Mitochondria-Derived Reactive Oxygen Species Dilate Cerebral Arteries by Activating Ca\(^{2+}\) Sparks

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Abstract—Mitochondria regulate intracellular calcium (Ca\(^{2+}\)) signals in smooth muscle cells, but mechanisms mediating these effects, and the functional relevance, are poorly understood. Similarly, antihypertensive ATP-sensitive potassium (K\(_{\text{ATP}}\)) channel openers (KCOs) activate plasma membrane K\(_{\text{ATP}}\) channels and depolarize mitochondria in several cell types, but the contribution of each of these mechanisms to vasodilation is unclear. Here, we show that cerebral artery smooth muscle cell mitochondria are most effectively depolarized by diazoxide (−15%, tetramethylrhodamine [TMRM]), less so by levocromakalim, and not depolarized by pinacidil. KCO-induced mitochondrial depolarization increased the generation of mitochondria-derived reactive oxygen species (ROS) that stimulated Ca\(^{2+}\) sparks and large-conductance Ca\(^{2+}\) channels, leading to transient K\(_{\text{Ca}}\) current activation. KCO-induced mitochondrial depolarization and transient K\(_{\text{Ca}}\) current activation were attenuated by 5-HD and glibenclamide, K\(_{\text{ATP}}\) channel blockers. MnTMPyP, an antioxidant, and Ca\(^{2+}\) spark and K\(_{\text{Ca}}\) channel blockers reduced diazoxide-induced vasodilations by >60%, but did not alter dilations induced by pinacidil, which did not elevate ROS. Data suggest diazoxide drives ROS generation by inducing a small mitochondrial depolarization, because nanomolar CCCP, a protonophore, similarly depolarized mitochondria, elevated ROS, and activated transient K\(_{\text{Ca}}\) currents. In contrast, micromolar CCCP, or rotenone, an electron transport chain blocker, induced a large mitochondrial depolarization (−84%, TMRM), reduced ROS, and inhibited transient K\(_{\text{Ca}}\) currents. In summary, data demonstrate that mitochondria-derived ROS dilate cerebral arteries by activating Ca\(^{2+}\) sparks, that some antihypertensive KCOs dilate by stimulating this pathway, and that small and large mitochondrial depolarizations lead to differential regulation of ROS and Ca\(^{2+}\) sparks. (Circ Res. 2005;97:354-362.)

Key Words: vascular smooth muscle ■ ryanodine receptor ■ vasodilation ■ redox

Systemic blood pressure and tissue blood flow are modulated by changes in the diameter of small arteries and arterioles. A fundamental determinant of arterial diameter is the intracellular calcium concentration ([Ca\(^{2+}\)]\(_{i}\)) of smooth muscle cells. This spatially averaged [Ca\(^{2+}\)]\(_{i}\), termed global [Ca\(^{2+}\)]\(_{i}\), arises due to extracellular Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) release.1 A nanomolar elevation in smooth muscle cell global [Ca\(^{2+}\)]\(_{i}\), stimulates Ca\(^{2+}\)/calmodulin-dependent myosin light chain kinase, leading to vasoconstriction, whereas a reduction in global [Ca\(^{2+}\)]\(_{i}\) results in dilation.1 Another intracellular Ca\(^{2+}\) signal, termed a Ca\(^{2+}\) spark, is a localized [Ca\(^{2+}\)]\(_{i}\), transient that is generated in response to the opening of multiple ryanodine-sensitive Ca\(^{2+}\) release (RyR) channels on the sarcoplasmic reticulum (SR).1 Although Ca\(^{2+}\) sparks elevate [Ca\(^{2+}\)]\(_{i}\), in the local vicinity of the release site to micromolar concentrations, Ca\(^{2+}\) sparks do not contribute directly to global [Ca\(^{2+}\)]\(_{i}\), because of their transient and localized nature.1,2 Rather, sparks activate several nearby sarclemma large-conductance Ca\(^{2+}\)-activated potassium (K\(_{\text{Ca}}\)) channels.1 Ca\(^{2+}\) spark-induced transient K\(_{\text{Ca}}\) currents induce membrane hyperpolarization, leading to a reduction in voltage-dependent Ca\(^{2+}\) channel activity, a decrease in global [Ca\(^{2+}\)]\(_{i}\), and relaxation.1 Several signaling elements, including protein kinases and carbon monoxide,3 modulate arterial diameter by regulating Ca\(^{2+}\) sparks and the effective coupling of Ca\(^{2+}\) sparks to K\(_{\text{Ca}}\) channels.1

Mitochondria also regulate local and global Ca\(^{2+}\) signaling in smooth muscle cells,4–7 although the underlying mechanisms and the physiological function of such modulation are poorly understood. Mitochondria potential is ~150 to 200 mV more negative than the cytosol, and changes spontaneously and in response to stimuli. Spontaneous changes in mitochondrial potential, termed flickers, occur in mitochondria of several cell types, including smooth muscle cells.8 Mitochondria potential is also altered by stimuli, including hypoxia.9 Thus, in arterial smooth muscle cells, changes in mitochondria potential may modulate local and global Ca\(^{2+}\) signals, leading to functional consequences. It has been known for several years that synthetic openers of ATP-sensitive K\(^{+}\) (K\(_{\text{ATP}}\)) channels depolarize mitochondria, including those in cardiac myocytes.10,11 Recently, diazoxide, a K\(_{\text{ATP}}\) channel opener, was shown to depolarize.
mitochondria and reduce cytosolic Ca\textsuperscript{2+} removal in femoral artery smooth muscle cells.\textsuperscript{6} Investigating the regulation of mitochondrial potential and local and global Ca\textsuperscript{2+} signaling in arterial smooth muscle cells by K\textsubscript{ATP} channel openers may provide insights into the mechanisms by which mitochondria and changes in mitochondria potential modulate contractility, particularly because these compounds are vasodilators.\textsuperscript{12}

Here, we demonstrate that a small mitochondrial depolarization, such as that induced by diazoxide, leads to the generation of reactive oxygen species (ROS) that elevate Ca\textsuperscript{2+} spark frequency and increase the effective coupling of Ca\textsuperscript{2+} sparks to K\textsubscript{Ca} channels in arterial smooth muscle cells, resulting in vasodilation. Data also indicate that small and large mitochondrial depolarizations lead to differential regulation of ROS and Ca\textsuperscript{2+} sparks. This study identifies a novel mechanism by which mitochondria regulate local and global Ca\textsuperscript{2+} signaling and arterial diameter.

**Materials and Methods**

**Tissue Preparation**

Animal procedures were approved by the Animal Care and Use Committee at the University of Tennessee. Sprague-Dawley rats (200 to 250 g) of either sex were euthanized and the brain removed. Cerebral arteries (200 to 250 µm in diameter) were harvested, cleaned, and maintained in ice-cold (4°C) physiological saline solution (PSS) containing (in m/mol/L): 112 NaCl, 4.8 KCl, 1.2 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, and 10 glucose, gassed with 74% N\textsubscript{2}–21% O\textsubscript{2}–5% CO\textsubscript{2} (pH 7.4). Smooth muscle cells were isolated as described previously.\textsuperscript{13}

**Tetramethylrhodamine, Methyl Ester Imaging**

Experiments were performed using HEPES-buffered PSS containing (in mM): 2 NaHCO\textsubscript{3}, 1.8 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, and 10 glucose, washed, and maintained in ice-cold (4°C) physiological saline solution (PSS) containing (in m/mol/L): 112 NaCl, 4.8 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 NaHCO\textsubscript{3}, 30 KCl, 10 NaCl, 1.8 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 10 HEPES, and 0.05 EGTA (pH 8.2). Bath solution was HEPES-buffered PSS. For perforated patch-clamp configuration, the pipette solution contained (in m/mol/L): 112 NaCl, 4.8 KCl, 26 NaHCO\textsubscript{3}, 1.8 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, and 10 glucose, gassed with 74% N\textsubscript{2}–21% O\textsubscript{2}–5% CO\textsubscript{2} (pH 7.4).

**Patch-Clamp Electrophysiology**

Potassium currents were measured using the conventional whole-cell or perforated patch-clamp configuration (Axopatch 200 B, Clampex 8.2). Bath solution was HEPES-buffered PSS. For perforated patch-clamp configuration, the pipette solution contained (in m/mol/L): 110 K\textsubscript{Asp}, 30 KCl, 10 NaCl, 1 MgCl\textsubscript{2}, 10 HEPES, and 0.05 EGTA (pH 7.2, KOH). For conventional whole-cell configuration, the pipette solution contained (in m/mol/L): 140 KCl, 1.9 MgCl\textsubscript{2}, 0.037 CaCl\textsubscript{2}, 0.1 EGTA, 10 HEPES, and 2 Na\textsubscript{ATP} (pH 7.2, KOH). Transient K\textsubscript{Ca} current and single K\textsubscript{Ca} channel activity (NP0) were measured at -40 and 0 mV, respectively. At least 5 minutes of continuous data were analyzed offline to calculate transient K\textsubscript{Ca} current properties, or K\textsubscript{Ca} channel NP0.

**Reactive Oxygen Species Measurements**

**Dichlorofluorescein**

Endothelium-denuded artery segments were incubated for 20 minutes at room temperature in HEPES-buffered PSS containing (100 µmol/L) dihydroethidium (DHE; 10 µmol/L) for 25 minutes at room temperature followed by a 30 minute wash. Cells were imaged using a Noran Oz laser scanning confocal microscope by illuminating with 488 nm light and collecting emitted light >500 nm. Images were recorded every 8.3 ms. Ca\textsuperscript{2+} sparks and global Ca\textsuperscript{2+} were analyzed offline as described elsewhere and in the online data supplement.\textsuperscript{4,14}

**Confocal Ca\textsuperscript{2+} Imaging**

Cerebral arteries (50 to 200 µm in diameter) were harvested, cleaned, and maintained in ice-cold (4°C) physiological saline solution (PSS) containing (in mM): 134 NaCl, 6 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, and 10 glucose (pH 7.4). For conventional whole-cell configuration, the pipette solution contained (in m/mol/L): 112 NaCl, 4.8 KCl, 26 NaHCO\textsubscript{3}, 1.8 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, and 10 glucose, gassed with 74% N\textsubscript{2}–21% O\textsubscript{2}–5% CO\textsubscript{2} (pH 7.4). Smooth muscle cells were isolated as described previously.\textsuperscript{13}

**Pressureized Artery Diameter Measurements**

Cerebral arteries were cannulated at each end in a perfusion chamber containing PSS (Living Systems Instrumentation). The chamber was continuously perfused with PSS and maintained at 37°C. Intravascular pressure was monitored using a pressure transducer. Wall diameter was measured at 1 Hz using a CCD camera attached to a Nikon TS100-F microscope and the edge-detection function of IonWizard.

**Statistical Analysis**

Values are expressed as mean±SEM. Student t test was used for comparing paired and unpaired data from two populations, and ANOVA and Student–Newman–Keuls tests were used for multiple group comparisons. Evaluation of whether distributions were Gaussian was by the method of Kolmogorov and Smirnov. Simultaneous spark and transient K\textsubscript{Ca} current amplitude data were fit with a first-order polynomial linear function and the slope±SEM of each fit was compared using a Student t test. No differences were observed between genders and data were pooled. P<0.05 was considered significant.

An expanded Materials and Methods section is provided in the online data supplement.

**Results**

**Regulation of Arterial Smooth Muscle Mitochondrial Potential by K\textsubscript{ATP} Channel Modulators**

Punctuate TMRM fluorescence in cerebral artery smooth muscle cells was reduced by rotenone, an electron transport chain (ETC) complex I blocker (10 µmol/L), indicating mitochondria specific loading (Figure 1). Rotenone (10 µmol/L), or CCCP, (10 µmol/L) rapidly reduced mean TMRM fluorescence by 69±5 (n=24) and 84±3 (n=40) %, respectively (P<0.05 for each), similarly to previous reports.\textsuperscript{4} Diazoxide (10 to 500 µmol/L) induced a concentration-dependent steady-state reduction in TMRM fluorescence of between 8±1 and 15±1% (P<0.05 for each). Levocurakalin (100 µmol/L), another K\textsubscript{ATP} channel opener, reduced TMRM fluorescence by 8±1% (P<0.05). In contrast, the K\textsubscript{ATP} channel opener pinacidil (100 µmol/L) or 15 mmol/L K\textsuperscript{+}, which induces a similar cell membrane hyperpolarization as sarcosommmal K\textsubscript{ATP} (sarcK\textsubscript{ATP}) channel activation (≈15 mV\textsuperscript{+}), did not alter TMRM fluorescence (P>0.05). Glibenclamide (10 µmol/L), a sarcK\textsubscript{ATP} channel and mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channel blocker, and 5-HD (500 µmol/L), a mitoK\textsubscript{ATP} channel blocker, did not alter TMRM fluorescence.

**Dihydroethidium**

Endothelium-denuded artery segments were incubated for 20 minutes in HEPES-buffered PSS with or without diazoxide (100 µmol/L). Arteries were embedded in OTC, snap-frozen, sectioned, and then incubated with dihydroethidium (DHE; 10 µmol/L) for 30 minutes at 37°C. Sections were illuminated with 488 nm light, and emitted light >590 nm was collected using a Noran Oz confocal microscope. Tissue processing did not elevate ROS (see online data supplement, available at http://circres.ahajournals.org).

**Mitochondrial ROS Activate Ca\textsuperscript{2+} Sparks**

Endothelium-denuded artery segments were cannulated at each end in a perfusion chamber containing PSS (Living Systems Instrumentation). The chamber was continuously perfused with PSS and maintained at 37°C. Intravascular pressure was monitored using a pressure transducer. Wall diameter was measured at 1 Hz using a CCD camera attached to a Nikon TS100-F microscope and the edge-detection function of IonWizard.
K<sub>ATP</sub> Channel Openers That Depolarize Mitochondria Activate Transient K<sub>Ca</sub> Currents

Diazoxide induced concentration-dependent (10 to 100 μmol/L) transient K<sub>Ca</sub> current activation in voltage-clamped (−40 mV) smooth muscle cells. For example, diazoxide (100 μmol/L) increased mean transient K<sub>Ca</sub> current frequency from 0.58±0.1 to 0.96±0.14 Hz, or to ≈203% of control (Figure 2). Diazoxide (100 μmol/L) also increased mean transient K<sub>Ca</sub> current amplitude from 27.5±3 to 45±4 pA, or to ≈135% of control (Figure 2). Levcromakalim (100 μmol/L) was a less effective transient K<sub>Ca</sub> current activator than diazoxide but increased frequency to ≈187% of control. Diazoxide similarly activated transient K<sub>Ca</sub> currents when applied in the presence of diltiazem (50 μmol/L), indicating stimulation was independent of voltage-dependent Ca<sup>2+</sup> channel activity. When applied alone, glibenclamide (10 μmol/L) and 5-HD (500 μmol/L) did not alter transient K<sub>Ca</sub> currents, but both K<sub>ATP</sub> channel blockers reduced diazoxide-induced transient K<sub>Ca</sub> current activation. In the continued presence of diazoxide, rotenone (10 μmol/L) rapidly reduced mean transient K<sub>Ca</sub> current frequency and amplitude to ≈22% and 48% of the control values before diazoxide application, respectively. In contrast to the stimulatory effects of diazoxide and levcromakalim, pinacidil (100 μmol/L) did not alter transient K<sub>Ca</sub> currents. These data indicate K<sub>ATP</sub> channel openers that depolarize mitochondria also stimulate transient K<sub>Ca</sub> currents, and this effect is reversed by K<sub>ATP</sub> channel inhibitors and an ETC blocker.

**Diazoxide Elevates Ca<sup>2+</sup> Spark Frequency and Effective Coupling to K<sub>Ca</sub> Channels**

To investigate mechanisms mediating transient K<sub>Ca</sub> current activation by K<sub>ATP</sub> channel openers, simultaneous measurements of sparks and transient K<sub>Ca</sub> currents were obtained in voltage-clamped (−40 mV) cells. Diazoxide (100 μmol/L) increased mean Ca<sup>2+</sup> spark frequency to ≈179% of control (Figure 3). Diazoxide also increased the effective coupling of Ca<sup>2+</sup> sparks to K<sub>Ca</sub> channels (P<0.05), although mean Ca<sup>2+</sup> spark amplitude and the percentage of Ca<sup>2+</sup> sparks that activated a transient K<sub>Ca</sub> current (control, 93±4%; diazoxide, 94±2%) did not change (P>0.05). In the same cells, diazoxide did not change global [Ca<sup>2+</sup>]<sub>i</sub>, (F/F<sub>0</sub>, 107±5% of control, P>0.05). Data indicate diazoxide elevates Ca<sup>2+</sup> spark, and thus, transient K<sub>Ca</sub> current frequency, by a mechanism that does not involve an elevation in global [Ca<sup>2+</sup>]<sub>i</sub>, and suggest diazoxide elevates transient K<sub>Ca</sub> current amplitude by increasing K<sub>Ca</sub> channel sensitivity to Ca<sup>2+</sup> sparks.

To determine whether diazoxide enhances K<sub>Ca</sub> channel coupling to Ca<sup>2+</sup> sparks due to a direct or indirect mechanism, K<sub>Ca</sub> channel activity (N<sub>P</sub>) was measured using the perforated patch-clamp configuration. Ca<sup>2+</sup> sparks, and thus, transient K<sub>Ca</sub> currents, were abolished with thapsigargin, a SR Ca<sup>2+</sup>-ATPase inhibitor. Diazoxide (100 μmol/L) increased mean K<sub>Ca</sub> channel N<sub>P</sub>, to ≈187% of control (online Figure I). In contrast, when applied in the continued presence of CCCP (1 μmol/L), to depolarize mitochondria, diazoxide did not alter K<sub>Ca</sub> channel activity. Data suggest diazoxide does not activate K<sub>Ca</sub> channels directly, but elevates the effective coupling of sparks to K<sub>Ca</sub> channels.

**Figure 1. Regulation of cerebral artery smooth muscle mitochondrial potential.** A, Confocal image illustrating TMRM fluorescence localization and reduction by rotenone. B, Original traces with arrow indicating time of drug application. C, Average changes in TMRM fluorescence with diazoxide (10 μmol/L, n=9; 100 μmol/L, n=5), diazoxide (100 μmol/L)+glibenclamide (1 μmol/L, n=5), diazoxide (100 μmol/L)+5-HD (500 μmol/L, n=6), levcromakalim (100 μmol/L, n=4), pinacidil (100 μmol/L, n=5), glibenclamide (10 μmol/L, n=6), 5-HD (500 μmol/L, n=4), and 15 mmol/L K<sup>+</sup> (n=5). *,#, and † illustrate P<0.05 when compared with control, diazoxide, (100 μmol/L), or levcromakalim, respectively.

When applied alone. However, glibenclamide (1 μmol/L) attenuated diazoxide- and levcromakalim-induced mitochondrial depolarization, and 5-HD (500 μmol/L) blocked diazoxide-induced mitochondrial depolarization. These data indicate that diazoxide and levcromakalim depolarize mitochondria in cerebral artery smooth muscle cells by a mechanism that does not involve sarcolemmal K<sub>ATP</sub> channel activation or membrane hyperpolarization, but by a mechanism that is inhibited by K<sub>ATP</sub> channel blockers.
coupling of Ca\(^{2+}\) sparks to K\(_{Ca}\) channels by a mitochondria-dependent mechanism.

**Diazoxide Elevates ROS, Whereas Rotenone and CCCP Reduce ROS**

Because mitochondria are a major source of ROS,\(^{15}\) we tested the hypothesis that mitochondrial depolarization alters ROS generation in cerebral artery smooth muscle cells. Diazoxide (100 \(\mu\)mol/L) elevated the fluorescence of DCF, an ROS indicator, in smooth muscle cells of endothelium-denuded cerebral arteries to 143±11% of control (Figure 4). DCF fluorescence did not change after vehicle (DMSO, time control) or pinacidil (100 \(\mu\)mol/L). Catalase (2000 U/mL), or MnTMPyP (10 \(\mu\)mol/L), a superoxide dismutase and catalase mimetic, blocked diazoxide-induced DCF fluorescence elevations. In contrast to the effect of diazoxide, rotenone (10 \(\mu\)mol/L), or CCCP (1 \(\mu\)mol/L), caused a profound reduction in DCF fluorescence to ≈53% and ≈37% of control, respectively. Rotenone also prevented diazoxide-induced DCF fluorescence elevations, indicating diazoxide generates mitochondria-derived ROS. In agreement, apocynin (25 \(\mu\)mol/L), an NAD(P)H oxidase blocker, oxyipurinol (10 \(\mu\)mol/L), a xanthine oxidase inhibitor, and 17-octadecanoic acid (10 \(\mu\)mol/L), a cytochrome P450 blocker, did not alter diazoxide (100 \(\mu\)mol/L)-induced DCF fluorescence elevations (see online data supplement). Diazoxide (100 \(\mu\)mol/L) also elevated fluorescence intensity of DHE, another ROS indicator, in smooth muscle cells to 137±9% of control (Figure 4). Data indicate diazoxide elevates mitochondria-derived ROS, pinacidil does not alter ROS, and rotenone and CCCP reduce ROS in cerebral artery smooth muscle cells.

**Diazoxide Stimulates Transient K\(_{Ca}\) Currents by Elevating ROS**

To determine whether diazoxide activates transient K\(_{Ca}\) currents by elevating ROS, the conventional whole-cell configuration of patch-clamp was used. Inclusion of the antioxidants superoxide dismutase (SOD), and catalase (300 U/mL of each) in the pipette solution abolished diazoxide-induced elevations in transient K\(_{Ca}\) current frequency and amplitude (Figure 5). In contrast, when boiled SOD and catalase (92°C, 30 minutes) were included in the pipette solution, diazoxide increased transient K\(_{Ca}\) current frequency to ≈213% of control. Collectively, data indicate that diazoxide elevates transient K\(_{Ca}\) current frequency and amplitude by inducing an elevation in mitochondria-derived ROS and suggest diazoxide does not activate RyR channels directly.

**Diazoxide Dilates Cerebral Arteries Because of RyR and K\(_{Ca}\) Channel Activation**

To investigate whether mitochondria-derived ROS induce vasodilation by activating RyR and K\(_{Ca}\) channels, diameter regulation of endothelium-denuded pressurized (60 mm Hg) cerebral arteries was measured. Arterial diameter regulation by a K\(_{ATP}\) channel opener was measured in control and then again in the same artery after MnTMPyP, thapsigargin, ryanodine, a RyR channel inhibitor, or iberiotoxin, a selective K\(_{Ca}\) channel blocker.

Diazoxide (100 \(\mu\)mol/L) and pinacidil (100 \(\mu\)mol/L) reversibly increased mean diameter by 25±3 \(\mu\)m (n=29) and 77±6 \(\mu\)m (n=21), respectively, from a baseline diameter of 142±3 \(\mu\)m, (Figure 6 and online data supplement). MnTMPyP (10 \(\mu\)mol/L), thapsigargin (100 nM), ryanodine (10 \(\mu\)mol/L), and iberiotoxin (100 nM) reduced mean diazoxide-induced dilations to between ≈23% and 36% of those obtained in control in the same arteries. In control, diazoxide induced reproducible dilations (second application was 98±4% of first, n=5, \(P<0.05\), online Figure II), indicating attenuated dilations with blockers were not attributable to receptor desensitization. In contrast, thapsigargin, ryanodine, iberiotoxin, or MnTMPyP did not alter dilations induced by pinacidil (100 \(\mu\)mol/L), which did not depolarize mitochondria, elevate ROS, or activate transient K\(_{Ca}\) currents. Data suggest diazoxide dilates pressurized cerebral arteries by inducing an elevation in ROS and by activating RyR and K\(_{Ca}\) channels. In contrast, pinacidil-induced dilations do not
occur because of ROS elevations, or RyR or KCa channel activation.

**Differential Regulation of Transient KCa Currents by Small and Large Mitochondrial Depolarizations**

We sought to determine mechanisms by which KATP channel openers elevate ROS, and to investigate mechanisms that lead to differential regulation of ROS and transient KCa currents by KATP channel openers and CCCP or rotenone. We tested the hypothesis that a small mitochondrial depolarization, such as that induced by diazoxide, elevates ROS and activates transient KCa currents, whereas a large mitochondrial depolarization reduces ROS and inhibits transient KCa currents. Thus, concentration-dependent regulation of mitochondrial potential, ROS, and transient KCa currents by CCCP, which elevates H+ permeability, were measured.

Figure 7A illustrates concentration-dependent regulation of TMRM fluorescence intensity by CCCP in a smooth muscle cell. A low concentration of CCCP (1 nM) decreased TMRM fluorescence intensity by 13±1%, similar to the effect of 100 μmol/L diazoxide (Figure 7B). In the same cell, a higher CCCP concentration (10 μmol/L) reduced TMRM...
fluorescence intensity by 84±3% (Figures 1 and 7A, n=40, P<0.05). In agreement with our hypothesis, 1 nM CCCP elevated DCF fluorescence intensity to 140±5% of control, whereas micromolar CCCP profoundly reduced DCF intensity (Figures 4 and 7C). Consistent with these observations, 1 nM CCCP increased mean transient KCa current frequency to 218±53% of control, whereas in the same cells, 10 μmol/L CCCP abolished transient KCa currents (Figure 7D and 7E). CCCP (1 nM) did not activate transient KCa currents by elevating global [Ca^{2+}]_i, because global [Ca^{2+}]_i did not change (see online data supplement). These data suggest a small mitochondrial depolarization activates transient KCa currents by elevating ROS, whereas a large mitochondrial depolarization inhibits these events by reducing ROS.

**Discussion**

The principal novel findings of this study are: (1) Mitochondria-derived ROS activate Ca^{2+} sparks and transient KCa currents in arterial smooth muscle cells. (2) This pathway is activated by antihypertensive KATP channel openers and leads to vasodilation. (3) Small and large mitochondrial depolarizations lead to differential regulation of ROS, Ca^{2+} sparks, and transient KCa currents.

KATP channel openers depolarize mitochondria in several cell types, including cardiac myocytes.\textsuperscript{10,11} We show that structurally distinct KATP channel openers depolarize mitochondria in arterial smooth muscle cells. Similarities and differences are apparent when comparing the effects of different KATP channel openers on mitochondrial potential in smooth muscle cells to findings in cardiac myocytes, where responses are well characterized. In heart cells, diazoxide, levromakalim, and pinacidil depolarize cardiac myocyte mitochondria, whereas in smooth muscle cells, pinacidil had no effect.\textsuperscript{11} However, KATP channel opener-induced mitochondrial depolarization is similarly reversed by glibenclamide and 5-HD in both cell types. One explanation for dissimilar mitochondrial pharmacology between cardiac and smooth muscle cells is that the identity or amino acid sequence of targets for KATP channel openers may differ slightly.

Inner mitochondrial membrane KATP channels have been isolated from several tissues, including heart and brain, and incorporated into proteoliposomes and lipid bilayers for characterization.\textsuperscript{10,11,16} MitoKATP channel-independent targets for KATP channel openers have also been described in mitochondria. In studies using submitochondrial particles isolated from pig heart, pinacidil and diazoxide did not alter mitochondrial potential but inhibited NADH dehydrogenase in ETC complex I and succinate dehydrogenase in ETC complex II, respectively.\textsuperscript{17} 5-HD may also be a substrate for acyl-CoA synthetase.\textsuperscript{17} Although mitoKATP has been characterized in isolated mitochondrial membranes from several cell types, there are no direct measurements of a smooth muscle mitoKATP channel. CCCP at 1 nM produced a similar mitochondrial depolarization, ROS elevation, and transient KCa current activation as 100 μmol/L diazoxide, indicating mitochondrial depolarization is the trigger for these changes. Rotenone reduced ROS, prevented the diazoxide-induced
ROS elevation, and blocked transient KCa currents, either when applied alone or together with diazoxide. Thus, the ETC appears to be the source of ROS generated in response to a small mitochondrial depolarization, and ETC inhibition reduces ROS and transient KCa currents. If diazoxide elevated ROS generation through complex II block, then pinacidil should have some effect by blocking the ETC upstream at complex I. In addition, KATP channel opener-induced mitochondrial depolarization and transient KCa current activation were inhibited by KATP channel blockers. Although effects mediated via ETC inhibition cannot be ruled out, diazoxide may act via an ETC-independent pathway in smooth muscle cells. Conceivably, diazoxide may activate mitoKATP channels to elicit mitochondrial depolarization, ROS generation, and transient KCa current activation.

Transient and sustained mitochondrial depolarizations occur under resting conditions and in response to stimuli. In the vasculature and in airway smooth muscle, mitochondrial generation of ROS can occur in response to several stimuli, including flow, temperature, and carbon monoxide, a heme-oxygenase-derived vasodilator. Data suggest regulation of Ca2+ sparks by mitochondria-derived ROS is an ongoing feedback process that can be up- or downregulated by changes in mitochondrial potential. A small mitochondrial depolarization leads to an increase in ROS generation and Ca2+ spark activation. In contrast, a large mitochondrial depolarization reduces ROS and inhibits Ca2+ sparks, consistent with the concept that under resting conditions, 1% to 2% of O2 used in the ETC is incompletely reduced, leading to O2/H2 generation. ETC blockers reduce ROS in pulmonary artery smooth muscle cells but increase ROS in renal artery smooth muscle cells, effects that may be attributable to different mitochondria in these cell types. Data presented here suggest the degree of mitochondrial depolarization also regulates both the magnitude and direction of ROS generation and thus, Ca2+ spark frequency in arterial smooth muscle cells.
Ca\(^{2+}\)-ATPases.\(^{21,24}\) Oxidizing and reducing agents have been shown to both activate and inhibit smooth muscle \(K_{Ca}\) channels.\(^{25-29}\) ROS also activate a number of other signal transduction pathways that may modulate RyR and \(K_{Ca}\) channels, including those mediated by protein kinases.\(^{22}\) At a physiological voltage of \(-40\) mV, mitochondria-derived ROS activated Ca\(^{2+}\) sparks and increased \(K_{Ca}\) channel sensitivity to Ca\(^{2+}\) sparks, resulting in transient \(K_{Ca}\) current frequency and amplitude elevations. Ca\(^{2+}\) spark activation occurred in the absence of a change in global Ca\(^{2+}\), suggesting ROS may mediate local communication between mitochondria and RyR channels. Supporting this local signaling concept, mitochondria and the SR are found within 20 nm of each other in smooth muscle cells.\(^{30}\)

A large mitochondrial depolarization blocks Ca\(^{2+}\) sparks and transient \(K_{Ca}\) currents because of permeability transition pore (PTP) opening.\(^{4}\) Data here suggest that a large mitochondrial depolarization also inhibits transient \(K_{Ca}\) currents by reducing mitochondrial-derived ROS, which explains why PTP blockers only partially attenuate rotenone-induced transient \(K_{Ca}\) current inhibition.\(^{4}\)

Previous studies that investigated the regulation of diazoxide-induced dilations by \(K_{Ca}\) channel blockers most commonly used vascular rings in which tone was established with a vasoconstrictor (eg, norepinephrine).\(^{31}\) In these investigations, \(K_{Ca}\) channel blockers did not significantly attenuate diazoxide-induced relaxations.\(^{31,32}\) However, vasoconstrictors strongly inhibit Ca\(^{2+}\) sparks, and intravascular pressure activates Ca\(^{2+}\) sparks.\(^{13,33,34}\) In a nonpressurized vascular ring exposed to a vasoconstrictor, Ca\(^{2+}\) spark frequency would be low, and mechanisms that dilate via Ca\(^{2+}\) spark activation would be blunted. In our study, cerebral arteries were pressurized to physiological pressure (60 mm Hg). Under these conditions, Ca\(^{2+}\) sparks occur frequently in smooth muscle cells (\(~1\) Hz), and Ca\(^{2+}\) spark and transient \(K_{Ca}\) current activation leads to vasodilation.\(^{1,13}\) Indeed, topical application of ROS dilates cerebral arteries in vivo via \(K_{Ca}\) channel activation.\(^{35}\) The remaining dilation induced by diazoxide in the presence of MnTMPyP, or Ca\(^{2+}\) spark or \(K_{Ca}\) channel blockers, is most likely attributable to sarcKATP channel activation, particularly because each blocker had similar effects.

\(K_{ATP}\) channel openers have been used in a wide variety of therapeutic applications.\(^{36}\) Because of relatively high specificity for pancreatic β-cell plasma membrane \(K_{ATP}\) channels, diazoxide is used to attenuate excessive insulin secretion but is also used to reduce blood pressure in severe hypertension. Nicorandil, a \(K_{ATP}\) channel opener and NO donor, is used in angina treatment. Our study reveals a novel vasodilatory pathway activated by \(K_{ATP}\) channel openers that could be exploited. Mitochondrial potential and transient \(K_{Ca}\) currents were most sensitive to diazoxide and insensitive to pinacidil, indicating distinct pharmacology when compared with arterial smooth muscle cell sarc\(K_{ATP}\) channels.\(^{12}\)

In summary, this study describes a novel dilatory signaling pathway activated by mitochondria-derived ROS. We show ROS activate Ca\(^{2+}\) sparks and transient \(K_{Ca}\) currents in arterial smooth muscle cells, leading to vasodilation, and we illustrate that this pathway can be stimulated by \(K_{ATP}\) channel openers. Data also suggest small and large mitochondrial depolarizations lead to differential regulation of ROS and Ca\(^{2+}\) sparks, indicating the degree to which mitochondria depolarize determines not only signal magnitude, but also direction.

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**References**


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**Figure 7.** Differential regulation of transient \(K_{Ca}\) currents by small and large mitochondrial depolarizations. A, Original recording illustrating TMRM intensity changes in the same cell with 1 nmol/L and 10 \(\mu\)mol/L CCCP. B, Mean changes in TMRM intensity induced by 1 nmol/L CCCP (n=5) and 100 \(\mu\)mol/L diazoxide (reproduced from Figure 1C for comparison). C, 1 nmol/L CCCP elevates DCF fluorescence (n=6). D, 1 nmol/L CCCP activates transient \(K_{Ca}\) currents, whereas 10 \(\mu\)mol/L CCCP blocks these events in a voltage-clamped (−40 mV) cell. E, Mean change in transient \(K_{Ca}\) currents induced by 1 nmol/L CCCP (n=8). *\(P<0.05\) when compared with control.


Mitochondria-Derived Reactive Oxygen Species Dilate Cerebral Arteries by Activating Ca^{2+} Sparks

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Supplemental Documentation

Results

DCF fluorescence measurements

In the presence of apocynin (25 µM), an NAD(P)H oxidase blocker, diazoxide elevated DCF fluorescence intensity to 145 ± 5% of control (n=10). Similarly, in the presence of oxypurinol (10 µM), a xanthine oxidase inhibitor, or 17-ODYA (10 µM), a cytochrome P450 blocker, diazoxide elevated DCF fluorescence intensity to 140 ± 5% (n=10) and 146 ± 7% (n=8) of control, respectively. P was >0.05 for each, when compared with diazoxide alone.

DHE fluorescence measurements

To establish whether the DHE preservation protocol elevated ROS in smooth muscle cells, arteries were pre-treated with MnTMPyP (10 µM) for 10 minutes prior to freezing and cryosectioning, and compared with control arteries that were not exposed to the antioxidant. When compared with controls, DHE fluorescence intensity of arteries pre-incubated with MnTMPyP were 103 ± 2% of those in control (P>0.05, n=10), indicating freezing and cryosectioning did not elevate ROS.

Intracellular Ca²⁺ concentration measurements

To investigate if 1 nM CCCP activated transient K_Ca currents by elevating global [Ca²⁺]i, ratiometric imaging of fura-2 in isolated cerebral artery smooth muscle cells was performed as previously described ¹. 1 nM CCCP did not alter global [Ca²⁺]i (101 ± 1% of control, n=10, P>0.05). Thus, 1 nM CCCP does not activate transient K_Ca currents by elevating global [Ca²⁺]i.
Pressurized artery diameter measurements

At 60 mmHg, arteries constricted to 59 ± 1 % of passive diameter (n=37). Passive diameter was 244 ±4 µm (n=37). Thapsigargin (100 nM), ryanodine (10 µM) and iberiotoxin (100 nM) reduced mean arterial diameter by 13 ± 2 (n=11), 16 ± 1 (n=10), and 10 ± 1 (n=15) µm, respectively (P<0.05 for each). MnTMPyP (10 µM) did not alter mean arterial diameter (2 ± 2 µm change, n=7, P>0.05), suggesting a reduction in total cellular ROS does not influence arterial contractility.
Detailed Materials and Methods

Tissue preparation

Animal procedures used were reviewed and approved by the Animal Care and Use Committee policies at the University of Tennessee. Sprague-Dawley rats (200-250 g) of either sex were euthanized by peritoneal injection of a sodium pentobarbital overdose (150 mg kg⁻¹). The brain was removed and placed into ice-cold (4°C), physiological saline solution (PSS) containing (in mM): 112 NaCl, 4.8 KCl, 26 NaHCO₃, 1.8 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 10 glucose, gassed with 74% N₂-21% O₂-5% CO₂ (pH 7.4). Posterior cerebral, cerebellar and middle cerebral arteries (50-200 µm in diameter) were removed, cleaned of connective tissue and maintained in ice-cold PSS. For single cell isolation, smooth muscle cells were dissociated from cerebral arteries using enzymes, as previously described².

TMRM imaging

Experiments were performed using a HEPES-buffered PSS containing (mM): 134 NaCl, 6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 10 glucose (pH 7.4, NaOH). Isolated cerebral artery smooth muscle cells were incubated in HEPES-buffered PSS containing TMRM (tetramethylrhodamine methyl ester, 100 nM) for 20 min followed by a 15 min wash. TMRM is a cationic fluorescent potentiometric indicator that accumulates in mitochondria due to their negative ΔΨₘ. To reduce spontaneous TMRM fluorescence decay, experiments were performed using HEPES-buffered PSS supplemented with 10 nM TMRM. Smooth muscle cells were excited with 535 nm light and background corrected fluorescence intensity was collected every 1 s at 610 nm using a Dage MTI integrating CCD camera and Ionwizard software (Ionoptix, Milton, MA).
Patch-clamp electrophysiology

Potassium currents were measured using either the conventional whole-cell or perforated-patch configuration of the patch-clamp technique using an Axopatch 200B amplifier and Clampex 8.2 (Axon Instruments, Union City, CA, USA). Bath solution was HEPES-buffered PSS (composition described above). For perforated-patch experiments, the pipette solution contained (mM): 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, 0.05 EGTA (pH 7.2 with KOH). For conventional whole-cell experiments, the pipette solution contained (mM): 140 KCl, 1.9 MgCl₂, 0.037 CaCl₂, 0.1 EGTA, 10 HEPES, 2 Na₂ATP (pH 7.2 with KOH); the calculated free Ca²⁺ and Mg²⁺ concentrations of this solution are 100 nM and 1 mM, respectively (WEBMAXC, Stanford University, CA, USA). The perforated-patch configuration was used, unless stated otherwise. Transient K₉⁺ currents were measured using a holding potential of −40 mV. Single K₉⁺ channel activity (number of channels multiplied by open probability, NPo) was measured at 0 mV. Membrane currents were filtered at 1 kHz and digitized at 4 kHz. In single K₉⁺ channel measurements, where appropriate CCCP (1 nM) was applied for at least 5 minutes prior to diazoxide (100 µM) application, to depolarize mitochondria. Transient K₉⁺ current analysis was performed off-line using methodology described elsewhere. A transient K₉⁺ current was defined as the simultaneous opening of three or more K₉⁺ channels. At least five minutes of continuous data was analyzed to calculate transient K₉⁺ current frequency and amplitude or K₉⁺ channel NPo under each condition.

ROS measurements

DCF (dichlorofluoroscein). Endothelium-denuded cerebral artery segments were incubated for 60 min at room temperature in HEPES-buffered PSS containing H₂DCFDA (10µM), followed by wash. DCF fluorescence in smooth muscle cells was excited with 488 nm light and emitted light greater than 530
nm was collected using a Zeiss LSM 5 laser-scanning confocal microscope. DCF fluorescence changes were measured in paired experiments. Images were acquired in the same arteries in control and then after 20 min with diazoxide (100µM) or pinacidil (100 µM) or 5 min with rotenone (10 µM) or CCCP (1 µM). Where appropriate, arteries were pre-incubated with catalase (2000 U/ml) for 2 h, or MnTMPyP (10 µM) or rotenone (10µM) for 5 min, prior to a 20 min application of diazoxide (100 µM). Since DCF fluorescence can increase spontaneously upon exposure to excitation light, the same smooth muscle cells were imaged only once. Vehicle time controls were also performed to ensure changes in fluorescence occurred due to test compounds and not due to spontaneous increases. ImageJ software was used to quantify changes in fluorescence. Images were background corrected. Data are presented as relative DCF intensity changes when compared with paired control.

*DHE (dihydroethidium)*. Endothelium-denuded cerebral artery segments were incubated for 20 min in HEPES-buffered PSS without (time-matched control) or with diazoxide (100 µM). Arteries were then embedded in OTC and snap frozen in liquid N2. 30 µm thick transverse sections were cut, mounted on glass slides, and incubated with DHE (10 µM) in a 100 % N2 atmosphere in the dark for 30 min at 37°C. Arterial sections were excited with 488 nm light and emitted light >590 was collected using a Noran Oz laser-scanning confocal microscope. Custom written software was used to measure DHE fluorescence intensity. Fluorescence intensity in diazoxide was divided by that in time-matched controls and multiplied by 100 to determine effects of diazoxide on DHE intensity.

**Confocal Ca^{2+} imaging**

Isolated smooth muscle cells were incubated in HEPES-buffered PSS containing fluo-4AM (10 µM) for 25 min at room temperature followed by a 30 min wash to allow indicator de-esterification. Smooth muscle cells were imaged using a Noran Oz laser scanning confocal microscope (Noran Instruments,
Middleton, WI, USA) and a 60 x water immersion objective (NA = 1.2) attached to a Nikon TE300 microscope. Cells were by illuminated at 488 nm using a krypton-argon laser and emitted light >500 nm was captured. 56.3 µm x 52.8 µm images were recorded every 8.3 ms (120 images s⁻¹). Laser intensity was set such that acquisition did not alter transient K_{Ca} current frequency or amplitude. Current and fluorescence measurements were synchronized using a light emitting diode placed above the recording chamber that was triggered during acquisition. Each cell was imaged for 15 s. Ca^{2+} sparks in smooth muscle cells were analyzed using analysis software written by Drs. A. Bonev and M.T. Nelson (University of Vermont). Detection of Ca^{2+} sparks was performed by dividing an area 1.54 µm (7 pixels) x 1.54 µm (7 pixels) (i.e. 2.37 µm²) in each image (F) by a baseline (F₀) which was determined by averaging 10 images without Ca^{2+} spark activity. The entire area of each image was analyzed to detect Ca^{2+} sparks. A Ca^{2+} spark was defined as a local increase in F/F₀ that was greater than 1.2. Global Ca^{2+} fluorescence was calculated from the same images used for Ca^{2+} spark analysis and was the mean pixel value of 100 different images acquired over 10 s.

**Pressurized artery diameter measurements**

An artery segment ~1-2 mm in length was cannulated at one end in a perfusion chamber containing PSS (Living Systems Instrumentation, Burlington, VT). The endothelium was removed by placing an air bubble into the lumen for 1 minute. After washout of the bubble with PSS, the other end of the artery was cannulated. The chamber was continuously perfused with 3-6 ml/min of PSS equilibrated with a mixture of 21% O₂-5% CO₂-74% N₂ to pH 7.4 and maintained at 37°C. The artery segment was observed with a charge-coupled device (CCD) camera attached to a Nikon TS 100-F inverted microscope. Arterial wall diameter was measured using the automatic edge-detection function of IonWizard software (IonOptix) and digitized at 1 Hz using a personal computer. Intravascular
pressure was adjusted by elevating an attached reservoir and monitored using a pressure transducer (Living Systems Instrumentation). Once a steady-state myogenic constriction had been obtained at 60 mmHg, tested compounds were applied by chamber perfusion. Where appropriate, MnTMP was applied for 15 minutes prior to diazoxide or pinacidil addition. Thapsigargin, ryanodine or iberiotoxin were applied for 10 minutes prior to $K_{\text{ATP}}$ channel opener addition. Endothelium denudation was confirmed by the absence of a response to bradykinin (10 µM), an endothelium-dependent dilator. Passive diameter was determined by perfusion with Ca$^{2+}$-free PSS containing EGTA (1 mM).

**Statistical Analysis**

Values are expressed as mean ± standard error of the mean (SEM). Students t-test was used for comparing paired and unpaired data from two populations, and ANOVA and Student-Newman-Keuls test were used for multiple group comparisons. Evaluation of whether distributions were Gaussian was by the method of Kolmogorov and Smirnov. Simultaneous spark and transient $K_{\text{Ca}}$ current amplitude data were fit with a first-order polynomial linear function and the slope ± SEM of each fit was compared using a Students t-test. No differences were observed between genders and data were pooled. P<0.05 was considered significant.
References


Supplemental Figure Legends

Supplemental Figure 1. Diazoxide activates $K_{\text{Ca}}$ channels by a mitochondria-dependent mechanism in cerebral artery smooth muscle cells. A, Original traces illustrating single $K_{\text{Ca}}$ channel activity measured at 0 mV in the same cell using the perforated patch-clamp configuration. $\text{Ca}^{2+}$ sparks and thus, transient $K_{\text{Ca}}$ currents were blocked with thapsigargin (100 nM). Diazoxide (100 µM) increased $K_{\text{Ca}}$ channel activity. B, mean effects of diazoxide (100 µM) on $K_{\text{Ca}}$ channel activity applied alone, or in the continued presence of CCCP (1 µM). Average $N_{\text{P}}$ values: for diazoxide alone; control, 0.037 ± 0.014, diazoxide, 0.065 ± 0.024 (n = 7); for diazoxide + CCCP; CCCP, 0.037 ± 0.014, diazoxide + CCCP, 0.026 ± 0.012 (n= 5).

Supplemental Figure 2. Consecutive diazoxide application produces reproducible dilations in pressurized (60 mmHg) cerebral arteries. Original traces illustrating changes in arterial diameter induced by an initial (black line) and a second (gray line, 10 minutes after washout of first) application of diazoxide (100 µM). These data indicate receptor desensitization cannot explain reductions in diazoxide-induced dilations induced by MnTMPyP, thapsigargin, ryanodine, or iberiotoxin.
Supplemental Figure 1

Panel A: Graphs showing the effect of Diazoxide (100 μM) on cell currents. The graph on the left shows the control conditions, and the graph on the right shows the effect of Diazoxide.

Panel B: Bar graph illustrating the effect of Diazoxide and Diazoxide + CCCP on NPo (% control). Diazoxide + CCCP shows a significant decrease compared to Diazoxide alone, indicated by the asterisk.