PC-PLC/sphingomyelin synthase activity plays a central role in the development of myogenic tone in murine resistance arteries

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Mauban JR, Zacharia J, Fairfax S, Wier WG. PC-PLC/sphingomyelin synthase activity plays a central role in the development of myogenic tone in murine resistance arteries. Am J Physiol Heart Circ Physiol 308: H1517–H1524, 2015. First published April 17, 2015; doi:10.1152/ajpheart.00594.2014.—Myogenic tone is an intrinsic property of the vasculature that contributes to blood pressure control and tissue perfusion. Earlier investigations assigned a key role in myogenic tone to phospholipase C (PLC) and its products, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). Here, we used the PLC inhibitor, U-73122, and two other, specific inhibitors of PLC subtypes (PI-PLC and PC-PLC) to delineate the role of PLC in myogenic tone of pressurized murine mesenteric arteries. U-73122 inhibited depolarization-induced contractions (high external K$^+$ concentration), thus confirming reports of nonspecific actions of U-73122 and its limited utility for studies of myogenic tone. Edelfosine, a specific inhibitor of PI-PLC, did not affect depolarization-induced contractions but modulated myogenic tone. Because PI-PLC produces IP$_3$, we investigated the effect of blocking IP$_3$ receptor-mediated Ca$^{2+}$ release on myogenic tone. Incubation of arteries with xestospongin C did not affect tone, consistent with the virtual absence of Ca$^{2+}$ waves in arteries with myogenic tone. D-609, an inhibitor of PC-PLC and sphingomyelin synthase, strongly inhibited myogenic tone and had no effect on depolarization-induced contraction. D-609 appeared to act by lowering cytoplasmic Ca$^{2+}$ concentration to levels below those that activate contraction. Importantly, incubation of pressurized arteries with a membrane-permeable analog of DAG induced vasoconstriction. The results therefore mandate a reexamination of the signaling pathways activated by the Bayliss mechanism. Our results suggest that PI-PLC and IP$_3$ are not required in maintaining myogenic tone, but DAG, produced by PC-PLC and/or SM synthase, is likely through multiple mechanisms to increase Ca$^{2+}$ entry and promote vasoconstriction.

Bayliss; calcium; phospholipase C; diacylglycerol

It is well accepted that phospholipase C (PLC) is involved in the development and maintenance of myogenic tone. Inhibition of PLC resulted in significant reduction of myogenic tone in studies using pressurized arteries from various arterial beds and species (4, 7, 21, 36). As shown in rat cerebral arteries, the loss of tone is accompanied by membrane hyperpolarization and decrease of cytosolic Ca$^{2+}$ (21). The functional subtype(s) of PLC involved in the maintenance of tone is, however, unclear. PLC may be generally grouped into two functional subtypes: those that catalyze cleavage of PIP$_2$ (phosphoinositide-specific, PI-PLC) and ones that use phosphatidylincholine as a substrate (phosphatidylincholine-specific, PC-PLC). Cleavage of PIP$_2$ yields inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). On the other hand, cleavage of phosphatidylincholine yields phosphocholine and DAG. Whether the maintenance of tone involves one specific subtype or both subtypes is not clear. Earlier studies have used U-73122, and it has since been determined that U-73122 has undesirable side effects that can affect interpretation of results (18). It is possible to use two different antagonists to discriminate between PLC subtypes. Edelfosine (Ro-14-5243), a synthetic ether lipid, is used to selectively inhibit phosphoinositide-specific PLC (40). D609 is used as an inhibitor for PC-PLC subtype(s) (31). The contribution of one or both types of PLC to the development of myogenic tone can therefore be tested.

We have previously observed that the maintenance of tone in mesenteric arteries in vitro (30, 49) and in mice cremaster arteries in vivo (28) is achieved in the apparent absence of Ca$^{2+}$ waves. Because waves oftentimes develop involving Ca$^{2+}$ release from internal stores via IP$_3$ receptors, we reasoned that formation of IP$_3$ and Ca$^{2+}$ release via IP$_3$ receptors may not be centrally involved in the maintenance of myogenic tone. To account for the virtual absence of Ca$^{2+}$ waves and the putative role of PLC in the development of tone, we therefore hypothesized that the cleavage of phosphatidylincholine by PC-PLC and formation of DAG plays a critical role in the Bayliss response. The results suggest a central role for phosphatidylincholine and formation of DAG in pressure-induced increases of smooth muscle Ca$^{2+}$ and myogenic vasoconstriction.

MATERIALS AND METHODS

Pressurized Murine Mesenteric Arteries

Experiments and procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Third- and fourth-order mesenteric arteries were dissected and pressurized (Living Systems Instrumentation, St. Albans, VT) following methods described previously (27, 30). Briefly, pressurized mesenteric arteries were perfused at ~2 ml/min at 35–37°C with a solution of the following composition (in mmol/l): 112 NaCl, 4.9 KCl, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 25.7 NaHCO$_3$, 2.0 CaCl$_2$, 10 HEPES, and 11.5 glucose (pH 7.4 at 22°C, bubbled with 5% CO$_2$, 12% O$_2$, and 83% N$_2$). Ca$^{2+}$-free solution (2 mmol/l EGTA) was used to determine the full passive diameter of arteries. A custom video edge detection system was used to determine diameter (Labview; National Instruments, Austin, TX). Distending pressure was varied from 30 to 110 mmHg to obtain myogenic response curves. The passive diameter at 110 mmHg was used to normalize diameter measurements. Tone was represented as: \[ \text{diameter} = \frac{\text{diameter (passive 110mmHg)}}{\text{110mmHg}} \times 100. \]

In cases where pressure was kept constant at 70 mmHg, diameter measurements were normalized to the passive diameter measured at 70 mmHg. Iberiotoxin, cyclopiazonic acid (CPA), U-73122, edelfosine, and xestospongin C were obtained from Sigma (St. Louis, MO). D609 was purchased from Enzo Life Sciences (Farmingdale, NY). 1,2-Dioctanoyl-sn-glycerol was from Calbiochem/EMD Millipore (Billerica, MA). Data are presented as means ± SE; n denotes the number of pressurized arteries. Data comparisons were performed using Student’s t-test or ANOVA, as appropriate. A P < 0.05 denoted significant differences.

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Confocal Ca\(^{2+}\) Imaging of Pressurized Murine Mesenteric Arteries

Loading of resistance arteries with Ca\(^{2+}\) indicators. Dissected segments of arteries were loaded with Ca\(^{2+}\) indicator fluo 2-AM using previously described methods (49). After being loaded, the arteries were continuously superfused with gassed Krebs solution and allowed to develop myogenic tone at 70 mmHg, 35–37°C. A Zeiss 5Live slit scanning microscope (Carl Zeiss MicroImaging, Gottingen, Germany) equipped with a ×40, W C-Apochromat, 1.2 numerical aperture (NA) objective was used for imaging. Excitation of fluo 2 indicator was 488 nm, and the emission was longpass filtered at 505 nm. X-y confocal scans at five frames per second were used to observe Ca\(^{2+}\) waves. Line scans were used to detect Ca\(^{2+}\) sparks. The frequency of sparks was analyzed using the Spark Master Plug-in of ImageJ (39). The frequency of sparks is calculated for a 100-μm wide section of a line scan, thus yielding the unit spark per 100 micrometers per second. The unit reflects the frequency of sparks in a defined physical space corresponding to a subarea inside a single smooth muscle.

Widefield Förster Resonance Energy Transfer/Ca\(^{2+}\) Imaging of Pressurized Murine Mesenteric Arteries

Förster resonance energy transfer (FRET) measurements with appropriate biosensors provide a robust ratiometric way to quantify cellular parameters without the complications presented by conventional nonratiometric indicators. Whereas nonratiometric measurements can be confounded by factors such as focal plane, specimen movement, and concentration of indicator molecule (to name a few), ratiometric FRET measurements are less affected by these factors. The exMLCK FRET biosensor can reliably report Ca\(^{2+}\) levels and myosin light chain kinase (MLCK) activity. Here we use mesenteric arteries from mice expressing the exMLCK biosensor in vascular smooth muscle (19, 45). Arteries were imaged using an Olympus MVX10 microscope (Olympus America, Center Valley, PA) (objective lens: 2× Plan Apochromat, 0.5 NA) equipped with “DualView” optics (Photometrics, Tucson, AZ) and a Hamamatsu Orca-ER camera (Japan) as described previously (48, 50). Briefly, cyan fluorescent protein (CFP) excitation was at 426–446 nm [CFP emission = 455–485 nm, yellow fluorescent protein (YFP) emission = 520–550 nm]. Data were corrected for spectral bleedthrough (26). The CFP-to-YFP ratio at 30 mmHg (R\(_{30}\)) under control conditions was used to normalize ratio measurements (R/R\(_{30}\)) (where R is the ratio at a particular pressure) thus allowing for grouping of data.

RESULTS

Myogenic Tone and PLC: “PI-PLC and PC-PLC”

U-73122 is routinely used as a general-purpose inhibitor of PLC. Treatment of arteries that have developed myogenic tone with 3 μM U-73122 (n = 8) resulted in a complete loss of myogenic reactivity (Fig. 1A). Arteries followed the passive
diameter curve after treatment with U-73122, in agreement with earlier studies (21, 36). Edelfosine is an ether lysophospholipid that effectively inhibits PLC activity and catalysis of PIP₂, but without undesirable side effects seen with U-73122 (18, 40). In this regard it can be considered a preferred inhibitor for “PI-PLC.” Treatment of arteries with edelfosine resulted in an increase of tone at pressures of 30–70 mmHg (n = 7). Myogenic tone was unaltered at the higher pressures (Fig. 1B). D609 is a xanthate compound that has been shown to competitively inhibit phosphatidylcholine-specific PLC (Kᵢ = 6.4 μM) without affecting phosphatidylinositol-specific PLC (2). D609 is known to inhibit catabolism of phosphatidylcholine via a PLC-type reaction (31). Incubation of pressurized arteries with 10 μM D609 resulted in significant loss of myogenic tone at the pressures tested (30–110 mmHg, n = 5). For example, at 110 mmHg, while arteries maintained a diameter of 67.6 ± 1.9% of passive under control conditions, incubation with D609 resulted in significant vasodilation to 94.8 ± 2.8% (Fig. 1C). Myogenic tone is therefore “D609 sensitive” in murine mesenteric arteries.

We also quantified the contraction of arteries to treatment with 60 mM KCl or 10 μM phenylephrine (PE) before and after incubation with PLC antagonists. Treatment with U-73122 significantly reduced the contraction of pressurized arteries to stimulation with KCl and PE (n = 5) (Fig. 2A). The response of arteries to PE and KCl remained unchanged in the presence of edelfosine (n = 6) (Fig. 2B). In contrast, D609 significantly reduced the contraction to PE (n = 4), whereas the contraction to bath application of 60 mM KCl remained unaffected (n = 7) (Fig. 2C). Thus, D609 treatment results in loss of myogenic tone without concomitant changes in the contraction of arteries via depolarization with 60 mM KCl.

Overall, the above results indicate that the development of myogenic tone is a D609-sensitive process. The results also suggest that the activity of PI-PLC can be modulatory to myogenic tone.

**Ca²⁺ signals during established myogenic tone.** The diameter of an artery during development of myogenic tone is shown in Fig. 3A. Arteries loaded with Ca²⁺ indicator (fluo 2-AM) were imaged before development of myogenic tone. **Fig. 3. Ca²⁺ signals in pressurized murine mesenteric arteries before and after development of myogenic tone.** A: the diameter of a pressurized (70 mmHg) mesenteric artery is shown. Ca²⁺ fluorescence signals were obtained before (i, arrow) and after (ii, arrow) development of myogenic tone as shown in the diameter trace. B: typical x–y confocal scans (first column) used to calculate fluorescence values from single arterial myocytes show a virtual absence of high-amplitude, long-duration Ca²⁺ waves after development of myogenic tone (Bii, bottom, fluo 2-AM). C: summary bar graph depicting the frequency of Ca²⁺ waves in pressurized arterial myocytes (n = 4, *P < 0.05) before and after development of myogenic tone.
tion events (Fig. 3B). Figure 3B shows typical x-y confocal scans used to derive the Ca\(^{2+}\) fluorescence signals in individual smooth muscle cells under no tone (Fig. 3Bi) and myogenic tone (Fig. 3Bii) conditions. Ca\(^{2+}\) waves appear as high-amplitude long-duration events (Fig. 3Bi). These prominent Ca\(^{2+}\) signals were not readily observed when myogenic tone was established (Fig. 3Bii). The frequencies of observed Ca\(^{2+}\) waves in mesenteric arterial smooth muscle cells are significantly reduced at all observed pressures after the development of myogenic tone. Whereas the frequencies of waves at 30, 70, and 110 mmHg before tone are (cell\(^{-1}\)/min) 0.69 ± 0.11, 0.83 ± 0.09, and 0.85 ± 0.08 (n = 4), respectively, the frequencies were significantly reduced to 0.19 ± 0.04, 0.06 ± 0.01, and 0.0 (Fig. 3, B and C) after tone has developed. Ca\(^{2+}\) waves are therefore virtually absent in mouse pressurized mesenteric arteries that have developed myogenic tone.

The frequency of Ca\(^{2+}\) sparks before and after development of myogenic tone was also determined. Figure 4, A and B, shows typical line scans of smooth muscle cells that illustrate spontaneous Ca\(^{2+}\) sparks before and after development of myogenic tone. Sparks are evident in both the line scan images and in the intensity profile plots. The current method used here employing Ca\(^{2+}\) indicator dyes can potentially underestimate the frequency of sparks because of generally elevated fluorescence levels corresponding to increased steady-state levels of Ca\(^{2+}\). Figure 4C shows the summary of spark frequencies at different distending pressures. At a pressure of 70 mmHg and no tone, spark frequency was 3.46 ± 0.38 sparks·100 \(\mu\)m\(^{-1}\)·s\(^{-1}\). After development of myogenic tone Ca\(^{2+}\) sparks were still present, but an apparent decrease in the frequencies was observed at all pressures (e.g., 70 mmHg, 1.80 ± 0.34 sparks·100 \(\mu\)m\(^{-1}\)·s\(^{-1}\)).

Modulation of tone via internal Ca\(^{2+}\) stores. Paired recordings of the myogenic response were obtained from mesenteric arteries under control conditions and in the presence of ryanodine (30 \(\mu\)M, n = 3) (Fig. 5A) and iberiotoxin [Ca\(^{2+}\)-dependent K\(^{+}\) (K\(_{Ca}\)) channel antagonist] (100 nM, n = 4) (Fig. 5B). Both ryanodine and iberiotoxin caused significant increases in myogenic tone at pressures of 30–110 mmHg. Likewise, reduction of Ca\(^{2+}\) content of sarcoplasmic reticulum via incubation of arteries with CPA (10 \(\mu\)M, n = 5) (Fig. 5D) significantly increases myogenic tone. Incubation of arteries with xestospongin C (5 \(\mu\)M, n = 2) (Fig. 5C) to block IP\(_{3}\) receptor-mediated Ca\(^{2+}\) release did not change myogenic tone but effectively blocked contraction in response to bath-applied PE (10 \(\mu\)M). Together, the above results indicate that myogenic tone can be modulated by internal Ca\(^{2+}\) stores.

Inhibition of PC-PLC/sphingomyelin synthase and smooth muscle Ca\(^{2+}\). Step increases in pressure resulted in increases of the FRET ratio (Fig. 6A) indicating step increases of smooth muscle Ca\(^{2+}\). The FRET ratio response in the same artery was significantly reduced after treatment with D609. Pressure-induced changes in the FRET ratio still occur, but ratios achieved are significantly attenuated (n = 3, Fig. 6B). A linear curve fit of both response curves shows a similar slope between the control and D609 curve. The dynamic response of eX-M-LCK biosensor is unaltered in the presence of D609 as shown by the FRET response to depolarization with 117 mM K\(^{+}\) (n = 3, Fig. 6D). Control peak response was 2.95 ± 0.12 and in D609 2.91 ± 0.11. Catalysis of phosphatidylcholine by PC-PLC will produce DAG. Because DAG is known to gate some transient receptor potential (TRP) channels, we determined the effect of inhibition of nonselective cation channels with SKF-96365 and found significant loss of myogenic tone (n = 3, Fig. 6C), similar to that seen for D609. The results with SKF-96365 compound suggest downstream involvement of TRP-type channels.

DAG analog and arterial diameter. Figure 7A shows that treatment of a pressurized murine mesenteric artery (50 mmHg) with 20 \(\mu\)M 1,2-dioctanoyl-sn-glycerol results in vasoconstriction of the artery. Figure 7B shows the significant constriction of arteries after exposure to the DAG analog (n = 5).

![Fig. 4. Frequency of Ca\(^{2+}\) sparks in isobaric murine mesenteric artery smooth muscle cells during maintained myogenic constriction. A and B: representative line scans of pressurized murine mesenteric arteries loaded with Ca\(^{2+}\) indicator illustrate the prevalence of Ca\(^{2+}\) sparks before and after the development of myogenic tone. The frequency of sparks is calculated for a 100-\(\mu\)m-wide section of a line scan, thus yielding the unit spark·100 \(\mu\)m\(^{-1}\)·s\(^{-1}\). C: summary bar graph indicating a decrease in the frequency of Ca\(^{2+}\) sparks in smooth muscle cells during established myogenic tone. A and B, bottom: fluorescence units (F); bar is equivalent to 800 units. *P < 0.05, no tone vs. myogenic tone.](http://ajpheart.physiology.org/10.1152/ajpheart.00594.2014)
DISCUSSION

Uncontrolled Side Effects of U-73122

The majority of earlier studies on the role of PLC in myogenic tone have used U-73122 to indicate that PLC and, by extension, IP$_3$ and DAG were involved in the development and maintenance of myogenic tone. U-73122 has other undesirable side effects that prevent clear and unequivocal interpretation of results. These have included inhibition of K$^+$ currents and a G$_{i/o}$-mediated activation of an inward rectifying K$^+$ current (18). The mechanism by which the side effects occur is likely via alkylation reactions of U-73122. Importantly, in the case of myogenic constriction, U-73122 is known to inhibit voltage-dependent Ca$^{2+}$ influx via dihydropyridine-sensitive Ca$^{2+}$ channels in a neuronal cell line (NG108-15) (22). The loss of myogenic tone, hyperpolarization, and decrease in cytosolic Ca$^{2+}$ of myogenically active arteries when treated with U-73122 may therefore take effect via inhibition of dihydropyridine-sensitive channels (L-type Ca$^{2+}$ channels) and not necessarily via mechanisms involving PLC. Our present results recapitulate this concern, since it is clear that U-73122 not only inhibits myogenic tone (Fig. 1A) but also depolarization-induced constriction of arteries (Fig. 2A).

Phosphatidylinositol-Specific PLC

Although PLC is classified as either PI-PLC or PC-PLC, the mammalian gene(s) that correspond to such enzymes are not cloned (1). The classification of PI-PLC and PC-PLC in mammalian cells is therefore a functional classification and does not necessarily translate to a particular gene product. The ability of

Fig. 5. Modulation of myogenic tone by sarcoplasmic reticulum Ca$^{2+}$ stores. The effects of blockade of ryanodine receptors (30 μM ryanodine) (A) and Ca$^{2+}$-dependent K$^+$ (KCa) channels [100 nM iberiotoxin (IbTx)] (B) on myogenic tone of pressurized murine mesenteric arteries were measured. C: xestospongin C (5 μM XeC) was used to block Ca$^{2+}$ release via inositol 1,4,5-trisphosphate (IP$_3$) receptors. XeC did not affect myogenic tone but eliminated the contractile response to bath-applied PE (10 μM). D: incubation of arteries with 10 μM cyclopiazonic acid (CPA) significantly increased myogenic tone. Passive diameter was measured with 0 Ca$^{2+}$ perfusate (*$P < 0.01$). ctr, Control.

Fig. 6. Effects of inhibition of PC-PLC/sphingomyelin synthase on pressure-induced Ca$^{2+}$ response of murine mesenteric smooth muscle cells. A: the Förster resonance energy transfer (FRET) ratio response in a pressurized mesenteric artery expressing exMLCK biosensor is shown under control and after treatment with D609 (10 μM, n = 3). B: summary of the pressure-induced FRET response of mesenteric arteries [normalized ratio at a particular pressure (R) to that at 30 mmHg (R$_{30}$)] before and after treatment with D609, n = 3. C: myogenic tone in mesenteric arteries under control and after treatment with SKF-96365, n = 3. *P < 0.05, control vs. SKF-96365. D: FRET response of exMLCK biosensor to depolarization with 117 mM K$^+$ in the absence and presence of D609 (n = 3).
The results with xestospongin C treatment suggest that IP3-mediated Ca$^{2+}$ modulation of myogenic tone via Ryanodine Receptor-increases in tone modulatory role since edelfosine was able to induce detectable resistance-sized mesenteric arteries. PI-PLC, however, exerts a fosine (20 understood. Given that we have used effective doses of edelfosine to elicit Ca$^{2+}$ release from internal stores and subsequent Ca$^{2+}$ entry (as seen in MDCK cells) (20) may account for the trend of increase in myogenic tone. The mechanism by which edelfosine may elicit Ca$^{2+}$ release from internal stores of vascular smooth muscle cells is incompletely understood. Given that we have used effective doses of edelfosine (20 μM), we conclude that PI-PLC enzymatic activity is not obligatory for the maintenance of myogenic tone in murine resistance-sized mesenteric arteries. PI-PLC, however, exerts a modulatory role since edelfosine was able to induce detectable increases in tone.

Modulation of Myogenic Tone via Ryanodine Receptor-Mediated Ca$^{2+}$ Release

Ca$^{2+}$ waves are virtually absent in myogenically active, pressurized murine mesenteric arteries (Fig. 3B), consistent with earlier findings (28, 30). Incubation of arteries with the membrane-permeable inhibitor of IP$_3$ receptors, xestospongin C (10), did not affect established myogenic tone at doses that were effective at inhibiting PE-induced contractions (Fig. 5C). The results with xestospongin C treatment suggest that IP$_3$ receptor-mediated Ca$^{2+}$ release and Ca$^{2+}$ waves do not exert strong influence on myogenic responses of murine mesenteric arteries. The results with inhibition of IP$_3$ receptors in murine mesenteric arteries are different from results obtained from rat cerebral arteries where Ca$^{2+}$ release via IP$_3$ receptors seem to hold a more prominent role in establishing myogenic responses (13). In contrast, Ca$^{2+}$ release via ryanodine receptor channels does have significant effects on myogenic tone in murine mesenteric arteries. Depletion of CPA-sensitive Ca$^{2+}$ stores with CPA and inhibition of ryanodine receptor-mediated Ca$^{2+}$ release with ryanodine both significantly increase myogenic constriction (Fig. 5, A and D). Whereas sparks are present in myogenically active arteries, incubation of arteries with iberiotoxin also resulted in a similar increase in tone as that seen for ryanodine treatment. The above results are in accord with earlier studies in cerebral arterioles (34, 38) whereby sparks are postulated to activate Ca$^{2+}$-dependent K$_{Ca}$ currents, thus exerting vasodilatory influence on arteries. Prevention of sparks or blockade of K$_{Ca}$ channels causes vasoconstriction because of inhibition of a hyperpolarizing K$_{Ca}$ current. The results with ryanodine and iberiotoxin treatment both suggest that K$_{Ca}$ channels play a similar role in the rat cerebral arteries and mouse mesenteric arteries. Alternatively, the decrease in tone seen with CPA treatment may also arise from the ability of CPA treatment to increase the activity of mechanosensitive cation channels in vascular smooth muscle (37). An increase in depolarizing cationic current would depolarize smooth muscle, thus promoting vasoconstriction. Overall, the results suggest a modulatory role for the sarcoplasmic reticulum and Ca$^{2+}$ release via ryanodine receptors in determining myogenic responses.

Phosphatidylcholine-Specific PLC and Sphingomyelin Synthase

Arteries treated with D609 lost myogenic tone (Fig. 1C) and attenuated pressure-induced Ca$^{2+}$ responses (Fig. 6). The loss of tone occurs without any concomitant effects on depolarization-induced contraction, and thus the ability of the artery to contract. Although pressure-induced Ca$^{2+}$ responses are still detectable, D609 treatment shifted the entire response curve downward, thus lowering Ca$^{2+}$ levels and preventing the elevation of cytosolic Ca$^{2+}$ to levels that are permissive for contraction of the pressurized artery. Interestingly, the result with D609 is in contrast to an earlier study in rat posterior cerebral arteries where D609 did not inhibit myogenic vasoconstrictions (21). It is therefore possible that differences in arterial beds (mesentery and cerebral) as well as species differences may account for the differences observed.

Whereas D609 is a selective inhibitor for PC-PLC (2), as opposed to PI-PLC, D609 is also known to inhibit sphingomyelin synthase (SMS) (24). It is of note that both PC-PLC and

Fig. 7. Effects of membrane-permeable diacylglycerol (DAG) analog on diameter of pressurized murine mesenteric arteries. A: the diameter response of a pressurized mesenteric artery in response to treatment with 1,2-dioctanoyl-sn-glycerol (DOG, 20 μM, 50 mmHg) is shown. B: summary of the response of arteries to treatment with DOG (n = 5).

Fig. 8. Proposed role of PC-PLC/sphingomyelin synthase in the myogenic response of murine mesenteric arteries. Mechanical stretch of the plasma membrane leads to ligand-independent activation of G protein-coupled receptors (GPCRs), including angiotensin type 1 (AT$_1$). D609-sensitive PC-PLC and/or sphingomyelin synthase activate and use phosphatidylcholine to yield DAG metabolite. DAG activates plasma membrane currents, including transient receptor potential C (TRPC) family members and protein kinase C (PKC). Inhibition of a K$^+$ current also contributes to depolarization. The resultant increase in Ca$^{2+}$ influx and Ca$^{2+}$ sensitivity thus leads to myogenic vasoconstriction.
SMS catalyze reactions that use PC and yield DAG as a downstream product. Inhibition of PC-PLC and/or SMS is expected to diminish formation of DAG. Investigators have even suggested that SMS may account for the D609-sensitive mammalian PC-PLC (24). SMS family members are integral membrane proteins, and SMS 2 has been observed in the plasma membrane (17). The steps involved in the regulation of SMS enzymatic activity are less understood at this point. However, it is interesting to note that hypoxia can induce a cellular accumulation of DAG in multiple cell types (12, 43, 47) and that this response can be sensitive to inhibition of PC-PLC/SMS with D609 (43). Thus it seems that the activity of PC-PLC/SMS may be regulated via multiple stimulus modalities. The results presented here are consistent with the hypothesis that the activity of PC-PLC/SMS is central for the development of stretch-induced Ca\(^{2+}\) responses and myogenic vasoconstriction in murine mesenteric arteries.

**Bayliss Mechanism and a Central Role for PC and DAG**

Mechanically induced, ligand-independent activation of certain \(G_\text{q/11}\)-coupled receptors may constitute an early step in the mechanotransduction process involved in the myogenic response (29, 51). More recently, investigators have reported that ligand-independent activation of angiotensin type 1 (AT\(_1\)) receptors plays a significant role in the myogenic responses of pressurized murine mesenteric arteries (6, 41). Whether it is \(AT_{1A}\) or \(AT_{1B}\) that is centrally involved is a subject of discussion, since the two abovementioned studies point to either \(AT_{1A}\) or \(AT_{1B}\). Other metabotropic receptors, including endothelin (ET\(_A\)) and \(\alpha_1\)-receptors, may also be involved in sensing mechanical stretch, and different arrays of G protein-coupled receptors may participate in sensing mechanical stretch in other arterial arcades (6, 28). In addition, the involvement of particular receptors/signaling cascade may vary not only with the arterial arcade but also with the physiological state of the animal (15). The signaling steps downstream of activation of \(G_\text{q/11}\)-coupled receptors are less understood. However, investigators have demonstrated that particular G protein-coupled receptors have detectable “gating currents” and may be activated/modulated by membrane depolarization itself (5, 25). Furthermore, dihydropyridine receptors may act as voltage sensors and trigger Ca\(^{2+}\) release from the sarcoplasmic reticulum (3, 9). In this manner, the receptors (G protein coupled or dihydropyridine) can potentially participate in the myogenic response, albeit downstream of earlier mechanotransduction steps.

Mechanical stretch can lead to activation of particular cation channels (8, 35, 42, 44, 46). Recordings of the mechanosensitive currents show biophysical properties akin to TRP family of proteins (8, 37, 44). Treatment of arteries with SKF-96365 abolishes myogenic tone, suggesting the probability of downstream involvement of TRP-type cation channels. Downstream inhibition of an XE991-sensitive K\(^+\) current has been demonstrated to also modulate myogenic responses, but the mechanism for inhibition remains unsorted (41). Importantly, mechanical stretch is known to increase DAG in smooth muscle cells (32). A membrane-permeable DAG analog can augment activation of the mechanosensitive currents in smooth muscle (37) and tone (7, 11). Critically, treatment of pressurized murine mesenteric arteries with a DAG analog does induce vasoconstriction. DAG action may include activation of TRP channels, protein kinase C (PKC) enzyme which can lead to modulation of Ca\(^{2+}\) sparklets (33), as well as Ca\(^{2+}\) sensitivity. Further studies will be required to further delineate the mechanisms involved.

The present results suggest that catalysis of PC and formation of DAG are critical steps for the Bayliss response as seen in murine mesenteric arteries. The spatiotemporal pattern of DAG formation in response to increases in distending pressure in arteries was not determined in this study and should be a subject of future inquiry. Because D609 can inhibit PC-PLC and SMS, studies to differentiate between the two enzymes will also be needed. Murine knockout models for SMS 1 and 2 are available and may aid in this respect (14, 23).

In summary, we hypothesize that the Bayliss response proceeds as follows (Fig. 8): ligand-independent, stretch-induced activation of G protein-coupled receptors (including AT\(_1\)) would be responsible for 1) activation of PC-PLC/SMS and formation of DAG and 2) inhibition of a K\(^+\) conductance (41). Downstream actions of DAG, which include activation of particular TRP-type channels (16) and PKC enzymatic activity, may then contribute to depolarization, Ca\(^{2+}\) influx, increases in intracellular Ca\(^{2+}\) concentration, Ca\(^{2+}\) sensitivity, and vasoconstriction.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


