Local Regulation of Arterial L-Type Calcium Channels by Reactive Oxygen Species

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Rationale: Reactive oxygen species (ROS) are implicated in the development of cardiovascular disease, and oxidants are important signaling molecules in many cell types. Recent evidence suggests that localized subcellular compartmentalization of ROS generation is an important feature of ROS signaling. However, mechanisms that transduce localized subcellular changes in redox status to functionally relevant changes in cellular processes such as Ca\(^{2+}\) influx are poorly understood.

Objective: To test the hypothesis that ROS regulate L-type Ca\(^{2+}\) channel activity in cerebral arterial smooth muscle.

Methods and Results: Using a total internal reflection fluorescence imaging–based approach, we found that highly localized subplasmalemmal generation of endogenous ROS preceded and colocalized with sites of enhanced L-type Ca\(^{2+}\) channel sparklet activity in isolated cerebral arterial smooth muscle cells. Consistent with this observation and our hypothesis, exogenous ROS increased localized L-type Ca\(^{2+}\) channel sparklet activity in isolated arterial myocytes via activation of protein kinase C\(\alpha\) and when applied to intact cerebral arterial segments, exogenous ROS increased arterial tone in an L-type Ca\(^{2+}\) channel–dependent fashion. Furthermore, angiotensin II–dependent stimulation of local L-type Ca\(^{2+}\) channel sparklet activity in isolated cells and contraction of intact arteries was abolished following inhibition of NADPH oxidase.

Conclusions: Our data support a novel model of local oxidative regulation of Ca\(^{2+}\) influx where vasoconstrictors coupled to NAPDH oxidase (eg, angiotensin II) induce discrete sites of ROS generation resulting in oxidative activation of adjacent protein kinase C\(\alpha\) molecules that in turn promote local sites of enhanced L-type Ca\(^{2+}\) channel activity, resulting in increased Ca\(^{2+}\) influx and contraction. (Circ Res. 2010;107:1002-1010.)

Key Words: reactive oxygen species ■ L-type calcium channels ■ calcium sparklets ■ protein kinase C

Oxidative and reductive biochemical processes are integral components of cellular biology. However, disruption of cellular redox status beyond physiological parameters is correlated with disorders ranging from cardiovascular disease to neurodegeneration to cancer. The cardiovascular system appears to be particularly sensitive to perturbations in redox balance with increased oxidative stress implicated in the development of cardiovascular diseases including heart failure, atherosclerosis, stroke, and hypertension. The apparent sensitivity of the cardiovascular system to redox imbalance may reside in the relative functional importance of redox signaling under physiological conditions. Redox biochemistry has been studied extensively in the vasculature, with specialized subcellular compartmentalization, regulation, and generation of reactive oxygen species (ROS) emerging a common theme. However, mechanisms that transduce localized subcellular changes in redox signaling to functionally relevant changes in cellular processes such as Ca\(^{2+}\) influx are less clear.

Peripheral arterial resistance is determined by the contractile state of arterial smooth muscle, which is tightly coupled to Ca\(^{2+}\) influx through L-type voltage-dependent Cav1.2 Ca\(^{2+}\) channels. Total internal reflection fluorescence (TIRF) microscopy has been used to image L-type Ca\(^{2+}\) channel (Ca\(^{2+}\) sparklet) activity in arterial smooth muscle cells with high temporal and spatial resolution. Using this approach, a high-activity mode of Cav1.2 Ca\(^{2+}\) channel function was identified. These studies revealed that high-activity Ca\(^{2+}\) sparklets resulted from spatially restricted enhancement of Cav1.2 Ca\(^{2+}\) channel function by protein kinase (PK)\(\alpha\). PK\(\alpha\)-dependent Ca\(^{2+}\) sparklet activity was found to be necessary for normal arterial function. In addition, increased PK\(\alpha\)-dependent Ca\(^{2+}\) sparklet activity was associated with altered gene expression and arterial dysfunction in exper-
mental models of hypertension. Despite these intriguing observations, the molecular mechanisms underlying high-activity localized PKCa-dependent L-type Ca\textsuperscript{2+} channel function are poorly understood.

Using a novel TIRF imaging–based approach, we identified and characterized a mechanism that functionally links local oxidant signaling to sustained colocalized L-type Ca\textsuperscript{2+} channel activity. We demonstrate for the first time that highly localized subplasmalemmal generation of endogenous ROS precede and colocalize with enhanced L-type Ca\textsuperscript{2+} channel activity in cerebral arterial smooth muscle. Consistent with this observation, ROS increased L-type Ca\textsuperscript{2+} channel sparklet activity via activation of PKCa and increased arterial tone in an L-type Ca\textsuperscript{2+} channel–dependent fashion. Taken together, our data suggest that local oxidative activation of PKCa-dependent L-type Ca\textsuperscript{2+} influx represents a functionally relevant convergence of oxidant and Ca\textsuperscript{2+} signaling pathways in cerebral arterial smooth muscle with physiological and pathological implications.

Methods

Male Sprague–Dawley rats were euthanized with sodium pentobarbital (200 mg/kg IP) as approved by the Institutional Animal Care and Use Committee of Colorado State University. Smooth muscle cells were isolated from basilar and cerebral arteries.

Arteries for intact tissue experiments were cannulated, pressurized with bicarbonate-based physiological saline solution, and superfused with aerated physiological saline solution at 37°C. To block the effects of endothelial-derived nitric oxide, we used the nitric oxide synthase inhibitor N\textsuperscript{ω}-nitro-L-arginine (L-NNA) (300 μmol/L). Intravascular pressure was maintained at 80 mm Hg, and inner diameter was continuously monitored.

We used the conventional whole-cell patch-clamp technique to voltage clamp freshly isolated arterial myocytes. L-type Ca\textsuperscript{2+} channel sparklets were recorded with a through-the-lens TIRF system with ×60 (numerical aperture=1.49) and ×100 (numerical aperture=1.45) TIRF oil immersion objectives. All TIRF experiments were performed in the presence of thapsigargin (1 μmol/L). To monitor Ca\textsuperscript{2+} influx, myocytes were loaded with the Ca\textsuperscript{2+} indicator fluo-5F (200 μmol/L) via dialysis through the patch pipette. L-type Ca\textsuperscript{2+} channel sparklets were visualized and recorded at a holding potential of −70 mV with elevated external [Ca\textsuperscript{2+}] (20 μmol/L) to facilitate the detection of Ca\textsuperscript{2+} sparklet events and provide fluorescent signals of sufficient amplitude to permit quantal analysis of Ca\textsuperscript{2+} sparklet activity. Fluo-5F fluorescence was converted to [Ca\textsuperscript{2+}] and analyzed as previously reported using a custom automated algorithm.\textsuperscript{17,21} Ca\textsuperscript{2+} sparklet activity was quantified\textsuperscript{17,18} by calculating the n\textsubscript{P}, of each sparklet site, where n is the number of quantal units and P\textsuperscript{P} is the probability that a given Ca\textsuperscript{2+} sparklet site is active. As with previous reports,\textsuperscript{17,18} Ca\textsuperscript{2+} sparklet activity was bimodally distributed with sites of low activity (n\textsubscript{P} 0 to 0.2) and high activity (n\textsubscript{P} >0.2). TIRF microscopy was also used to visualize subplasmalemmal ROS generation in isolated arterial myocytes using the cell-permeant ROS indicator 5-(and-6)-chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate acetyl ester (DCF) (10 μmol/L).

For our immunofluorescence studies, we used a mouse monoclonal PKCa antibody (Abcam) with an Alexa Fluor 488–conjugated rabbit anti-mouse secondary antibody on freshly prepared myocytes. Fluorescence was undetectable in control experiments where either the primary or the secondary antibody was omitted (data not shown). Membrane and cytosolic PKCa-associated fluorescence was quantified by measuring the intensity of pixels above a set threshold. We determined the ratio of plasma membrane to cytosolic PKCa-associated fluorescence and used this as an indicator of PKCa translocation and activity.\textsuperscript{19,22}

Normally distributed data are presented as the means±SEM with comparisons performed using parametric tests. For bimodally distributed Ca\textsuperscript{2+} sparklet activity (ie, n\textsubscript{P} datasets, comparisons were performed using nonparametric tests. Arithmetic means of n\textsubscript{P} datasets are indicated in the figures (solid red horizontal lines) for nonstatistical purposes. Probability values of less than 0.05 were considered significant, and asterisks (*) in the figures indicate a significant difference between groups.

Results

To test the overall hypothesis that ROS regulate L-type Ca\textsuperscript{2+} channel activity in cerebral arterial smooth muscle, we formulated 4 specific requisite criteria: (1) exogenous ROS should increase arterial tone in a L-type Ca\textsuperscript{2+} channel–dependent manner; (2) exogenous ROS should stimulate Ca\textsuperscript{2+} sparklet activity in isolated myocytes; (3) inhibition of endogenous ROS generation should limit arterial contraction and prevent stimulation of Ca\textsuperscript{2+} sparklet activity; and (4) endogenous ROS production should colocalize with Ca\textsuperscript{2+} sparklet activity.

Exogenous ROS Increase Arterial Tone in Pressurized Cerebral Arteries

We examined the effects of ROS on arterial smooth muscle function by exposing pressurized cerebral arteries (80 mm Hg at 37°C) to the ROS-generating system xanthine oxidase (XO) (0.2 mU/mL) plus hypoxanthine (HX) (250 μmol/L) (Figure 1). The nitric oxide synthase inhibitor L-NNA (300 μmol/L) was present to preclude contractile responses from ROS-dependent reductions in nitric oxide.\textsuperscript{23} Following the development of a stable level of myogenic tone (24.8±2.5%, n=8 arteries), we exposed arteries to HX followed by XO/HX and monitored changes in luminal diameter. Although HX alone had no effect (P>0.05, n=8 arteries), addition of XO reversibly reduced luminal diameter by 18.1±6.9 μm, corresponding to a 10.7±4.1% increase in arterial tone (Figure 1A and 1B; P<0.05, n=8 arteries). Transient dilations were occasionally observed (in 3 of 8 arteries) following addition of XO. In the presence of the L-type Ca\textsuperscript{2+} channel antagonist diltiazem (10 μmol/L), which nearly
abolished arterial tone (from 23.6±7.1% to 4.4±1.7%), XO/HX was without effect (Figure 1C and 1D; \( P > 0.05, n = 3 \) arteries). Although multiple mechanisms are certainly involved in arterial contractile responses to XO/HX, these data are consistent with the hypothesis that ROS increase arterial tone by stimulating L-type \( \text{Ca}^{2+} \) channel function.

**Exogenous ROS Stimulate L-Type \( \text{Ca}^{2+} \) Channel Sparklet Activity in Isolated Cerebral Arterial Smooth Muscle Cells**

To test the hypothesis that ROS stimulate L-type \( \text{Ca}^{2+} \) channel activity, we recorded \( \text{Ca}^{2+} \) sparklets in isolated voltage-clamped (−70 mV) arterial myocytes before and after XO (2 mU/mL) + HX (250 \( \mu \text{mol/L} \)). As shown in Figure 2A, XO/HX exposure increased \( \text{Ca}^{2+} \) sparklet activity. To further characterize the effects of ROS on \( \text{Ca}^{2+} \) sparklets, we constructed \( \text{Ca}^{2+} \) sparklet amplitude histograms under control conditions and after XO/HX (Figure 2B). Fitting these distributions with a multicomponent Gaussian function revealed that \( \text{Ca}^{2+} \) sparklet amplitudes were quantal and that stimulation by ROS increased the number of quanta activated but not the amplitude of the quantal event (34 nmol/L [\( \text{Ca}^{2+} \)] for control and 36 nmol/L [\( \text{Ca}^{2+} \)] for XO/HX). Note that the \( \text{Ca}^{2+} \) sparklet quantal amplitudes before and after XO/HX approximate those previously reported for arterial smooth muscle L-type \( \text{Ca}^{2+} \) channels and for heterologously expressed Cav1.2 L-type \( \text{Ca}^{2+} \) channels.\(^{17,18,20,24}\)

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![Figure 1. ROS increase tone in pressurized cerebral arteries. A, Representative time course showing the luminal diameter of a pressurized (80 mm Hg) cerebral artery exposed to HX (250 \( \mu \text{mol/L} \)) followed by XO (0.2 mU/mL) plus HX. B, Plot of the mean±SEM change in arterial tone (% change in arterial tone) during HX and XO/HX exposure (n = 7 arteries). C, Representative time course showing the luminal diameter of a pressurized (80 mm Hg) cerebral artery exposed to HX followed by XO/HX in the presence of the L-type \( \text{Ca}^{2+} \) channel blocker diltiazem (10 \( \mu \text{mol/L} \)). D, Plot of the mean±SEM change in arterial tone (% change in arterial tone) during diltiazem+HX and diltiazem+XO/HX exposure (n = 3 arteries). \( * P < 0.05 \).](http://circres.ahajournals.org/)

![Figure 2. ROS increase L-type \( \text{Ca}^{2+} \) channel sparklet activity in isolated cerebral arterial smooth muscle cells. A, Representative TIRF images showing \( \text{Ca}^{2+} \) influx in an arterial myocyte before and after application of XO/HX (2 mU/mL and 250 \( \mu \text{mol/L} \), respectively). Traces show the time course of \( \text{Ca}^{2+} \) influx at the 3 circled sites before and after XO/HX. B, \( \text{Ca}^{2+} \) sparklet amplitude histograms before (white) and after XO/HX exposure (red). The solid black lines are best fits to the control (\( q = 34 \text{nmol/mol} \)) and to the XO/HX (\( q = 36 \text{nmol/mol} \)) histograms with a multicomponent Gaussian function, where \( q \) is the quantal unit of \( \text{Ca}^{2+} \) influx (see the Online Data Supplement). C, Plot of \( \text{Ca}^{2+} \) sparklet site activities (nps) before and after XO/HX exposure (n = 8 cells). The red solid lines are the arithmetic means of each group, and the dashed line marks the threshold for high-activity \( \text{Ca}^{2+} \) sparklet sites (nps = 0.2). D, Plot of the mean±SEM, \( \text{Ca}^{2+} \) sparklet density (\( \text{Ca}^{2+} \) sparklet sites/\( \mu \text{m}^2 \)) before and after XO/HX (n = 8 cells). \( * P < 0.05 \).](http://circres.ahajournals.org/)
these data, we conclude that ROS increase Ca\(^{2+}\) influx by stimulating localized L-type Ca\(^{2+}\) channel activity.

Next, we quantified L-type Ca\(^{2+}\) channel activity by determining the nP\(_s\) of each Ca\(^{2+}\) sparklet site, where n is the number of quantal levels detected and P\(_s\) is the probability that a given Ca\(^{2+}\) sparklet site is active. As evident in the histogram in Figure 2B, Ca\(^{2+}\) sparklet activity (nP\(_s\)) increased after XO/HX exposure with the number of high-activity Ca\(^{2+}\) sparklet sites (nP\(_s\)≥2) increasing from 2 to 16 (Figure 2C; P<0.05, n=8 cells). In addition to increasing Ca\(^{2+}\) sparklet activity, XO/HX also increased Ca\(^{2+}\) sparklet site density by 3.5-fold by promoting Ca\(^{2+}\) influx at regions previously devoid of activity (Figure 2D; P<0.05, n=8 cells). These data suggest that ROS stimulate localized L-type Ca\(^{2+}\) channel function by increasing the occurrence of high-activity Ca\(^{2+}\) sparklet sites and by stimulating nascent Ca\(^{2+}\) sparklet activity.

Stimulation of L-Type Ca\(^{2+}\) Channel Sparklet Activity by Exogenous ROS Is PKCa-Dependent
PKCa, which is subject to oxidative activation,\(^{26}\) stimulates L-type Ca\(^{2+}\) channel sparklet activity.\(^{17,18}\) Accordingly, we tested the effects of XO/HX on PKCa activation in arterial myocytes with immunofluorescence by using the level of plasma membrane-associated PKCa as an indicator of activation (Figure 3A).\(^{19,22}\) Under control conditions (HX; 250 μmol/L), PKCa-associated fluorescence was diffusely cytosolic with few discrete elevations along the plasma membrane. In contrast, following XO (2 μU/mL)+HX (250 μmol/L) exposure, and indicative of activation, PKCa-associated fluorescence was characterized by prominent focal elevations near (<1 μm) the plasma membrane (Figure 3B; P<0.05, n=15 cells).

Figure 3. ROS increase L-type Ca\(^{2+}\) channel sparklet activity via stimulation of PKCa. A, Representative surface plots of PKCa-associated immunofluorescence in cerebral arterial myocytes exposed to either HX alone (250 μmol/L) or XO and HX (2 μU/mL and 250 μmol/L, respectively). B, Bar plot of the mean±SEM membrane-to-cytosol PKCa-associated fluorescence ratios in HX- and XO/HX-exposed myocytes (n=15 cells from 3 independent experiments). C, Representative TIRF images showing Ca\(^{2+}\) influx in an arterial myocyte under control conditions and after exposure to XO and HX (2 μU/mL and 250 μmol/L, respectively) in the presence of the PKC inhibitor Go6976 (100 nmol/L). D, Plot of Ca\(^{2+}\) sparklet site activities (nP\(_s\)) and mean±SEM. Ca\(^{2+}\) sparklet density (Ca\(^{2+}\) sparklet sites/μm\(^2\)) under control conditions and after exposure to XO and HX in the presence of Go6976 (n=5 cells). *P<0.05.
Our PKCα immunofluorescence data suggest that ROS could increase L-type Ca\(^{2+}\) channel sparklet activity by activating PKCα. To examine this possibility, we tested the effects of XO/HX on Ca\(^{2+}\) sparklets in the presence of the PKC inhibitor Gö6976 (100 nmol/L). In contrast to experiments in the absence of Gö6976 (eg, Figure 2), XO/HX had no effect on Ca\(^{2+}\) sparklet activity or density following inhibition of PKCα (Figure 3C and 3D; \(P>0.05\), n=5 cells). These data suggest that ROS stimulate L-type Ca\(^{2+}\) channel sparklet activity via activation of PKCα.

### NADPH Oxidase Inhibition Prevents Angiotensin II–Dependent Stimulation of L-Type Ca\(^{2+}\) Channels and Arterial Constriction

Angiotensin (Ang) II stimulates ROS-generating NADPH oxidase signaling complexes. Therefore, we used Ang II to examine the role of endogenous ROS generation on Ca\(^{2+}\) sparklet activity and arterial constriction. As shown previously, Ang II (100 nmol/L) increased L-type Ca\(^{2+}\) channel sparklet activity (\(nP_{\text{r}}\)) and the number of active Ca\(^{2+}\) sparklet sites (Figure 4A and 4B; \(P<0.05\), n=8 cells). This effect was blocked by the Ang II type 1 (AT1) receptor antagonist ZD7155 (500 nmol/L; control \(nP_{\text{r}}=0.35\pm0.32\), Ang II+ZD7155 \(nP_{\text{r}}=0.30\pm0.11\), \(P>0.05\), n=4 cells). Inhibition of NADPH oxidase–mediated ROS generation with apocynin (25 μmol/L) abolished contractile responses to Ang II (1 nmol/L; Figure 5B through 5D; \(P<0.05\), n=3 arteries). Taken together, these data support our hypothesis that generation of endogenous ROS is necessary for AT1-dependent Ang II stimulation of L-type Ca\(^{2+}\) channels and arterial constriction.

### Ang II Stimulates L-Type Ca\(^{2+}\) Channel Sparklet Activity via Local Production of ROS

If generation of endogenous ROS were necessary for Ang II–dependent stimulation of L-type Ca\(^{2+}\) channels, then sites of Ca\(^{2+}\) sparklet activity should colocalize with endogenous sites of ROS production. We used TIRF microscopy to visualize subplasmalemmal generation of ROS in response to Ang II. Isolated myocytes were loaded with the ROS indicator DCF (10 μmol/L). Figure 6A shows TIRF images of DCF fluorescence under control conditions and after application Ang II (100 nmol/L; both in Ca\(^{2+}\)-free buffer). Interestingly, discrete sites (puncta) of elevated DCF fluorescence were apparent under control conditions (0.006±0.002 puncta per μm\(^2\)) and increased in number 3.5-fold with Ang II (0.021±0.002 puncta per μm\(^2\); \(P<0.05\), n=6 cells). The amplitude of the DCF elevations before and after Ang II were not different (\(P<0.05\); n=6 cells). Ca\(^{2+}\) sparklet site and ROS puncta densities (0.025±0.005 and 0.021±0.002, respectively) were similar (\(P>0.05\)).
Having demonstrated that increased ROS production (as with Ca$^{2+}$ sparklet activity) in response to Ang II is localized, we developed an experimental approach to visualize the spatial distributions of ROS generation (with DCF) and L-type Ca$^{2+}$ channel sparklet activity (with fluo-5F) in the same cell. First, we loaded isolated myocytes with DCF in Ca$^{2+}$-free buffer (as above), placed a patch pipette on the cell, formed a GΩ seal (ie, established the cell-attached patch configuration), and applied Ang II (100 nmol/L). On visualization of sites of punctate elevations in DCF fluorescence (Figure 7A, panel 1, time course ii), negative pressure was applied to the interior of the pipette to rupture the plasma membrane (ie, establish the whole-cell patch configuration), thus dialyzing the cell with fluo-5F. Next, we replaced the Ca$^{2+}$-free external solution with one containing 20 mmol/L Ca$^{2+}$ and monitored for Ca$^{2+}$ sparklet activity. Strikingly, we found that the sites of ROS generation colocalized with high-activity (n$P = 0.56 \pm 0.26$) Ca$^{2+}$ sparklet sites (Figure 7A, panel 2, time course ii; n = 6 sites from 5 cells). Although Ca$^{2+}$ sparklet sites were always associated with a preceding site of ROS generation, we did observe 3 sites of ROS generation that were not associated with subsequent Ca$^{2+}$ sparklet activity (data not shown; n = 3 ROS sites in 7 cells).

To further establish the proximity of punctate ROS generation and Ca$^{2+}$ sparklet activity, we thresholded (mean basal fluo-5F fluorescence plus 3 times its SD) our fluo-5F fluorescence images to isolate Ca$^{2+}$ sparklet fluorescence and merged them (Figure 7A, panel 3) with the DCF images (Figure 7A, panel 1). As evident in the composite image (Figure 7A, panel 4), sites of punctate ROS generation and Ca$^{2+}$ sparklet activity were closely apposed. Indeed, the average distance between the peaks of ROS puncta and Ca$^{2+}$ sparklet sites was <1 μm (0.91 ± 0.24 μm; n = 6 sites from 5 cells). To place this distance into perspective, the average cell area imaged during these experiments was 99.2 ± 13.7 μm$^2$ and the probability, by chance alone, that we would observe 6 ROS puncta and 6 Ca$^{2+}$ sparklet sites <1 μm apart in this area is <1 in 1 000 000 (see the Online Data Supplement for details). From these data, we conclude that sites of ROS generation precede and colocalize with sites of L-type Ca$^{2+}$ channel sparklet activity.

**Discussion**

In this study, we combined TIRF imaging with conventional techniques to test the hypothesis that ROS regulate L-type Ca$^{2+}$ influx in cerebral arterial smooth muscle cells. A, Representative TIRF images showing punctate elevations of DCF fluorescence (indicating ROS formation) in an arterial myocyte before and after application of Ang II (100 nmol/L). B, Plot of the mean±SEM ROS puncta density (ROS puncta/μm$^2$) before and after Ang II (n = 6 cells). C, Plot of the mean±SEM ROS puncta amplitude (arbitrary units [AU]) before and after Ang II (n = 6 cells). *P < 0.05.

**Figure 6. Ang II promotes spatially restricted generation of ROS in isolated cerebral arterial smooth muscle cells.** A, Representative TIRF images showing punctate elevations of DCF fluorescence (indicating ROS formation) in an arterial myocyte before and after application of Ang II (100 nmol/L). B, Plot of the mean±SEM ROS puncta density (ROS puncta/μm$^2$) before and after Ang II (n = 6 cells). C, Plot of the mean±SEM ROS puncta amplitude (arbitrary units [AU]) before and after Ang II (n = 6 cells). *P < 0.05.

**Figure 7. Local sites of elevated ROS generation precede and colocalize with Ca$^{2+}$ sparklet activity in cerebral arterial smooth muscle cells.** A, Representative TIRF images of an arterial myocyte exposed to Ang II showing sequential DCF fluorescence (indicating ROS formation) (panel 1) and fluo-5F fluorescence (indicating Ca$^{2+}$ influx) (panel 2). B, Plot of the mean±SEM distance (in μm) between the peaks of ROS puncta and adjacent Ca$^{2+}$ sparklet sites (n = 6 ROS/Ca$^{2+}$ sparklet sites from 5 cells). C, Plot of the mean±SEM cell area imaged (in μm$^2$) during the DCF/fluo-5F experiments (n = 5 cells). D, Proposed mechanism by which local generation of ROS stimulate PKC-dependent L-type Ca$^{2+}$ channel sparklet activity in cerebral arterial smooth muscle cells (see Discussion).

**Figure 7.**
Ca\(^{2+}\) channel activity in cerebral arterial smooth muscle. The major findings in support of this hypothesis are: (1) exogenous ROS generated by XO constrict pressurized cerebral arteries in an L-type Ca\(^{2+}\) channel–dependent manner; (2) exogenous ROS increase PKC\(\alpha\)-dependent L-type Ca\(^{2+}\) channel sparklet activity in isolated arterial myocytes; (3) generation of endogenous ROS by NADPH oxidase is necessary for stimulation of Ca\(^{2+}\) sparklets and contraction by Ang II; and (4) Ang II induces localized sites of ROS production that precede and colocalize with subsequent Ca\(^{2+}\) sparklet activity. These observations support a model (see Figure 7D) of local oxidative regulation of Ca\(^{2+}\) influx where vasocostricators coupled to NADPH oxidase (eg, Ang II) induce discrete sites of ROS generation, resulting in activation of adjacent PKC\(\alpha\) molecules. PKC\(\alpha\) activation in turn promotes localized L-type Ca\(^{2+}\) channel (ie, sparklet) activity, resulting in increased Ca\(^{2+}\) influx and arterial contraction. To the best of our knowledge, our model provides the first experimentally based mechanistic framework whereby local changes in redox signaling result in changes in Ca\(^{2+}\) influx and arterial function.

ROS impairment of endothelial function leads to increased arterial tone.\(^{23,29}\) Our observation that XO/HX constricts cerebral arteries (presumably via PKC activation) after endothelial nitric oxide synthase inhibition with L-NNA suggests that ROS also increase tone through smooth muscle–specific mechanisms. Activation of arterial smooth muscle PKC by ROS could induce contraction by a minimum of 4 non–mutually exclusive mechanisms: (1) increased localized L-type Ca\(^{2+}\) channel sparklet activity as characterized here; (2) modulation of other plasmalemmal ion channels (eg, inhibition of hyperpolarizing potassium channels\(^{30}\) or activation of depolarizing transient receptor potential channels\(^{31}\)); (3) decreased hyperpolarizing ryanodine receptor–dependent Ca\(^{2+}\) spark activity\(^{32}\); and (4) sensitization of the arterial smooth muscle contractile apparatus to Ca\(^{2+}\).3\(^{33}\) The discrete subcellular nature of Ang II–dependent ROS generation demonstrated here suggests that the relationship between ROS and these diverse regulatory mechanisms could be determined in part by coincident localization of ROS-generating and smooth muscle regulatory signaling complexes. In addition, differential subcellular distributions of signaling complexes and ROS-generating enzymes could account for contradictory data regarding the effect of ROS on arterial tone (ie, contraction\(^{34}\) versus relaxation\(^{35}\)).

PKC stimulation of NADPH oxidase is well-documented.\(^{36,37}\) However, reciprocal activation of PKC by ROS in arterial smooth muscle, as shown here, has not been reported. Oxidative activation of PKC\(\alpha\) leads to sustained cofactor-independent kinase activity.\(^{25,38}\) Sustained PKC activity is consistent with the observation that established high-activity L-type Ca\(^{2+}\) channel sparklet sites are abolished by PKC inhibition.\(^{17}\) Application of exogenous ROS did not produce a uniform translocation of PKC\(\alpha\) to the plasma membrane. Rather, accumulation of PKC\(\alpha\) at the plasma membrane was irregular with punctate elevations in PKC\(\alpha\)-associated fluorescence (Figure 3). Similarly, the increase in Ca\(^{2+}\) sparklet site density by exogenous ROS was far less than predicted if ROS exposure resulted in a generalized nonspecific translocation of PKC to the plasma membrane. Importantly, previous work in arterial smooth muscle cells has shown that the scaffold protein AKAP150 is necessary for punctate membrane localization of PKC\(\alpha\) and Ca\(^{2+}\) sparklet activity.\(^{19}\) In this context, our observation that ROS exposure produced spatially restricted PKC\(\alpha\)-dependent Ca\(^{2+}\) sparklet activity suggests that although ROS may induce PKC activation, additional components such as AKAP150 are necessary for efficient targeting of the kinase to the plasma membrane to stimulate localized L-type Ca\(^{2+}\) channel activity.

Our imaging of punctate sites of endogenous ROS generation in isolated cells with DCF indicates that stimulation of L-type Ca\(^{2+}\) channels by ROS would also be restricted by the localized nature of ROS production and not just by limited membrane targeting of PKC\(\alpha\) per se. However, PKC phosphorylation of p47phox is an important step in the initial activation of NADPH oxidase by Ang II.\(^{39}\) Thus, PKC regulation of L-type Ca\(^{2+}\) channels likely involves (at least) 2 events where PKC first stimulates the production of NADPH oxidase–derived ROS, which in turn oxidatively activates PKC\(\alpha\) resulting in enhanced Ca\(^{2+}\) sparklet activity. Our data do not address this hypothesis directly. However, during our ROS imaging experiments the external solution was free of Ca\(^{2+}\) and we incubated the cells with the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin. Thus, neither Ca\(^{2+}\) entry into the cell nor release from of Ca\(^{2+}\) from internal stores appears to be necessary for ROS generation in response to Ang II in isolated arterial myocytes.

In combination with supporting data, our sequential ROS/Ca\(^{2+}\) imaging experiments provide compelling evidence suggesting that endogenous ROS locally regulate L-type Ca\(^{2+}\) channel activity. Using this unique approach, we found that ROS production preceded and colocalized with sites of L-type Ca\(^{2+}\) channel sparklet activity (respective peaks <1 \(\mu\)m apart). It is possible that the molecular components necessary for ROS production and L-type Ca\(^{2+}\) channel activity share the same subcellular location but are independent and not functionally linked. However, this possibility is rendered unlikely by our data showing that: (1) exogenous ROS promote PKC\(\alpha\) activation and translocation to the plasma membrane; (2) exogenous ROS stimulate PKC\(\alpha\)-dependent Ca\(^{2+}\) sparklet activity; and (3) inhibition of endogenous ROS generation with apocynin abolished stimulation of Ca\(^{2+}\) sparklets by Ang II. Note that whether apocynin is acting as a bone fide inhibitor of NADPH oxidase or as an antioxidant\(^{40}\) does not contradict our overall hypothesis that localized generation of ROS stimulate L-type Ca\(^{2+}\) channel activity.

To conclude, in this study, we provide compelling evidence in support of the hypothesis that local changes in redox status are transduced to sustained Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (ie, high-activity Ca\(^{2+}\) sparklets). The implications and questions raised by our observations are intriguing. What molecular components are necessary for the formation of this novel signalosome? Are other signaling pathways subject to or influenced by similar local regulation...
by ROS? Does localized oxidative activation of L-type Ca\(^{2+}\) channels contribute to enhanced Ca\(^{2+}\) influx during hypertension? The data presented here may provide a mechanistic starting point for future efforts aimed at answering these important questions.

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**Disclosures**
None.

**References**


**Novelty and Significance**

**What Is Known?**

- Reactive oxygen species (ROS) are important signaling molecules in cardiovascular cells.
- Because of their reactive nature and as a mechanism conferring specificity, ROS production is thought to be localized for them to function effectively.
- Highly localized L-type calcium channel activity has been observed in arterial smooth muscle cells and shown to contribute to arterial contraction.

**What New Information Does This Article Contribute?**

- Exogenous ROS increase localized protein kinase C–dependent L-type calcium channel activity in isolated arterial smooth muscle cells and constrict intact arteries in an L-type calcium channel–dependent manner.
- Generation of endogenous ROS by NADPH oxidase is necessary for stimulation of L-type calcium channels and arterial contraction in response to the vasoconstrictor angiotensin II.
- Angiotensin II induces punctate sites of ROS generation that precede and colocalize with L-type calcium channel activity in isolated arterial smooth muscle cells.

ROS and calcium are essential components of arterial function under physiological and pathophysiological conditions. However, the relationship between these 2 signaling modalities is unclear. In this study, we investigated the functional and spatial relationship between ROS and L-type calcium channels, a major source of cytoplasmic calcium in arterial smooth muscle. We found that ROS stimulated local sites of L-type calcium channel activity in isolated arterial smooth muscle cells and induced contraction of intact arterial segments. Using a novel imaging-based approach, we visualized punctate sites of endogenous ROS formation in isolated arterial smooth muscle cells. We also show, for the first time, that the spatial distribution of these ROS puncta overlaps with that of local L-type calcium channel activity. These observations indicate that discrete sites of ROS generation are functionally and spatially coupled to local calcium influx through single L-type calcium channels in arterial smooth muscle cells. ROS are widely implicated in the pathogenesis of hypertension. Because the relationship between ROS and L-type calcium channels results in increased channel function, our findings should provide mechanistic insight into events underlying increased calcium influx during hypertension and lead to the development of new rational therapies for managing and preventing disease.
Local Regulation of Arterial L-Type Calcium Channels by Reactive Oxygen Species
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Detailed Methods

Isolation of cerebral arterial myocytes

Male Sprague-Dawley rats (250 to 350 g; Harlan, Indianapolis, IN) were euthanized with sodium pentobarbital (200 mg/kg intraperitoneally; Fort Dodge Animal Health, Fort Dodge, IA) in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of Colorado State University. Smooth muscle cells were prepared from basilar and cerebral arteries. Arteries were removed, cleaned of connective tissue, and placed in ice-cold Ca2+-free buffer containing (in mmol/L): 140 NaCl, 5 KCl, 2 MgCl₂, 10 glucose, and 10 HEPES (adjusted to pH 7.4 with NaOH). Arterial segments were incubated for 15 minutes at 37°C in Ca2+-free buffer supplemented with papain (10 U/mL; Worthington Biochemical, Lakewood, NJ) and dithiothreitol (1 mg/mL) followed by a second incubation (15 minutes at 37°C) in Ca2+-free buffer supplemented with Sigma Blend collagenases Types F and H (0.5 mg/mL each). Arteries were then washed with and placed in unsupplemented Ca2+-free buffer and kept on ice for 30 minutes after which gentle trituration with a fire-polished Pasteur pipette was used to create a cell suspension. Cells were used within 6 hours of dispersion.

Intact arterial diameter measurements

Cerebral arteries for intact tissue experiments were isolated and stored in ice-cold MOPS buffer containing (in mmol/L): 145 NaCl, 5 KCl, 1 MgSO₄, 2.5 CaCl₂, 1 KH₂PO₄, 0.02 EDTA, 2 pyruvate, 5 glucose, 1% bovine serum albumin, and 3 MOPS (adjusted to pH 7.4 with NaOH). Arteries were cleaned of connective tissue and transferred to a vessel chamber (Living Systems, Burlington, VT). One end of the artery was cannulated with a glass micropipette and secured with monofilament thread. Luminal contents were gently rinsed and the other end was cannulated and secured. Arteries were pressurized to 10 mmHg with a bicarbonate-based physiological saline solution (B-PSS) containing (in mmol/L): 119 NaCl, 4.7 KCl, 1.8 CaCl₂, 1.2 MgSO₄, 24 NaHCO₃, 0.2 KH₂PO₄, 10.6 glucose, 1.1 EDTA, and superfused (5 mL/min) with warmed B-PSS (37°C) aerated with a normoxic gas mixture (21% O₂, 6% CO₂, balance N₂). To block the effects of endothelial-derived nitric oxide, the nitric oxide synthase inhibitor NG-nitro-L-arginine (300 µmol/L) was included in all B-PSS superfusates.

Following a 15-minute equilibration period, intraluminal pressure was increased to 110 mmHg, the arteries were stretched to remove bends, and the pressure was reduced to 10 mmHg for an additional 15-minute equilibration period. Intravascular pressure was increased to 80 mmHg and inner diameter was continuously monitored using video microscopy and edge-detection software (Ionoptix, Milton, MA). To assess viability of the preparation, arteries were exposed to isotonic B-PSS containing 60 mmol/L KCl. Experiments began after a stable level of myogenic tone was obtained at 80 mmHg and were terminated by superfusing with Ca²⁺-free B-PSS to obtain the passive diameter of the artery. Xanthine oxidase (XO; 0.2 mU/mL) and hypoxanthine (HX; 250 µmol/L) were added to warm B-PSS (37°C) at the onset of experimentation to minimize excessive accumulation of reactive oxygen species. The L-type Ca²⁺ channel blocker diltiazem (10 µmol/L) was included in some experiments. Arterial tone was calculated as the percentage difference in active luminal diameter versus passive luminal diameter. For our experiments with Ang II (1 nmol/L), apocynin (25 µmol/L), and Gö6976 (100 nmol/L), in addition to NG-nitro-L-arginine (300 µmol/L), we included the AT2 receptor antagonist PD 123319 (1 µmol/L) to preclude endothelial AT2 receptor responses.
**Electrophysiology**

We used the conventional whole-cell patch-clamp technique to control isolated arterial myocyte membrane potential using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Freshly prepared cerebral arterial myocyte suspensions (in Ca²⁺-free buffer) were pipetted into a recording chamber and the cells allowed to adhere to the glass cover slip for 20 minutes at room temperature. During experiments, cells were superfused with a solution containing (in mmol/L): 120 NMDG⁺, 5 CsCl, 1 MgCl₂, 10, glucose, 10 HEPES, and 20 CaCl₂ adjusted to pH 7.4 with HCl. Pipettes were filled with a solution composed of (in mmol/L): 87 Cs-aspartate, 20 CsCl, 1 MgCl₂, 5 MgATP, 0.1 Na₂GTP, 1 NADPH, 10 HEPES, 10 EGTA, and 0.2 fluo-5F adjusted to pH 7.2 with CsOH. Macroscopic Ca²⁺ currents at a test potential of +30 mV from holding potential of -70 mV were acquired at 10 kHz and filtered at 1 kHz. All electrophysiological experiments were performed at room temperature (22 - 25°C).

**Total internal reflection fluorescence (TIRF) microscopy**

L-type Ca²⁺ channel sparklets were recorded with a TILL Photonics (Victor, NY) through-the-lens TIRF system built around an inverted Olympus IX-71 (Center Valley, PA) microscope equipped with Olympus 60X (numerical aperture = 1.49) and 100X (numerical aperture = 1.45) TIRF oil-immersion objectives and an Andor iXON EMCCD camera (Andor Technology, South Windsor, CT). Pixel sizes were 0.26 µm (60 X) and 0.16 µm (100X). To monitor Ca²⁺ influx, myocytes were loaded with the Ca²⁺ indicator fluo-5F (200 µmol/L; pentapotassium salt; Invitrogen, Carlsbad, CA) and an excess of the non-fluorescent Ca²⁺ buffer EGTA (10 mmol/L) via dialysis through the patch pipette as previously described 1, 2. L-type Ca²⁺ channel sparklets, but not macroscopic Ca²⁺ currents as above, were performed in the presence of the Ca²⁺-ATPase inhibitor thapsigargin (1 µmol/L). Excitation of fluo-5F was achieved with a 491 nm laser and excitation and emission light was separated with appropriate filters. Images were acquired at 50 - 100 Hz. Ca²⁺ sparklets were visualized and recorded at a holding potential of -70 mV with elevated external [Ca²⁺] (20 mmol/L) to facilitate the detection of Ca²⁺ sparklet events and provide fluorescent signals of sufficient amplitude to permit quantal analysis of Ca²⁺ sparklet activity.

XO (2 mU/mL) and HX (250 µmol/L) were combined at the onset of experimentation. Note that we used a greater amount of XO (2 mU/mL) in our electrophysiological experiments performed at room temperature (≈ 22°C) than in our intact artery experiments (0.2 mU/mL) performed at 37°C to compensate for differences in solution transit time and temperature-dependent effects on XO activity 3. Drug treatments (XO/HX and angiotensin II) in the presence or absence of inhibitors (Gö6976, apocynin, or ZD7155) were allowed to progress between 5 and 10 minutes. Only recordings with stable GΩ seals were analyzed.

**L-type Ca²⁺ channel sparklet analysis**

Background-subtracted fluo-5F fluorescence signals were converted to [Ca²⁺] as previously reported 1, 4 using the equation

\[
[Ca^{2+}] = K_d \frac{F/F_{max} - 1/R_f}{1 - F/F_{max}}
\]

where \(F\) is fluorescence, \(F_{max}\) is the fluorescence intensity of fluo-5F in the presence of saturating free Ca²⁺, \(F_{min}\) is the fluorescence intensity of fluo-5F in a solution where [Ca²⁺] is 0, \(K_d\) is the dissociation constant of fluo-5F (1280 nmol/L), and \(R_f\) is the \(F_{max}/F_{min}\) of fluo-5F (286). \(K_d\) and \(R_f\) values for fluo-5F were determined in vitro and are similar to those reported previously 5. \(F_{max}\) was determined at the end of each experiment by exposing cells to a solution containing ionomycin (10 µmol/L) and 20 mM Ca²⁺.
sparklets were detected and defined for analysis using a custom automated algorithm\textsuperscript{1} written in Interactive Data Language (IDL; ITT Visual Information Solutions, Boulder, CO). Ca\textsuperscript{2+} sparklets were defined as having a minimal amplitude equal to or larger than the mean basal [Ca\textsuperscript{2+}]\textsubscript{i} plus three times its standard deviation (\(\approx 18 \text{ nmol/L under our experimental conditions}\)). For an elevation in [Ca\textsuperscript{2+}], to be considered a Ca\textsuperscript{2+} sparklet, a grid of 3 x 3 contiguous pixels had to have a [Ca\textsuperscript{2+}] value at or above the amplitude threshold and the location of the highest-amplitude pixel within this grid was defined as the location (peak) of the Ca\textsuperscript{2+} sparklet site.

We performed a quantal analysis of Ca\textsuperscript{2+} sparklet activity\textsuperscript{1,2} using histograms generated from the amplitudes of individual Ca\textsuperscript{2+} sparklet events. The resulting histograms were fitted with the multicomponent Gaussian function

\[
N = \sum_{j=1}^{n} a_j \exp \left[ -\frac{(\text{[Ca}^{2+}\text{]} - jq)^2}{2jb} \right]
\]

where \(a\) and \(b\) are constants, [Ca\textsuperscript{2+}]\textsubscript{i} is intracellular Ca\textsuperscript{2+}, and \(q\) is the quantal unit of Ca\textsuperscript{2+} influx.

Ca\textsuperscript{2+} sparklet activity was determined\textsuperscript{1,2} by calculating the \(nP_s\) of each sparklet site, where \(n\) is the number of quantal levels detected, and \(P_s\) is the probability that a given Ca\textsuperscript{2+} sparklet site is active. \(nP_s\) values were obtained using the single channel analysis module of pCLAMP 10.0 (Molecular Devices, Sunnyvale, CA) on imported [Ca\textsuperscript{2+}]\textsubscript{i} time course records from established sparklet sites. Ca\textsuperscript{2+} sparklet events were detected with no duration constraints and an initial unitary [Ca\textsuperscript{2+}] elevation of 38 nmol/L\textsuperscript{2}. Detected events were then cross-referenced with the original TIRF image stack to verify that they met the amplitude and spatial criteria for Ca\textsuperscript{2+} sparklets described above. Consistent with previous reports\textsuperscript{1,2}, Ca\textsuperscript{2+} sparklet activity was bimodally distributed with sites of low activity (\(nP_s\) between 0 and 0.2) and high activity (\(nP_s\) greater than 0.2). Ca\textsuperscript{2+} sparklet densities (Ca\textsuperscript{2+} sparklet sites per \(\mu m^2\)) were calculated by dividing the number of Ca\textsuperscript{2+} sparklet sites detected by the area of cell membrane visible in the TIRF images.

**Detection of localized reactive oxygen species generation**

TIRF microscopy was also to visualize subplasmalemmal generation of reactive oxygen species (ROS) in isolated arterial myocytes. Cells were loaded in Ca\textsuperscript{2+}-free buffer supplemented with the cell-permeant ROS indicator 5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester (DCF; 10 \(\mu mol/L\); Invitrogen, Carlsbad, CA) for 20 minutes at room temperature. Following removal of excess DCF with Ca\textsuperscript{2+}-free buffer, excitation of subplasmalemmal intracellular DCF was achieved with a 491 nm laser and excitation and emission light was separated with appropriate filters. Similar to Ca\textsuperscript{2+} sparklet sites\textsuperscript{2}, for an area of elevated DCF fluorescence to be considered a site of increased ROS generation, a grid of 3 x 3 contiguous pixels had to have a fluorescent amplitude equal to or larger than the mean basal DCF fluorescence plus three times its standard deviation and the location of the highest-amplitude pixel within this grid was defined as the location (peak) of the site. ROS puncta densities (ROS puncta per \(\mu m^2\)) were calculated by dividing the number of sites detected by the area of cell membrane visible in the TIRF images.
Sequential imaging of reactive oxygen species generation and Ca\(^{2+}\) sparklet activity

To examine the spatial distribution of ROS generation and Ca\(^{2+}\) sparklet activity in isolated arterial myocytes, we used TIRF microscopy to sequentially image DCF (i.e., ROS) and fluo-5F (i.e., Ca\(^{2+}\)) fluorescence. First, we loaded arterial myocytes with the cell-permeant ROS indicator DCF in Ca\(^{2+}\)-free buffer for 20 minutes as described above. Following washout of excess DCF with Ca\(^{2+}\)-free buffer, a patch pipette was lowered onto a candidate myocyte, a GΩ seal was established (cell-attached configuration), and angiotensin II (Ang II; 100 nmol/L) was applied to the external solution to stimulate ROS generation. Changes in DCF fluorescence were then monitored with 491 nm laser excitation. Upon visual confirmation of punctate elevations in DCF fluorescence (see Figure 7A, image 1), negative pressure was applied to the patch pipette to rupture the plasma membrane and dialyze the cell with the Ca\(^{2+}\) indicator fluo-5F (whole-cell configuration). We then superfused the cell with the same external solution containing 20 mmol/L Ca\(^{2+}\) (with no Ang II) used to record Ca\(^{2+}\) sparklets described above and monitored for Ca\(^{2+}\) sparklet activity (also with 491 nm laser excitation; see Figure 7A, image 2). Note that the markedly dissimilar kinetics of DCF fluorescence (Figure 7A, time course i at right) and fluo-5F fluorescence (Figure 7A, time course ii at right) demonstrate sufficient dilution of intracellular DCF during dialysis with fluo-5F to permit sequential usage of these two spectrally overlapping indicators under these experimental conditions.

ROS/ Ca\(^{2+}\) sparklet colocalization analysis

The average distance between the peaks (pre-defined as the pixels of highest intensity) of ROS puncta and Ca\(^{2+}\) sparklet sites was 0.91 ± 0.24 µm (n = 6 sites from 5 cells). The probability, by chance alone, that we would observe 6 ROS puncta and 6 Ca\(^{2+}\) sparklet sites approximately 1 µm apart is less than 1 in 1,000,000. We reached this value with the following approach: On average, the cell area imaged in our ROS/Ca\(^{2+}\) sparklet experiments was approximately 100 µm\(^2\). If this area is assumed to be a grid of 1 µm x 1 µm pixels then the chance of complete overlap between the peak ROS and Ca\(^{2+}\) sparklet sites (i.e., they occur at the same pixel) would be 1 in 100. However, we found that the measured peaks between these two events were approximately 1 µm apart. Thus, we need to include the 8 pixels surrounding the ROS peak pixel in our estimation (now 9 pixels out of 100). Rounding up to 10 total pixels for convenience indicates that for each experiment the chance of observing a ROS puncta peak within 1 µm of a Ca\(^{2+}\) sparklet site peak would be 10 per 100 pixels imaged (or 1:10). We found that Ca\(^{2+}\) sparklet peaks were on average 1 µm away from ROS peaks (less than a 1:10 probability) in 6 independent recordings. Thus, the probability of such an observation by chance alone is at a minimum 0.1 to the sixth power, or \(P < 0.000001\).

Immunohistochemistry

For our immunofluorescence studies, we used a mouse monoclonal PKCα antibody (Abcam, Cambridge, MA) with an Alexa Fluor 488-conjugated rabbit anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) as used previously \(^6,7\). Freshly prepared cerebral arterial myocyte suspensions were pipetted onto cover slips and the cells allowed to adhere for 20 minutes at room temperature. The adhered cells were then subjected in parallel to 20 minutes of either control or experimental conditions at room temperature in the same Ca\(^{2+}\)-free buffer used for cell isolation. Cells were then fixed in 4 % formaldehyde for 10 minutes at room temperature, rinsed three times with phosphate buffered saline (PBS), and permeabilized with cooled (-80 °C) methanol for 10 minutes. Three methanol washes were applied with the final wash being allowed to evaporate. The fixed cells were then placed in blocking buffer (2% BSA in PBS) for 20 minutes and incubated overnight at 4°C with the PKCα (1:250) primary antibody. The next day cells were washed three times with blocking buffer and probed with secondary antibody [1:250] for 45 minutes at room temperature. Following three final washes with blocking buffer, the cover slips were mounted onto slides using UltraCruz™ mounting medium (Santa Cruz Biotechnology, Santa Cruz, CA). Images were...
obtained on a Zeiss LSM 510 Meta confocal microscope (Thornwood, NY). Excitation of the Alexa Fluor 488-conjugated secondary antibody was achieved with a 488 nm laser and excitation and emission light was separated with appropriate filters. Fluorescence was undetectable in control experiments where either the primary or the secondary antibody was omitted (data not shown). Membrane and cytosolic PKCα-associated fluorescence was quantified by measuring the intensity of pixels above a set threshold defined as the mean fluorescence intensity outside the cells (i.e., background) plus three times its standard deviation. For each cell analyzed, pixel intensities of the plasma membrane (≈ 1 µm in width) and a spatially equivalent section of cytosol were obtained and averaged. From these measurements, we determined the ratio of plasma membrane to cytosolic PKCα-associated fluorescence and used this as an indicator of PKCα translocation and activity as previously reported. Two-dimensional PKCα-associated fluorescence images were converted to three-dimensional surface plots with ImageJ (version 1.42q; National Institutes of Health, USA).

**Chemicals and statistics**

Gö6976 and the PKC inhibitory peptide (PKCi) were from EMD (Gibbstown, NJ), ZD7155 and PD123319 were from Tocris Bioscience (Ellisville, MO); all other chemicals were from Sigma (St. Louis, MO) unless stated otherwise. Normally distributed data are presented as the mean ± standard error of the mean (SEM). Two-sample comparisons of these data were performed using either a paired or unpaired (as appropriate) two-tailed Student’s t test and comparisons between more than two groups were performed using a repeated measures ANOVA with Newman-Keuls multiple comparison post-test. Ca2+ sparklet activity (i.e. nP50) datasets were bimodally distributed, thus two-sample comparisons of nP50 data were performed using the non-parametric Wilcoxon matched pairs test (two-tailed) and comparisons between more than two groups were performed using the non-parametric Friedman test with Dunn’s multiple comparison post-test. Arithmetic means of nP50 datasets are indicated in the figures (solid red horizontal lines) for non-statistical visual purposes and dashed lines mark the threshold for high-activity Ca2+ sparklet sites (nP50 ≥ 0.2). P values less than 0.05 were considered significant and asterisks (*) used in the figures indicate a significant difference between groups.

**Supplemental References**


Online Figure I. Reactive oxygen species increase PKC-dependent macroscopic L-type Ca\(^{2+}\) currents in isolated cerebral arterial smooth muscle cells. A, Representative macroscopic L-type currents in response to a step depolarization to +30 mV from a holding potential of -70 mV before and after XO plus HX (2mU/mL and 250 µmol/L, respectively) under control conditions (top) and in the presence of a PKC inhibitory peptide (PKCi; 100 µmol/L in the patch pipette; bottom). B, Plot of the mean ± SEM macroscopic L-type Ca\(^{2+}\) current densities (pA / pF) before and after XO/HX under control conditions and in the presence of PKCi (n = 6 cells). C, Representative macroscopic L-type currents before and after Ang II (100 nmol/L) under control conditions (top) and in the presence of the NADPH oxidase inhibitor apocynin (25 µmol/L; bottom). D, Plot of the mean ± SEM macroscopic L-type Ca\(^{2+}\) current densities (pA / pF) before and after Ang II under control conditions and in the presence of apocynin (n = 5 cells). \(* P < 0.05\)