ1 Signaling

Integrin receptors can recruit and activate PLCγ1.19,22 Consistent with αβ3 integrin mobilizing this phospholipase, elevated pressure augmented PLCγ1-Y783 phosphorylation, an effect attenuated by RGD but not RAD peptide (Figure 4A). The general PLC inhibitor U73122 was subsequently shown to diminish Ca2+ waves, whereas the inactive analog (U73433) was without effect (Figure 4B and 4C). PLC inhibition by U73122 also markedly reduced MLC20 phosphorylation and myogenic tone development (Figure IV in the online-only Data Supplement), whereas its inactive analog (U73433) did not influence these parameters. Phosphorylation of PLCγ1 will result in diacylglycerol and IP3 production, the latter of which triggers Ca2+ waves via IP3Rs. Three sequential data sets connect these elements, the first being that IP3R inhibitors (xestospongin C or 2-APB) decreased the frequency and number of cells firing Ca2+ waves in arteries pressurized to 80 mm Hg (Figure 5A–5D). Next, bath application of xestospongin C and 2-APB (Figure 5E–5H) decreased that component of the myogenic response insensitive to diltiazem, an L-type Ca2+ channel inhibitor. Finally, this study showed that while pressure-induced increases in MLC20 phosphorylation were moderated by diltiazem, further attenuation was induced by adding xestospongin C on top of this L-type Ca2+ channel blocker (Figure 6A). Xestospongin C’s ability to reduce MLC20 phosphorylation can be ascribed, in part, to MLCP inhibition, as this IP3R inhibitor reduced MYPT1-T855 phosphorylation in diltiazem-treated arteries (Figure 6B). MYPT1-T697 phosphorylation was unaffected, neither by changes in intravascular pressure nor by the addition of diltiazem or xestospongin C (Figure 6C).

### Discussion

A century ago, Bayliss described the intrinsic ability of arteries to respond to intravascular pressure.4 The myogenic response is prominent in the cerebral circulation and is enabled by a global rise in cytosolic [Ca2+] that drives MLC20 phosphorylation and also actin polymerization.3,10 Although pressure-induced increases in cytosolic [Ca2+] are principally dependent on membrane depolarization and L-type Ca2+ channel activation, recent studies have noted a modest role for SR Ca2+ release.3,8–10,23 Ca2+ waves are asynchronous, voltage-insensitive events that propagate from end-to-end, enhancing MLC20 phosphorylation through MLCK activation and by inhibiting MLCP via MYPT1-T855 phosphorylation in diltiazem-treated arteries (Figure 6B). MYPT1-T697 phosphorylation was unaffected, neither by changes in intravascular pressure nor by the addition of diltiazem or xestospongin C (Figure 6C).

Consistent with this perspective, we observed that cerebral arteries treated with RGD but not RAD peptide reduced the ability of pressure to induce MYPT1-T855 phosphorylation (Figure 3B). In addition, we found that MYPT1-T855 phosphorylation in RGD-treated arteries was reduced by the further perturbed MYPT1-T697 phosphorylation.

of intravascular pressure to induce MLC20 phosphorylation (Figure 3A). Exposing RGD-treated arteries to diltiazem further reduced pressure-induced MLC20 phosphorylation, whereas ryanodine application had no effect. The latter observation is consistent with αβ3 integrin signaling directing the phosphorylation of this key contractile protein through Ca2+ wave generation. The ability of αβ3 integrin and Ca2+ waves to enhance MLC20 phosphorylation could arise, in part, to MLCP inhibition, as mediated through MYPT1-T855 phosphorylation (Figure III in the online-only Data Supplement).10 Consistent with this perspective, we observed that cerebral arteries treated with RGD but not RAD peptide reduced the ability of pressure to induce MYPT1-T855 phosphorylation (Figure 3B). In addition, we found that MYPT1-T855 phosphorylation in RGD-treated arteries was reduced by the further...
subunits are expressed in cerebral arteries (Figure IA and IB in the online-only Data Supplement) and past studies using skeletal muscle arteries have shown that $\alpha V \beta 3$, along with $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$, are key players in pressure-induced responses. Integrin receptors are part of focal adhesion complexes, which when stimulated, recruit a full complement of signaling proteins. The recruited proteins include PLCγ1, a phospholipase that could in theory initiate Ca$^{2+}$ waves by generating IP$_3$ and stimulating IP$_3$Rs. In keeping with this historical narrative, we began to explore the linkage between luminal pressure, integrin signaling, Ca$^{2+}$ wave generation, and myogenic tone in cerebral arteries. We focused on $\alpha V \beta 3$ integrin considering (1) mRNA expression in cerebral arteries, (2) the availability of specific pharmacological tools, and (3) its ability to influence myogenic tone independent of voltage and L-type Ca$^{2+}$ channel activity, as in the case of $\alpha \beta 1$ and $\alpha \beta 1$. A functional assay was initially developed where cerebral arteries were rapidly pressurized (15–80 mm Hg) and tone assessed during and after a 5-minute test period; this protocol was repeated in the absence and presence of an increasing concentration of RGD or RAD peptides. Analogous to skeletal muscle resistance arteries, we observed that the RGD peptide, which preferentially targets $\alpha V \beta 3$ integrin, elicited a concentration-dependent dilation. The control RAD peptide was without effect except at the highest concentration where a constriction was observed (Figure I in the online-only Data Supplement). Having established a working peptide concentration of 70 $\mu$ mol/L, we next addressed whether $\alpha V \beta 3$ integrin blockade attenuated myogenic tone by altering L-type Ca$^{2+}$ channel activity or SR Ca$^{2+}$ wave generation. In this regard, cerebral arteries were pretreated with the peptide and tone was assessed before or...
after blockade of L-type Ca\textsuperscript{2+} channels or Ca\textsuperscript{2+} wave generation. It was rationalized that if RGD-treated arteries failed to dilate either to diltiazem or to ryanodine, then α\textsubscript{v}β\textsubscript{3} integrin blockade was impairing that particular aspect of Ca\textsuperscript{2+} regulation. Control experiments revealed that RAD-treated arteries developed less myogenic tone after the addition of diltiazem or ryanodine (Figure 1). In contrast, ryanodine’s ability to attenuate myogenic tone was selectively lost in RGD-treated arteries, consistent with α\textsubscript{v}β\textsubscript{3} integrins triggering SR Ca\textsuperscript{2+} waves. This result was verified by loading cerebral arteries with Fluo-4 AM and pressurizing vessels to 80 mm Hg, inducing production of Ca\textsuperscript{2+} waves. These asynchronous events (frequency, ≈0.2 Hz) were evident in ≈80% of smooth muscle cells and blocking α\textsubscript{v}β\textsubscript{3} induced a notable reduction in Ca\textsuperscript{2+} waves; the control RAD peptide was without effect. Together, these observations indicate that α\textsubscript{v}β\textsubscript{3} integrin and its associated signaling pathway play a primary role in the triggering of Ca\textsuperscript{2+} waves. This analysis assumes that RGD peptides have few nonmyogenic effects.

Mufti et al\textsuperscript{10} previously showed that Ca\textsuperscript{2+} waves augment myogenic tone by providing a proportion of the Ca\textsuperscript{2+} required to enhance MLC\textsubscript{20} phosphorylation. Consequently, it followed that this key parameter should be measured before and after α\textsubscript{v}β\textsubscript{3} inhibition. Findings in Figure 3 showed that RGD but not the control RAD peptide lowered MLC\textsubscript{20} phosphorylation in pressurized cerebral arteries. It also showed that in RGD-treated arteries, the subsequent application of diltiazem but not ryanodine (an SR-depleting agent) further reduced MLC\textsubscript{20} phosphorylation. This finding is consistent with α\textsubscript{v}β\textsubscript{3} integrin driving this key phosphorylation step through Ca\textsuperscript{2+} wave suppression rather than through substantive changes in L-type Ca\textsuperscript{2+} channel activity. In theory, Ca\textsuperscript{2+} waves could facilitate MLC\textsubscript{20} phosphorylation by (1) binding to calmodulin and relieving auto inhibition of MLCK \textsuperscript{24} or (2) inhibiting MLCP through a Rho kinase–linked pathway previously shown to promote MYPT1-T855 phosphorylation (control data reprinted in Figure III in the online-only Data Supplement).\textsuperscript{10} In regard to the latter observation, we confirmed that the α\textsubscript{v}β\textsubscript{3}-blocking peptide RGD impaired pressure-induced phosphorylation of MYPT1-T855. We also showed that in RGD-treated arteries, diltiazem but not ryanodine attenuated MYPT1-T855 phosphorylation, consistent with α\textsubscript{v}β\textsubscript{3} integrin–linked Ca\textsuperscript{2+} waves modulating the targeting subunit of MLCP. Unlike the T855 site, MYPT1-T697 phosphorylation was unaffected by peptide treatment or the application of L-type Ca\textsuperscript{2+} channel blockers or Ca\textsuperscript{2+} wave inhibitors.

The inherent ability of α\textsubscript{v}β\textsubscript{3} to target myogenic mechanisms largely independent of L-type Ca\textsuperscript{2+} channels suggests...
that >1 integrin is involved in smooth muscle mechanotransduction. This perspective is consistent with Figure II in the online-only Data Supplement, which highlights the ability of α4β1 peptide blockers to moderate pressure-induced constriction. Similarly, Martinez-Lemus et al14 have shown in skeletal muscle arteries that peptide/antibody inhibitors of αvβ3 and α4β1 both negatively affected myogenic tone. Recent work has also implied a role for α5β1 in the regulation of L-type Ca2+ channel and other ionic conductances involved in the control of arterial VM.16,30,31 Such effects seem to be mediated through signaling pathways linked to the SRC (sarcoma) family of tyrosine kinases.30,32

Cell culture work focused on integrin signaling has noted that these receptors can mobilize focal adhesion kinase. 33 Through a high-affinity binding site, this kinase can phosphorylate PLCγ1 at Y783, a response that results in activation and induction of IP3 production. 19 Two key sets of experiments link PLC, in particular PLCγ1, to αvβ3 integrins and the induction of pressure-induced Ca2+ waves. First, biochemical measures conducted on paired cerebral arteries revealed that...
intravascular pressure enhanced PLCβ1-Y783 phosphorylation, a response diminished by RGD but not by the control RAD peptide (Figure 4A). Second, we found that the percentage of cells firing Ca²⁺ waves along with event frequency decreased in arteries treated with U73122, a broad-spectrum PLC inhibitor (Figure 4B–4E). Similar changes were absent in vessels treated with the inactive analogue U73343. Although Figure III in the online-only Data Supplement shows that U73122 markedly attenuated MLC₂₀ phosphorylation and myogenic tone, it is important to recognize that these robust effects cannot be singularly attributed to IP₃ production and the loss of Ca²⁺ waves. Diacylglycerol generation will be equally impaired by PLC inhibition and this effect will, in turn, affect TRP (transient receptor potential) channel activation, arterial depolarization, and Ca²⁺ influx via the voltage-gated Ca²⁺ channels.14,35

Given the preceding findings, we hypothesized that IP₃Rs are essential to the triggering of Ca²⁺ waves, MLC₂₀ phosphorylation and consequently myogenic tone in the cerebral circulation. Three sets of experiments strengthen this linkage, the first centered on Ca²⁺ waves and the inherent ability of IP₃R inhibitors (xestospongin C or 2-APB) to reduce the frequency and number of cells firing these events in cerebral arteries (Figure 5A–5D). The second involved assessing myogenic tone over a full pressure range and showing that the same inhibitors attenuated the residual component of the myogenic response insensitive to L-type Ca²⁺ channel blockade (Figure 5E–5H). Finally, Western blot analysis confirmed that in diltiazem-treated arteries, IP₃R inhibition further reduced residual MLC₂₀ phosphorylation (Figure 6A). This finding is consistent with Ca²⁺ waves modulating MLCK activation and secondarily MLCP inhibition through the MYPT1 T-855 phosphorylation site (Figure 6B and 6C).

The preceding findings highlight for the first time the importance of α₁β₁ signaling in activating IP₃R, to facilitate Ca²⁺ wave generation, MLC₂₀/MYPT1 phosphorylation, and in part myogenic tone development. This work compliments the findings by Westcott et al.,36 who previously implicated the involvement of PLC and IP₃R in pressure-induced Ca²⁺ waves. The presence of pressure-induced Ca²⁺ waves raises an important question centered on why these transient events, along with extracellular Ca²⁺ influx are both required to maintain the myogenic tone by providing a proportion of the Ca²⁺ needed to drive MLC₂₀ phosphorylation. By elucidating the mechanosensitive component of myogenic tone in the cerebral circulation, this study advances the foundational knowledge in Ca²⁺ dynamics, vascular tone development, and ultimately the regulation of brain blood flow.

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Disclosures
None.

References

Summary
Myogenic tone is driven by changes in cytosolic [Ca²⁺], an event intimately tied to L-type Ca²⁺ channel activity and secondarily to Ca²⁺ wave generation.3,10 Focusing on Ca²⁺ waves, this study elucidated an unknown role for α₁β₁, integrins and downstream PLCβ1 signaling in the induction of pressure-sensitive events. It further showed that Ca²⁺ waves enhanced myogenic tone by providing a proportion of the Ca²⁺ needed to drive MLC₂₀ phosphorylation. By elucidating the mechanosensitive component of myogenic tone in the cerebral circulation, this study advances the foundational knowledge in Ca²⁺ dynamics, vascular tone development, and ultimately the regulation of brain blood flow.
In the cerebral circulation, the myogenic response plays a critical role in maintaining constant tissue perfusion in a dynamically changing environment. This response is intimately tied to changes in cytosolic $[Ca^{2+}]$, primarily driven by $L$-type $Ca^{2+}$ channels and secondarily by $SR Ca^{2+}$ waves. In this study, we show that integrin signaling is essential to the induction of pressure-induced $Ca^{2+}$ waves and ultimately to tone development in cerebral vasculature. The modulation of integrin signaling could provide a means of moderating vessel hypercontractility associated with hypertension and diabetes mellitus.

**Significance**

In the cerebral circulation, the myogenic response plays a critical role in maintaining constant tissue perfusion in a dynamically changing environment. This response is intimately tied to changes in cytosolic $[Ca^{2+}]$, primarily driven by $L$-type $Ca^{2+}$ channels and secondarily by $SR Ca^{2+}$ waves. In this study, we show that integrin signaling is essential to the induction of pressure-induced $Ca^{2+}$ waves and ultimately to tone development in cerebral vasculature. The modulation of integrin signaling could provide a means of moderating vessel hypercontractility associated with hypertension and diabetes mellitus.
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Supplemental Material

Implications of $\alpha_v\beta_3$ Integrin signalling in the Regulation of $\text{Ca}^{2+}$ Waves and Myogenic Tone in Cerebral Arteries

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Supplemental Figure I. Integrin mRNA expression and the effects of α₃β₃ blocking peptide on myogenic tone. A) Illustrative gel of the primary integrin mRNA products expressed in cerebral arteries; actin sample was diluted 20X. B) Quantitative PCR analysis of integrin mRNA
expression in cerebral arteries. C-F) Cerebral arteries were rapidly pressurized from 15 to 80 mmHg for 5 min and diameter was assessed. This experiment was performed under control conditions and following treatment with $\alpha_\beta_3$ blocking- (RGD, 20, 70, 210 µM) or $\alpha_\beta_3$ control-(RAD, 20 70, 210 µM) peptide. Representative traces and summary data (n=6 arteries from 6 animals for each group) are presented for endothelial intact (C and D) and endothelial denuded (E and F) arteries. * denotes significant increase from the preceding group.
Supplemental Figure II. Concentration dependent effects of α₄β₁ blocking peptide on myogenic tone. Cerebral arteries were rapidly pressurized from 15 to 80 mmHg for 5 min and diameter was assessed. This experiment was performed under control conditions and following treatment with α₄β₁ blocking- (LDV, 20, 70, 210 µM) or α₃β₃ control- (LEV, 20, 70, 210 µM) peptide. Representative traces and summary data (n=6 arteries from 6 animals for each group) are presented in A and B, respectively. * denotes significant increase from the preceding group.
Supplemental Figure III. Effects of elevated pressure on MLC$_{20}$ and MYPT1 phosphorylation. Paired cerebral arteries were pressurized from 20 to 80 mmHg; vessels were subsequently frozen in acetone and processed for phosphorylation assessment of MLC$_{20}$, MYPT1-T855 or MYPT1-T697. Representative western blots and summary data (n=4-5 arterial pairs from 4-5 animals) are present in A-C. * denotes significant increase from 20 mmHg. This control data was originally published in Mufti et al., 2010 and has been reprinted to provide comparative context for Figure 3.
Supplemental Figure IV. Effects of Phospholipase C inhibition on MLC\textsubscript{20} phosphorylation and myogenic tone. A) Paired cerebral arteries were pressurized to 80 mmHg for 5 min under control conditions or following treatment with U73433 (500 nM, control analog) or U73122 (500 nM, general PLC inhibitor). Vessels were subsequently frozen in acetone and processed for MLC\textsubscript{20} phosphorylation. Representative western blots and summary data (n=4 arterial pairs from 4 animals for each group) are presented. B) Cerebral arteries were pressurized (20 to 100 mmHg) and arterial diameter measured in the absence and presence of U733343 or U73122. Representative traces and summary data (n=6 arteries from 6 animals) are presented in B and C, respectively. * denotes significant increase from the preceding experimental step.
Materials and Methods

Implications of α₃β₃ Integrin signalling in the Regulation of Ca²⁺ Waves and Myogenic Tone in Cerebral Arteries

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Animal Procedures

Animal procedures were approved by the Animal Care and Use Committee at the University of Calgary. Briefly, female Sprague-Dawley rats (10-12 weeks of age) were euthanized via carbon dioxide asphyxiation. The brains were carefully removed and placed in cold phosphate-buffered saline solution (pH 7.4) containing (in mM): 138 NaCl, 3 KCl, 10 Na₂HPO₄, 2 NaH₂PO₄, 5 glucose, 0.1 CaCl₂, and 0.1 MgSO₄. Middle and posterior cerebral arteries were carefully dissected out of surrounding tissue and cut into 2-3 mm segments.

Quantitative PCR

Total RNA was isolated from rat cerebral arteries using the RNeasy plus micro kit following manufacturer's recommendations. Reverse transcription was performed using the Quantitect reverse transcription kit. For the negative control groups, all components except the reverse transcriptase were included in the reaction mixtures. Real-Time PCR using validated Prime Time qPCR primers was performed using the Kapa SYBR Fast Universal qPCR Kit. Rat beta actin was utilized as the reference gene. Control reactions and those containing cDNA from cerebral arteries were performed with 1 ng of template per reaction. The running protocol extended to 45 cycles consisting of 95°C for 5 seconds (s), 55°C for 15 s and 72°C for 10 s using an Eppendorf Realplex 4 Mastercycler. PCR specificity was checked by dissociation curve analysis. No fluorescence was detected from template control samples. The mRNA abundance of the various integrin isoforms was determined by normalizing the cycle threshold (Ct) values to that of beta actin.

Vessel Myography

Arterial segments were mounted in a customized arteriograph and superfused with warm (37°C) physiological salt solution (PSS; pH 7.4; 21% O₂, 5% CO₂, balance N₂) containing (in mM): 119 NaCl, 4.7 KCl, 20 NaHCO₃, 1.1 KH₂PO₄, 1.2 MgSO₄, 1.6 CaCl₂, and 10 glucose.¹ Mounted arteries were equilibrated for 30 min at 15 mmHg and contractile responsiveness assessed by brief (~10 s) exposure to 60 mM KCl. Next, mounted arteries were rapidly
pressed from 15 to 80 mmHg; external diameter was monitored for 5 minutes (automated edge detection system; IonOptix, MA, USA). Arteries were then returned to 15 mmHg and equilibrated (20 min) with a low volume of PSS containing: 1) RGD or RAD (20, 70 or 210 μM); 2) RGD or RAD (70 μM) followed by ryanodine (10 μM), diltiazem (30 μM) and zero externally added Ca^{2+} + 2 mM EGTA (referred to as Ca^{2+} free PSS); or 3) LDV or LEV (20, 70 or 210 μM); or 4) a αvβ3 integrin monoclonal antibody (1:50). Most, but not all experiments were performed on endothelial denuded arteries. Endothelial cells were removed by passing air bubbles through the lumen \(^2\), \(^3\); successful removal was confirmed by the loss of bradykinin-induced dilation in pressure constricted vessels (80 mmHg). Functional experiments were also performed on endothelial denuded arteries exposed to a pressure step protocol (20-100 mmHg, 20 mmHg steps) under control conditions and in the presence of: 1) diltiazem (30 μM) followed by xestospongin C (10 μM) and Ca^{2+} free PSS; or 2) diltiazem (30 μM) followed by 2-APB (50 μM) and Ca^{2+} free PSS.

### Ca^{2+} Wave Measurements and Analysis

Whole arteries denuded of endothelium, were equilibrated for 20 min in HEPES buffer containing (in mM): 134 NaCl, 6 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 HEPES and 10 glucose.\(^4\) Arteries were then exposed (45 min) to HEPES buffer containing 10 μM fluo-4 AM and 10 μM pluronic acid. Loaded arteries were then washed (30 min) in PSS and mounted for vessel myography as described.\(^3\), \(^4\) To visualize Ca^{2+} waves, Fluo-4 loaded arteries were excited at 488 nm using a krypton-argon laser (power, 5-8 mW). Emission spectra (510 nM) were then viewed through a 63X water immersion objective (1.2 NA) coupled in series with a dual Nipkow Spinning Disk Confocal head (Solamere Technology Group, UT, USA) and a Mega-10 ICCD camera (Stanford Photonics, CA, USA). In order to limit laser-induced tissue injury, image acquisition was set to 30 second periods (10-20 frames/second) and movie files were analyzed offline with software provided by Stanford Photonics (CA, USA). A series of square boxes (~1.5–1.5 μm), created within the analysis software were placed on 10 successive cells that were in sharp focus using the first visibly loaded smooth muscle cell as a starting point. Changes in Fluo-4 emission spectra were assessed at these fixed positions and data were normalized to baseline fluorescence (F\(_0\)). Ca^{2+} waves were defined as an event that: 1) spreads from end-to-end in a single cell; 2) demonstrates a peak fluorescence of at least 15% above baseline; 3) lasts longer than 200 milliseconds. Ca^{2+} wave generation was quantified, both in terms of the percentage of cells firing waves and event frequency per firing cell, under each experimental condition. Ca^{2+} wave development was assessed under control conditions and in the presence of: 1) RGD (70 μM) or RAD (70 μM); 2) xestospongin C (10 μM) or 4) 2-APB (50 μM); and 3) U73433 (500 nM) or U73122 (500 nM).

### Measurement of MLC\(_{20}\) Activity

A 2-3 mm segment of endothelial denuded cerebral artery was cut in two with each segment being mounted in an arteriograph. One half of each pair was then exposed either to: 1) 15-20 or 80 mmHg of intravascular pressure; 2) 80 mmHg intravascular pressure ± RAD/RGD (70 μM) ± diltiazem (30 μM) or ryanodine (10 μM); 3) 80 mmHg of intravascular pressure ± U73343 (500 nM) or U73122 (500 nM); 4) 80 mmHg of intravascular pressure ± diltiazem (30 μM) ± xestospongin C (10 μM). All agents were initially applied to vessels at 10 mmHg (10 min) before pressurization to 80 mmHg. Pressurized arteries were flash-frozen in an ice-cold mixture of 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) in acetone. Fixed arteries were washed in ice-cold acetone containing 10 mM DTT and lyophilized overnight. The cannulated ends of the lyophilized vessel were removed to exclude tissue not exposed to test pressures.\(^5\), \(^6\) MLC\(_{20}\) phosphorylation was then ascertained using a two-step western blot technique.\(^5\), \(^6\) Briefly, cerebral artery extracts were electrophoresed (30 mA) on a Phos-tag SDS-PAGE gel and proteins were transferred to a PVDF membrane, washed in 0.5% glutaraldehyde. Subsequently,
membranes were blocked with 1.0% ECL blocking agent (GE Healthcare, Canada), containing 0.02% Tween-20. The membranes were incubated overnight with a rabbit anti-MLC$_{20}$ antibody (1:1000), washed and then again with a HRP anti-Rabbit antibody (1:10,000). Following incubation chemiluminescence was detected using an Amersham ECL advance detection kit (GE HealthCare), analyzed on a phosphor-imager (ImageQuant LAS 4000) and expressed relative to total MLC$_{20}$.

**Measurement of MYPT1 and PLC$_{\gamma}$1 Activity**

Extracted proteins from cerebral arteries were electrophoresed (30 mA, 10% polyacrylamide gel) and upon completion, the gel was cut below the 70 kDa marker band; proteins below and above were then transferred to PVDF and nitrocellulose membranes, respectively. PVDF membranes were washed in PBS, exposed to 0.5% gluteraldehyde and blocked in 0.1% TBST (Tris-Buffered Saline + Tween 20) containing 1.0% ECL blocking agent. The membranes were then washed and incubated overnight with a rabbit anti-α smooth muscle actin antibody (1:1000). After incubation the membranes were exposed to an anti-HRP rabbit antibody (1:10000) and bands was detected by chemiluminescence. Similarly, the nitrocellulose membrane was blocked in 0.1% TBST containing 1.0% skim milk, stained with Ponceau S solution, washed in distilled water and dried overnight on a filter paper. Subsequently, membranes were blocked with 0.5% l-block and were incubated with a rabbit anti-phospho-MYPT1 antibody (1:1000) directed against T687 or T855 site. Upon completion, the membranes were washed and incubated again with an anti-rabbit-IgG biotin conjugate (1:40,000), which was followed by an exposure to HRP-streptavidin (1:200,000). PLC$_{\gamma}$1 phosphorylation was detected by chemiluminescence, standardized to α-actin and expressed relative to the appropriate control.

MLC$_{20}$, MYPT1 (T855) and MYPT1 (T697) phosphorylation were assessed on paired pressurized arteries prior to and following treatment with 1) α$_{v}$β$_{3}$ control (RAD, 70 µM) or blocking peptide (RGD, 70 µM) + diltiazem (30 µM) or ryanodine (10 µM); or 2) diltiazem (30 µM) + xestospongin C (10 µM). PLC$_{\gamma}$1 phosphorylation was assessed on paired pressurized arteries prior to and following treatment with α$_{v}$β$_{3}$ control (RAD, 70 µM) or blocking peptide (RGD, 70 µM).

**Chemicals and Drugs**

RNeasy plus micro kit and reverse transcription kit were obtained from Qiagen (ON, CA) while Prime Time qPCR primers and Kapa SYBR Fast Universal qPCR Kit were obtained from Integrated DNA Technologies (ON, CA) and Kapa Biosystems (MA, USA), respectively. Bradykinin, GRADSP-, GRGDSP-, ELDIVSPT, EILEVSPT, diltiazem, 2-APB, xestospongin C and other reagents were purchased from Sigma-Aldrich. Fluo-4, and pluronic acid were acquired from Molecular Probes (OR, USA), monoclonal antibody against αvβ3 integrin was bought from Novus Biologicals (ON, CA) and ryanodine was obtained from Ascent Chemicals (NJ, USA). Concentrations were chosen based on of their known pharmacological properties and use in the published literature. Stock reagents were solubilized in DMSO and final solvent concentration did not exceed 0.05%. Primary, secondary and tertiary antibodies/substrates were obtained from the following sources: rabbit anti-MLC$_{20}$ (Santa Cruz, CA, USA); rabbit anti-α actin (Abcam, MA, USA); rabbit anti-phospho-MYPT1 and rabbit anti-phospho-PLC$_{\gamma}$1 (Millipore,
MA, USA); anti-rabbit HRP (Thermo Scientific, ON, CA); anti-rabbit IgG biotin conjugate (Jackson ImmunoResearch, PA, USA); HRP-streptavidin (Thermo Scientific).

Statistical Analysis
Data are expressed as means ± S.E., and n indicates the number of vessels or cells. No more than 2 experiments were performed on vessels from any given animal. Paired t-tests were performed to compare the effects of a given condition/treatment on arterial diameter, Ca\(^{2+}\) wave frequency or protein phosphorylation. P values ≤ 0.05 were considered statistically significant. Minimum n value calculations determined that 3-4 paired replicates to be sufficient for statistical comparisons.

References


