Genetic ablation of calcium-independent phospholipase A$_2$B causes hypercontractility and markedly attenuates endothelium-dependent relaxation to acetylcholine


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Am J Physiol Heart Circ Physiol 298: H2208–H2220, 2010. First published April 9, 2010; doi:10.1152/ajpheart.00839.2009.—Activation of phospholipases leads to the release of arachidonic acid and lysophospholipids that play prominent roles in regulating vasomotor tone. To identify the role of calcium-independent phospholipase A$_2$B (iPLA$_2$B) in vasomotor function, we measured vascular responses to phenylephrine (PE) and ACh in mesenteric arteries from wild-type (WT; iPLA$_2$B$^{+/+}$) mice and those lacking the $\beta$-isoform (iPLA$_2$B$^{-/-}$) both ex vivo and in vivo. Vessels isolated from iPLA$_2$B$^{-/-}$ mice demonstrated increased constriction to PE, despite lower basal smooth muscle calcium levels, and decreased vasodilation to ACh compared with iPLA$_2$B$^{+/+}$ mice. PE constriction resulted in initial intracellular calcium release with subsequent steady-state constriction that depended on extracellular calcium influx. Endothelial denudation had no effect on vessel tone or PE-induced constriction although the dilution to ACh was significantly reduced in iPLA$_2$B$^{+/+}$ vessels. In contrast, vessels from iPLA$_2$B$^{-/-}$ constrained by 54% after denudation, indicating smooth muscle hypercontractility. In vivo, blood pressure, resting vessel diameter, and constriction of mesenteric vessels to PE were not different in iPLA$_2$B$^{-/-}$ vessels compared with WT mouse vessels. However, relaxation after ACh administration in situ was attenuated, indicating an endothelial inability to induce dilation in response to ACh. In cultured endothelial cells, inhibition of iPLA$_2$B with (S)-(E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BEL) decreased endothelial nitric oxide synthase phosphorylation and reduced endothelial agonist-induced intracellular calcium release as well as extracellular calcium influx. We conclude that iPLA$_2$B is an important mediator of vascular relaxation and intracellular calcium homeostasis in both smooth muscle and endothelial cells and that ablation of iPLA$_2$B causes agonist-induced smooth muscle hypercontractility and reduced agonist-induced endothelial dilation.

vascular smooth muscle; vascular endothelium; calcium signaling; 12-hydroxyicosatetraenoic acid; lysophosphatidylcholine

THE PATHOPHYSIOLOGICAL MECHANISMS underlying hypertension and vascular complications of diabetes relate, in part, to the impaired relaxation of vascular smooth muscle. For instance, ACh-induced mouse or rat mesenteric vessel dilation is reduced in conditions of oxidative stress, diabetes, or hypertension (11, 19, 23, 24). ACh-mediated endothelium-dependent vascular relaxation has been attributed primarily to the activation of endothelial nitric oxide (NO) synthase (eNOS) and the formation of NO to relax smooth muscle cells. In rodent mesenteric vessels NO contributes 50% to 80% of the ACh-mediated dilatory response (4, 23, 24, 33). However, a substantial portion of the vasodilatory response to ACh cannot be accounted for by NO alone and has previously been demonstrated to result from the action(s) of an endothelium-derived hyperpolarizing factor (EDHF) (23, 31). Although the precise chemical identity of EDHF is unknown, substantial evidence indicates that it is an oxygenated eicosanoid metabolite, such as epoxyeicosatrienoic acid, which is formed by a cytochrome $P$-450 monoxygenase following the release of arachidonic acid (AA) from its endogenous storage pools. Thus EDHF could also cause endothelial membrane hyperpolarization, which spreads to adjacent smooth muscle cells via heterocellular gap junctions to cause further vessel relaxation (9). Importantly, epoxyeicosatrienoic acids have been shown to induce vasodilation in rat mesenteric vessels (4, 31) in the absence of alterations in NO. The strongest evidence for the importance of NO-independent effects in vascular relaxation is that mice deficient in eNOS still exhibit ACh-mediated vasodilation (38).

The majority of arachidonic acid released in endothelial cells is mediated by intracellular PLA$_2$, which catalyze the hydrolysis of fatty acid substrates, e.g., arachidonic acid, from glycerophospholipid substrates (25, 40). Although both Ca$_{2+}$-dependent PLA$_2$ (cPLA$_2$) and Ca$_{2+}$-independent PLA$_2$ (iPLA$_2$) forms of the enzyme are present in mammalian cells (34), the predominant activity in endothelium is derived from iPLA$_2$ (25) and is sensitive to inhibition by (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BEL) (20). iPLA$_2$ activities are broadly distributed in mammalian tissues (22), and nine members of the iPLA$_2$ family have been identified. This family of enzymes is defined by the presence of a patatin-like homology domain (20 kDa), which contains consensus sequences for a nucleotide binding fold (GXGXXXG) and a serine $\alpha$/$\beta$-hydrolase motif (GXGXXG) (21). These enzymes are classified as PNPLA1–9 (HUGO nomenclature), reflecting the nine genes encoding this family of proteins in the human genome.
In vascular smooth muscle cells, iPLA₂ contributes to the regulation of calcium homeostasis. Depletion of intracellular calcium stores activates iPLA₂ (20, 35, 39), which activates store-operated channels that facilitate calcium influx resulting in smooth muscle contraction (30). Recently, we reported that the iPLA₂-generated arachidonic acid was essential to vasodilation resulting from application of ACh to rat mesenteric arteries in vitro (33), although iPLA₂ did not appear to contribute to purine-induced dilation of rat middle cerebral arteries (41). In addition, calcium-independent isoforms of PLA₂ were found to regulate calcium entry in aortic endothelial cells (5).

To further clarify the action of iPLA₂β in vascular contraction and relaxation in resistance vessels, we generated mice null for iPLA₂β (iPLA₂β⁻/⁻) by homologous recombination (2, 3). The goals of the present study were to determine whether genetic ablation of iPLA₂β attenuates phenylephrine (PE)-induced constriction and ACh-mediated relaxation in resistance vessels. Using a well-accepted ex vivo assay of vascular reactivity in isolated mesenteric arterioles together with in vivo assessment of vascular relaxation, we demonstrated an important role for iPLA₂β in rendering vascular smooth muscle from iPLA₂β⁻/⁻ animals hypercontractile, despite lower calcium levels in these cells. In addition, vessels from these animals exhibited reduced endothelium-dependent agonist-induced relaxation likely due to reduced eNOS phosphorylation and agonist-induced calcium influx into the endothelial cells.

MATERIALS AND METHODS

Preparation of mice and pressurized vessels. All protocols were approved by the Animal Studies Committee at Washington University. Mice homozygous for disruption of the iPLA₂β allele (iPLA₂β⁻/⁻) were generated as described previously and established in a C57BL/6 background (2, 3). Animals were genotyped as described previously (2, 3). Male knockout mice over 12 wk of age (25–35 g) and age-matched wild-type controls (iPLA₂βᵒˢᵉᵇ/O) were anesthetized with ketamine (87 mg/kg ip) and xylazine (13 mg/kg ip). The abdomen was shaved and incised in the midline to access the small intestine. A 5- to 7-cm loop of the jejunum was withdrawn from the incision into a petri dish and kept moist with saline. Adipose and loose connective tissues were carefully removed from two to three second or third order mesenteric arterioles with the aid of a dissecting microscope. For ex vivo analysis of vascular reactivity, 2- to 3-mm lengths of the exposed vessels without branches were excised and transferred to a petri dish with 2.0 mol/l MOPS buffer containing 10% wt/vol of dialyzed bovine serum albumin and (in mmol/l) 144 NaCl, 3.0 KCl, 2.5 CaCl₂, 1.5 MgSO₄, 2.0 pyruvate, 5.0 glucose, 0.02 ethylenediaminetetraacetic acid, and 1.21 NaH₂PO₄ at 20°C. The mouse was then euthanized by anesthetic overdose. For in vivo video-microscopic imaging of the mesenteric vessels, the mouse was placed on the stage of an inverted microscope (Zeiss Axiovert S100TV; Zeiss, Thornwood, NY). The vessel was cannulated on one side with a perfusion pipette and occluded on the other side with a collecting pipette (17). No luminal flow was allowed in the experiment. The vessels were observed with a video camera system (MTI CCD-72; Dage-MTI, Michigan City, IN), and the video images were recorded on digital video tape. Diameter measurements were made in response to agonists added to the bath (17). Briefly, the internal diameters were measured online at 60 mmHg and 37°C with a computerized diameter tracking system (sampling rate of 10 Hz; Diamtrak 3 Plus; Montech, Melbourne, Australia) that also stored the digital still images for analysis. After a 20-min equilibration period, the vessel diameter was measured and the bath solution was replaced with warmed MOPS buffer containing freshly dissolved PE (Sigma, St. Louis, MO) titrated to induce maximal constriction. Preliminary data showed maximal constriction with 100 μmol/l PE, which was then used in all subsequent experiments. Since mesenteric vessels of this caliber do not develop spontaneous tone (38), we used the PE stimulation as an indicator of viability and discarded vessels that yielded <90% change in diameter. The diameter of the arteriole was measured after both maximal and steady-state constriction with PE (within 2 min) after which the bath solution was changed to MOPS buffer containing 100 μmol/l PE plus 0.1 μmol/l ACh (Sigma). After relaxation of the vessel to a new steady state, the diameter was measured and the bath solution exchanged with MOPS buffer containing 100 μmol/l PE and 1.0 μmol/l ACh. The steady-state diameter was then measured again. The bath solution was changed to MOPS buffer alone to achieve baseline relaxation to the original (pre-PE) vessel diameter. In wild-type vessels, the experiment was then repeated with buffers containing the mechanism-based inhibitor of iPLA₂ BEL (10 μmol/l). In preliminary experiments, we confirmed that the BEL vehicle (ethanol, 0.1%) had no effect on vessel responses. In a subset of experiments, we tested the capacity of the vessels to constrict to a depolarizing stimulus by exposing vessels from both wild-type and iPLA₂β knockout mice to MOPS buffer containing 40 mmol/l potassium chloride.

Measurement of intracellular calcium in arteriolar smooth muscle cells. Isolated and cannulated vessels from either wild-type or iPLA₂β knockout mice were loaded extraluminally with the Ca²⁺-sensitive dye fura-2 according to methods reported previously (8). Briefly, fura-2-acetoxymethyl ester (50 μg of fura-2 AM; Molecular Probes, Eugene, OR) was presoaked in 10 μl of dry DMSO containing 20% of Pluronic (Molecular Probes) as a dispersing agent diluted to 100 μl with distilled H₂O and then to a final volume of 10 μl with MOPS buffer (5 μmol/l fura-2 AM final concentration). The vessels were incubated with the fura-2 AM solution for 15 min at 37°C in the dark, followed by washing and equilibration with MOPS buffer for 20 min. Light emissions following excitation at 340 and 380 nm (sampling rate of 10 Hz) were compared ratiometrically following application of 100 μmol/l PE, PE plus 10 μmol/l ACh, and after washout with MOPS buffer using a photon multiplier-based detection system (C&L Instruments, Hershey, PA). Simultaneous video measurements of vessel diameters were made utilizing a 730-nm laser and a Diamtrak system. Estimates of the intracellular calcium levels were determined after system calibration as described previously (8, 13). Extraluminal fura-2 application has been reported to specifically load the smooth muscle cells with the endothelium not contributing to the fura-2 signal (10, 26). To confirm this in our preparation, in some vessels we measured the fura-2 ratio before and after endothelial denudation using air embolus as described previously (18). In PE preconstricted vessels endothelial denudation reduced the dilatory response to ACh (10 μmol/l) by 74% in wild-type vessels (P < 0.05, n = 3), indicating successful endothelial impairment. However, the fura-2 signal was unchanged after denudation (wild-type vessels, ratio 0.71 ± 0.06 before and 0.69 ± 0.06 after embolus, n = 4; iPLA₂β knockout vessels, ratio 0.53 ± 0.02 before and 0.52 ± 0.01 after embolus), confirming that the endothelium did not contribute to the fura-2 signal.
Videocontrast imaging of in vivo mesenteric vessels. The mesenteric arcade was superfused with 10 mmol/l HEPES buffer containing (in mmol/l) 135 NaCl, 2.6 NaHCO3, 0.34 NaH2PO4, 0.44 KH2PO4, 5 KCL, 1.4 CaCl2, 1.17 MgSO4, 0.025 EDTA, and 5.5 glucose at pH 7.35–7.4. The solution was heated to 37°C (Radnoti heat exchanger; Radnoti, Monrovia, CA) before being dripped on the exposed vessels. One of the dissected vessels was located at 40X magnification with a digital camera (Nikon Coolpix 5000, maximum zoom) attached to a color monitor (Sony PVM-1342Q; Sony, San Diego, CA). A baseline image of the vessel was then saved for analysis of the inner diameter. The superfusate buffer was switched to HEPES buffer containing fresh 100 μmol/l PE (shown to yield maximal constriction in preliminary studies), and an image of the constricted vessel was saved after 5 min. Superfusion with PE was continued, and 16 μmol/l ACh in buffer (0.2 ml) was injected into the jugular venous catheter. Preliminary studies showed that bolus injection of 16 μmol/l ACh induced relaxation of the mesenteric arterioles without affecting heart function (as determined by measuring heart rate from the surface ECG). Images of the vessel were saved every 30 s for 5 min after injection of ACh. The vessel was then superfused with buffer alone for at least 4 min to restore the original diameter before moving to another vessel to repeat the process. Images stored in the digital camera were uploaded to a personal computer containing Jasc Paintshop Pro 6.0 software (Corel; Ottawa, ON, Canada). With the use of the 40X image micrometer scale, the pixel coordinates provided by Paintshop were expressed as a pixel-to-micron ratio that was used to analyze vessel images. The distance between points (X,Y) on the opposite edges of the vessel was calculated in pixels with use of the Pythagorean theorem: pixel distance = [(X2-X1)2 + (Y2-Y1)2]0.5. The resulting distance was multiplied by the pixel-to-micron ratio to yield the diameter of the vessel in microns.

Measurements of blood pressure. In a separate set of experiments, blood pressure was measured in intact animals anesthetized with 1.5% isoflurane in oxygen since this anesthetic causes minimal cardiac depression in mice. A 1.4-F catheter pressure probe (Millar Instruments, Houston, TX) was passed into the ascending aorta via a cutdown of the right common carotid artery. Mean arterial blood pressure and heart rate were recorded with use of a Powerlab/4sp data acquisition system (ADInstruments, New Castle, Australia).

Determination of iPLA2β mRNA levels. Tissue-specific expression of iPLA2β was analyzed using RT-PCR as described previously (3). Briefly, PCR conditions typically employed a 30-cycle reaction with steps at 53°C for 30 s, 72°C for 2 min, and 94°C for 30 s per cycle. PCR products were resolved by agarose gel electrophoresis. The following primer sets were used for amplification from cDNA encoding iPLA2β: OR, 5'-CTGCGAAATTTCCATGTCGAAAGATAACATGGGAG-3'; ER, 5'-CCGAGGGCCTTCTTACATCGGAAGTACAC-3'; FF, 5'-ATGATTACATCGATGACAGCA3'- R, 5'-ACACAGTTACACGGCATTGGG-3'. Primer sets were utilized to amplify PCR products from iPLA2β and mesenteric cDNA.

Cell culture of endothelial cells. EA.hy 926 endothelial cells derived from human umbilical vein endothelium were kindly provided by Dr. Cora-Jean S. Edgell (Pathology Department, University of North Carolina, Chapel Hill, NC). Cell cultures were maintained in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, HT supplement (100 μmol/l hypoxanthine, 16 μmol/l thymidine) and 10% heat-inactivated fetal bovine serum. These cells were seeded, grown in an atmosphere of 5% CO2 at 37°C to confluence, subcultured routinely using 0.25% trypsin/EDTA, and used for experiments within nine passages.

Fluorometric determination of nitric oxide. Confluent cells in 6-mm tissue culture dishes were washed twice with HBSS supplemented with 0.35 g/l NaHCO3, 1.8 mmol/l CaCl2, 100 μmol/l L-arginine, and 0.8 mmol/l MgSO4 (pH 7.4) and were preincubated in the same buffer for 4 h to allow cells to become quiescent. The buffer solution was then aspirated, and the cells were incubated for 10 min in fresh HBSS in the presence or absence of agonists. For iPLA2β inhibition experiments, cells were pretreated with the indicated concentration of BEL [racemic-, (R)- or (S)-BEL] or ethanol vehicle (<0.1% vol/vol in final concentration) for 10 min at 37°C and the buffer solution was replaced at the time of agonist stimulation. Racemic BEL is a generic inhibitor of iPLA2β, (S)-BEL is a specific inhibitor for the iPLA2β, and (R)-BEL for the iPLA2γ isofrom (20).

After stimulation with 100 μmol/l ATP, the supernatants were collected for spectrofluorometric quantification of NO release. The NO assay was done according to the manufacturer's instructions with minor modifications. Briefly, aliquots (80 μl) of the collected supernatants were placed in a white 96-well microplate. Ten microliters of nitrate reductase solution and 10 μl of cofactor solution were prepared and added to each well for conversion of nitrate (NO3⁻) into nitrite (NO2⁻). After a 2-h incubation at room temperature, samples were further incubated for 40 min in the presence of the fluorescent probe 2,3-diaminonaphthalene (Cayman Chemical, used as provided by the manufacturer). To terminate the reaction and enhance fluorometric sensitivity, 10 μl of 2.8 mol/l NaOH was added and the fluorescence of each well was then measured at room temperature using a Spectra Max Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA) with 365 nm excitation and 450 nm emission wavelengths.

Intracellular calcium measurements in endothelial cells. Similar to the vessel experiments, intracellular calcium concentrations were measured in cultured endothelial cells using fura-2 AM. Confluent cells were split 1:4 onto Delta TPG dishes (0.17 mm thick; Bioteps, Butler, PA) and grown for 24 h. After cells were washed with HBSS twice, they were incubated for 90 min in the dark in HBSS (pH 7.4) containing 5 μmol/l fura-2 AM [pre-dissolved in DMSO containing F-127 Pluronic (0.02% final concentration)]. The cells were washed again to remove unincorporated fura-2 AM. For BEL inhibition experiments, cells were incubated in HBSS containing the indicated concentrations of BEL or ethanol vehicle (0.1% vol/vol) alone for 15 min. After excess BEL was washed away, fresh buffer solution (including 100 μmol/l ETGA for Ca2⁺-free media) was placed into the dishes, followed by a 10-min preincubation period before fluorescence measurements. The dishes were mounted on the stage of a Zeiss inverted digital microscope (40X) and cells were illuminated alternatively with 340 nm/380 nm light for excitation, and data were collected at 510 nm as the fixed emission wavelength. The concentration of intracellular Ca2⁺ was calculated based upon the fluorescence intensity ratio (Ratio F340/F380). Image data were analyzed by using Slidebook software (Intelligent Imaging Innovations, Denver, CO). Chemicals used were racBEL, (S)-, (R)-BEL (10 μmol/l), AAME (20 μmol/l), 1-palmityl-l-lysophosphatidylcholine (LPC, 10 μmol/l), 12-hydroxyecosatetraenoic acid (12-HETE, 4 μmol/l), ATP 100 μmol/l, 5,8,11,14-ecicosatetraenoic acid (ETYA, 20 μmol/l, a nonmetabolizable analog of AA, which blocks cyclooxygenase, lipoxigenase, and cytochrome P-450 epoxide pathways), 17-octadecenoic acid (17-ODYA; 10 μmol/l, a cytochrome P-450 epoxide and ω-hydroxylase inhibitor), ibuprofen (20 μmol/l, a cyclooxygenase inhibitor), and nordihydroguaiaretic acid (NDGA; 20 μmol/l, a lipoxigenase inhibitor).

eNOS phosphorylation Western blotting analysis. EA.hy 926 cells utilized for Western blotting were washed with ice-cold PBS and lysed in 500 μl of 50 mmol/l Tris, pH 7.4, containing 150 mmol/l NaCl, 1 mmol/l EDTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mmol/l phenylmethanesulfonfyl fluoride, 2 μg/ml aprotinin, and 1 μg/ml leupeptin. Protein concentration was determined with a Bio-Rad protein assay utilizing bovine serum albumin as standard. Western blotting with an antibody against phospho-eNOS at Ser1177 was performed following the manufacturer’s protocol (Cell Signaling Technology, Danvers, MA) using 7% SDS-PAGE gels with
Results

Characterization of iPLA\(_2\)\(_B\) expression. The presence of iPLA\(_2\)\(_B\) in the mesenteric arteries under study in iPLA\(_2\)\(_B^{+/+}\) animals was confirmed by RT-PCR. As anticipated, genetic ablation of iPLA\(_2\)\(_B\) by homologous recombination resulted in the complete absence of iPLA\(_2\)\(_B\) message in the mesenteric vessels (Fig. 1) as well as in all other tissues examined previously (2, 3).

Vascular responses of mesenteric vessels to PE and ACh using pressurized ex vivo preparations. After anesthesia, mesenteric vessels were harvested and subjected to analysis using quantitative video microscopy. First, the effects of PE-mediated constriction and ACh-induced dilation of mesenteric vessels from iPLA\(_2\)\(_B^{+/+}\) and iPLA\(_2\)\(_B^{-/-}\) mice were examined. Examples of the vasomotor responses and representative traces of diameter changes are shown in Figs. 2 and 3, A and B, respectively. Vessels isolated from iPLA\(_2\)\(_B^{-/-}\) mice had an average diameter of 168 ± 28 \(\mu m\) (n = 5) that was not different from the diameter of vessels from wild-type animals (165 ± 4 \(\mu m\), n = 5; Mann-Whitney test). Vessels from both groups of mice constricted significantly to PE (100 \(\mu mol/l\)). However, vessels from iPLA\(_2\)\(_B^{-/-}\) mice showed significantly higher maximal and steady-state constriction responses to PE than wild-type vessels (48 ± 11 vs. 83 ± 4 \(\mu m\) and 48 ± 9 vs. 91 ± 7 \(\mu m\), respectively). Addition of ACh 10\(^{-5}\) \(\mu mol/l\) dilated wild-type vessels but had no significant effect on vessels from iPLA\(_2\)\(_B^{-/-}\) mice. Replacing the PE containing buffer with MOPS buffer resulted in all examined vessels returning to their baseline diameters. To elucidate the contribution of the endothelium to the diameter responses, we denuded an additional three vessels each from wild-type and iPLA\(_2\)\(_B^{-/-}\) animals. Denudation did not affect the resting diameter of wild-type vessels (240 ± 11 vs. 240 ± 10 \(\mu m\)). Constriction to PE (100 \(\mu mol/l\)) was not significantly enhanced (101 ± 12 vs. 84 ± 7 \(\mu m\)), but the dilation to ACh (10 \(\mu mol/l\)) was reduced by 74% (P < 0.05, paired t-test), indicating endothelial damage. Vessels from iPLA\(_2\)\(_B^{-/-}\) mice, however, constricted from 234 ± 27 to 111 ± 13 \(\mu m\) (P < 0.05, paired t-test) following denudation. In preliminary experiments, we also tested 100 \(\mu mol/l\) PE, which enhanced the vessel constriction (average 123 vs. 54 \(\mu m\)), whereas addition of ACh (10 \(\mu mol/l\)) did not change the diameter (average 55 \(\mu m\)).

Vessels from wild-type and iPLA\(_2\)\(_B^{-/-}\) mice similarly constricted to depolarizing potassium chloride (40 mmol/l). The vessel from the wild-type mice decreased their diameter from 212 ± 2 to 128 ± 2 \(\mu m\), a decrease of 60 ± 2% (n = 3) with vessels from iPLA\(_2\)\(_B^{-/-}\) mice constricting from a diameter of 196 ± 1 \(\mu m\) before to 121 ± 1 \(\mu m\) in response to depolarization, a decrease of 64 ± 2% (n = 3).

Ratiometric calcium measurements in isolated vessels. To determine the role of intracellular free calcium in mediating the observed effects, concomitant measurements of vessel diameter and intracellular calcium ratios were made in vessels from iPLA\(_2\)\(_B^{+/+}\) and iPLA\(_2\)\(_B^{-/-}\) animals. Vessels from iPLA\(_2\)\(_B^{-/-}\) mice had a significantly lower basal fluorescence ratio (R = 0.47 ± 0.02 or 41 nmol/l Ca\(^{2+}\)) compared with vessels from wild-type mice (0.80 ± 0.01 or 172 nmol/l Ca\(^{2+}\)), indicating a lower calcium level in mesenteric vessels from iPLA\(_2\)\(_B^{-/-}\) mice. Mesenteric arterioles isolated from iPLA\(_2\)\(_B^{-/-}\) animals demonstrated an increase in intracellular calcium after addition of 100 \(\mu mol/l\) PE (R = 0.80 ± 0.03 or 172 nmol/l Ca\(^{2+}\)) that coincided with vessel constriction of the vessel. Vessels from iPLA\(_2\)\(_B^{+/+}\) animals also showed an increase in calcium fluorescence ratio (1.17 ± 0.03 or 355 nmol/l Ca\(^{2+}\)) consistent with the presence of iPLA\(_2\)\(_B\) in these vessels. Addition of ACh (10 \(\mu mol/l\)) to vessels from iPLA\(_2\)\(_B^{+/+}\) mice increased intracellular calcium by 1.6 ± 0.3 whereas vessels from iPLA\(_2\)\(_B^{-/-}\) mice did not change the intracellular calcium levels.

Statistical analysis. Results are expressed as means ± SE. N indicates the number of animals, with one or two vessels per animal studied for ex vivo and two or three vessels studied per animal for in vivo experiments. Results from multiple vessels were averaged. Differences between means were determined using the paired or unpaired Student’s t-test as appropriate. Differences in responses to agonists and inhibitors between knockout and wild-type animals were determined by ANOVA or repeated-measures ANOVA as appropriate. For non-parametric evaluation we used the Mann-Whitney test. For cell culture experiments, only one treatment was applied per culture. Results from multiple cultures were averaged, and the effects of agonists and antagonists on calcium responses were determined by Student’s t-test. A level of P ≤ 0.05 was considered significant.

Fig. 1. PCR amplification of calcium-independent phospholipase A\(_2\) (iPLA\(_2\)\(_B\)) message from iPLA\(_2\)\(_B^{-/-}\) and iPLA\(_2\)\(_B^{+/+}\) mouse tissues. Three primer pair sets (see MATERIALS AND METHODS) were utilized to amplify iPLA\(_2\)\(_B\) message from cDNA libraries from heart (lanes 1 and 2) and mesenteric arterioles (Mesent lanes 3 and 4). A: OF and OR amplified a 431 nt product. B: RR and OR amplified a 683 nt product. C: OF and R amplified an 898 nt product. D: PCR amplification of iPLA\(_2\)\(_B\) genomic DNA utilizing primers 5′-TCAGCAGCAGTCGTTGAGGTA-3′ and 5′-CAGGCTCCAAAGAATCTGGTGATCG-3′ to amplify a 978 nt in iPLA\(_2\)\(_B^{+/+}\) (lane 2) but not iPLA\(_2\)\(_B^{-/-}\) mice (lane 1).
constriction. Although the absolute increase in the calcium fluorescence ratio was similar (ΔR = 0.36 for wild-type vessels and ΔR = 0.34 for iPLA2β-/-), vessels from iPLA2β-/- animals demonstrated substantially greater vasoconstriction. In both iPLA2β+/+ and iPLA2β-/- vessels, the fluorescence ratio decreased after an initial peak, which coincided with reaching an increased steady-state diameter in wild-type vessels. However, the diameter in iPLA2β-/- vessels did not increase even after the decrease in fluorescence ratio. Addition of ACh 10^-5 mol/l significantly dilated the wild-type vessels coincident with a significant decrease in calcium level. In stark contrast, addition of ACh did not dilate vessels from iPLA2β-/- mice or decrease their calcium fluorescence ratio.

Comparisons between the PE responses of iPLA2β+/+ and iPLA2β-/- vessels demonstrated that the relative increase of the calcium fluorescence ratio was higher in iPLA2β-/- vessels (169 ± 16%) compared with iPLA2β+/+ vessels (145 ± 5%; P < 0.05). In seven vessels from iPLA2β-/- animals, the calcium containing MOPS buffer was replaced with calcium free buffer by replacing the calcium chloride with equimolar sodium chloride. After 10 min of incubation, the PE stimulation (PE dissolved in Ca²⁺-free MOPS) was repeated. Despite removal of the extracellular calcium, PE stimulation resulted in an initial constriction and a simultaneous Ca²⁺-signal increase, which was not different from that observed in the calcium control, suggesting the release of intracellular calcium. This constriction was followed by a rapid relaxation and decrease in calcium level with the steady-state constriction greatly reduced compared with calcium control, indicating that the steady-state constriction is depending upon the influx of extracellular calcium. A second PE stimulation further reduced the initial PE-induced constriction and calcium level, indicating that intracellular calcium stores had been greatly depleted. Furthermore, within seconds, the vessels returned to a steady-state diameter that was not different from their control diameter. We repeated this experiment in four vessels from wild-type animals (diameter of 215 ± 24 μm) and found that the decreases in diameter and calcium responses paralleled those observed in iPLA2β-/- vessels (data not shown). Collectively, these results indicate the participation of both intracellular and extracellular calcium in facilitating calcium-mediated, agonist-induced contraction. In particular, sustained contraction requires a stimulus-mediated sustained influx of extracellular calcium. Moreover, a second stimulation with PE reduced both the initial and steady-state vasoconstriction, in parallel with the calcium signal, which would suggest that the initial PE response relies predominantly on calcium release from intracellular, depletable stores, whereas the steady-state constriction largely depends on the influx of extracellular calcium (Fig. 4).

Inhibition of iPLA2β with BEL attenuates PE-mediated vasoconstriction and ACh-mediated vasorelaxation. The baseline diameter of iPLA2β+/+ vessels was 181 ± 8 μm (n = 7) and was not significantly changed after treatment with BEL (184 ± 9 μm). Application of 100 μmol/l PE resulted in a marked constriction of the mesenteric vessels (106 ± 8 μm or 58% of control diameter) that was significantly attenuated in the presence of BEL (125 ± 6 μm or 68% of control diameter; Fig. 5). However, a spontaneous, partial relaxation to a steady-state constriction during continued exposure to PE was not different either before (136 ± 6 μm) or after (129 ± 4 μm) BEL treatment. Moreover, application of increasing doses of ACh to the constricted vessels induced a nearly complete relaxation.

Fig. 2. Video microscopic images demonstrating typical responses of mesenteric arterioles from wild-type and iPLA2β-/- (knockout) mice to phenylephrine (PE) and ACh. Isolated mesenteric vessels were pressurized as described in MATERIALS AND METHODS. The mesenteric arteriole isolated from iPLA2β-/- mouse shows enhanced constriction to PE (100 μmol/l) and reduced dilation to ACh (0.1 and 1 μmol/l) compared with a vessel isolated from a wild-type animal, which fully dilates in response to the same concentration of ACh.
response for untreated iPLA2 β/−/− vessels with diameters returning to 176 ± 7 μm (97% of control diameter), but only resulted in partial relaxation to 157 ± 9 μm (85% of control diameter; P < 0.05 vs. control) for BEL-treated vessels. Replacement of the PE-containing buffer with MOPS buffer resulted in complete relaxation to control diameters in all examined vessels (Fig. 5).

Vascular responses to agonists in vivo and arterial blood pressure measurements. To confirm the importance of these results in intact animals, intravital microscopy was performed on mesenteric arteries from iPLA2 β/−/− and iPLA2 β/−/− mice. Similar results to those obtained with excised vessels were observed during contraction and relaxation of mesenteric arteries in vivo. Application of 100 μmol/l PE to the surface of
the mesentery led to a 25% reduction in diameter for iPLA2/H9252/H11001 vessels (180 ± 5 to 135 ± 5 μm; n = 10) and a 27% reduction in diameter for iPLA2/H9252/H11002 vessels (170 ± 5 to 125 ± 6 μm; n = 10) with the average control diameters not different (unpaired t-test; Fig. 6). However, although intravenous injection of 16 μmol/l ACh induced 11% relaxation over 60 s in vessels from iPLA2/H9252/H11001 animals, only 5% relaxation occurred in vessels from iPLA2/H9252/H11002 animals (P < 0.05 vs. iPLA2/H9252/H11001; Fig. 6), indicating reduced endothelium-dependent dilation. Aortic blood pressure was not significantly different between wild-type and knockout animals averaging 76.5 ± 4.7 mmHg in iPLA2β+/+ wild-type and 78.6 ± 4.6 mmHg in iPLA2β−/− mice (n = 6 in each group).

Inhibition of endothelial NO production and eNOS phosphorylation by BEL. To further elucidate the contribution of iPLA2β on vascular regulation and its possible contribution to endothelial function, we studied the effect of iPLA2β inhibition in cultured endothelial cells. As shown in Fig. 7A, inhibition of iPLA2β with racemic BEL (5 μmol/l) caused a significant decrease in nitrite production that was partially restored...
by adding the membrane permeable arachidonic acid analog arachidonyl methyl ester (AAME; 20 μmol/l) as substrate. These results indicate that arachidonic acid produced by iPLA2β contributes to NO production in endothelial cells. Since phosphorylation of eNOS at Ser1177 regulates eNOS activity (27) we measured the phosphorylation state of eNOS by Western blot using an antibody against phospho-Ser1177. The iPLA2β-enantioselective mechanism-based inhibitor (S)-BEL reduced phosphorylation at Ser1177 by more than 50% (Fig. 7B), indicating that iPLA2β activity contributes to the activation of eNOS.

**Endothelial iPLA2β mediated calcium responses in cultured cells.** Similar to the vessel experiments, agonist-induced calcium increases consisted of both intracellular calcium release and extracellular calcium influx (Fig. 8A). Inhibition with racemic BEL (10 μmol/l) reduced and delayed endothelial calcium response similar to the specific inhibition of iPLA2β with (S)-BEL (10 μmol/l; Fig. 8B), indicating that iPLA2β reaction products and/or their downstream metabolites likely contribute to agonist-induced intracellular calcium release and extracellular calcium influx.

**Identification of downstream iPLA2β generated lipid mediators modulating endothelial cell calcium responses.** To further investigate the putative role(s) of iPLA2β in endothelial cell Ca2+ responses, we tested multiple lipid mediators either generated directly by the iPLA2β reaction or through downstream products that may be generated after the release of arachidonic acid by iPLA2β activity. To deliver arachidonic acid across the cell membrane without the typical obligatory sequential coupling to acyl-CoA synthesis, AAME (20 μmol/l) was used, which readily crosses cell membranes allowing access to multiple intracellular membrane compartments before hydrolysis by intracellular esterases to generate free arachidonic acid. The addition of AAME to endothelial cells resulted in a rapid release of calcium from internal stores in calcium free medium and also caused a rise in the calcium signal when calcium was added to the medium (Fig. 9A). We next examined the effects of ETYA, a generic inhibitor of all AA oxidative pathways (12), and found that it reduced ATP-induced cellular calcium increase both from intracellular and extracellular sources (Fig. 9B). Inhibition of arachidonic acid ω-oxidation and epoxygenase-dependent epoxyeicosatrienoic acid (EET) formation by 17-octadecynoic acid (17-ODYA; 10 μmol/l) had no effect on ATP (100 μmol/l)-induced calcium release from intracellular stores, indicating that neither 20-HETE (ω-hydroxylase product) nor EETs (epoxygenase products) are involved in the cascade of events leading to calcium release. Inhibition with ibuprofen (20 μmol/l) did not alter ATP-induced calcium influx, indicating that prostanoids are also not involved in this pathway (Fig. 9, C and D). However, lipoxygenase inhibition with nordihydroguaiaretic acid (NDGA; 20 μmol/l) decreased the calcium response indicating that lipoxygenase products such as hydroxyeicosatetraenoic acids (HETEs) contribute to the calcium response (Fig. 9, C and D). We found that 12-HETE (4 μmol/l; Fig. 9E), but not 5-HETE or 15-HETE (data not shown), caused increases in intracellular calcium ion similar to AAME, indicating that HETEs may be the downstream product involved in intracellular calcium release. In contrast, administration of LPC (10 μmol/l), a byproduct of iPLA2β activity, caused a strong extracellular calcium influx response but did not contribute to intracellular calcium release (Fig. 9F). Previous studies have demonstrated that HETEs contribute to microvascular calcium regulation (28).

**Fig. 7. Effects of BEL and analog arachidonyl methyl ester (AAME) on ATP-stimulated nitric oxide (NO) production and endothelial NO synthase (eNOS) phosphorylation in endothelial cells.** Cultured EA.hy926 endothelial cells were stimulated with ATP (100 μmol/l) and iPLA2β was inhibited by either racemic BEL (racBEL) or the (S) and (R) enantiomers of BEL. A: RacBEL (5 μmol/l) significantly inhibited NO production (measured as nitrite) that was partially restored by administration of AAME (20 μmol/l). *P < 0.01 compared with ATP-stimulated controls (n = 3). B: representative Western blot of eNOS phosphorylation at Ser1177 (top) with the corresponding quantification of the density data (bottom) demonstrating that (S)-BEL (5 μmol/l, specific for iPLA2β), but not (R)-BEL (5 μmol/l), dose-dependently reduced eNOS phosphorylation at Ser1177. These results indicate that iPLA2β contributes to agonist-stimulated NO production by regulating eNOS phosphorylation. *P < 0.01 compared with ATP-stimulated controls (n = 3).

**Fig. 8. Effects of BEL and BEL enantiomers on ATP-stimulated intracellular calcium responses in cultured EA.hy926 endothelial cells.** Cells were stimulated with ATP (100 μmol/l, specific for iPLA2) and iPLA2β activity (27) was partially restored by administration of AAME (20 μmol/l). Inhibition with racemic BEL (10 μmol/l) reduced and delayed endothelial calcium response similar to the specific inhibition of iPLA2β with (S)-BEL (10 μmol/l; Fig. 8B), indicating that iPLA2β reaction products and/or their downstream metabolites likely contribute to agonist-induced intracellular calcium release and extracellular calcium influx.

**Fig. 9. Effects of ETYA and its enantiomers on intracellular calcium responses in cultured EA.hy926 endothelial cells.** Cells were stimulated with ATP (100 μmol/l) and iPLA2β activity (27) was partially restored by administration of AAME (20 μmol/l). A: Representative Western blot showing the phosphorylation state of eNOS at Ser1177 (top) with the corresponding quantification of the density data (bottom) demonstrating that ETYA (20 μmol/l) did not alter ATP-stimulated calcium influx, indicating that prostanoids are also not involved in this pathway (Fig. 9, C and D). However, lipoxygenase inhibition with nordihydroguaiaretic acid (NDGA; 20 μmol/l) decreased the calcium response indicating that lipoxygenase products such as hydroxyeicosatetraenoic acids (HETEs) contribute to the calcium response (Fig. 9, C and D). We found that 12-HETE (4 μmol/l; Fig. 9E), but not 5-HETE or 15-HETE (data not shown), caused increases in intracellular calcium ion similar to AAME, indicating that HETEs may be the downstream product involved in intracellular calcium release. In contrast, administration of LPC (10 μmol/l), a byproduct of iPLA2β activity, caused a strong extracellular calcium influx response but did not contribute to intracellular calcium release (Fig. 9F). Previous studies have demonstrated that HETEs contribute to microvascular calcium regulation (28).
DISCUSSION

The results of this study demonstrate multiple important roles of iPLA$_2$B in regulating vasomotor function and calcium signaling in vascular cells. First, it confirms that iPLA$_2$B-generated arachidonic acid and its downstream metabolites are essential to ACh-induced vascular relaxation in vivo and ex vivo. Second, iPLA$_2$B plays a significant role in the agonist-induced constriction of isolated mesenteric arterioles with genetic ablation of iPLA$_2$B in mice resulting in enhanced agonist-induced smooth muscle contraction in isolated vessels. Third, genetic ablation of iPLA$_2$B lowered basal smooth muscle calcium levels, indicating that iPLA$_2$B regulates cytosolic calcium levels and modulates smooth muscle contractility. Fourth, iPLA$_2$B contributes to endothelial-dependent vasomotor responses by regulating eNOS phosphorylation and changes in intracellular calcium concentration in response to agonists. Finally, the iPLA$_2$B-initiated production of eicosanoids leads to the generation of downstream metabolites such as 12-HETE that modulate intracellular calcium homeostasis, whereas the generation of both eicosanoids and LPC contribute to the regulation of agonist-induced extracellular calcium influx in endothelial cells. Collectively, these results underscore the importance of multiple pathways of signaling mediated by iPLA$_2$B that emanate from the concomitant release of arachidonic acid and lysolipids by this enzyme during cellular signaling and demonstrate that iPLA$_2$B-mediated pathways modulate both smooth muscle cell basal calcium homeostasis and stimulus-provoked alterations in calcium concentration, contraction, and relaxation.

Genetic ablation of iPLA$_2$B leads to alterations in vascular responsivity and changes in calcium homeostasis. In arterioles from mice lacking iPLA$_2$B$^{-/-}$ (Fig. 3), the response to PE-mediated constriction was enhanced compared with that of wild-type controls. Moreover, ACh-mediated vasodilation of the PE-constricted vessels was significantly attenuated. The lack of a vasodilatory response to ACh in PE-constricted vessels from iPLA$_2$B$^{-/-}$ mice indicates that iPLA$_2$B is an essential component mediating vasodilatory responses to ACh (Fig. 3). Remarkably, vessels isolated from iPLA$_2$B$^{-/-}$ mice displayed a significantly lower basal calcium level compared with wild-type vessels (Fig. 3). These findings demonstrate that iPLA$_2$B ablation results in a disruption of calcium homeostasis and sensitivity of downstream calcium-mediated signaling in these vessels. The mechanism(s) responsible for the decreased cytosolic calcium level in iPLA$_2$B$^{-/-}$ vessels with enhanced responsivity to a given concentration of calcium remains to be elucidated. Although the PE-induced absolute calcium alterations were consistently higher in wild-type vessels, the constriction response to added PE was higher in iPLA$_2$B$^{-/-}$ vessels compared with vessels from iPLA$_2$B$^{+/+}$ animals (Fig. 3C). One possibility is that iPLA$_2$B$^{-/-}$ vessels were more sensitive to small calcium increases through increased kinase activation of contractile proteins that contributed to the increased constriction response to a given calcium concentration. Alternatively, a decreased capacity of the endothelium to cause vasodilation may enhance PE-induced vasoconstriction since the final vessel diameter is the sum of smooth muscle constriction and endothelium-dependent dilation. Denudation of wild-type vessels did not result in an enhanced tone, although the dilation to ACh was significantly reduced. This indicates that
under resting conditions, the endothelium did not contribute significantly to the resting diameter. Denudation also did not significantly enhance the constriction to PE, indicating that denudation does not result in agonist-induced smooth muscle hypercontractility in wild-type vessels. However, denudation of iPLA₂⁻/⁻ vessels induced a significant constriction, indicating that in these vessels the vascular smooth muscle has a significant tone under resting conditions and that an endothelial...
contribution significantly offsets that tone. The latter observation may be an adaptive response, which would explain the lack of differences in either control vessel diameters and blood pressures in vivo. Since inhibition of iPLA2β in endothelial cells resulted in a decline of the eNOS activity, the endothelium could compensate by producing other relaxation factors such as prostacyclin or EDHF (1, 37). Alternatively, dilatory mechanisms depending on NO activity may be enhanced to compensate for the reduced NO availability. The mechanism by which the endothelium compensated for the increased smooth muscle tone in iPLA2β−/− vessels remains to be established in further studies. In preliminary experiments, we studied the response to PE in two iPLA2β−/− vessels and found that after denudation the constriction to PE was increased by 56%, further indicating hypercontractility of the smooth muscle. Subsequent ACh did not change the vessel diameter. It is interesting to note that depolarizing KCl did not enhance constriction in vessels from iPLA2β−/− mice. This may indicate a difference in agonist-induced constriction versus constriction induced by a depolarizing agent. Further studies will be needed to elucidate this difference. Taken together, these results would suggest that iPLA2β−/− vessels agonist-induced smooth muscle hypercontractility and not an inability of the endothelium to dilate the vessels is responsible for the enhanced constriction to PE.

After removal of extracellular calcium, vessels from iPLA2β−/− mice still showed a significant initial constriction to PE, indicating that during this phase, calcium is released from intracellular stores (Fig. 4). The subsequent steady-state constriction, however, was markedly less than that observed before calcium removal, suggesting that extracellular calcium contributes to this phase. Subsequent restimulation with PE showed a significantly reduced constriction, indicating that intracellular calcium stores were likely depleted (Fig. 4). This would suggest that PE causes an initial calcium release from intracellular stores resulting in a maximal constriction with the subsequent steady-state constriction dependent upon the influx of extracellular calcium.

These data, taken together, indicate that iPLA2β has a significant role in cellular calcium homeostasis and calcium-contractile coupling. The profound effect of iPLA2β ablation on the basal calcium level (in iPLA2β−/− mice) may explain the lack of blood pressure differences between the wild-type and knockout animals since the lower calcium level in knock-out vessels presumably results in a decreased basal vessel tone, thereby representing a compensatory mechanism for preventing hypertension. In addition, the endothelium may provide a compensatory mechanism to offset smooth muscle hypercontractility.

**Inhibition of iPLA2β with BEL.** We reported previously that in vitro pharmacological inhibition of iPLA2β with BEL (1 μmol/l) did not affect the PE-induced constriction of rat mesenteric arterioles but greatly decreased the vessel dilation in response to ACh (33). These results have been confirmed in our present ex vivo analysis of arterioles from wild-type mice in which BEL significantly reduced the dilatory response to ACh (Fig. 5). Our data also show that inhibition of iPLA2β with BEL can affect the maximum constrictor response to PE, which was not observed in mouse aorta (5). Nevertheless, the steady-state vessel diameter following treatment with PE was not affected by BEL. Using an ex vivo preparation similar to ours, Park et al. (30) also studied the effect of iPLA2β inhibition with BEL and found that it eliminated the PE-induced constriction in mouse mesenteric vessels. Previous studies from this group have reported that 25 μmol/l BEL diluted PE-constricted vessels (30). The reason for these differences compared with our data is not clear. Park et al. (30) and Smahi et al. (35) both used 25 μmol/l BEL, whereas we have routinely used 10 μmol/l, which is five- to 10-fold above the IC50 of BEL for iPLA2β at 2 μmol/l in vitro (20) and 1 μmol/l ex vivo (33). Remarkably, iPLA2β−/− mice demonstrated a greater constriction after PE administration compared with WT mice. Taken together, the results using mice in which iPLA2β was genetically ablated, in conjunction with pharmacological inhibition by BEL, provide new insights into the contribution of iPLA2β on vasomotor regulation and identify the differences between chronic compensated removal of iPLA2β activity through genetic ablation and those which occur following acute pharmacological inhibition with mechanism-based inhibition by BEL. It seems reasonable that pharmacological inhibition with BEL, which blunted the PE effect, may have also blunted contributions from the multiple other iPLA2 family members as well as other enzymes that are targets for BEL. The effects of iPLA2β ablation on calcium levels, vascular contraction, and vascular relaxation have not been reported previously.

**Role of iPLA2-dependent mediators in vascular calcium signaling.** Increased cytosolic calcium is an important signal for the production of endothelial dilators including NO (38). Thus decreased iPLA2β activity may not only result in reduced concentrations of arachidonic acid-dependent vasodilators such as prostanooids or cytochrome P-450-derived EDHFs but also NO production due to reduced intracellular calcium availability.

To further study the contribution of iPLA2β activity to vascular regulation, we measured the agonist-induced phosphorylation of eNOS in cultured endothelial cells. Cell lines originating from a variety of vessels including EA.hy926 cells have previously served as a reliable model to study endothelial cell (6) and PLA2 biology (14). Although iPLA2 enzyme activity has been shown in human umbilical vein endothelial cells (16), such activity has not been shown in EA.hy926 cells. Our data indicate, for the first time, specific iPLA2β activity in these cells, making them a suitable model to study iPLA2β-related mechanisms in vascular endothelium. We found that inhibition of iPLA2β by BEL strongly decreased eNOS phosphorylation at Ser1177, thus decreasing eNOS activity and production of NO (Fig. 7). Therefore, this decrease in eNOS activity could, in part, explain the reduced dilation response of the iPLA2β−/− blood vessels to endothelium-dependent agonists. Furthermore, we found that lipid products of iPLA2β catalytic activity can mediate both intracellular calcium release as well as extracellular calcium influx. Since eNOS activity is dependent upon calcium-activated calmodulin, a reduction in intracellular calcium would also result in an attenuation of NO-mediated signaling. Moreover, acute inhibition of iPLA2β with BEL may result in a decrease in the concentration of free arachidonic acid, thereby decreasing the availability of vaso-active eicosanoids and reducing phospholipase C stimulation and subsequent diglyceride generation. Thus other endothelium-dependent vasodilatory factors such as PG12 or EDHF whose action or production depend on intracellular calcium signaling would be affected. General inhibition of arachidonic acid...
metabolic pathways with ETYA showed that arachidonic acid oxidative products contribute to both intracranial and extracellular calcium fluxes (Fig. 9). Inhibition of cyclooxygenase with ibuprofen or inhibition of cytochrome P-450 epoxygenase(s) with 17-ODYA did not affect the calcium response as measured by fura-2 fluorescence (Fig. 9C), indicating that their respective mediators PG2 or EETs are not likely involved in endothelial calcium regulation. In contrast, in this model application of 12-HETE resulted in intracellular calcium release similar to AAME, indicating that HETEs may contribute to endothelial calcium regulation similar to results found in other cells (15, 29). Indeed, inhibiting lipoxigenases with nordihydroguaiaretic acid reduced the intracellular calcium response to ATP, indicating that lipoxigenase products such as HETEs may be involved (Fig. 9C). 12-HETE production in endothelial cells has been described previously (32), but its effect on endothelial calcium regulation has not been reported. AAME caused calcium release from intracellular stores in our experiments. The present results identify a prominent downstream AA metabolite (e.g., 12-HETE) in endothelial cells that could mediate the previously observed vasodilatory effects of AA in rat mesenteric arterioles (33). These results differ from observations in mouse intact aorta where AA caused vasoconstriction (5) consistent with the release of an AA-derived vasoconstriction factor (36), indicating that the released active AA metabolite and its subsequent vasomotor activity may differ between vascular beds. Our results are in agreement with previous reports demonstrating L-PCL-induced increases in extracellular calcium influx into endothelial cells (5, 7).

In summary, we have demonstrated that ablation of iPLA2beta in mice effectively eliminated the dilation of mesenteric arterioles in response to ACh, indicating that iPLA2beta activity may be a major upstream regulator of vascular reactivity. Although vessels from knockout animals had lower basal calcium levels, PE-induced vasoconstriction was enhanced compared with vessels from wild-type animals. Endothelial denudation in iPLA2beta knock-out vessels caused significant tone development, indicating that the endothelium may have adapted by a yet unknown mechanism to compensate for the increased agonist-induced smooth muscle hypercontractility enabling resting tone and blood pressure to be maintained. However, agonist-induced endothelium-dependent dilation was reduced likely due to decreased eNOS phosphorylation and agonist-induced calcium responses. Our results demonstrate an important role of iPLA2beta activity in vascular smooth muscle cell calcium homeostasis and regulation of contractility as well as regulation of eNOS activity and endothelial agonist-induced calcium responses.

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