Detection of differentially regulated subsarcolemmal calcium signals activated by vasoactive agonists in rat pulmonary artery smooth muscle cells

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Subedi KP, Paudel O, Sham JS. Detection of differentially regulated subsarcolemmal calcium signals activated by vasoactive agonists in rat pulmonary arterial smooth muscle cells. Am J Physiol Cell Physiol 306: C659–C669, 2014. First published December 18, 2013; doi:10.1152/ajpcell.00341.2013.—Intracellular calcium (Ca$^{2+}$) plays pivotal roles in distinct cellular functions through global and local signaling in various subcellular compartments, and subcellular Ca$^{2+}$ signal is the key factor for independent regulation of different cellular functions. In vascular smooth muscle cells, subsarcolemmal Ca$^{2+}$ is an important regulator of excitation-contraction coupling, and nucleoplasmic Ca$^{2+}$ is crucial for excitation-transcription coupling. However, information on Ca$^{2+}$ signals in these subcellular compartments is limited. To study the regulation of the subcellular Ca$^{2+}$ signals, genetically encoded Ca$^{2+}$ indicators (cameleon), D3cpv, targeting the plasma membrane (PM), cytoplasm, and nucleoplasm were transfected into rat pulmonary arterial smooth muscle cells (PASMCs) and Ca$^{2+}$ signals were monitored using laser scanning confocal microscopy. In situ calibration showed that the $K_d$ for Ca$^{2+}$ of D3cpv was comparable in the cytoplasm and nucleoplasm, but it was slightly higher in the PM. Stimulation of digitonin-permeabilized cells with 1,4,5-trisphosphate (IP$_3$) elicited a transient elevation of nucleoplasmic Ca$^{2+}$ concentration with similar amplitude and kinetics in the nucleoplasm and cytoplasm. Activation of G protein-coupled receptors by endothelin-1 and angiotensin II preferentially elevated the subsarcolemmal Ca$^{2+}$ signal with higher amplitude in the PM region than the nucleoplasmic and cytoplasmic Ca$^{2+}$. In contrast, the receptor tyrosine kinase activator, platelet-derived growth factor, elicited Ca$^{2+}$ signals with similar amplitudes in all three regions, except that the rise-time and decay-time were slightly slower in the PM region. These data clearly revealed compartmentalization of Ca$^{2+}$ signals in the subcellular compartments and provide the basis for further investigations of differential regulation of subcellular Ca$^{2+}$ signals in PASMCs.

cameleon; FRET; Ca$^{2+}$ signaling; endothelin; angiotensin

Ca$^{2+}$ is a ubiquitous second messenger that plays crucial roles in almost every cellular process from egg fertilization, cell proliferation, muscle contraction, transcriptional regulation to apoptosis (7). Specific Ca$^{2+}$ signals are encoded in amplitude and frequency depending on the activities and dynamic properties of the Ca$^{2+}$ influx/release channels and transporters. They are decoded locally by specific effectors in various subcellular compartments and organelles (5, 7, 11, 12). The local concentration of Ca$^{2+}$ ($[Ca^{2+}]_i$) in various organelles may differ during cell activation, and subcellular Ca$^{2+}$ signal is the key factor for independent regulation of different cellular functions. Hence, elucidating local Ca$^{2+}$ signals in various subcellular compartments is important for the understanding of the physiological regulation and pathophysiological dysregulation of specific cellular processes.

In vascular smooth muscle cells (VSMCs), subsarcolemmal Ca$^{2+}$ signals play pivotal roles in the regulation of vascular functions. Ca$^{2+}$ influx through plasmalemmal Ca$^{2+}$-permeating channels generates local Ca$^{2+}$ microdomains that develop rapidly under plasma membrane (PM) and trigger a wide range of physiological responses, including Ca$^{2+}$-induced Ca$^{2+}$ release for excitation-contraction coupling, and activation of Ca$^{2+}$-dependent proteins/enzymes to modulate channel activities and transcriptional factors (6, 40, 44). Local Ca$^{2+}$ release events from the peripherally coupled sarcoplasmic reticulum (SR) can also generate Ca$^{2+}$ signals in the subsarcolemmal space to activate Ca$^{2+}$-activated K$^+$ channel to generate spontaneous transient outward currents (21, 37) and/or Ca$^{2+}$-activated Cl$^-$ channels to elicit spontaneous transient inward currents to cause vasorelaxation and vasoconstriction, respectively (16, 55, 56). Besides subsarcolemmal Ca$^{2+}$ signals, nucleoplasmic Ca$^{2+}$ is known to play crucial roles in gene transcription and cell proliferation (2, 41). It is regulated by the propagation of cytoplasmic Ca$^{2+}$ into the nucleoplasm and by local Ca$^{2+}$ release via 1,4,5-trisphosphate (IP$_3$) receptors (IP$_3$Rs) and ryanodine receptors of perinuclear Ca$^{2+}$ stores, nuclear envelope, and nucleoplasmic reticulum (13, 20, 30, 31, 33, 37). However, information regarding subsarcolemmal and nucleoplasmic Ca$^{2+}$ signals in VSMCs is limited, and direct comparison of Ca$^{2+}$ signals between the subcellular compartments during agonist stimulation is unavailable.

Although small molecule fluorescent dyes like rhod-2 and Fluo-5N have been used successfully to measure Ca$^{2+}$ signals in mitochondria (23, 53) and sarcoplasmic reticulum (24, 47), respectively, under certain conditions, the study of Ca$^{2+}$ signaling in other specific intracellular compartments has been limited by technical difficulties. For example, many subcellular organelles in live cells are not readily discernible by fluorescence microscopy, hence the commonly used fluorescent Ca$^{2+}$ indicators, including Fluo-3, Fluo-4, Fura-2, and Indo-1, are unable to distinguish Ca$^{2+}$ signals of specific organelles from the neighboring cytoplasmic Ca$^{2+}$ signals. These limitations can be overcome by using genetically encoded Ca$^{2+}$ indicators with targeting sequences to express selectively into specific organelles. As our first effort to study local Ca$^{2+}$ signals in subcellular compartments, we used the second-generation fluorescence resonance energy transfer (FRET)-based Ca$^{2+}$-sensor (cameleon) D3cpv (38, 39) to generate specific probes targeting the plasma membrane and the nucleus. We verified their subcellular locations and performed in situ calibration of the Ca$^{2+}$ biosensors in rat pulmonary arterial smooth muscle
cells (PASMCs). Simultaneous detection of subsarcolemmal, nucleoplasmic, and cytoplasmic Ca$^{2+}$ signals was performed by coexpression of the Ca$^{2+}$ biosensors. We successfully recorded Ca$^{2+}$ signals in these subcellular compartments activated by IP$_3$, endothelin-1 (ET-1), angiotensin II (ANG II), and platelet-derived growth factor (PDGF) and reveal for the first time the differentially regulated subcellular Ca$^{2+}$ signals activated by the vasoactive agonists in PASMCs.\textsuperscript{1}

MATERIALS AND METHODS

Generation of targeted and nontargeted constructs. The nontargeted cameleon (D3cpv) was generated by using 4mtD3cpv (kindly provided by Dr. R. Tsien) (38). The mitochondria targeting sequence (4mt) of 4mtD3cpv was excised by restriction digestion (HindIII). The digested construct was purified and religated to generate D3cpv. Nuclear targeted (3NLS-D3cpv) and membrane targeted (Lyn-D3cpv) cameleons were generated by cloning synthetic oligonucleotides coding for three tandem repeats of the nuclear localization signal from simian virus large T-antigen (3NLS, DPKKKKRVDPKKKRRVPDKKKKKKV) (25), and the myristoylation-palmitoylation sequence from lyn kinase (Lyn, MGCIKSRKDNLDDGVDMDKT) (60) specifically, to the NH$_2$ terminus of D3cpv (Fig. 1A). The oligonucleotide inserts were designed in such a way that the translation initiation codon (ATG) of targeted sequences was surrounded by Kozak consensus sequence, GCCACCATGG, for optimal expression (28). Synthetic oligonucleotides encompassing the Kozak consensus sequence, coding sequence of targets (3NLS and Lyn) and sticky ends for HindIII site were synthesized and annealed. The annealed inserts were directly ligated into the HindIII-restricted D3cpv and transformed into Escherichia coli. Clones were selected after screening by PCR and restriction digestion, and the inserted sequences were confirmed by automated sequencing.

Isolation and culture of rat PASMCs. All experiments were performed under protocols approved by the Johns Hopkins University Animal Care and Use Committee. Rat PASMCs were enzymatically isolated and cultured as described earlier (59). In brief, male Wistar rats (150–250 g) were anesthetized with pentobarbital sodium (130 mg/kg ip). Lungs were removed quickly and immersed in ice-cold HBSS containing (in mM) 130 NaCl, 5 KH$_2$PO$_4$, 0.75 MgCl$_2$, 1.5 CaCl$_2$, 10 HEPES, and dextran (MW: 40,000) at room temperature. Cells were then washed twice with HHBSS, and images were taken. Both of the dyes were excited with an argon laser line (543 nm), and emission was recorded at 560–615 nm. To eliminate the possibility of signal contamination by cameleon fluorescence, the laser intensity was decreased to the level at which cameleon fluorescence was undetectable. The whole cell staining was done by using excess amounts of SYTO 83 Orange and the plasma membrane marker CellMask Orange (Invitrogen) at room temperature. Cells were then washed twice with HHBSS, and images were taken.

For Ca$^{2+}$ imaging experiments, cells were rinsed thrice and then maintained in HHBSS for at least 10 min at room temperature. Cells were exposed to different agonists, and images were recorded for different time courses. For IP$_3$-induced Ca$^{2+}$ signals, cells were permeabilized and maintained in an internal medium before agonist treatment. Cell permeabilization was done as described earlier (14) with some modification. Briefly, cells were exposed to 15 \mu M digitonin in standard solution containing (in mM) 100 K$^+$ aspartate, 15 KCl, 0.75 MgCl$_2$, 10 HEPES, and 10 glucose (pH 7.2, adjusted with NaOH). Second- and third-generation intrapulmonary arteries were isolated from the lungs. The surrounding connective tissue was carefully removed. The arteries were cut opened to expose the endothelial surface, and the endothelium was removed by gentle rubbing with a cotton swab. The tissues were allowed to recover for 30 min in ice-cold HBSS, followed by 20 min in reduced-Ca$^{2+}$ (20 \mu M) HBSS at room temperature. The tissues were digested at 37°C for 20 min in 20 \mu mol-Ca$^{2+}$-free HBSS containing type I collagenase (1,750 U/ml), papain (9.6 U/ml), bovine serum albumin (2 mg/ml), and DTT (1 mM). After digestion was stopped by washing the tissue with nominal Ca$^{2+}$-free HBSS, PASMCs were mechanically dispersed by gentle trituration with a wide bore pipette tip in Ca$^{2+}$-free HBSS at room temperature. Cells were then placed on 100-mm petri dishes, allowed to settle for 20 min at room temperature, and cultured overnight in Ham’s F-12 medium (with l-glutamine) supplemented with 0.5% FCS, 100 U/ml streptomycin, and 0.1 mg/ml penicillin at 37°C in a humidified atmosphere containing 5% CO$_2$. The Ham’s medium was replaced with smooth muscle cell growth basal medium (SmBM, Lonza) supplemented with growth factors, 5% FCS, 100 U/ml streptomycin, and 0.1 mg/ml penicillin.

The cells were cultured for 2–3 days before passage. Cells at passages 2-3 were used for the experiments.

Transfection. Rat PASMCs were transiently transfected with 0.5–1 \mu g of cameleon plasmids using an Amaxa Nucleofector kit for primary smooth muscle cells (Lonza). The transfected cells were then seeded onto 25-mm glass coverslips in petri dishes containing prewarmed SmBM supplemented with growth factors, FCS, and antibiotics and incubated in a humidified atmosphere containing 5% CO$_2$. Fluorescence experiments were performed 48 h after transfection. For the experiments on agonist-induced Ca$^{2+}$ response, PASMCs were starved overnight by replacing the complete medium with SmBM without growth factors and FCS.

Confocal microscopy. Rat PASMCs were transfected with D3cpv, 3NLS-D3cpv, and/or Lyn-D3cpv and cultured for 24–48 h before imaging. They were washed thrice with Hanks’ balanced salt solution (Invitrogen/Life Technologies) buffered with 20 mM HEPES and containing 2 g/l D-glucose (HHBSS, pH 7.4). Confocal images were acquired under a Zeiss LSM-510 inverted confocal microscope (Zeiss) with a Zeiss Plan-Neofluor ×40 oil immersion objective (numerical aperture 1.3). To confirm the targeted expression, cameleons were excited by the 458 nm line of a HeNe laser, and the emitted fluorescence signal was captured at both 475–515 nm [cyan fluorescent protein (CFP)] and 530 nm (cpY). The cells were then loaded with the cell-permeant fluorescent nucleic acid stain SYTO 83 Orange or the plasma membrane marker CellMask Orange (Invitrogen) at room temperature. Cells were then washed twice with HHBSS, and images were taken. Both of the dyes were excited with an argon laser line (543 nm), and emission was recorded at 560–615 nm. To eliminate the possibility of signal contamination by cameleon fluorescence, the laser intensity was decreased to the level at which cameleon fluorescence was undetectable. The whole cell staining was done by using excess amounts of SYTO 83 Orange, and the image was taken before the dyes diffused out of the cell or moved completely into the nucleus.

For Ca$^{2+}$ imaging experiments, cells were rinsed thrice and then maintained in HHBSS for at least 10 min at room temperature. Cells were exposed to different agonists, and images were recorded for different time courses. For IP$_3$-induced Ca$^{2+}$ signals, cells were permeabilized and maintained in an internal medium before agonist treatment. Cell permeabilization was done as described earlier (14) with some modification. Briefly, cells were exposed to 15 \mu M digitonin in standard solution containing (in mM) 100 K$^+$ aspartate, 15 KCl, 0.75 MgCl$_2$, 10 HEPES, and 10 glucose (pH 7.2, adjusted with NaOH). Second- and third-generation intrapulmonary arteries were isolated from the lungs. The surrounding connective tissue was carefully removed. The arteries were cut opened to expose the endothelial surface, and the endothelium was removed by gentle rubbing with a cotton swab. The tissues were allowed to recover for 30 min in ice-cold HBSS, followed by 20 min in reduced-Ca$^{2+}$ (20 \mu M) HBSS at room temperature. The tissues were digested at 37°C for 20 min in 20 \mu mol-Ca$^{2+}$-free HBSS containing type I collagenase (1,750 U/ml), papain (9.6 U/ml), bovine serum albumin (2 mg/ml), and DTT (1 mM). After digestion was stopped by washing the tissue with nominal Ca$^{2+}$-free HBSS, PASMCs were mechanically dispersed by gentle trituration with a wide bore pipette tip in Ca$^{2+}$-free HBSS at room temperature. Cells were then placed on 100-mm petri dishes, allowed to settle for 20 min at room temperature, and cultured overnight in Ham’s F-12 medium (with l-glutamine) supplemented with 0.5% FCS, 100 U/ml streptomycin, and 0.1 mg/ml penicillin at 37°C in a humidified atmosphere containing 5% CO$_2$. The Ham’s medium was replaced with smooth muscle cell growth basal medium (SmBM, Lonza) supplemented with growth factors, 5% FCS, 100 U/ml streptomycin, and 0.1 mg/ml penicillin.

\textsuperscript{1} This article is the topic of an Editorial Focus by Ningyong Xu, Michael Francis, Donna L. Cioffi, and Troy Stevens (58).

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ratios ($R_{\text{min}}$ and $R_{\text{max}}$) were determined by adding 3 mM EGTA and 10 mM CaCl$_2$, respectively, in the presence of 5 μM Ca$^{2+}$ ionophore (4-bromo-Ca$^{2+}$ ionophore, A23187, Sigma) at the end of the experiment, and the proportionality factor was determined by the ratio of emission intensities of the Ca$^{2+}$-free to the Ca$^{2+}$-bound cameleon (34).

Data analysis. Results are presented as means ± SE with n equal to the number of cells. Statistical comparisons were conducted with one-way ANOVA or paired t-test, according to the experimental design. Statistical significance was defined as $P < 0.05$.

RESULTS

Verification of targeted Ca$^{2+}$ indicators. The nontargeted D3cpv, nucleus-targeted 3NLS-D3cpv, and plasma membrane-targeted Lyn-D3cpv were transfected into rat PASMCs. Confocal imaging showed that D3cpv was expressed in a diffuse pattern indistinguishable from that of cytoplasmic dye, and with a lower expression in the nucleus region (Fig. 1B, a–d, and 1C). In contrast, 3NLS-D3cpv was exclusively...
localized in the nucleus (Fig. 1B, f and 1C), and Lyn-D3cpv was expressed solely in the plasma membrane of PASMCs (Fig. 1B, j and 1C). Specific targeting of the organelles by the cameleons was verified by staining the same cells with the fluorescent nucleic acid stain SYTO 83 and the plasma membrane marker CellMask. The SYTO 83 and CellMask fluorescence were colocalized with 3NLS-D3cpv (Fig. 1B, e–h) and Lyn-D3cpv (Fig. 1B, i–l) respectively. When the cells were cotransfected with 3NLS-D3cpv and Lyn-D3cpv, the nuclear and PM targeting cameleons were clearly distinguishable and highly localized (Fig. 1B, m–p, and 1C), indicating that they can be employed for simultaneous measurement of nucleoplasmic and subsarcolemmal Ca$^{2+}$ signals in the same cell.

In situ calibration of D3cpv, 3NLS-D3cpv, and Lyn-D3cpv in rat PASMCs. The $K_d$ for Ca$^{2+}$ of genetically encoded Ca$^{2+}$ indicators can be different in vivo and in vitro (19) and may vary in different subcellular compartments (14, 32). Hence, we performed in situ calibration of the targeted and nontargeted cameleons. Rat PASMCs expressing 3NLS-D3cpv were permeabilized with digitonin (25 $\mu$M for 45 s) and exposed to different [Ca$^{2+}$]$_{free}$. Figure 2A shows original images of two nuclei of PASMCs obtained before and during the calibration procedure at various [Ca$^{2+}$]$_{free}$. Increase in [Ca$^{2+}$]$_{free}$ caused a rapid decrease in CFP fluorescence and increase in cpV fluorescence (Fig. 2B), resulting in an overall increase in FRET fluorescence ratio (Fig. 2C), which was saturated at [Ca$^{2+}$]$_{free}$ of $\gtrsim$10 $\mu$M. The same permeabilization procedure, however, resulted in rapid loss of D3cpv and Lyn-D3cpv in the transfected cells. The leak of cyttoplasmic D3cpv was prevented by using a lower concentration of digitonin at a reduced exposure time (12.5 $\mu$M for 30 s), and the loss of Lyn-D3cpv from the plasma membrane was avoided by using S. aureus α-toxin for membrane perforation (8). The apparent $K_d$ for Ca$^{2+}$ binding was estimated by fitting the FRET ratio as a function of [Ca$^{2+}$]$_{free}$ using the Hill equation (Fig. 2D). The apparent $K_d$ of 3NLS-D3cpv for Ca$^{2+}$ (digitonin-permeabilized cells: 0.22 ± 0.02 $\mu$M, n = 34) was similar to that of D3cpv (digitonin-permeabilized cells: 0.22 ± 0.01 $\mu$M, n = 11) (Fig. 2E). However, the $K_d$ value of Lyn-D3cpv ($\pm$-toxin-permeabilized cells: 0.56 ± 0.03 $\mu$M, n = 12, $P < 0.001$) was significantly higher than those of D3cpv and 3NLS-D3cpv (Fig. 2E). The $K_d$ of 3NLS-D3cpv obtained by $\pm$-toxin and digitonin permeabilization were basically the same ($\pm$-toxin-permeabilized cells: 0.27 ± 0.05 $\mu$M, n = 5). These results indicate that Ca$^{2+}$ binding affinity of D3cpv is similar in nucleoplasmic and cytoplasmic compartments but is lower in subsarcolemmal regions of rat PASMCs.

*IP$_3$-induced nucleoplasmic and cytoplasmic Ca$^{2+}$ signals in permeabilized rat PASMCs. IP$_3$Rs are expressed on the nuclear envelope and nucleoplasmic reticulum and have been implicated in nucleoplasmic Ca$^{2+}$ signaling. We first characterized the nucleoplasmic Ca$^{2+}$ signals by examining IP$_3$-induced Ca$^{2+}$ release in digitonin-permeabilized PASMCs transfected with 3NLS-D3cpv. Application of IP$_3$ (10 $\mu$M) elicited a transient elevation of nucleoplasmic [Ca$^{2+}$]$_{free}$ (Fig. 3A, a–c, and 3B), which lasted for a brief period of time (time to 75% of peak: 2.0 ± 0.3 s and time of half-decay: 7.1 ± 1.7 s, n = 9, Fig. 3D). The transient nucleoplasmic Ca$^{2+}$ signals in fully permeabilized PASMCs indicate rapid Ca$^{2+}$ released and re-
removal from the nucleoplasm. To examine whether direct activation of IP₃Rs differentially regulates nuclear and cytoplasmic Ca²⁺ signals, PASMCs were cotransfected with D3cpv and 3NLS-D3cpv and partially permeabilized with digitonin to retain cytoplasmic D3cpv. Under this condition, application of IP₃ elicited cytoplasmic and nucleoplasmic Ca²⁺ signals with significantly longer durations (Fig. 3A, d–f, and 3C). The peak change in fluorescence ratio (cytoplasmic = 0.098 ± 0.008, nucleoplasmic = 0.112 ± 0.013, n = 5), rise-time (time to 75% of peak, cytoplasmic = 10.2 ± 1.1 s, nucleoplasmic = 9.7 ± 0.6 s, n = 5), and time of half-decay (cytoplasmic = 55.4 ± 7.0 s, nucleoplasmic = 59.3 ± 8.0 s, n = 5) (Fig. 3E) of the nucleoplasmic and cytoplasmic Ca²⁺ transients were similar, suggesting efficient Ca²⁺ diffusion between the nucleus and cytoplasm.

Fig. 3. Effects of inositol 1,4,5-trisphosphate (IP₃) on the nucleoplasmic and cytoplasmic Ca²⁺ increase in digitonin-permeabilized rat PASMCs. A: 2D confocal images of PASMCs cotransfected with D3cpv and 3NLS-D3cpv and permeabilized with digitonin in excess (a–c) and under controlled conditions (d–f). IP₃ (10 μM)-induced FRET ratio changes were analyzed in different regions of interest (ROIs 1–3) in the nucleus, and the cytoplasm. Cells showing only cpV fluorescence (a and d), and Ca²⁺ mobilization before (b and e) and after (c and f) IP₃ treatment. B: ΔFRET fluorescence ratio traces showing Ca²⁺ mobilization in the nucleoplasm of rat PASMCs (ROI 1) after IP₃ treatment. C: ΔFRET fluorescence ratio traces showing Ca²⁺ mobilization in the cytoplasm (●) and nucleoplasm (○) of rat PASMCs (ROI 2 and 3, respectively) after IP₃ treatment. D: statistical analysis of the peak normalized ΔFRET fluorescence ratio, time to 75% of the peak, and time of half-decay of IP₃-induced Ca²⁺ transients in the nucleoplasm of rat PASMCs under excess permeabilization conditions. E: statistical analysis of the peak normalized ΔFRET fluorescence ratio, time to 75% of the peak, and time of half-decay of IP₃-induced Ca²⁺ transients in the nucleoplasm and cytoplasm of rat PASMCs under moderate permeabilization conditions.
**ET-1 and ANG II preferentially triggered subsarcolemmal Ca\(^{2+}\) increase in PASMCs.** We next compared Ca\(^{2+}\) signals in the PM region, cytoplasm, and nucleoplasm activated by the physiological agonists ET-1 and ANG II. Rat PASMCs were cotransfected either with 3NL3-D3cpv and Lyn-D3cpv or with D3cpv and 3NL3-D3cpv. ET-1 (30 nM) and ANG II (100 nM) both elicited nucleoplasmic and PM Ca\(^{2+}\) signals in the 3NL3-D3cpv and Lyn-D3cpv cotransfected cells (Fig. 4, A–H). The Ca\(^{2+}\) signal was significantly stronger in the PM region (Fig. 4, A–H), with the peak change in FRET fluorescence ratio approximately threefold higher in the PM compared with the nucleoplasm (ET-1: PM = 0.237 ± 0.021, nucleoplasm = 0.076 ± 0.011, n = 8, P < 0.001; ANG II: PM = 0.255 ± 0.020; nucleoplasm = 0.093 ± 0.009, n = 12, P < 0.001) (Fig. 4, C and F, shown as peak normalized intensity values). Significant difference between [Ca\(^{2+}\)] within nucleoplasm and the PM region was verified by converting the FRET signals to absolute [Ca\(^{2+}\)], according to Grynkiewicz et al. (17) (Fig. 4, G and H). In contrast, both agonists caused similar changes in fluorescence ratio in the nucleoplasm and the cytoplasm of D3cpv and 3NL3-D3cpv cotransfected PASMCs (Fig. 5, A–F). The kinetics of Ca\(^{2+}\) signals were not different between nucleoplasm and PM, or between nucleoplasm and cytoplasm (data not shown). These data indicate that ET-1 and ANG II preferentially activate PM Ca\(^{2+}\) signals compared with nucleoplasmic and cytoplasmic Ca\(^{2+}\) and for the first time demonstrate the differential regulation of subcellular Ca\(^{2+}\) signals in PASMCs.

**PDGF-induced comparable Ca\(^{2+}\) increase in the PM, nucleoplasm, and cytoplasm.** We further examined the subcellular Ca\(^{2+}\) signals induced by the receptor tyrosine kinase agonist PDGF. PDGF elicited Ca\(^{2+}\) transients with a slower kinetics compared with ET-1 and ANG II-treated cells. Time to 75% of peak was 33.8 ± 3.2 s for PDGF compared with 13.1 ± 1.9 s for ET-1 or 11.1 ± 1.1 s for ANG II; the time of half-decay was 81.2 ± 12.4 s for PDGF compared with 25.7 ± 4.1 s for ET-1 or 23.6 ± 4.90 s for ANG II. There was no clear difference in the amplitude of the Ca\(^{2+}\) signals in PM (peak ΔFRET ratio: 0.079 ± 0.011, n = 8) versus nucleoplasm (0.083 ± 0.009, n = 8) and the cytoplasm (0.090 ± 0.009, n = 6) versus nucleoplasm (0.086 ± 0.009, n = 8) (Fig. 6, A–F). The kinetics of PDGF-induced Ca\(^{2+}\) signals in the PM region, however, was significantly slower in terms of the rise-time and the half-decay time compared with those of nucleoplasm and cytoplasm (Fig. 6E). Our data suggest that PDGF regulates subcellular Ca\(^{2+}\) signals in a manner essentially different from that of ET-1 and ANG II.

**DISCUSSION**

Intracellular Ca\(^{2+}\) is a second messenger for the regulation of numerous cellular functions. Despite being a key player of cellular processes, the regulation of local and compartmentalized Ca\(^{2+}\) signal remains particularly difficult to study. It is because Ca\(^{2+}\) probes with adequate dynamic range, suitable Ca\(^{2+}\) sensitivity, and specific subcellular localization are required to detect and quantify the Ca\(^{2+}\) signals (2). In the present study, we used the FRET-based cameleon D3cpv to generate specific Ca\(^{2+}\) probes to measure cytoplasmic, nucleoplasmic, and subsarcolemmal Ca\(^{2+}\) signals in PASMCs. D3cpv was chosen among the other genetically encoded Ca\(^{2+}\) probes because it has a wide dynamic range of emission fluorescence and a $K_D$ (0.6 μM) for Ca\(^{2+}\) that is suitable for monitoring the moderate Ca\(^{2+}\) elevations in cytoplasm and nucleoplasm (35, 38, 39). Since the in vivo and in vitro $K_D$ of genetically encoded Ca\(^{2+}\) indicators can be different (19), we performed in situ calibration of the targeted and nontargeted cameleons in our PASMCs. The apparent $K_D$ for Ca\(^{2+}\) of D3cpv and 3NL3-D3cpv determined in situ are similar, but they are slightly lower than the $K_D$ of 0.6 μM determined in vitro using recombinant D3cpv (38). This is in agreement with earlier findings on several types of cameleons showing a decrease in the $K_D$ value when calibrated in vivo compared with in vitro (19). In contrast, the PM-targeted Lyn-D3cpv has a slightly higher in situ $K_D$ compared with those of D3cpv and 3NL3-D3cpv. This could be related to the local ionic environment of the membrane bound cameleon. The higher apparent $K_D$ of Lyn-D3cpv is not due to the use of α-toxin for membrane permeabilization because $K_D$ values of the nucleoplasmic targeted 3NL3-D3cpv are similar when calibrated using digitonin or α-toxin. Our finding is congruent with previous observations that the same cameleon may show different $K_D$ values in different subcellular compartments (14, 32). One potential limitation of D3cpv is the slower kinetics compared with the Ca\(^{2+}\) fluorescent dyes (19), so it may miss the detection of very rapid transient events. However, D3cpv has been used successfully to detect single Ca\(^{2+}\) spikes in neuronal cells (34), suggesting that it should be adequate for reporting the usually slower Ca\(^{2+}\) response in PASMCs.

Using the targeted Ca\(^{2+}\) probes, we compared the Ca\(^{2+}\) signals induced by exogenous IP\(_3\) in the cytoplasmic and nucleoplasmic compartments. IP\(_3\)Rs are expressed in SR of PASMCs, and photorelease of caged-IP\(_3\) can generate Ca\(^{2+}\) sparks and regenerative global Ca\(^{2+}\) release through cross-activation of ryanodine receptors (61). In PASMCs permeabilized with a mild treatment of digitonin, exogenous application of IP\(_3\) induced cytoplasmic and nucleoplasmic Ca\(^{2+}\) signals, which had no clear difference in magnitude and kinetics, suggesting efficient Ca\(^{2+}\) diffusion between the cytosol and the nucleus under our experimental conditions. This is consistent with the notion that the nuclear pore complexes are freely permeable to small ions and molecules (1, 49), even though cytoplasmic and nucleoplasmic Ca\(^{2+}\) can be regulated independently by specific signaling pathways (4, 30). When PASMCs were treated under excessive permeabilization conditions, exogenous IP\(_3\) elicited a rapid nucleoplasmic Ca\(^{2+}\) response with a much shorter duration. This could be related to the shorter diffusion time of IP\(_3\) and faster dispersal of released Ca\(^{2+}\) in the fully permeabilized cells. It is interesting, however, to note that the rapid transient nucleoplasmic Ca\(^{2+}\) response is similar to Ca\(^{2+}\) release from IP\(_3\)Rs in the nuclear envelope and/or nucleoplasmic reticulum reported in isolated nucleus of other cell types (9, 15, 63). Since the expression of functional IP\(_3\)Rs in the nuclear envelope or nucleoplasmic reticulum of PASMCs has not been examined, their relative contribution to nuclear Ca\(^{2+}\) signaling requires further investigation.

An important observation of the present study is the differential regulation of Ca\(^{2+}\) signals in subcellular regions by the agonists of G protein-coupled receptors. ET-1 and ANG II elicited Ca\(^{2+}\) signals were significantly stronger in the subsarcolemmal region than in the cytoplasm or nucleoplasm of PASMCs. Since the PM-targeted Lyn-D3cpv is inserted into...
Fig. 4. Effects of endothelin-1 (ET-1) and angiotensin II (ANG II) on the nucleoplasmic and PM Ca\textsuperscript{2+} increase in rat PASMCs. A: 2D confocal images of PASMCs cotransfected with Lyn-D3cpv and 3NLS-D3cpv, showing cpV fluorescence (a), and Ca\textsuperscript{2+} mobilization in the PM and nucleoplasm before (b) and after (c) ET-1 (30 nM) treatment. B: ΔFRET fluorescence ratio traces showing Ca\textsuperscript{2+} mobilization in the PM (ROI 1 and 3) and nucleoplasm (ROI 2 and 4) of rat PASMCs (from A) after ET-1 treatment. C: statistical analysis of the peak normalized ΔFRET fluorescence ratio of ET-1-induced Ca\textsuperscript{2+} transients in the nucleoplasm and PM of rat PASMCs. One-way ANOVA was used to calculate *P values. **P < 0.001; n = 8. Error bars denote SE. D: 2D confocal images of the cells cotransfected with Lyn-D3cpv and 3NLS-D3cpv, showing cpV fluorescence (a), and Ca\textsuperscript{2+} mobilization in the PM and nucleoplasm before (b) and after (c) ANG II (100 nM) treatment. E: ΔFRET fluorescence ratio traces showing Ca\textsuperscript{2+} mobilization in the PM (ROI 1 and 3) and nucleoplasm (ROI 2 and 4) of rat PASMCs (from D) after ANG II treatment. F: statistical analysis of the peak normalized ΔFRET fluorescence ratio of ANG II-induced Ca\textsuperscript{2+} transients in the nucleoplasm and PM of rat PASMCs. One-way ANOVA was used to calculate *P values. **P < 0.001; n = 12. Error bars denote SE. G and H: time course showing changes in nucleoplasmic and PM [Ca\textsuperscript{2+}] in a single cell upon treatment with ET-1 and ANG II.
PDGF (20 ng/ml) treatment. 

Ca\(^{2+}\) mobilization in the cytoplasm and nucleoplasm before (b) and after (c) ET-1 (30 nM) treatment. E: FRET fluorescence ratio traces showing Ca\(^{2+}\) mobilization in the cytoplasm (ROI 1 and 3) and nucleoplasm (ROI 2 and 4) of rat PASMCs (from A) after ET-1 treatment. C: statistical analysis of the peak normalized FRET fluorescence ratio of ET-1 induced Ca\(^{2+}\) transients in the nucleoplasm and cytoplasm of rat PASMCs. One-way ANOVA was used to calculate \(P\) values. \(P < 0.05; n = 10\). Error bars denote SE. D: 2D confocal images of a PASMC cotransfected with D3cpv and 3NLS-D3cpv, showing cpV fluorescence (a), and Ca\(^{2+}\) mobilization in the cytoplasm and nucleoplasm before (b) and after (c) ANG II (100 nM) treatment. F: statistical analysis of the peak normalized FRET fluorescence ratio of ANG II-induced Ca\(^{2+}\) transients in the nucleoplasm and cytoplasm of rat PASMCs. One-way ANOVA was used to calculate \(P\) values. \(P > 0.05; n = 12\). Error bars denote SE.

In contrast, PDGF-induced Ca\(^{2+}\) signal in the plasma membrane region was similar in amplitude compared with those of

Subcellular Ca\(^{2+}\) dynamics in PASMCs: 

The plasma membrane, it detects Ca\(^{2+}\) signals within a few nanometers underneath the plasma membrane. This is the site where numerous Ca\(^{2+}\) signaling processes occur, including Ca\(^{2+}\) entry via voltage-dependent and voltage-independent Ca\(^{2+}\) channels, and cross-signaling between Ca\(^{2+}\) release channels and membrane channels. ET-1 and ANG II mobilize intracellular Ca\(^{2+}\) through G protein stimulation of PLC-\(\beta\) to generate IP\(_3\) and diacylglycerol (DAG), leading to the activation of multiple Ca\(^{2+}\)-permeating cation channels (29, 43, 62) and Ca\(^{2+}\) release channels (22, 48, 61) in VSMCs. Since [Ca\(^{2+}\)] in the microdomain of an opened Ca\(^{2+}\)-permeating channel can reach hundreds of micromolar (36, 45, 50), the remarkably high [Ca\(^{2+}\)] detected by Lyn-D3cpv in the PM region likely reflects Ca\(^{2+}\) signals generated near the membrane Ca\(^{2+}\) channels and perhaps from Ca\(^{2+}\) release channels of peripherally coupled SR during agonist stimulation (10, 59). The lesser Ca\(^{2+}\) signals in the cytoplasm and nucleus might relate to Ca\(^{2+}\) release from the centrally located SR and lysosomal Ca\(^{2+}\) stores, which are known to be activated by ET-1 in PASMCs (22, 61).

Fig. 6. Effects of platelet-derived growth factor (PDGF) on PM, cytoplasmic, and nucleoplasmic Ca\(^{2+}\) increase in rat PASMCs. A: 2D confocal images of a PASMC cotransfected with Lyn-D3cpv and 3NLS-D3cpv, showing cpV fluorescence (a), and Ca\(^{2+}\) mobilization in the PM and nucleoplasm before (b) and after (c) PDGF (20 ng/ml) treatment. B: FRET fluorescence ratio traces showing Ca\(^{2+}\) mobilization in the PM (ROI 1) and nucleoplasm (ROI 2) of rat PASMCs (from A) after PDGF treatment. C: 2D confocal images of a PASMC cotransfected with D3cpv and 3NLS-D3cpv, showing cpV fluorescence (a), and Ca\(^{2+}\) mobilization in the cytoplasm and nucleoplasm before (b) and after (c) PDGF treatment. D: FRET fluorescence ratio traces showing Ca\(^{2+}\) mobilization in the cytoplasm (ROI 3) and nucleoplasm (ROI 4) of rat PASMCs (from C) after PDGF treatment. E: statistical analysis of the peak normalized FRET fluorescence ratio, time to 75% of peak, and time of half-decay of PDGF-induced Ca\(^{2+}\) transients in the nucleoplasm and PM of rat PASMCs. F: statistical analysis of the normalized FRET fluorescence ratio peak intensity, time to 75% of peak, and time of half-decay of PDGF-induced Ca\(^{2+}\) transients in the nucleoplasm and cytoplasm of rat PASMCs. Paired \(t\)-test was used to calculate \(P\) values. \(*P < 0.05\) compared with nucleoplasmic. Error bars denote SE.
cytoplasm and nucleoplasm, except with slower onset and decay. The cytoplasmic and nucleoplasmic Ca\(^{2+}\) transients induced by PDGF were also significantly slower than those activated by ET-1 and ANG II. This is consistent with previous reports that ANG II elicits larger and faster increases in IP\(_3\) than PDGF in vascular smooth muscle cells (26) and that ET-1 and PDGF show different profiles of intracellular Ca\(^{2+}\) responses (27). These contrasting Ca\(^{2+}\) responses are probably related to the differences in signaling mechanisms of the tyrosine kinase receptor agonist PDGF and the G protein-coupled receptor agonists (26, 27, 42, 51). PDGF activates PDGF receptors (PDGFR-\(\alpha\) and PDGFR-\(\beta\)), causing tyrosine phosphorylation of PLC-\(\gamma\) to generate DAG and IP\(_3\), as well as activation of other signaling pathways including Ras-MAPK and PI3K (3). Depending on the subcellular location and spatial association of the signaling molecules and their effectors, PDGF may preferentially activate Ca\(^{2+}\) release from the centrally located Ca\(^{2+}\) stores, instead of the membrane Ca\(^{2+}\)-permeating channels. It is consistent with reports that mitogens and growth factors stimulate Ca\(^{2+}\) release from Ca\(^{2+}\) stores in the perinuclear regions and/or nucleoplasmic reticulum to regulate gene transcription and cell proliferation in other cell types (2, 9, 18, 20, 41, 57). The faster PDGF-induced Ca\(^{2+}\) signals in the cytoplasm and nucleus compared with those in the PM region observed in this study suggest that PDGF-induced Ca\(^{2+}\) increase is initiated in the central Ca\(^{2+}\) sources of PASMCs. However, there was no clear disparity between the PDGF-induced cytoplasmic and nucleoplasmic Ca\(^{2+}\) signals in PASMCs under our experimental conditions. It is likely that the Ca\(^{2+}\) signal of IP\(_3\)Rs in the nuclear envelope and/or nucleoplasmic reticulum was masked by cytoplasmic Ca\(^{2+}\) release, which can easily diffuse through nuclear pore complexes. Since fast transient local Ca\(^{2+}\) signals may escape detection due to the limitations in the imaging speed (2 frames/s) of our experiments, future investigations using techniques with improved spatial and temporal resolution are needed for further elucidation of the local regulation of nucleoplasmic Ca\(^{2+}\) signals by the growth factors.

Taken together, we have successfully applied the genetically encoded cameleon D3cpv to probe specific Ca\(^{2+}\) signals in the subsarcolemmal, cytosolic, and nucleoplasmic compartments in PASMCs. Our results provide the first experimental evidence that subsarcolemmal Ca\(^{2+}\) signals is preferentially stimulated by the G protein-coupled receptor agonists ET-1 and ANG II in PASMCs. The use of organelle-specific Ca\(^{2+}\) biosensors and the observation of compartmentalized Ca\(^{2+}\) signals in this study lay the foundation for further investigation of the differential regulation of subcellular Ca\(^{2+}\) signals in PASMCs, as well as other cell types.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

K.P.S. and J.S.K.S. conception and design of research; K.P.S. and O.P. performed experiments; K.P.S. analyzed data; K.P.S. and J.S.K.S. interpreted results of experiments; K.P.S. prepared figures; K.P.S. drafted manuscript; K.P.S. and J.S.K.S. edited and revised manuscript; J.S.K.S. approved final version of manuscript.

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