Stimulation of arterial smooth muscle L-type calcium channels by hydrogen peroxide requires protein kinase C

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Changes in intracellular calcium regulate countless biological processes. In arterial smooth muscle, voltage-dependent L-type calcium channels are major conduits for calcium entry with the primary function being determination of arterial diameter. Similarly, changes in intracellular redox status, either discrete controlled changes or global pathological perturbations, are also critical determinants of cell function. We recently reported that in arterial smooth muscle cells, local generation of hydrogen peroxide leads to colocalized calcium entry through L-type calcium channels. Here we extend our investigation into mechanisms linking hydrogen peroxide to calcium influx through L-type calcium channels by focusing on the role of protein kinase C (PKC). Our data indicate that stimulation of L-type calcium channels by hydrogen peroxide requires oxidant-dependent increases in PKC catalytic activity. This effect is independent of classical cofactor-dependent activation of PKC by diacylglycerol. These data provide additional experimental evidence supporting the concept of oxidative stimulation of L-type calcium channels.

Introduction

Voltage-dependent Cav1.2 L-type Ca\textsuperscript{2+} channels are the primary point of Ca\textsuperscript{2+} entry in mammalian arterial smooth muscle. Therefore, changes in the open probability of L-type Ca\textsuperscript{2+} channels in arterial smooth muscle cells in response to vasoconstrictors or vasodilators correlates with changes arterial diameter. Our group and others have used total internal reflection fluorescence (TIRF) microscopy to investigate mechanisms governing L-type Ca\textsuperscript{2+} channel function with high temporal and spatial resolution.\textsuperscript{1-6} These studies revealed that L-type Ca\textsuperscript{2+} channel functionality is heterogeneously dispersed throughout the smooth muscle cell sarcolemma as a consequence of spatially limited distributions of key regulatory molecules and subcellular processes.

Recently, our group reported that the vasoconstrictor angiotensin II (Ang II) leads to punctate generation of reactive oxygen species (ROS) by NADPH oxidase.\textsuperscript{1,3} This increase in local ROS production, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) to be more precise, in turn promotes discrete colocalized Ca\textsuperscript{2+} influx events via protein kinase C (PKC)-dependent activation of L-type Ca\textsuperscript{2+} channels. Our observations suggest that oxidative activation of PKC is involved with ROS-dependent stimulation of L-type Ca\textsuperscript{2+} channels in arterial smooth muscle cells.\textsuperscript{1,3} Indeed, the PKC inhibitor Gö6976 prevented oxidant-dependent stimulation of L-type Ca\textsuperscript{2+} channels by exogenous ROS produced by xanthine oxidase.\textsuperscript{1}

Here we expand on this topic by furthering our investigation into the mechanisms underlying oxidative activation of L-type Ca\textsuperscript{2+} channels by PKC. Our data indicate that H\textsubscript{2}O\textsubscript{2}, as with ROS generated enzymatically by xanthine oxidase (which includes superoxide and H\textsubscript{2}O\textsubscript{2}) stimulates L-type Ca\textsuperscript{2+} channels in a PKC-sensitive manner. Consistent with the importance of oxidative activation of PKC, as opposed to classical activation mechanisms such as diacylglycerol (DAG), we found that...
isolated arterial smooth muscle cells (see Figure 1). Ang II increased the density of L-type channel Ca\textsuperscript{2+} sparklet sites as well as the activity (nP\textsubscript{s}) of those sites (p < 0.05, n = 5 cells). To test if oxidative stimulation of L-type Ca\textsuperscript{2+} channels requires DAG-dependent activation of PKC we repeated our Ang II experiments in the presence of the PKC inhibitor safingol, which inhibits PKC activation by competitively interacting with the regulatory DAG/phorbol binding domain of the kinase.10 Interestingly, we found that safingol (50 μM) had no effect on Ang II-dependent stimulation of L-type Ca\textsuperscript{2+} channels. As noted above, the PKC catalytic site inhibitor Gö6976 abolished stimulation of L-type Ca\textsuperscript{2+} channels by exogenous ROS generated by xanthine oxidase.3 From these accumulated data we conclude that oxidative stimulation of L-type Ca\textsuperscript{2+} channels does not require DAG-dependent activation of PKC. Rather, we suggest that during increased oxidative stress, activation of

Results

For our hypothesis that oxidative activation of PKC is necessary for H\textsubscript{2}O\textsubscript{2}-dependent stimulation of L-type Ca\textsuperscript{2+} channels to be valid, following oxidant exposure, inhibition of cofactor-dependent activation of PKC (i.e., via DAG) should be without effect while inhibition of PKC catalytic activity should prevent channel stimulation.

To begin, we tested the hypothesis that inhibiting the interaction between DAG and PKC (i.e., cofactor-dependent activation of PKC) would not impede oxidant-dependent stimulation of arterial smooth muscle L-type Ca\textsuperscript{2+} channels. We recorded L-type Ca\textsuperscript{2+} channel activity optically using a combination of voltage-clamp electrophysiology and TIRF microscopy as previously described.1,3 L-type Ca\textsuperscript{2+} channel activity was quantified in two ways: (1) the number of active L-type channel Ca\textsuperscript{2+} influx sites per μm\textsuperscript{2} (Ca\textsuperscript{2+} sparklet site density) and (2) the activity of the L-type Ca\textsuperscript{2+} channels at these sites as determined by their calculated nP\textsubscript{s} values (n is the number of quantal levels observed and P\textsubscript{s} is the probability that the site is active).5

For these experiments we generated endogenous ROS by activating NADPH oxidase with Ang II.7 We demonstrated previously that ROS, specifically H\textsubscript{2}O\textsubscript{2}, is necessary for oxidant-dependent stimulation of L-type Ca\textsuperscript{2+} channels in arterial smooth muscle cells.3 In agreement with earlier findings,1,3,5,9 Ang II (100 nM) increased L-type Ca\textsuperscript{2+} channel activity in isolated arterial smooth muscle cells (see Figure 1). Ang II increased the density of L-type channel Ca\textsuperscript{2+} sparklet sites as well as the activity (nP\textsubscript{s}) of those sites (p < 0.05, n = 5 cells). To test if oxidative stimulation of L-type Ca\textsuperscript{2+} channels requires DAG-dependent activation of PKC we repeated our Ang II experiments in the presence of the PKC inhibitor safingol, which inhibits PKC activation by competitively interacting with the regulatory DAG/phorbol binding domain of the kinase.10 Interestingly, we found that safingol (50 μM) had no effect on Ang II-dependent stimulation of L-type Ca\textsuperscript{2+} channels. In the context of our prior work,1,3 these data indicate that oxidant-dependent activation of arterial smooth muscle L-type Ca\textsuperscript{2+} channels by H\textsubscript{2}O\textsubscript{2} following Ang II exposure involves oxidative activation of PKC.

Figure 1. Inhibition of diacylglycerol-protein kinase C interactions with safingol does not prevent angiotensin II-dependent stimulation of arterial smooth muscle L-type Ca\textsuperscript{2+} channels. (A) Representative traces showing the time course of Ca\textsuperscript{2+} influx under control conditions (left), in the presence of Ang II (100 nM; middle) and in the presence of Ang II plus safingol (10 μM; right). (B) Plot of L-type Ca\textsuperscript{2+} channel sparklet site activities (nP\textsubscript{s}) under control conditions, in the presence of Ang II and in the presence of Ang II plus safingol (n = 5 cells each). The solid gray lines are the arithmetic means of each group and the dashed line marks the threshold for high-activity Ca\textsuperscript{2+} sparklet sites (nP\textsubscript{s} ≥ 0.2). (C) Plot of the mean ± SEM L-type Ca\textsuperscript{2+} channel sparklet site densities (Ca\textsuperscript{2+} sparklet sites/μm\textsuperscript{2}) under control conditions, in the presence of Ang II and in the presence of Ang II plus safingol (n = 5 cells each). * p < 0.05.
PKC, which is necessary for stimulation of L-type Ca\(^{2+}\) channels, could occur via an oxidant-dependent mechanism.\(^1\)

Next, we examined if H\(_2\)O\(_2\)-dependent stimulation of arterial smooth muscle L-type Ca\(^{2+}\) channels requires PKC catalytic activity by testing the effect of the PKC catalytic site inhibitor Gö6976 (100 nM) on H\(_2\)O\(_2\)-dependent activation of L-type Ca\(^{2+}\) channels. Consistent with our previous observations,\(^3\) in the absence of Gö6976, H\(_2\)O\(_2\) (100 μM) produced robust L-type Ca\(^{2+}\) channel activity (see Figure 2). In contrast, in the presence of Gö6976, H\(_2\)O\(_2\) had minimal effect on L-type Ca\(^{2+}\) channel function. Specifically, Gö6976 abolished the increase in the number of active L-type Ca\(^{2+}\) channel sites observed following H\(_2\)O\(_2\) exposure under control conditions (p < 0.05, n = 5 cells). Surprisingly, the activity of L-type Ca\(^{2+}\) channel that were observed (i.e., nP) following H\(_2\)O\(_2\) were not statistically different in the absence or presence of Gö6976 (p > 0.05, n = 5 cells). Note, however, that the number of L-type channel Ca\(^{2+}\) influx events observed was reduced ≈ 4-fold from 17 events under control conditions to only four events in the presence Gö6976 (n = 5 cells). Thus, we conclude that stimulation of L-type Ca\(^{2+}\) channels by H\(_2\)O\(_2\) requires catalytic PKC activity.

**Discussion**

Here we continued our characterization of the role of PKC in the oxidative stimulation of arterial smooth muscle L-type Ca\(^{2+}\) channels. We observed that: (1) Inhibition of the interaction between DAG and PKC did not prevent Ang II from stimulating L-type Ca\(^{2+}\) channels, which we had previously shown to be oxidant-dependent;\(^1,3\) and (2) stimulation of L-type Ca\(^{2+}\) channels by H\(_2\)O\(_2\) was abolished by inhibition of PKC catalytic activity. From these data and our published work\(^1,3\) we conclude that stimulation of arterial smooth muscle L-type Ca\(^{2+}\) channels by H\(_2\)O\(_2\) involves oxidative activation of PKC.

Previous studies have shown that ROS increase the activity of PKC isoforms (such as PKC\(\alpha\)) by oxidizing reactive cysteine residues in the kinase regulatory domain leading to constitutive cofactor-independent activity.\(^11,13\) Conversely, oxidation of cysteine residues in the catalytic domain leads to enzyme inactivation. Importantly, oxidative modification of the PKC regulatory and enzymatic domains is concentration dependent: Cysteine residues in the regulatory domain are more sensitive to oxidative modification than those in the catalytic domain.\(^11\) As a result, limited exposure to mild oxidative conditions increases kinase activity while prolonged pathological oxidative insults promote inhibition.

We have shown that Ang II produces punctate elevations in ROS production in isolated arterial myocytes.\(^1,3\) Our results presented here therefore suggest a potential mechanistic basis for differential activation of discrete functional pools of PKC in arterial smooth muscle cells: PKC molecules in close proximity to sites of localized ROS generation would be subject to oxidative activation while those distal to these sites would not. Thus, targeting of PKC to specific subcellular sites (e.g., through interacting with the scaffolding protein AKAP150)\(^8\) could result in initiation of oxidant-dependent and independent PKC signaling cascades with different physiological outcomes.

To conclude, our data indicate that local oxidative activation of PKC and subsequent stimulation of adjacent L-type Ca\(^{2+}\) channels gives rise to coordinated sites of ROS generation and Ca\(^{2+}\) entry. We suggest that oxidative regulation of arterial smooth muscle PKC represents a critical intracellular signaling nexus where perturbations in oxidative status translate into changes in arterial smooth muscle function via regulation of L-type Ca\(^{2+}\) channel activity. Our data clearly indicate that local ROS production stimulates colocalized Ca\(^{2+}\) influx through L-type channels.\(^1,3\) Interestingly, Ca\(^{2+}\) is known to stimulate NADPH oxidase activity via PKC.\(^14\) Thus, it is reasonable to propose that colocalized ROS and Ca\(^{2+}\) microdomains may form a reciprocal coupling mechanism leading to sustained ROS generation and Ca\(^{2+}\) influx via NADPH oxidase and L-type Ca\(^{2+}\) channels, respectively. Future studies should address this intriguing hypothesis.
Materials and Methods

Isolation of rat cerebral arterial myocytes. Adult male Sprague-Dawley rats (Harlan) were euthanized with sodium pentobarbital (200 mg/kg intraperitoneally; Fort Dodge Animal Health) 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 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 collagenase (300 U/mL; Worthington Biochemical) and dithiothreitol (1 mg/mL) followed by a second incubation (15 min at 37°C) in Ca²⁺-free buffer supplemented with papain (10 U/mL; Worthington Biochemical) 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 and total internal reflection fluorescence (TIRF) microscopy. 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). 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 Cs₂-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).

Ca²⁺ influx through L-type channels was visualized with a TILL Photonics through-the-lens TIRF system built around an inverted Olympus IX-71 microscope using a 100X (numerical aperture = 1.45) TIRF oil-immersion objective and an Andor iXON EMCCD camera (Andor Technology). To monitor Ca²⁺ influx, myocytes were loaded with the Ca²⁺ indicator fluo-5F (200 μM; pentapotassium salt; Invitrogen) and an excess of EGTA (10 mM) via the patch pipette. 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 to permit quantal analysis. All experiments were allowed to progress between 5 and 10 min and only recordings with stable GΩ seals were analyzed. All experiments were performed at room temperature (22–25°C).

L-type Ca²⁺ channel sparklet analysis. Background-subtracted fluo-5F fluorescence signals were converted to [Ca²⁺] using the equation

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

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 and \( R_f \) is the \( F_{max} / F_{min} \) of fluo-5F. \( K_d \) and \( R_f \) values for fluo-5F were determined in vitro and \( F_{max} \) was determined at the conclusion of each experiment with ionomycin (10 μM). Fluo-5F fluorescence images were analyzed with custom software. For an elevation in [Ca²⁺], to be considered an L-type Ca²⁺ channel sparklet event, a grid of 3 x 3 contiguous pixels had to have a [Ca²⁺] amplitude equal to or larger than the mean basal [Ca²⁺], plus three times its standard deviation.

L-type Ca²⁺ channel sparklet activity was determined by calculating the nP value 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) on imported [Ca²⁺], time course records. L-type Ca²⁺ channel sparklet activity was quantified using an initial unitary [Ca²⁺], elevation of 38 nM as determined experimentally. Consistent with previous reports, L-type Ca²⁺ channel sparklet activity was bimodally distributed with sites of low activity (nP between 0 and 0.2) and high activity (nP greater than 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.

Chemicals and statistics. All chemicals were from Sigma 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 one-way ANOVA with Tukey's multiple comparison post-test. L-type Ca²⁺ channel sparklet activity (i.e., nP) data sets were bimodally distributed, thus two-sample comparisons of nP values were examined with 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 nP data sets are indicated in the figures (solid gray horizontal lines) for non-statistical visual purposes and dashed gray lines mark the threshold for high-activity Ca²⁺ sparklet sites (nP ≥ 0.2). Values less than 0.05 were considered significant and asterisks (*) used in the figures indicate a significant difference between groups.

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