Hydrogen peroxide mediates oxidant-dependent stimulation of arterial smooth muscle L-type calcium channels

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Chaplin NL, Amberg GC. Hydrogen peroxide mediates oxidant-dependent stimulation of arterial smooth muscle L-type calcium channels. Am J Physiol Cell Physiol 302: C1382–C1393, 2012. First published February 8, 2012; doi:10.1152/ajpcell.00222.2011.—Changes in calcium and redox homeostasis influence multiple cellular processes. Dysregulation of these signaling modalities is associated with pathology in cardiovascular, neuronal, endocrine, and other physiological systems. Calcium and oxidant signaling mechanisms are frequently inferred to be functionally related. To address and clarify this clinically relevant issue in the vasculature we tested the hypothesis that the ubiquitous reactive oxygen molecule hydrogen peroxide mediates oxidant-dependent stimulation of cerebral arterial smooth muscle L-type calcium channels. Using a combinatorial approach including intact arterial manipulations, electrophysiology, and total internal reflection fluorescence imaging, we found that application of physiological levels of hydrogen peroxide to isolated arterial smooth muscle cells increased localized calcium influx through L-type calcium channels. Similarly, oxidant-dependent stimulation of L-type calcium channels by the vasoconstrictor ANG II was abolished by intracellular application of catalase. Catalase also prevented ANG II from increasing localized subplasmalemmal sites of increased oxidation previously associated with localized calcium influx through L-type channels. Furthermore, catalase largely attenuated the contractile response of intact cerebral arterial segments to ANG II. In contrast, enhanced dismutation of superoxide to hydrogen peroxide with SOD had no effect on ANG II-dependent stimulation of L-type calcium channels. From these data we conclude that hydrogen peroxide (H₂O₂) had no effect on ROS puncta formation or ANG II-dependent stimulation of L-type Ca²⁺ channels. Consistent with this, concomitant increases in Ca²⁺ influx through L-type Ca²⁺ channels (i.e., Ca²⁺ sparklets). Furthermore, the vasoconstrictor ANG II induced punctate generation of subplasmalemmal ROS that preceded and colocalized with subsequent L-type Ca²⁺ channel activity. While these observations demonstrate that ROS promote L-type Ca²⁺ channel function and that the spatial distribution of local ROS generation mirrors that of local Ca²⁺ influx through L-type Ca²⁺ channels, the identity of the participating ROS linking these Ca²⁺ and redox signaling events is unclear.

Hydrogen peroxide (H₂O₂) is an important ROS signaling molecule (7, 24, 42). H₂O₂-dependent modulation of protein function is thought to occur by oxidation of key amino acids (e.g., cysteine) that operate as allosteric redox-dependent switches. As with most cell types, major sources of H₂O₂ in arterial smooth muscle cells include NADPH oxidase (Nox) enzyme complexes and the mitochondrial electron transport chain. Previous work demonstrated that H₂O₂ regulates macroscopic recombinant L-type Ca²⁺ currents (11). However, the role of H₂O₂ in oxidative stimulation of localized arterial smooth muscle L-type Ca²⁺ channels has not been investigated.

In this study, we tested the hypothesis that H₂O₂ mediates oxidant-dependent stimulation of L-type Ca²⁺ channels in rat cerebral arterial smooth muscle cells. We found that exogenous H₂O₂ increased not only macroscopic L-type Ca²⁺ channel currents but, more importantly, H₂O₂-dependent increases in L-type Ca²⁺ channel function were localized. Conversely, removal of endogenous H₂O₂ with catalase abolished ANG II-dependent stimulation of L-type Ca²⁺ channels. Consistent with these observations, catalase also prevented increases in punctate subplasmalemmal ROS generation following ANG II exposure and attenuated ANG II-dependent constriction of pressurized cerebral arterial segments. In contrast to removal of H₂O₂ with catalase, enhanced dismutation of superoxide (to H₂O₂) had no effect on ROS puncta formation or ANG II-dependent stimulation of L-type Ca²⁺ channels. Taken together, these data provide compelling evidence that in rat cerebral arterial smooth muscle cells, physiologically relevant oxidant-dependent stimulation of L-type Ca²⁺ channels is mediated by locally produced H₂O₂.

MATERIALS AND METHODS

Isolation of rat cerebral arterial myocytes. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) were euthanized with pentobarbital sodium (200 mg/kg ip; 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. Isolated smooth muscle cells were prepared from basilar and cerebral arteries. Arteries were removed, cleaned, and placed in ice-cold Ca²⁺-free buffer containing (in mM): 140 NaCl, 5

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KCl, 2 MgCl2, 10 glucose, and 10 HEPES (adjusted to pH 7.4 with NaOH). Arteries were incubated for 15 min 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 min at 37°C) in Ca2+-free buffer supplemented with collagenase (300 U/ml; Type II, Worthington Biochemical). Arteries were then washed with and placed in Ca2+-free buffer and kept on ice for 30 min after which trituration with a fire-polished Pasteur pipette was used to create a cell suspension; cells were used within 6 h of dispersion.

Electrophysiology. Freshly prepared smooth muscle cell suspensions were pipetted into a glass-bottomed recording chamber, and the cells were allowed to adhere for 20 min. Membrane potential was controlled with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The perforated whole cell patch-clamp technique was used to record macroscopic L-type Ca2+ channel currents with barium (Ba2+) as the charge carrier. For these experiments, the amphotericin B (250 µg/ml) supplemented pipette solution contained (in mM): 120 CsCl, 20 TEA-Cl, 1 EGTA, and 20 HEPS (adjusted to pH 7.4 with CsOHa) and cells were superfused with an external solution composed of (in mM): 115 NaCl, 10 TEA-Cl, 0.5 MgCl2, 5.5 glucose, 5 CsCl, 20 BaCl2, and 20 HEPS (adjusted to pH 7.4 with CsOH). The mean capacitance of the cells used for macroscopic L-type Ca2+ channel current experiments was 18.2 ± 0.9 pF (n = 12 cells). For our Ca2+ imaging experiments, we used the conventional dialyzed whole cell patch-clamp technique. During these experiments, cells were superfused with a solution containing (in mM): 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 mM): 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). All electrophysiological experiments were performed at room temperature (22–25°C) and were allowed to progress between 5 and 10 min. Only recordings with stable GΩ seals were analyzed.

TIRF microscopy. Ca2+ influx through L-type channels was visualized with a TILL Photonics (Victor, NY) through-the-lens TIRF microscopy using a TILL Photonics (Victor, NY) through-the-lens TIRF microscopy and an Andor iXON EMCCD camera (Andor Technology, South Windsor, CT). To monitor Ca2+ influx, myocytes were loaded with the Ca2+ indicator fluo-5F (200 µM; pentapotassium salt; Invitrogen, Carlsbad, CA) and an excess of EGTA (10 mM) via the patch pipette. To preclude potential contaminating Ca2+ release events from the sarcoplasmic reticulum, the Ca2+-ATPase inhibitor thapsigargin (1 µM) was present during all experiments. Excitation of fluo-5F was achieved with a 491-nm laser and excitation, and emission light was separated with appropriate filters. Ca2+ influx was recorded at 50 Hz at a holding potential of −70 mV and elevated external [Ca2+] (20 mM) to facilitate the detection of events and provide fluorescent signals of sufficient amplitude (25) to permit quantal analysis.

L-type Ca2+ channel sparklet analysis. Background-subtracted fluo-5F fluorescence signals were detected and quantified by calculating the F/Fmax where F is fluorescence, Fmax is the fluorescence intensity of fluo-5F in a solution where [Ca2+] is 0, KD is the dissociation constant of fluo-5F, and Fmax values were determined in vitro and Fmax was determined at the conclusion of each experiment with ionomycin (10 µM). Fluo-5F fluorescence images were analyzed with custom software (kindly supplied by Dr. L. Fernando Santana) (25).

For an elevation in [Ca2+]i, to be considered an L-type Ca2+ channel sparklet event, a grid of 3 × 3 contiguous pixels had to have a [Ca2+]i amplitude equal or larger than the mean basal [Ca2+]i, plus three times its standard deviation.

Quantal analysis of L-type Ca2+ channel sparklet activity (2, 25, 26) was performed on histograms generated from individual event amplitudes. The resulting histograms were fitted with the multicomponent Gaussian function

\[ N = \sum_{i=1}^{n} \alpha_i \exp \left( -\frac{(C_{Ca^{2+}} - j_{Ca^{2+}} i)^2}{2 \beta^2} \right) \]

where C and j are constants, [Ca2+]i is intracellular Ca2+, and q is the quantal unit of Ca2+ influx.

L-type Ca2+ channel sparklet activity was determined (2, 25, 26) by calculating the nP, of each site, where n is the number of quantal levels detected, and P, is the probability that the site is active. nP, values were obtained using pCLAMP 10.0 (Molecular Devices, Sunnyvale, CA) on imported [Ca2+]i time course records. L-type Ca2+ channel sparklet activity was quantified using an initial unitary [Ca2+]i elevation of 38 nM as determined experimentally (26). Consistent with previous reports (2, 3, 25, 26), L-type Ca2+ channel sparklet activity was bimodally distributed with sites of low activity (nP, between 0 and 0.2) and high activity (nP, > 0.2). Active L-type Ca2+ channel densities (Ca2+ sparklet sites per µm2) were calculated by dividing the number of active sites by the area of cell membrane visible in the TIRF images.

Detection of ROS generation. TIRF microscopy was also used to visualize subplasmalemmal ROS generation as described previously by us (2). Cells were loaded in Ca2+-free buffer supplemented with the cell-permeant ROS indicator 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate ester (DCF; 10 µM; Invitrogen, Carlsbad, CA) for 20 min at room temperature. Following removal of excess DCF with un-supplemented Ca2+-free buffer, excitation of subplasmalemmal DCF was achieved with a 491-nm laser and excitation and emission light was separated with appropriate filters. Analogous to L-type Ca2+ channel influx, for an area of elevated DCF fluorescence to be considered a localized site of increased ROS generation (an ROS puncta), a grid of 3 × 3 contiguous pixels had to have a fluorescence amplitude equal to or larger than the mean basal DCF fluorescence plus three times its standard deviation (26). ROS puncta densities (ROS puncta per µm2) were calculated by dividing the number of sites detected by the area of cell membrane visible in the TIRF images. Changes in DCF fluorescence (ΔDCF) were calculated from the mean pixel intensities of the total intracellular submembranous slice visible in the TIRF images (average ΔDCF) and the areas confined to nascent ROS puncta (puncta ΔDCF).

Intact arterial diameter measurements. Middle cerebral arteries for intact tissue experiments were isolated and stored in ice-cold MOPS buffer containing (in mM): 145 NaCl, 5 KCl, 1 MgSO4, 2.5 CaCl2, 1 KH2PO4, 0.02 EDTA, 2 pyruvate, 5 glucose, 1% BSA, 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 onto a glass micropipette and secured with monofilament suture. Luminal contents were rinsed, and the other end was cannulated onto an opposing micropipette and secured. Arteries were pressurized to 20 mmHg with a bicarbonate-based physiological saline solution (B-PSS) containing (in mM): 119 NaCl, 4.7 KCl, 1.8 CaCl2, 1.2 MgSO4, 24 NaHCO3, 0.2 KH2PO4, 10.6 glucose, and superfused (3 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 µM) was included in the B-PSS superfusate.

Following a 15-min equilibration period, intraluminal pressure was increased to 60 mmHg, arteries were stretched appropriately to remove bends, and the pressure was lowered back to 20 mmHg for a
second 15-min equilibration period. Intravascular pressure was then again increased to 60 mmHg and the inner diameter continuously monitored using video microscopy and edge detection software (Ion-optix, Milton, MA). To assess viability of the preparation, all arteries were exposed to isotonic B-PS containing 60 mM KCl. Experiments with ANG II (10 nM) and/or polyethylene glycol (PEG)-catalase (500 U/ml) were initiated after a stable level of tone was obtained at 60 mmHg and were terminated by superfusing with Ca\(^{2+}\)-free B-PS supplemented with the L-type Ca\(^{2+}\) blocker diltiazem (10 μM) to obtain the passive diameter of the artery. Arterial tone (% constriction) was calculated as the percentage difference in active luminal diameter versus passive luminal diameter.

Passive arterial diameters were not different for experiments with ANG II alone (180.6 ± 11.5 μm, n = 5 arteries) and for ANG II plus PEG-catalase (193.3 ± 20.1 μm, n = 4 arteries; P > 0.05). Similarly, myogenic tone at 60 mmHg under control conditions (i.e., with only N\(^{\text{G}}\)-nitro-L-arginine present) was also similar for experiments with ANG II alone (39.2 ± 3.0%, n = 5 arteries) and for ANG II plus PEG-catalase (32.2 ± 6.8%, n = 4 arteries; P > 0.05). A small increase in arterial diameter was observed following PEG-catalase exposure, 7.7 ± 0.75 μm which corresponded to a 8.5 ± 3.8% loss of tone, but this did not approach statistical significance (P = 0.41, n = 4 arteries).

Chemicals and statistics. Manganese tetrakis (4-N-methylpyridyl) porphyrin (MnTMPyP) and tempol were from EMD (Gibbstown, NJ); all other chemicals were from Sigma (St. Louis, MO) unless stated otherwise. Normally distributed data are presented as means ± SE. 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 one-way ANOVA with Tukey’s multiple comparison posttest. L-type Ca\(^{2+}\) channel sparklet activity (i.e., n\(P_s\)) datasets were bimodally distributed, thus two-sample comparisons of n\(P_s\) data were examined with the nonparametric Wilcoxon matched pairs test (two-tailed) and comparisons between more than two groups were performed using the nonparametric Friedman test with Dunn’s multiple comparison posttest. Arithmetic means of n\(P_s\) datasets are indicated in the figures (solid grey horizontal lines) for nonstatistical visual purposes, and dashed grey lines mark the threshold for high-activity Ca\(^{2+}\) sparklet sites (n\(P_s\) ≥ 0.2) (2, 25, 26). P values < 0.05 were considered significant and asterisks (*) used in the figures indicate a significant difference between groups.

RESULTS

To test our hypothesis that H\(_2\)O\(_2\) mediates oxidative stimulation of L-type Ca\(^{2+}\) channels we proposed four experimental criteria: 1) application of exogenous H\(_2\)O\(_2\) should increase L-type Ca\(^{2+}\) channel activity, 2) lowering endogenous H\(_2\)O\(_2\) should reduce L-type Ca\(^{2+}\) channel activity; 3) lowering endogenous H\(_2\)O\(_2\) should also limit arterial constriction in response to oxidative stimuli, and 4) manipulation of other candidate ROS, such as superoxide, should minimally effect L-type Ca\(^{2+}\) channel function.

Catalase attenuates contraction of intact rat cerebral arteries in response to ANG II. We previously reported that ROS generation by ANG II, a known activator of arterial NADPH oxidase complexes (16, 39), constricted rat cerebral arteries in an L-type Ca\(^{2+}\) channel-dependent manner (2). To continue this line of investigation and establish the importance of H\(_2\)O\(_2\) as a participating ROS we tested the effect of catalase on pressurized (60 mmHg) middle cerebral arterial responses to ANG II. Similar to previous reports (2, 40, 43), ANG II (10 nM) produced a moderate constriction (19.3 ± 4.0 μm, n = 5 arteries) of rat cerebral arterial segments (Fig. 1A). However, in the presence of cell-permeable PEG-catalase (500 U/ml), the contractile response to ANG II (3.5 ± 2.2 μm) was nearly abolished (Fig. 1, B and C; P < 0.05, n = 4 arteries). These data are consistent with our hypothesis that H\(_2\)O\(_2\) is involved in oxidative-dependent contraction of arterial smooth muscle (e.g., stimulation of L-type Ca\(^{2+}\) channels).

Exogenous H\(_2\)O\(_2\) increases macroscopic L-type Ca\(^{2+}\) channel currents in isolated cerebral arterial smooth muscle cells. To investigate whether stimulation of L-type Ca\(^{2+}\) channels could contribute to the catalase-sensitive component of ANG II-induced arterial constriction we applied a physiologically relevant concentration of H\(_2\)O\(_2\) (100 μM) (35, 36) to isolated arterial myocytes. We found that exogenous H\(_2\)O\(_2\) increased macroscopic L-type Ca\(^{2+}\) channel currents obtained with the amphotericin B perforated patch technique using Ba\(^{2+}\) as the charge carrier. We arrived at this conclusion with two voltage-clamp protocols. First, we tested the effect of H\(_2\)O\(_2\) on macroscopic L-type Ca\(^{2+}\) channel currents in response to step depolarizations to 0 mV from a holding potential of −70 mV. As shown in Fig. 2, A and B, exogenous H\(_2\)O\(_2\) increased diltiazem-sensitive macroscopic L-type Ca\(^{2+}\) channel currents (P < 0.05, n = 5 cells). Second, to confirm and illustrate the voltage-dependency of the H\(_2\)O\(_2\)-induced current, we recorded diltiazem-sensitive macroscopic Ba\(^{2+}\) currents during ramp depolarizations from −70 to +40 mV before and after application of H\(_2\)O\(_2\) (see Fig. 2, C–E). As with our recordings obtained during step depolarizations, diltiazem-sensitive currents evoked during continuous ramp depolarizations were...
Hydrogen peroxide (H₂O₂) increases macroscopic L-type Ca²⁺ channel currents in isolated rat cerebral arterial smooth muscle cells. A: representative L-type Ca²⁺ channel currents with barium (Ba²⁺) traces during step depolarizations to 0 mV from a holding potential of −70 mV before (left) and after application of H₂O₂ (100 μM; middle) and H₂O₂ + diltiazem (10 μM; right). B: plot of the mean ± SE peak Ba²⁺ current densities before and after H₂O₂ and H₂O₂ + diltiazem (n = 5 cells). C: representative diltiazem-sensitive (10 μM) currents evoked during voltage ramps from −70 to +40 mV with a depolarization rate of 220 mV/s before (left) and after application of H₂O₂ (100 μM; right); the dotted grey line marks the zero current level. D: plot showing the amplitude (pA) of a representative H₂O₂-induced current as a function of voltage (mV) during a ramp depolarization as described in C. E: plot of the mean ± SE net area under the curves associated with the diltiazem-sensitive currents recorded during the ramp protocol before and after H₂O₂ (n = 7 cells). *P < 0.05.

Exogenous H₂O₂ stimulates localized L-type Ca²⁺ channel activity. Our macroscopic current recordings indicate that H₂O₂ stimulates L-type Ca²⁺ channels. However, these data provide no information with regard to the spatial regulation of L-type Ca²⁺ channel function by H₂O₂. To overcome this deficiency we used TIRF microscopy to visualize Ca²⁺ influx through single L-type Ca²⁺ channels with high temporal and spatial resolution. First, however, we used TIRF microscopy to image subplasmalemmal changes in intracellular oxidation in arterial myocytes exposed to exogenous H₂O₂ with the cell-permeable fluorescent ROS indicator DCF (10 μM; see MATERIALS AND METHODS). Important for this study, H₂O₂, but not superoxide, is implicated as an experimentally relevant DCF oxidant (17, 33, 47). As shown in Fig. 3, exogenous H₂O₂ (100 μM) produced a homogenous (i.e., nonpunctate by our criteria) increase in intracellular oxidation as evident in the uniform increase in subplasmalemmal DCF fluorescence (average Δ DCF) throughout the visualized intracellular space (P < 0.05, n = 5 cells). These observations suggest that increased DCF fluorescence in our TIRF imaging experiments faithfully reports changes in intracellular oxidation throughout the imaged subplasmalemmal space without spatially erroneous confounding biases, such as nonuniform intracellular compartmentalization or oxidation of the ROS indicator.

Having demonstrated that H₂O₂ increased intracellular oxidation throughout the imaged subplasmalemmal space, we then used TIRF microscopy to image Ca²⁺ influx through single L-type channels. To do so, we applied H₂O₂ (100 μM) to isolated voltage-clamped (at −70 mV) cerebral arterial myocytes dialyzed with the Ca²⁺ indicator fluo-5F and monitored for changes in Ca²⁺ influx. Consistent with the hypothesis that H₂O₂ stimulates local L-type Ca²⁺ channel activity, punctate sites of Ca²⁺ entry (i.e., Ca²⁺ sparklets) increased following H₂O₂ application (see Fig. 4A). To link these image stacks to L-type Ca²⁺ channel function we constructed and analyzed event amplitude histograms from fluo-5F fluorescence time courses as shown in Fig. 4A, right. Similar to previous reports (2, 25, 26, 30), the resulting event distributions (see Fig. 4B) revealed that localized Ca²⁺ influx was quantal and that addition of H₂O₂ increased the number of quanta activated (P < 0.05, n = 5 cells) but not the quantal amplitude (q = 35 nM [Ca²⁺], for control and q = 34 nM [Ca²⁺], for H₂O₂; P > 0.05, n = 5 cells). Importantly, the quantal amplitudes before and after H₂O₂ were indistinguishable from each other and approximated those previously reported for arterial smooth muscle L-type Ca²⁺ channels and for Ca.1.2 L-type Ca²⁺ channels in heterologous expression systems (2, 25–27). From these data, and consistent with our conventional macroscopic electrophysiological recordings, we conclude that H₂O₂ increases Ca²⁺ influx in rat cerebral arterial smooth muscle cells by stimulating local L-type Ca²⁺ channels.

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To further characterize the effect of H₂O₂ on L-type Ca²⁺ channel function we quantified the L-type Ca²⁺ channel activity represented in the fluo-5F fluorescence recordings by calculating \( nP_s \) values for each Ca²⁺ influx site where \( n \) is the number of quantal levels observed and \( P_s \) is the probability that the site is active. As evident in the event amplitude histograms shown in Fig. 4B, H₂O₂ increased L-type Ca²⁺ channel function (as quantified by \( nP_s \)) with the number of high-activity Ca²⁺ influx sites \((nP_s \geq 0.2)\) (26) increasing from two to fourteen (Fig. 4C; \( P < 0.05, n = 5 \) cells). Furthermore, the density (sites per \( \mu m^2 \)) of L-type Ca²⁺ channel influx increased 3.9-fold (Fig. 4D; \( P < 0.05, n = 5 \) cells). These data...
indicate that H$_2$O$_2$ stimulated L-type Ca$^{2+}$ channel function by increasing the number of channels active and by increasing the overall activity of these channels.

**Oxidative-dependent stimulation of L-type Ca$^{2+}$ channels is prevented by H$_2$O$_2$ removal.** Next, we examined whether endogenous H$_2$O$_2$ contributes to oxidative stimulation of L-type Ca$^{2+}$ channels by manipulating H$_2$O$_2$ within arterial smooth muscle cells with exogenous catalase. For these experiments, we used ANG II to generate ROS in isolated arterial smooth muscle cells. While superoxide is the de novo product of NADPH oxidase, superoxide is rapidly converted to H$_2$O$_2$ and oxygen by SOD catalysis or spontaneous dismutation (7).

We recently demonstrated that ANG II promotes localized ROS generation in isolated arterial smooth muscle cells (2). To further investigate local oxidant signaling in arterial smooth muscle we again used TIRF microscopy to image subplasmalemmal ROS production in myocytes loaded with the ROS indicator DCF. Similar to our previous findings (2), ANG II (100 nM) increased the observed incidence of ROS puncta as illustrated in the DCF fluorescence images shown in Fig. 5, A and B ($P < 0.05, n = 5$ cells). Indicative of a localized ROS signaling mechanism, ANG II failed to induce a significant change in the average $\Delta$ DCF fluorescence in submembranous slices visible in the TIRF images of these cells (Fig. 5C; $P > 0.05, n = 5$ cells). However, in contrast to the average $\Delta$ DCF, the increase in DCF fluorescence of nascent ROS puncta was approximately four times greater than the average $\Delta$ DCF ($P < 0.05, n = 5$ cells) after ANG II application. Interestingly, the $\Delta$ DCF fluorescence of ROS puncta (Fig. 5C) was not statistically different from the average $\Delta$ DCF produced by exogenous (100 $\mu$M) H$_2$O$_2$ as shown in Fig. 3 above ($P > 0.05, n = 5$ cells). These data indicate that acute ANG II exposure induces localized changes in intracellular oxidation in isolated arterial myocytes and that application of exogenous H$_2$O$_2$ (100 $\mu$M) replicates this effect by creating an experimentally equivalent degree (albeit homogenously) of subplasmalemmal oxidation.

To examine whether increased ROS puncta density involves NADPH oxidase we tested the effect of the putative NADPH oxidase inhibitor apocynin on ANG II-dependent ROS generation. Suggesting NADPH oxidase involvement, apocynin (10 $\mu$M) abolished the effect of ANG II on localized ROS generation (Fig. 5, D and E; $P > 0.05, n = 5$ cells). This finding is consistent with our previous observation (2) that apocynin prevented ANG II stimulation of localized L-type Ca$^{2+}$ channel activity and further supports the general concept of oxidative regulation of Ca$^{2+}$ influx in arterial smooth muscle via L-type Ca$^{2+}$ channels. ANG II in combination with apocynin had no effect on either the average $\Delta$ DCF fluorescence or nascent puncta $\Delta$ DCF fluorescence (Fig. 5F; $P > 0.05, n = 5$ cells). Note that in the TIRF images presented in Fig. 5 (and Fig. 8 below), only sites of DCF fluorescence circled in yellow satisfied the criteria to be defined as a site of increased local ROS generation (see MATERIALS AND METHODS).

To examine the necessity of H$_2$O$_2$ for oxidant-dependent regulation of L-type Ca$^{2+}$ channels we used catalase to convert endogenous H$_2$O$_2$ to oxygen and water. The TIRF images in Fig. 6A show the effect of ANG II on arterial L-type Ca$^{2+}$ channel function. As reported previously (2, 29, 31), ANG II (100 nM) increased L-type Ca$^{2+}$ channel activity by increasing

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**Fig. 5.** Apocynin prevents ANG II-dependent stimulation of localized subplasmalemmal reactive oxygen species (ROS) generation. A: representative TIRF images showing punctate DCF fluorescence (indicating ROS generation) in an arterial myocyte before and after ANG II (100 nM). B: plot of the mean $\pm$ SE ROS puncta density before and after ANG II (n = 5 cells). C: plot of the mean $\pm$ SE change in the spatially averaged DCF fluorescence (Avg $\Delta$ DCF) and that of nascent ROS puncta in cells exposed to ANG II (n = 5 cells). D: representative TIRF images showing punctate DCF fluorescence before and after ANG II in the presence of apocynin (25 $\mu$M). E: plot of the mean $\pm$ SE ROS puncta density before and after ANG II + apocynin (n = 5 cells). F: plot of the mean $\pm$ SE change in the spatially averaged DCF fluorescence (Avg $\Delta$ DCF) and that of nascent ROS puncta in cells exposed to ANG II + apocynin (n = 5 cells). Note that only sites of DCF fluorescence circled in yellow satisfied the criteria to be considered as a site of increased local ROS generation (see MATERIALS AND METHODS). *P < 0.05; ns†, not significant relative to control cells presented in Fig. 3.

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the number of active channel sites and by increasing the activity of the channels at those sites (Fig. 6B; \( P < 0.05, n = 5 \) cells). Dialyzing cells with catalase by including 500 U/ml of the enzyme in the pipette solution abolished stimulation of L-type Ca\(^{2+}\) channels by ANG II (Fig. 6C and D; \( P < 0.05, n = 5 \) cells). Catalase dialysis had no effect on basal L-type Ca\(^{2+}\) channel activity (\( P > 0.05 \)). From these data we conclude that H\(_2\)O\(_2\) is necessary for ANG II-dependent stimulation of arterial smooth muscle L-type Ca\(^{2+}\) channels.

**ANG II-dependent stimulation of L-type Ca\(^{2+}\) channels is unaffected by enhanced superoxide dismutation.** Thus far we have demonstrated that H\(_2\)O\(_2\) stimulates L-type Ca\(^{2+}\) channels and is necessary for increased channel activity in response to ANG II. As noted above, H\(_2\)O\(_2\) produced by NADPH oxidase results from dismutation of catalytically produced superoxide (7). However, our data does not necessarily preclude a direct contributory role for superoxide. To address this possibility we examined the effect of enhanced superoxide dismutation on stimulation of L-type Ca\(^{2+}\) channels by ANG II. First, we included SOD (50 U/ml) in the pipette solution and recorded L-type Ca\(^{2+}\) channel activity before and after exposure to ANG II. Unlike catalase, dialysis of arterial smooth muscle cells with SOD did not prevent ANG II-dependent stimulation of L-type Ca\(^{2+}\) channels (Fig. 7A). Indeed, the density of L-type Ca\(^{2+}\) channel sparklet sites and the activity at those sites following application of ANG II was unaffected by the presence of exogenous SOD (Fig. 7B; \( P < 0.05, n = 5 \) cells).

Similar to catalase, SOD had no effect on basal L-type Ca\(^{2+}\) channel activity (\( P > 0.05 \)).

Second, we tested the effect two structurally unrelated cell-permeable small molecule SOD mimetics on ANG II-dependent stimulation of L-type Ca\(^{2+}\) channels. The metalloporphyrin MnTMPyP and the nitroxide tempol. Similar to SOD itself, MnTMPyP (10 \( \mu \)M) and tempol (5 mM) had no effect on ANG II-dependent stimulation of L-type Ca\(^{2+}\) channels (Fig. 7, C–F; \( P < 0.05, n = 5 \) cells each); basal L-type Ca\(^{2+}\) channel activity was also unaffected by these two compounds (\( P > 0.05 \)). These data are consistent with the hypothesis that, in contrast to H\(_2\)O\(_2\), superoxide in and of itself does not directly contribute to oxidative stimulation of arterial smooth muscle L-type Ca\(^{2+}\) channels.

As noted above, we previously found that localized sites of ROS generation precede and colocalize with subsequence L-type Ca\(^{2+}\) channel activity (2). To relate the effectiveness of catalase and ineffectiveness of SOD on preventing ANG II from stimulating L-type Ca\(^{2+}\) channels to ROS, we examined the effect of these two enzymes on subplasmalemmal ROS generation with TIRF microscopy. Figure 8A shows near-membrane DCF fluorescence in a myocyte exposed to cell-permeant PEG-catalase (500 U/ml) before and after ANG II, and Fig. 8D shows a cell exposed to PEG-SOD (100 U/ml) before and after ANG II. While neither enzyme had an effect (\( P > 0.05 \)) on baseline ROS production, which was minimal to begin with, PEG-catalase, but not PEG-SOD, abolished the
increase in ROS puncta density in response to ANG II (Fig. 8, B and E; P < 0.05, n = 5 cells each).

Similar to ANG II alone or with apocynin (see Fig. 5), the average Δ DCF fluorescence was not increased by ANG II in the presence of PEG-catalase (Fig. 8C; P > 0.05, n = 5 cells). In contrast, in the presence of PEG-SOD, ANG II increased the average Δ DCF fluorescence (Fig. 8F; P < 0.05, n = 5 cells). This increase was not significantly different (P > 0.05) than that induced by exogenous H₂O₂ (see Fig. 2). Note however, that nonpunctal increases in oxidation observed with H₂O₂ and PEG-SOD plus ANG II were not associated with uniform increases in L-type Ca²⁺ channel function (see Figs. 4 and 7). Indeed, L-type Ca²⁺ channel activity was independent of the average Δ DCF as Ca²⁺ sparklet site densities in cells exposed to either H₂O₂ or ANG II plus SOD (where average Δ DCF fluorescence increased) were not different from cells exposed to ANG II alone (where average Δ DCF fluorescence was unchanged; P > 0.05; n = 5 cells each). ANG II in combination with PEG-catalase and PEG-SOD had no effect on puncta Δ DCF fluorescence (Fig. 8, C and F; P > 0.05, n = 5 cells each). In context with our other experiments, these data support our hypothesis that localized generation of H₂O₂ stimulates L-type Ca²⁺ channels in arterial smooth muscle cells.

**DISCUSSION**

In this study, experiments were performed to test the hypothesis that H₂O₂ mediates oxidant-dependent stimulation of rat cerebral arterial smooth muscle L-type Ca²⁺ channels. Using a combination of arterial diameter measurements, conventional electrophysiology, and TIRF microscopy, in agreement with this hypothesis, we found that 1) depletion of H₂O₂ with catalase all but abolished oxidative-dependent contraction of cerebral arterial segments by ANG II; 2) application of exogenous H₂O₂ increased L-type Ca²⁺ channel activity in isolated arterial smooth muscle cells; 3) catalase reduced oxidant-dependent stimulation of L-type Ca²⁺ channels; 4) catalase also attenuated sites of localized ROS production associated with L-type Ca²⁺ channel activity; and 5) enhanced superoxide dismutation with SOD or small molecule SOD mimetic compounds were without effect. From these data we conclude that H₂O₂ is an important local mediator of oxidant signaling in arterial smooth muscle by stimulating L-type Ca²⁺ channels.

We reported previously (2) that exogenous ROS generated by xanthine oxidase stimulated L-type Ca²⁺ channels in arterial smooth muscle cells. Similar to NADPH oxidase, xanthine oxidase produces superoxide and H₂O₂ (14) with H₂O₂ repre-
resenting the majority of the detectable ROS (13). From a biochemical perspective, H$_2$O$_2$ is well-suited to function as a classical second messenger, while superoxide is not (7). Here we observed that the effect of exogenous H$_2$O$_2$ on L-type Ca$^{2+}$ channels recapitulated our previous results with xanthine oxidase where superoxide and H$_2$O$_2$ were produced (2). Consistent with this, removal of endogenous H$_2$O$_2$ with catalase abolished L-type channel stimulation by ANG II while enhanced superoxide dismutation was without effect. These data suggest that H$_2$O$_2$ is sufficient in mediating oxidant-dependent stimulation of arterial smooth muscle L-type Ca$^{2+}$ channels and that any role of superoxide is likely to be indirect (i.e., as a biochemical precursor to H$_2$O$_2$ via dismutation).

H$_2$O$_2$ has been shown to stimulate native macroscopic L-type Ca$^{2+}$ channel currents in cultured neurons (1) and L-type Ca$^{2+}$ currents produced by recombinant channel subunits expressed in HEK-293 cells (11). Similarly, we found that exogenous ROS generated by xanthine oxidase (where H$_2$O$_2$ and superoxide are produced) increased whole cell L-type Ca$^{2+}$ channel currents in rat cerebral arterial myocytes (2). Consistent with these published reports, here we found that exogenous H$_2$O$_2$ stimulated arterial smooth muscle L-type Ca$^{2+}$ channel currents. In these experiments, H$_2$O$_2$ increased diltiazem-sensitive L-type Ca$^{2+}$ channels (with Ba$^{2+}$ as the charge carrier) using the amphotericin-perforated patch technique to preserve intracellular constituents such as endogenous antioxidants. H$_2$O$_2$ increased L-type Ca$^{2+}$ channel currents in step and continuous ramp depolarizations. These currents displayed kinetic and voltage-dependent properties consistent with L-type Ca$^{2+}$ channels (4). From these observations we conclude that exogenous H$_2$O$_2$ stimulates L-type Ca$^{2+}$ channels in cerebral arterial smooth muscle cells. Although of significant interest in and of themselves, these data provide no information regarding the spatial regulation of arterial smooth muscle L-type Ca$^{2+}$ channels by H$_2$O$_2$.

We previously reported (2) that oxidative stimulation of L-type Ca$^{2+}$ channels in arterial smooth muscle cells is highly localized, not homogenously distributed throughout the plasma membrane. As with ROS generated by xanthine oxidase, here we found that application of exogenous H$_2$O$_2$ increased L-type Ca$^{2+}$ channel activity that was limited to approximately six sites of Ca$^{2+}$ influx per 100 mm$^2$ of membrane imaged. Importantly, the majority of oxidant-induced Ca$^{2+}$ influx events at these sites were produced by the opening of single L-type Ca$^{2+}$ channels (see Fig. 4B and Ref. 2). This observation provides empirical evidence in direct opposition to the alternative hypothesis that intracellular oxidation with H$_2$O$_2$ produces nonuniform Ca$^{2+}$ influx by stimulating spatially heterogeneous clusters of L-type Ca$^{2+}$ channels. To highlight the spatial sequestration of this Ca$^{2+}$ influx, rat cerebral arterial smooth muscle cells are reported to have ~5 L-type Ca$^{2+}$ channels per mm$^2$ (34). Thus, the number of channels activated by exogenous H$_2$O$_2$ is much less than expected based on conventional electrophysiological recordings. This does not preclude a contribution from direct channel stimulation by H$_2$O$_2$. However, prior evidence (2, 25, 29) indicates that additional proteins with limited plasmalemmal distributions such as AKAP150 and PKC$\alpha$ are necessary for oxidant-
dependent stimulation of L-type Ca\(^{2+}\) channels in arterial smooth muscle cells. Indeed, in our previous report we found that exogenous ROS generated by xanthine oxidase (which includes H\(_2\)O\(_2\)) induced irregular punctate translocation of PKC\(\alpha\) to the plasma membrane and that PKC\(\alpha\) activity was necessary for ROS-dependent stimulation of L-type Ca\(^{2+}\) channels (2).

In arterial smooth muscle cells NADPH oxidase signaling complexes and the mitochondrial electron transport chain are two potential sources of H\(_2\)O\(_2\) generation (18, 20). In each case, H\(_2\)O\(_2\) is generally produced indirectly by the dismutation of superoxide. NADPH oxidase-derived H\(_2\)O\(_2\) is postulated to remain within a submembranous compartment a few micrometers from its site of generation (24, 46). Our visualization of discrete subplasmalemmal catalase-sensitive punctate ROS generation following ANG II exposure with TIRF microscopy is in agreement with these findings (this paper and Ref. 2). Furthermore, while ANG II increased the density of ROS puncta approximately fourfold, we found no change in the average \(\Delta\) DCF fluorescence in these cells. We did, however, find that exogenous H\(_2\)O\(_2\) and ANG II plus PEG-SOD resulted in an increase in average \(\Delta\) DCF fluorescence. Importantly, these diffuse increases in intracellular oxidation were not associated with concomitant spatially dispersed increases in L-type Ca\(^{2+}\) channel activity. Thus, in agreement with prior work (2, 25, 26, 28, 29), these data strongly suggest that oxidative stimulation of L-type Ca\(^{2+}\) channels in arterial smooth muscle requires additional molecular participants (e.g., AKAP150 and/or PKC\(\alpha\) as noted above) thus giving rise to its local nature.

Our TIRF imaging also suggests that NADPH oxidase is involved in ROS-dependent stimulation of L-type Ca\(^{2+}\) channels in arterial smooth muscle: inhibition of NADPH oxidase with apocynin prevented ANG II-dependent stimulation of punctate ROS generation (Fig. 5) as well as localized L-type Ca\(^{2+}\) channel function (2). However, apocynin is reported to have intrinsic antioxidant properties that are independent of NADPH oxidase inhibition (9). Although these effects were shown at concentrations greater than that used in this study (25 \(\mu\)M), it is possible that a portion of the observed inhibitory effect of apocynin on ROS puncta generation and L-type Ca\(^{2+}\) channel function could result from antioxidant activity rather than NADPH oxidase inhibition. This implies that other sources of H\(_2\)O\(_2\) (e.g., mitochondria) could participate in oxidant-dependent regulation of arterial smooth muscle L-type Ca\(^{2+}\) channels. Future studies should address this hypothesis. Catalase was effective in preventing increased localized ROS production and stimulation of L-type Ca\(^{2+}\) channels by ANG II. In contrast to H\(_2\)O\(_2\) removal with catalase, SOD did not prevent ANG II from increasing localized ROS generation and stimulating L-type Ca\(^{2+}\) channels. The small molecule SOD mimetics MnTMPyP and tempol were also ineffective in preventing L-type Ca\(^{2+}\) channel stimulation. The outcome of the SOD experiments are consistent with our hypothesis that H\(_2\)O\(_2\) is the ROS involved in oxidant-dependent stimulation of L-type Ca\(^{2+}\) channels. However, the ineffectiveness of MnTMPyP and tempol is somewhat unexpected as these two compounds are reported to have catalase-like activity in addition to their effects on superoxide (5, 45). The reason for this apparent discrepancy is not entirely clear. However, evidence suggests that nitroxides including tempol can cause transient increases in vascular H\(_2\)O\(_2\) and that catalase-like activity of these compounds require other intracellular molecular constituents (e.g., heme proteins or peroxidases) (15, 23) that could be sufficiently diluted by cell dialysis with the pipette solution. Alternatively, it is possible that the catalase-like activity of these agents is temporally insufficient to remove H\(_2\)O\(_2\) before stimulation L-type Ca\(^{2+}\) channels.

As SOD increases the formation of H\(_2\)O\(_2\) it is reasonable to predict that pharmacologically enhanced dismutation of superoxide to H\(_2\)O\(_2\) would result in increased oxidant-dependent L-type Ca\(^{2+}\) channel activity and ROS puncta formation. Although there was a trend for increased channel activity and ROS puncta formation with enhanced superoxide dismutase these differences did not reach statistical significance. This was the case for basal and ANG II-induced L-type Ca\(^{2+}\) channel activity and ROS puncta formation. These observations suggest, at least under our experimental conditions, that superoxide production in response to ANG II is sufficiently dismutated to H\(_2\)O\(_2\) to an extent that additional dismutation has little or no effect on L-type Ca\(^{2+}\) channel function. Importantly, this appears to be the case in intact cells (i.e., ROS puncta experiments) and in cells following intracellular dialysis with an exogenous solution (i.e., L-type Ca\(^{2+}\) channel sparklet experiments). Note, however, that SOD did increase the average \(\Delta\) DCF although this was not associated with an increase in L-type Ca\(^{2+}\) channel activity.

Our data obtained from isolated arterial smooth muscle cells showing H\(_2\)O\(_2\)-dependent Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels is in agreement with prior work examining the effect of H\(_2\)O\(_2\) on intact arterial preparations. In our previous publication (2) we found that ROS generated by xanthine oxidase (which includes H\(_2\)O\(_2\)) induced arterial contraction. Others have shown that application of H\(_2\)O\(_2\) also resulted in arterial contraction (37), an effect which was enhanced in tissues collected from hypertensive animals and dependent on Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels. H\(_2\)O\(_2\) may also participate in the generation of the arterial myogenic contractile response (32). Consistent with these reports, here we found that cell-permeable PEG-catalase largely attenuated the contractile response of pressurized middle cerebral arterial segments to ANG II.

In contrast to the reports referenced above and data presented in this paper, H\(_2\)O\(_2\) has also been shown to be an endothelium-derived relaxation factor that acts at least in part by hyperpolarization of arterial smooth muscle via activation of large-conductance Ca\(^{2+}\)-activated potassium (BK) channels (8, 12, 19, 22). At a minimum, this discrepancy could arise from different experimental conditions and the arterial bed under investigation. However, the highly localized nature of subplasmalemmal H\(_2\)O\(_2\) generation and subsequent coupling to L-type Ca\(^{2+}\) channels demonstrated in our work (Ref. 2 and this paper) provides a possible mechanistic explanation for these apparently contradictory observations: local sites of H\(_2\)O\(_2\) generation functionally coupled to proteins associated with contraction (e.g., L-type Ca\(^{2+}\) channels) lead to vasoconstriction, while those coupled to proteins associated with relaxation (e.g., BK channels) lead to vasodilation. Thus, H\(_2\)O\(_2\) may function in vivo as either a vasoconstrictor or vasodilator depending on the cellular processes to which it is functionally coupled. Similarly, during in vitro experimentation, H\(_2\)O\(_2\) could cause either vasodilation or vasoconstriction depending
on the relative balance of vasodilatory and vasoconstrictive processes stimulated by the different experimental circumstances, including but not limited to, the presence of a functional endothelium, concentration of H2O2 used, and method of delivery.

To conclude, in this manuscript, we provide strong evidence supporting our hypothesis that H2O2 is the ROS responsible for oxidant-dependent stimulation of arterial smooth muscle L-type Ca2+ channels. In addition, our data further support the concept of local crosstalk between ROS and Ca2+ signaling in arterial smooth muscle. These observations also add to a growing body of evidence indicating that H2O2 acts as a local second messenger regulated in time and space with defined functions in multiple biological systems.

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AUTHOR CONTRIBUTIONS
Author contributions: N.L.C. and G.C.A. performed experiments; N.L.C. and G.C.A. analyzed data; N.L.C. and G.C.A. interpreted results of experiments; N.L.C. and G.C.A. approved final version of manuscript; G.C.A. drafted manuscript; G.C.A. edited and revised manuscript.

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