Phosphatidylinositol 3,5-bisphosphate increases intracellular free Ca\(^{2+}\) in arterial smooth muscle cells and elicits vasoconstriction

Neerupma Silswal,1 Nikhil K. Parelkar,1 Michael J. Wacker,1 Marco Broutto,1,2 and Jon Andresen1

1Basic Medical Science Department and 2Schools of Nursing and Medicine, Muscle Biology Research Group, University of Missouri, Kansas City, Missouri

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Silswal N, Parelkar NK, Wacker MJ, Broutto M, Andresen J. Phosphatidylinositol 3,5-bisphosphate increases intracellular free Ca\(^{2+}\) in arterial smooth muscle cells and elicits vasoconstriction. Am J Physiol Heart Circ Physiol 300: H2016–H2026, 2011. First published March 18, 2011; doi:10.1152/ajpheart.01011.2010.—Phosphatidylinositol (3,5)-bisphosphate [PI(3,5)P\(_2\)] is a newly identified phosphoinositide that modulates intracellular Ca\(^{2+}\) by activating ryanodine receptors (RyRs). Since the contractile state of arterial smooth muscle depends on the concentration of intracellular Ca\(^{2+}\), we hypothesized that by mobilizing sarcoplasmic reticulum (SR) Ca\(^{2+}\) stores PI(3,5)P\(_2\) would increase intracellular Ca\(^{2+}\) in arterial smooth muscle cells and cause vasoconstriction. Using immunohistochemistry, we found that PI(3,5)P\(_2\) was present in the mouse aorta and that exogenously applied PI(3,5)P\(_2\) readily entered aortic smooth muscle cells. In isolated aortic smooth muscle cells, exogenous PI(3,5)P\(_2\) elevated intracellular Ca\(^{2+}\), and it also contracted aortic rings. Both the rise in intracellular Ca\(^{2+}\) and the contraction caused by PI(3,5)P\(_2\) were prevented by antagonizing RyRs, while the majority of the PI(3,5)P\(_2\) response was intact after blockade of inositol (1,4,5)-trisphosphate receptors. Depletion of SR Ca\(^{2+}\) stores with thapsigargin or caffeine and/or ryanodine blunted the Ca\(^{2+}\) response and greatly attenuated the contraction elicited by PI(3,5)P\(_2\). The removal of extracellular Ca\(^{2+}\) or addition of verapamil to inhibit voltage-dependent Ca\(^{2+}\) channels reduced but did not eliminate the Ca\(^{2+}\) or contractile responses to PI(3,5)P\(_2\). We also found that PI(3,5)P\(_2\) depolarized aortic smooth muscle cells and that LaCl\(_3\) inhibited those aspects of the PI(3,5)P\(_2\) response attributable to extracellular Ca\(^{2+}\). Thus, full and sustained aortic contractions to PI(3,5)P\(_2\) required the release of SR Ca\(^{2+}\), probably via the activation of RyR, and also extracellular Ca\(^{2+}\) entry via voltage-dependent Ca\(^{2+}\) channels.

aortic smooth muscle cells; calcium; ryanodine receptors; sarcoplasmic reticulum

PHOSPHOINOSITIDE (PI) lipids are located on the cytoplasmic face of intracellular membranes, and different isomers have specific subcellular localizations. The hydroxyl groups of the inositol ring of phosphatidylinositol can be variably phosphorylated to form seven distinct Pls. PI (3,5)-bisphosphate [PI(3,5)P\(_2\)] is the most recently identified PI phosphate isomer (9, 19, 75), and it is concentrated on intracellular organelles, particularly those of late endosomes, lysosomes, and the sarcoplasmic reticulum (SR) (41, 59). Synthesis of PI(3,5)P\(_2\) from PI (3)-phosphate [PI(3)P] is catalyzed by a complex of proteins including FAB1, VAC14, and FIG4, which can also remove the 5'-phosphate to regenerate PI(3)P (20). A family of proteins known as myotubularin and myopathy-related (MTMR) phosphatases removes the 3'-phosphate to generate PI (5)-phosphate [PI(5)P] (31).

Dysfunctions in PI(3,5)P\(_2\) metabolism lead to pathologies (50, 70). For example, deletion of Vac14 or mutation of FIG4 in mice results in neurodegeneration and premature death (77). In addition, deletion of Mtmr14 in mice disrupts Ca\(^{2+}\) signaling, causing the muscle weakness and exercise intolerance that is characteristic of centronuclear myopathy (51, 59). In humans, a deleterious allele of FIG4 is a risk factor for amyotrophic and primary lateral sclerosis, and mutations in MTMR genes are associated with myopathies and Charcot-Marie-Tooth diseases (10, 70). Thus, PI(3,5)P\(_2\) appears important in several diseases, although much remains to be learned regarding its function in eukaryotes.

Interestingly, PI(3,5)P\(_2\) binds to and activates ryanodine receptors (RyRs), thereby increasing intracellular Ca\(^{2+}\) by emptying SR stores (59, 71). In arterial smooth muscle, SR Ca\(^{2+}\) contributes to the development and maintenance of vascular tone (52, 73), but the role of PI(3,5)P\(_2\) in arterial vascular Ca\(^{2+}\) signaling remains undefined. We hypothesized that PI(3,5)P\(_2\) would mobilize SR Ca\(^{2+}\) stores, causing vasocontraction. Therefore, we examined the effects of PI(3,5)P\(_2\) in vitro in isolated mouse aortic smooth muscle cells using ratiometric Ca\(^{2+}\) imaging and in isolated arteries using microscopy. Our results demonstrated that PI(3,5)P\(_2\) caused contraction of aortic rings by activating RyRs to release SR Ca\(^{2+}\) followed by the opening of voltage-dependent Ca\(^{2+}\) channels (VDCCs). If extracellular Ca\(^{2+}\) entry was prevented, either by blockade of VDCC, by removal of extracellular Ca\(^{2+}\), or by the inclusion of LaCl\(_3\), PI(3,5)P\(_2\) still generated transient contractions. Prior depletion of SR Ca\(^{2+}\) stores, however, prevented PI(3,5)P\(_2\) from having any effect. Thus, the activation of RyRs by PI(3,5)P\(_2\) was sufficient to release SR Ca\(^{2+}\) and cause small contractions, whereas VDCCs were required to achieve full increases in intracellular Ca\(^{2+}\) and sustained aortic contractions.

METHODS

Animals and reagents. Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 12 wk of age. Mice were killed by CO\(_2\) inhalation. The Animal Care and Use Committee of the University of Missouri-Kansas City approved all protocols. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. PI lipids, including PI(3,5)P\(_2\), PI(3)P, and PI(5)P (Echelon Biosciences, Salt Lake City, UT), were prepared by dissolving them in chloroform, methanol, and water at a ratio of 1:2:0.8 as per the manufacturer’s instructions, and just before addition to the bath, they were mixed with histone (Echelon Biosciences) solution at a ratio of 1:0.8. Histone carrier protein, which is required for the intracellular delivery of Pls (46), stock solution was prepared by dissolving the protein in double-distilled H\(_2\)O to a concentration of 1 mM. Solvents never exceeded 0.1% (vol/vol), and vehicle controls included the solvents and histone carrier protein but not the PI lipid.
Immunohistochemistry. The thoracic aorta was removed, cleaned of excess fat and connective tissue, immediately frozen in tissue freezing medium, and sectioned at 8 μm using a cryostat. Sections were mounted two to three to a slide and fixed in 4% formaldehyde for 10 min at room temperature. Slides were washed with 0.1% Triton X-100 in PBS (PBS-T) followed by blockade with 5% normal goat serum and 1% BSA (blocking buffer) containing 0.05 μg/ml of AffiniPure Fab fragment goat-anti mouse IgG (H+L) for 1 h. Sections were incubated overnight at 4°C with either anti-PI(3,5)P2 mouse monoclonal IgG2b antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:4,000 in blocking buffer for 1 h at room temperature. Sections were then incubated with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml) in blocking buffer for 5 min. Before being coverslipped, sections were washed three times in PBS-T and dehydrated with increasing concentrations of ethanol. Sections were dried and coverslipped using Vectorshard HardSet mounting medium (Vector Labs). Slides were imaged by epifluorescence microscopy using an Olympus IX71 (Olympus America, Center Valley, PA) inverted microscope fitted with a Hamamatsu ORCA-R2 charge-coupled device (CCD) camera (Hamamatsu, Bridgewater, NJ) and a Sutter LB-XL light source (Novato, CA). Images were processed with Slidebook software (version 5.0.0.9, Intelligent Imaging Innovations, Denver, CO).

Aorta digestion and smooth muscle cell isolation. Thoracic aortas were excised and cleaned of fat and excess connective tissue in ice-cold HBSS. Sections of aortas 2–3 mm in length were placed in ice-cold (4°C) solution I [127 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl2, 11.8 mM glucose, 10 mM HEPES (pH 7.4), and 2.4 mM CaCl2]. After rings had been washed once in solution I, aortas were cut open longitudinally and placed into a new solution containing albumin (5 mg/ml), papain (3.5 mg/ml), and dithiothreitol (5 mg/ml) in solution I for 25 min at 37°C. Tissue was washed with solution I (2 × 3 min each) and afterward incubated at 37°C in another enzyme solution containing albumin (5 mg/ml), collagenase (2.5 mg/ml), and hyaluronidase (2.5 mg/ml) in solution I for 6 min. Dissociated tissue was washed twice in digestion buffer and then triturated by passing the digest 10 times through the tip of a flame-polished Pasteur pipette. The digest was stored on ice until use during the same day.

Visualization of PI(3,5)P2 entry into aortic smooth muscle cells. Aortic smooth muscle cells were plated into a glass-bottomed perfusion chamber and placed on the stage of an inverted Olympus IX71 microscope, and cells were bathed in Ca2+-free HBSS (Invitrogen, Carlsbad, CA). Fluorescently tagged PI(3,5)P2 in the form of Bodipy-TMR-PL (5,5′-dichloro-1,1′-dihydrindolamine) was mixed with the histone carrier protein and added to the cells at 3 μM. After being incubated for 5 min, smooth muscle cells were washed three times with Ca2+-free HBSS and imaged by epifluorescence and phase-contrast microscopy. No fluorescence was observed in aortic smooth muscle cells treated only with vehicle.

Ratiometric Ca2+ imaging. Aortic smooth muscle cells were plated onto glass-bottomed plastic tissue culture dishes (Warner Instruments, Hamden, CT) and incubated with fura 2-AM (2 μM, Invitrogen) in HBSS buffer for 30 min and then were washed with HBSS buffer to remove any unincorporated dye. The dishes were then placed on the stage of an inverted Olympus IX71 microscope and superfused with HBSS or Ca2+-free HBSS (with 0.4 mM EGTA) as indicated. Images were collected using Hamamatsu ORCA-R2 CCD camera and a Sutter LB-XL light source and processed with Slidebook ratiometric software. Photo toxicity was minimized using a neutral density filter and by keeping exposure times as short as possible. All experiments were performed at room temperature. Data are expressed as a ratio (340/380 nm) of emitted fluorescence at 510 nm in cells excited at 340 and 380 nm. Responses to PI(3,5)P2 (1 or 3 μM) alone or in the presence of ryanodine (0.2–50 μM), thapsigargin (10 μM), verapamil (10 μM), caffeine (10 mM), or appropriate vehicle controls were analyzed by directly adding the agonist/antagonist to the bath. Responses of 5–10 cells were averaged to form one observation per treatment per animal (where n is the number of animals). Isometric tension myography. The thoracic aorta was rapidly excised and placed in ice-cold HBSS, where blood, fat, and excess connective tissues were carefully removed. Segments 3–4 mm in length were mounted on pins in chambers of a DMT 610M wire myograph system (Danish Myo Technology, Aarhus, Denmark) containing Krebs buffer [which contained (in mM) 119 NaCl, 4.7 KCl, 0.24 NaHCO3, 1.18 KH2PO4, 1.19 MgSO4, 5.5 glucose, and 1.6 CaCl2] saturated at 37°C with a gas mixture containing 20% O2–5% CO2–75% N2 (Airgas Mid South, Tulsa, OK). Replacing CaCl2 with 0.4 mM EGTA in standard Krebs buffer produced Ca2+-free buffer. Arterial rings were progressively stretched to 0.75 g of equivalent force passive tension in 0.1-g steps and allowed to equilibrate for 45 min. To assess the quality of the preparation before the concentration-response curve to PI(3,5)P2 was determined, aortic rings were exposed to isometric KCl (40 and 80 mM) and also to 10 μM PGE2, followed by 1 μM ACh. Vessels were rinsed once with fresh Krebs buffer every 15 min and several times after concentration-response curves. The role of extracellular Ca2+ in PI(3,5)P2-mediated contraction was examined by washing aortic segments with Ca2+-free Krebs buffer for 2 min before the responses to PI(3,5)P2 were determined. Aortic segments were pretreated with verapamil (10 μM) for 3 min while with ryanodine (10 or 100 μM, Ascent Scientific, Princeton, NJ), thapsigargin (10 μM, Alexis, San Diego, CA) alone or together for 20 min before the addition of PI(3,5)P2. Preincubation with caffeine (10 mM) was performed for 5 min to determine its effect on PI(3,5)P2-mediated contraction. Force changes were recorded using an ADInstruments (Colorado Springs, CO) PowerLab 4/30 and associated LabChart Pro software (version 6.1) running on a standard Windows XP computer platform.

Electrophysiology. The membrane potential of freshly dissociated aortic smooth muscle cells was determined by patch-clamp electrophysiology similar to a previous study by our laboratory (47). Briefly, aortas were enzymatically digested, and smooth muscle cells were isolated as described above. Cells were plated onto the bottom of a laminar perfusion chamber (Bioscience Tools, San Diego, CA) placed on the stage of an Olympus IX71 inverted microscope and bathed in buffer consisting of (in mM) 140 NaCl, 4.2 KCl, 1.2 mM MgCl2, 11.8 mM glucose, 10 mM HEPES (pH 7.4), and 2.4 mM CaCl2]. Aortic segments were pretreated with verapamil (10 μM) for 3 min while with ryanodine (10 or 100 μM, Ascent Scientific, Princeton, NJ), thapsigargin (10 μM, Alexis, San Diego, CA) alone or together for 20 min before the addition of PI(3,5)P2. Preincubation with caffeine (10 mM) was performed for 5 min to determine its effect on PI(3,5)P2-mediated contraction. Force changes were recorded using an ADInstruments (Colorado Springs, CO) PowerLab 4/30 and associated LabChart Pro software (version 6.1) running on a standard Windows XP computer platform.

Statistics. Data are plotted and expressed as means ± SE, with n indicating the number of animals studied for a given treatment. Changes in isometric tension are expressed in grams. In myograph experiments, two-factor ANOVA was used to determine differences in the concentration-response curves. In Ca2+ imaging experiments, Student’s t-test was used to determine the significance of differences between two observations, whereas one-factor ANOVA with Tukey’s post hoc test was used for multiple comparisons. In isobaric experiments, percent contractions were expressed as the percent decrease

PI(3,5)P2 EFFECT IN AORTIC SMOOTH MUSCLE CELLS

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from the baseline diameter of the middle cerebral artery. Data were plotted and statistics computed with Graphpad Prism (version 5.01, San Diego, CA). Significance was accepted at $P < 0.05$.

RESULTS

PI(3,5)P$_2$ in aortic smooth muscle cells. In sections of the mouse aorta, we detected red immunofluorescence for PI(3,5)P$_2$ that overlapped with blue DAPI staining of nuclei in between layers of internal elastic laminae. Magnification: $\times 40$. B: isolated mouse aortic smooth muscle cells imaged by phase contrast or by Cy3 epifluorescence to reveal fluorescence caused by the addition of 3 $\mu$M BODIPY-TMR-PI(3,5)P$_2$. The phase-contrast and fluorescent images were superimposed (merge) to demonstrate that BODIPY-TMR-PI(3,5)P$_2$ was present only in the interior of the cells. Magnification: $\times 30$.

PI(3,5)P$_2$ increases intracellular Ca$^{2+}$ in aortic smooth muscle cells. In sections of the mouse aorta, we detected red immunofluorescence for PI(3,5)P$_2$ that overlapped with blue DAPI staining of nuclei in between layers of internal elastic laminae that autofluoresced green (Fig. 1A). Thus, using epifluorescence microscopy, we found that PI(3,5)P$_2$ was present at least in the smooth muscle layers of the mouse aorta under basal conditions. Since we planned to determine if PI(3,5)P$_2$ mobilized internal Ca$^{2+}$, we first verified that exogenous PI(3,5)P$_2$ would enter arterial smooth muscle cells. Similar to a previous report (69), we used epifluorescence microscopy to show that fluorescently tagged PI(3,5)P$_2$ [BODIPY-TMR PI(3,5)P$_2$] complexed with the histone carrier protein entered isolated aortic smooth muscle cells (Fig. 1B). Thus, PI(3,5)P$_2$ was normally present in aortic smooth muscle cells, and we could introduce exogenous PI(3,5)P$_2$ into isolated aortic smooth muscle cells to study its effects.

Ca$^{2+}$ and contractile responses to PI(3,5)P$_2$. In isolated aortic smooth muscle cells, PI(3,5)P$_2$ increased intracellular Ca$^{2+}$ in a concentration-dependent ($P < 0.0001$) manner, whereas the vehicle was without any effect (Fig. 2A and B). The rise in internal Ca$^{2+}$ was likely specific to PI(3,5)P$_2$ because little to no effect was observed using the PI(3,5)P$_2$ metabolites PI(5)P and PI(3)P. The response to 1 $\mu$M PI(5)P was indistinguishable from that of vehicle alone, whereas 1 $\mu$M PI(3)P increased intracellular Ca$^{2+}$ no more than one-third as effectively as 1 $\mu$M PI(3,5)P$_2$. The 340/380 ratio trace shown in Fig. 2A also demonstrates that 100 mM KCl reliably elicited a maximal Ca$^{2+}$ response in healthy aortic smooth muscle cells, and only data from cells that responded to KCl were included in the analysis. Since PI(3,5)P$_2$ increased intracellular Ca$^{2+}$ in isolated aortic smooth muscle cells, we next determined whether or not PI(3,5)P$_2$ could also contract the aorta. Using aortic rings held under isometric tension, we found that PI(3,5)P$_2$, but not vehicle, caused vasocontraction in a concentration-dependent ($P < 0.0001$) manner (Fig. 3). In addition to this, using isobaric myography, we also found that PI(3,5)P$_2$ caused concentration-dependent vasoconstriction of isolated middle cerebral arteries (Supplemental Material, Supplemental Fig. S1).

1 Supplemental Material for this article is available at the American Journal of Physiology-Heart and Circulatory Physiology website.
To investigate the source of the Ca\(^{2+}\), we returned to ratiometric Ca\(^{2+}\) imaging of isolated aortic smooth muscle cells. Both RyR1 and RyR2, the latter of which is the predominate subtype found in the vasculature (23, 76), are directly activated by PI(3,5)P\(_2\) (59, 71). As previously reported (21, 78), we found that ryanodine up to 10 \(\mu\)M released SR Ca\(^{2+}\), whereas higher concentrations blocked RyRs (Fig. 4A). Interestingly, both 1 \(\mu\)M PI(3,5)P\(_2\) and 1 \(\mu\)M ryanodine increased intracellular Ca\(^{2+}\) in a similar manner (Fig. 4B). Likewise, caffeine, which is a RyR agonist, concentration dependently increased intracellular Ca\(^{2+}\) (Supplemental Fig. S2A), and an inhibitory concentration of ryanodine completely blocked the caffeine response (Fig. 4D). Similarly, an inhibitory concentration of ryanodine effectively blocked both the Ca\(^{2+}\) and contractile responses to PI(3,5)P\(_2\) (Fig. 4C and E).

To further investigate the effect of SR Ca\(^{2+}\) stores in the PI(3,5)P\(_2\) response, mouse aortic smooth muscle cells were treated with thapsigargin [an inhibitor of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)] or caffeine and/or ryanodine. In ratiometric Ca\(^{2+}\) imaging experiments, preincubation with 10 \(\mu\)M thapsigargin, 10 \(\mu\)M ryanodine, or 10 mM caffeine essentially eliminated \((P < 0.0001)\) the PI(3,5)P\(_2\) Ca\(^{2+}\) response (Fig. 5A). Preincubation of aortic rings with thapsigargin (10 \(\mu\)M) likewise prevented PI(3,5)P\(_2\) from causing contractions (Fig. 5B). Similarly, the application of ryanodine (10 \(\mu\)M) and thapsigargin (10 \(\mu\)M) together also kept PI(3,5)P\(_2\) from contracting the aorta (Fig. 5B). Pretreatment with only ryanodine (10 \(\mu\)M) to deplete the SR, however, greatly attenuated \((P = 0.0136)\) but did not totally abolish the PI(3,5)P\(_2\) response (Fig. 5B). For instance, in the presence of 10 \(\mu\)M ryanodine, 3 \(\mu\)M PI(3,5)P\(_2\) elicited some contraction (Fig. 5B); however, it was 60% less than the maximal control response. Furthermore, preincubation with 10 mM caffeine to deplete the SR also prevented PI(3,5)P\(_2\) from contracting the aorta (Supplemental Fig. S2C).

In addition, the inositol (1,4,5)trisphosphate (IP\(_3\)) receptor (IP\(_3\)R) blocker xestospongin C (25 \(\mu\)M) reduced \((P < 0.0001)\) the Ca\(^{2+}\) response to 1 \(\mu\)M PI(3,5)P\(_2\) by 29%, indicating that IP\(_3\)Rs could also have been activated by PI(3,5)P\(_2\) (Fig. 6).

Next, we investigated the role of extracellular Ca\(^{2+}\) in the PI(3,5)P\(_2\) response. Interestingly, the removal of extracellular Ca\(^{2+}\) or addition of verapamil (10 \(\mu\)M) to block VDCCs similarly reduced, but did not abolish, the response to PI(3,5)P\(_2\) (Fig. 7A and B). As shown in Fig. 7B, the Ca\(^{2+}\) response to 1 \(\mu\)M PI(3,5)P\(_2\) was reduced by 27% \((P < 0.0001)\) in Ca\(^{2+}\)-free buffer and by 42% \((P = 0.0002)\) in the presence of verapamil. This could not have simply been due to depletion of Ca\(^{2+}\) stores, but rather was due to inhibition of VDCCs.
SR Ca\(^{2+}\) by superfusion with Ca\(^{2+}\)-free buffer because, as shown in Supplemental Fig. S2B, the absence of extracellular Ca\(^{2+}\) did not affect the caffeine response. Similarly, verapamil also did not alter \((P = 0.7701)\) the Ca\(^{2+}\) response to 10 mM caffeine, indicating that verapamil had no effect on SR Ca\(^{2+}\) stores (Supplemental Fig. S2B). Again, similar to the Ca\(^{2+}\) imaging experiments, the removal of extracellular Ca\(^{2+}\) largely, although not completely, inhibited aortic contractions to PI(3,5)P\(_2\) (data not shown). Interestingly, the addition of 10 \(\mu M\) verapamil to Ca\(^{2+}\)-containing Krebs buffer allowed only transient contractions to PI(3,5)P\(_2\) \((P = 0.0014)\) that were not sustained at any concentration of PI(3,5)P\(_2\) (Fig. 7, C and D).

Given that PI(3,5)P\(_2\) appeared to open VDCCs subsequent to RyR activation, we determined whether or not PI(3,5)P\(_2\) also caused membrane depolarization. To do so, we used patch-clamp electrophysiology in current clamp mode with freshly isolated aortic smooth muscle cells. We found that the application of 3 \(\mu M\) PI(3,5)P\(_2\) elicited depolarizations in 83% of the cells tested that averaged 6.3 mV (Fig. 7, C and D).

**Fig. 4.** Effect of ryanodine blockade on PI(3,5)P\(_2\) responses. A: ryanodine up to 10 \(\mu M\) concentration dependently increased intracellular Ca\(^{2+}\), whereas 50 \(\mu M\) ryanodine was without effect \((n = 3)\). B: both 1 \(\mu M\) Ryanodine and 1 \(\mu M\) PI(3,5)P\(_2\) released Ca\(^{2+}\) from sarcoplasmic reticulum (SR) ryanodine receptors almost in the same way. Arrows indicate the times when the drug was given. C: an inhibitory concentration \((50 \mu M)\) of ryanodine prevented PI(3,5)P\(_2\) from increasing intracellular Ca\(^{2+}\) in isolated aortic smooth muscle cells. D: summary data showing that 50 \(\mu M\) ryanodine effectively blocked the response to caffeine and PI(3,5)P\(_2\) \((n = 3)\). E: raw trace showing that pretreatment with 100 \(\mu M\) ryanodine also abolished aortic contractions to PI(3,5)P\(_2\). Data are means ± SE.

*Statistically significant difference \((P ≤ 0.05)\).
While examining the effects of inhibiting various pathways that could lead to the depolarization of vascular smooth muscle, we found that LaCl₃ blocked the effects of PI(3,5)P₂ related to entry of extracellular Ca²⁺. Using ratiometric Ca²⁺ imaging, we found that LaCl₃ inhibited (P < 0.0001) but did not abolish the rise in Ca²⁺ caused by PI(3,5)P₂ (Fig. 8, B and C), whereas it had no effect on the caffeine response (Supplemental Fig. 2B). Interestingly, the Ca²⁺ response to PI(3,5)P₂ in the presence of verapamil was indistinguishable (P = 0.7055) from the response with LaCl₃. In addition, 100 μM LaCl₃ impaired (P = 0.0085) but did not eliminate aortic contractions to PI(3,5)P₂ (Fig. 8A). For example, contraction to 3 μM PI(3,5)P₂ was 0.17 g in control rings and 0.04 g in the presence of LaCl₃ (Fig. 8A). Importantly, we did not observe inhibition of VDCCs by LaCl₃. This was demonstrated in aortic smooth muscle cells by showing that in the presence of LaCl₃, 100 mM KCl still elevated intracellular Ca²⁺ (Fig. 8B).

DISCUSSION

In this study, we examined the hypothesis that PI(3,5)P₂ would stimulate Ca²⁺ release from the SR of arterial smooth muscle cells and cause vasoconstriction. Below, we present three major novel findings. First, PI(3,5)P₂ is present in arterial smooth muscle under basal conditions. Second, using ratiometric Ca²⁺ imaging, we demonstrated that PI(3,5)P₂ elevates intracellular Ca²⁺ in two steps, beginning with the activation of RyRs, which mobilized SR Ca²⁺, followed by membrane depolarization and the activation of VDCCs to admit extracellular Ca²⁺. Third, PI(3,5)P₂ contracted the mouse aorta in a manner that required RyR-dependent SR Ca²⁺ release. For contraction to PI(3,5)P₂ to be sustained, extracellular Ca²⁺ entry via VDCCs was also required. Therefore, we propose that PI(3,5)P₂ stimulates RyRs to release SR Ca²⁺, which, by itself, can cause transient contractions, but for contractions to be sustained, additional Ca²⁺ entry from outside the cell is required. Similar to the aorta, we also found that PI(3,5)P₂ caused vasoconstriction of the mouse middle cerebral artery, which indicates that PI(3,5)P₂ can also constrict resistance arteries. Together, these results expand our understanding of vascular PI lipid signaling by showing that a newly identified PI lipid, PI(3,5)P₂, can modulate SR Ca²⁺ release and thus influence the contractile state of arterial smooth muscle.

PI(3,5)P₂ has only recently been discovered, and its presence in different tissues is still being understood. We found that PI(3,5)P₂ is present under basal conditions in the smooth muscle cell layers of the mouse aorta. Since there is no known pharmacological way to specifically and selectively increase PI(3,5)P₂ levels, we applied exogenous PI(3,5)P₂ to study its effects. First, however, we verified that PI(3,5)P₂ would enter arterial smooth muscle cells. Using fluorescently tagged PI(3,5)P₂ and epifluorescence microscopy, we found that freshly isolated arterial smooth muscle cells effectively take up PI(3,5)P₂ to the extent that every cell examined was fluorescent. This is identical to a previous study (69) in neutrophils that demonstrated that a variety of PI lipids, including PI(3,5)P₂, entered cells when complexed to the histone carrier protein. Although it was not possible for us to determine exactly where inside the cell that exogenous PI(3,5)P₂ became concentrated, it appeared to cluster in the interior of the cell and did not highlight the cell membrane. This is consistent with...
the known localization of PI(3,5)P2 in the membranes of intracellular organelles, including the SR (59).

The Ca2+/H11001 and contractile responses to exogenous PI(3,5)P2 were likely specific to PI(3,5)P2 because exogenous application of the PI(3,5)P2 metabolites PI(3)P or PI(5)P was largely without effect. The Ca2+/H11001 response to PI(3)P, although minor, was predictable because PI(3)P and PI(3,5)P2 can be interconverted. These data perhaps indicate that, within the time frame of our experiments, metabolism of PIs did not significantly contribute to the observed responses. Thus, the effects of exogenous PI(3,5)P2 were most likely due to PI(3,5)P2 itself and not a metabolite. The aortic contractions caused by PI(3,5)P2 appeared dependent on both intracellular and extracellular Ca2+/H11001. One of the mechanisms by which Ca2+/H11001 is released into the cytoplasm to elicit contraction occurs through RyR channels located in the SR membrane (35). There are three molecularly distinct subtypes of RyR channels (RyR1, RyR2, and RyR3), and all three are present in the mouse aorta (24). Ryanodine, at ≤10 μM, activates RyR channels and empties SR Ca2+/H11001 pools, whereas >10 μM ryanodine blocks RyR channels (40, 78), which we confirmed in our experiments. Interestingly, the Ca2+/H11001 release caused by 1 μM ryanodine was very similar to that of 1 μM PI(3,5)P2, suggesting that ryanodine and PI(3,5)P2 release SR Ca2+/H11001 in the same manner. We found that inhibitory concentrations of ryanodine prevented PI(3,5)P2 from eliciting a Ca2+/H11001 response in isolated aortic smooth muscle cells and also blocked contraction of the mouse aorta. Depletion of SR stores with thapsigargin, ryanodine, and/or caffeine greatly reduced the Ca2+/H11001 response to PI(3,5)P2 and abolished PI(3,5)P2-induced contractions. Therefore, we hypothesize that in aortic smooth muscle PI(3,5)P2 releases SR Ca2+/H11001 by activating RyRs.

In support of this hypothesis, Shen et al. (59) found that PI(3,5)P2 bound to and directly activated RyR1. Importantly, we (71) recently found that PI(3,5)P2 also binds to and activates RyR2. Because ryanodine completely blocked both the Ca2+/H11001 and contractile responses to PI(3,5)P2, it was unlikely that the other SR Ca2+/H11001-release mechanism, IP3Rs, were involved in the PI(3,5)P2 response. Regardless, we investigated the possibility that PI(3,5)P2 could also activate IP3Rs by blocking them with xestospongin C. In the presence of xestospongin C, the majority of the Ca2+/H11001 response to PI(3,5)P2 was intact. Although selective for IP3R, xestospongin C also inhibits SERCA; thus, it may be that SR stores were partially depleted by the inhibitor (13). If so, xestospongin C would be expected to decrease the PI(3,5)P2 response. In this way, xestospongin C would be acting similarly to thapsigargin, which itself reduced, but did not eliminate, the rise in intracel-
lular Ca\(^{2+}\) caused by PI(3,5)P\(_2\). Considering that ryanodine alone, which is highly specific for RyRs without affinity for IP\(_3\)Rs, abolished the responses to PI(3,5)P\(_2\), our data overall suggest that in aortic smooth muscle the ryanodine-sensitive SR Ca\(^{2+}\) pool may be mobilized by PI(3,5)P\(_2\) to increase intracellular Ca\(^{2+}\). Nevertheless, the possibility that IP\(_3\)Rs may also contribute to the PI(3,5)P\(_2\) response deserves future attention.

In the vasculature, L-type VDCCs are the major pathway for extracellular Ca\(^{2+}\) entry and are important regulators of vascular tone (38). In the absence of extracellular Ca\(^{2+}\) or in the presence of the VDCC inhibitor verapamil, PI(3,5)P\(_2\) elicited only transient contractions. Blockade of the sustained phase of the PI(3,5)P\(_2\)-mediated contractions by verapamil indicated that maximal contractions to PI(3,5)P\(_2\) required VDCCs and extracellular Ca\(^{2+}\). Also, in isolated aortic smooth muscle cells, the removal of extracellular Ca\(^{2+}\) or the addition of verapamil similarly decreased the rise in intracellular Ca\(^{2+}\) caused by PI(3,5)P\(_2\). This was not, however, simply an effect of removing extracellular Ca\(^{2+}\) or of verapamil on SR stores because neither of these conditions altered the rise in intracellular Ca\(^{2+}\) caused by caffeine. It seems then that the PI(3,5)P\(_2\)-mediated increase in intracellular Ca\(^{2+}\) observed in the presence of verapamil or Ca\(^{2+}\)-free buffer was due to Ca\(^{2+}\) release from the SR. This conclusion is supported by the observation that the increase in intracellular Ca\(^{2+}\) was substantially attenuated when the SR Ca\(^{2+}\) pool was depleted by pretreatment with thapsigargin or caffeine. An earlier study (34), which found that PI(3,5)P\(_2\) did not directly stimulate L-type VDCCs in vascular myocytes, also reinforces this conclusion.

Using patch-clamp electrophysiology, we found that PI(3,5)P\(_2\) elicited membrane depolarization. Although not large, a 6-mV depolarization is substantial enough to increase the open probability of VDCCs, elevate intracellular Ca\(^{2+}\), and cause vasorelaxation (28, 29, 43). In cerebral arteries, for example, a 9-mV depolarization opens VDCCs, admits extracellular Ca\(^{2+}\), and causes 25% constriction (28), which is on par with our results. In vascular smooth muscle, depolarization may occur by one of three main mechanisms, including inhibition of K\(^+\) channels, activation of Cl\(^-\) channels, or activation of cationic transient receptor potential (TRP) channels.

Since PI(3,5)P\(_2\) did not cause the entry of extracellular Ca\(^{2+}\) (via VDCCs) without first acting on the SR, we conclude that PI(3,5)P\(_2\) did not appreciably act on any ion channel within the plasma membrane. In addition, since PI(3,5)P\(_2\) first acts by raising intracellular Ca\(^{2+}\), we considered it unlikely that K\(^+\) channel inhibition could have been responsible for the depolarization because the open probability of many K\(^+\) channels is actually enhanced by increases in intracellular Ca\(^{2+}\).

The fact that PI(3,5)P\(_2\) raised intracellular Ca\(^{2+}\) levels makes the Ca\(^{2+}\)-activated Cl\(^-\) channel an attractive candidate for the depolarization because in vascular smooth muscle the equilibrium potential for Cl\(^-\) is depolarized compared with the resting membrane potential (1). Although there are no specific inhibitors of Ca\(^{2+}\)-activated Cl\(^-\) channels, we used perhaps one of the best, or at least one of the most commonly used inhibitors, DIDS, to determine if PI(3,5)P\(_2\) activated a Cl\(^-\) channel. Consistent with numerous reports (16–18, 27, 33, 74) detailing nonspecific effects of Cl\(^-\) channel inhibitors, we found that DIDS alone increased intracellular Ca\(^{2+}\) in isolated aortic smooth muscle cells (data not shown), which is the opposite of what we predicted. Thus, we did not find DIDS to be a useful tool and consider that a complete investigation of Ca\(^{2+}\)-activated Cl\(^-\) channels as a component of the PI(3,5)P\(_2\) response is a subject best left for future investigation.

In contrast, the pan-TRP channel inhibitor LaCl\(_3\) acted very much like verapamil or zero extracellular Ca\(^{2+}\). That is, LaCl\(_3\) reduced, but did not eliminate, the rise in intracellular Ca\(^{2+}\) or the contractions caused by PI(3,5)P\(_2\). Because LaCl\(_3\) is not specific for TRP channels, it is important to point out that we did not observe nonspecific inhibition of VDCCs by LaCl\(_3\). In vascular smooth muscle, TRP channels can be activated by...
depletion of SR Ca\(^{2+}\) stores and may interact directly with the SR via RyRs and IP\(_3\)R. Thus, we believe it is possible, but not assured, that the activation of RyRs by PI(3,5)P\(_2\) causes cationic TRP channels to open, thereby depolarizing the membrane and activating VDCCs. At this time, however, we are not prepared to definitively claim that this mechanism accounts for the activation of VDCCs by PI(3,5)P\(_2\). This is because the available pharmacology is suboptimal and also because of the great variety of TRP channels that could possibly be involved. In addition, we have not been able to rule out a role for Ca\(^{2+}\)-activated Cl\(^{-}\) channels, the molecular identity of which is still uncertain. Therefore, we believe that full elucidation of the mechanism by which PI(3,5)P\(_2\) causes depolarization is beyond the scope of the present study.

Abnormalities in Ca\(^{2+}\) signaling have been described in cardiovascular diseases, and pharmacological blockers of VDCCs are used in the treatment of hypertension and angina (30, 37, 42). As our experiments demonstrate, PI(3,5)P\(_2\) modulates Ca\(^{2+}\) signaling in vascular smooth muscle, and, therefore, it may regulate cardiovascular function. In this way, PI(3,5)P\(_2\) joins rank with the better-known PI bisphosphate lipid PI (4,5)-bisphosphate [PI(4,5)P\(_2\)], which is often referred to simply asPIP\(_2\). Perhaps the best-known effect of PI(4,5)P\(_2\) is its degradation by receptor-activated phospholipase C to generate IP\(_3\) and diacylglycerol (DAG). While the signal transduction cascades initiated by the cleavage of PI(4,5)P\(_2\) into IP\(_3\) and DAG are well appreciated, the direct actions of PI(4,5)P\(_2\) are less well known. As a component of the plasma membrane, PI(4,5)P\(_2\) has access to membrane-bound proteins including ion channels, and it has both direct and indirect actions on a variety of ion channels. For example, KCNQ channels require PI(4,5)P\(_2\), and its depletion inhibits the channel (62, 64, 66, 67). In addition, in vascular smooth muscle, TRPC1 is activated by PI(4,5)P\(_2\), whereas TRPC6 is inhibited by it (2, 32, 53). Opening of VDCCs may also be modulated by PI(4,5)P\(_2\) via a complicated interaction with arachidonic acid (49). Typically, however, PI(4,5)P\(_2\) acts to increase ion channel activity, although that is far from the limit to PI(4,5)P\(_2\)’s actions, which have been well reviewed by Suh and Hille in 2005 and 2008 (63, 65).

Compared with PI(4,5)P\(_2\), much less is known about the actions of PI(3,5)P\(_2\). The synthesis of PI(3,5)P\(_2\) is tightly regulated, and changes in its levels may be protective or deleterious. In yeast, there is a dramatic and transient rise in PI(3,5)P\(_2\) levels of up to 20-fold in response to hyperosmotic shock (9). In the mammalian cell line of differentiated 3T3-L1 adipocytes, PI(3,5)P\(_2\) levels also increased with hyperosmotic stress (58). Similarly, IL-2 and ultraviolet exposure also stimulated PI(3,5)P\(_2\) formation in T lymphocytes (26). Insulin also appears to stimulate the production of PI(3,5)P\(_2\), which causes glucose transporter 4 to translocate to the plasma membrane (6). Thus, it appears that, in general, PI(3,5)P\(_2\) levels are increased as a protective mechanism to deal with cellular stress (19).

Mutations in genes that affect enzymes responsible for PI(3,5)P\(_2\) metabolism are also linked to human diseases (12, 15, 39, 45, 72). For example, in humans, mutations of PI-3-kinase results in Francois-Neetens-Mouchette fleck corneal dystrophy (36), mutations in MTMR2 cause Charcot-Marie-Tooth type 4B (7, 8), and mutations of FIG4/SAC3 cause Charcot-Marie-Tooth type 4J (11). PI(3,5)P\(_2\) levels are also elevated in skeletal muscles of MIP/MTMR14 phosphatase knockout mice, resulting in basal Ca\(^{2+}\) leakage from the SR by the direct activation of RyR1 (59). Therefore, control of PI(3,5)P\(_2\) appears critical for the proper regulation of intracellular Ca\(^{2+}\) levels in skeletal muscle. While it is too soon to speculate how increases in PI(3,5)P\(_2\) might be protective in the vasculature, or how decreased levels may contribute to disease, it may be that PI(3,5)P\(_2\) levels are increased in cardiovascular disease states. Our experiments are, therefore, a first step toward understanding the physiological role for PI(3,5)P\(_2\) in the cardiovascular system.

In summary, we demonstrated, for the first time, that PI(3,5)P\(_2\) elevates intracellular Ca\(^{2+}\) in aortic smooth muscle cells and induces vasconstriction of aortic rings. The increase in intracellular Ca\(^{2+}\) and contraction was caused first by SR Ca\(^{2+}\) release via the activation of RyRs followed by membrane depolarization that opened VDCCs, thereby admitting extracellular Ca\(^{2+}\). While it is possible to speculate how the activation of RyRs by PI(3,5)P\(_2\) could lead to membrane depolarization, there are challenges, such as limited pharmacological and genetic tools for the study of TRP channels and Cl\(^{-}\) channels, in particular, that make full exploration of this aspect of the mechanism outside the extent of the present investigation. Future studies will address this as well as the consequences of altered PI(3,5)P\(_2\) signaling on arterial smooth muscle and its implications in cardiovascular health and disease.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


PI(3,5)P₂ EFFECT IN AORTIC SMOOTH MUSCLE CELLS


