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Rotenone-stimulated superoxide release from mitochondrial complex I acutely augments L-type Ca^{2+} current in A7r5 aortic smooth muscle cells

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Ochi R, Dhagia V, Lakhkar A, Patel D, Wolin MS, Gupte SA. Rotenone-stimulated superoxide release from mitochondrial complex I acutely augments L-type Ca^{2+} current in A7r5 aortic smooth muscle cells. Am J Physiol Heart Circ Physiol 310: H1118–H1128, 2016. First published February 12, 2016; doi:10.1152/ajpheart.00889.2015.—Voltage-gated L-type Ca^{2+} current (I_{Ca,L}) induces contraction of arterial smooth muscle cells (ASMCs), and I_{Ca,L} is increased by H_{2}O_{2} in ASMCs. Superoxide released from the mitochondrial respiratory chain (MRC) is dismutated to H_{2}O_{2}. We studied whether superoxide per se acutely modulates I_{Ca,L} in ASMCs using cultured A7r5 cells derived from rat aorta. Rotenone is a toxin that inhibits complex I of the MRC and increases mitochondrial superoxide release. The superoxide content of mitochondria was estimated using mitochondrial-specific MitoSOX and HPLC methods, and was shown to be increased by a brief exposure to 10 μM rotenone. I_{Ca,L} was recorded with 5 mM BAPTA in the pipette solution. Rotenone administration (10 nM to 10 μM) resulted in a greater I_{Ca,L} increase in a dose-dependent manner to a maximum of 22.1% at 10 μM for 1 min, which gradually decreased to 9% after 5 min. The rotenone-induced I_{Ca,L} increase was associated with a shift in the current-voltage relationship (I-V) to a hyperpolarizing direction. DTT administration resulted in a 17.9% increase in I_{Ca,L} without a negative shift in I-V, and rotenone produced an additional increase with a shift. H_{2}O_{2} (0.3 mM) inhibited I_{Ca,L} by 13%, and additional rotenone induced an increase with a negative shift. Sustained treatment with Tempol (4-hydroxy tempo) led to a significant I_{Ca,L} increase but it inhibited the rotenone-induced increase. Staurosporine, a broad-spectrum protein kinase inhibitor, partially inhibited I_{Ca,L} and completely suppressed the rotenone-induced increase. Superoxide released from mitochondria affected protein kinases and resulted in stronger I_{Ca,L} preceding its dismutation to H_{2}O_{2}. The removal of nitric oxide is a likely mechanism for the increase in I_{Ca,L}.

L-type calcium channel; mitochondria; rotenone; smooth muscle; superoxide

NEW & NOTEWORTHY

Rotenone-induced superoxide release in mitochondria and acutely increased L-type Ca^{2+} channel currents (I_{Ca,L}) in A7r5 arterial smooth muscle cells. Rotenone is dismutated to hydrogen peroxide (H_{2}O_{2}), which increases I_{Ca,L}. However, the increase occurred in the presence of dithiothreitol or H_{2}O_{2}, indicating that superoxide per se caused an increase in I_{Ca,L}.

The increase was suppressed by protein kinase inhibitor staurosporine.

INFLUX OF CALCIUM IONS THROUGH L-TYPE Ca^{2+} CHANNEL CURRENTS (I_{Ca,L}) is the major source of higher [Ca^{2+}], which initiates contraction of arterial smooth muscle cells (ASMCs) and regulates blood pressure (30). I_{Ca,L} is modulated by neurotransmitters, hormones, and autacoids in the control of cardiovascular function (21, 24, 32). Regulation of hypoxic vasodilatation of systemic arteries and hypoxic vasoconstriction of the pulmonary artery are fundamental regulatory mechanisms to ensure the efficient supply of O_{2} throughout the human body. Hypoxia in tissues is detected by a change in reactive oxygen species (ROS) (18). Superoxide and H_{2}O_{2} are produced in tissues and regulate cellular function in various physiological and pathophysiological conditions (8, 14, 18, 22, 40, 42, 50). The main sources of superoxide are NADPH oxidase (NOX) localized in the plasma membrane and the mitochondrial respiratory chain (MRC) in the mitochondrial inner membrane (11, 14, 33, 51, 56).

ANG II binds to the angiotensin type-1 receptor and augments the release of superoxide by activating NOX in the vasculature (33). H_{2}O_{2} dismutated from superoxide generated by ANG II-stimulated NOX and from superoxide produced by exogenous xanthine oxidase plus hypoxanthine augments I_{Ca,L} in rat cerebral ASMCs (5); this increase is mediated by PKCα activation (5, 12, 32). H_{2}O_{2} also increases I_{Ca,L} via CaMKII-induced phosphorylation of Cav1.2 channels in rabbit (52) and rat ventricular myocytes (41). Activation of Cav1.2 channels regulates mitochondrial metabolism in guinea pig ventricular myocytes (46) via the actin cytoskeleton, which is connected to voltage-dependent anion channels (VDACs) in the mitochondrial outer membrane (45). In rat cerebral ASMCs, ROS generated from a subpopulation of mitochondria increases H_{2}O_{2} in microdomains adjacent to Cav1.2 channels, thus increasing Cav1.2b channel opening (13).

Complex I, a proton-pumping NADH:ubiquinone oxidoreductase with a molecular mass of approximately 1 MDa (54), is the first complex of the MRC in the inner membrane. MRC releases a considerable amount of superoxide during normal oxidative metabolism (11, 56). Rotenone, a phytoxicin, binds to complex I and increases the release of superoxide into the mitochondrial matrix (44, 56). In bovine coronary ASMC mitochondria, rotenone stimulates the release of superoxide, detectable by MitoSOX fluorescence (16). Chronic...
hypoxia and rotenone treatment increases \( I_{\text{Ca,L}} \) in HEK 293 cells that express a human cardiac L-type \( \text{Ca}^{2+} \) channel \( \alpha_1 \) subunit (9). Pararaut-generated superoxide-acutely increases high-K\(^+\)-induced \( I_{\text{Ca,L}} \)-dependent intracellular \( \text{Ca}^{2+} \) increase in rat renal afferent arterioles (47). However, it remains unclear how superoxide acutely regulates \( I_{\text{Ca,L}} \), probably because modulation by superoxide partly overlaps with \( \text{H}_2\text{O}_2 \)-induced modulation.

We studied acute superoxide-induced modulation of \( I_{\text{Ca,L}} \) using rotenone as a stimulator of superoxide release from mitochondria, inhibitors of \( \text{H}_2\text{O}_2 \)-mediated modulation, and \( \text{H}_2\text{O}_2 \) in cultured A7r5 ASMCs derived from embryonic rat thoracic aorta (25). Rotenone resulted in greater mitochondrial superoxide release, as estimated by the oxidation product of MitoSOX using HPLC, and an acute increase in \( I_{\text{Ca,L}} \). The increase was associated with a characteristic shift of the current-voltage (\( I-V \)) relationship to the hyperpolarizing direction. Rotenone-induced increase in \( I_{\text{Ca,L}} \) was strongly inhibited by Tempol, a SOD mimetic (48), and by staurosporine, a broad-spectrum protein-kinase inhibitor (31, 36). The increase was not affected by DTT or \( \text{H}_2\text{O}_2 \), indicating that per se was responsible for the increase.

**MATERIALS AND METHODS**

**Cell culture.** A7r5 smooth muscle cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained under 5% \( \text{CO}_2 \) at 37°C in DMEM supplemented with L-glutamine, 4.5 g/l glucose, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells at ~70% confluence were subcultured weekly using 0.05% trypsin-EDTA (Gibco 25300-054; Thermo Fischer Scientific, Grand Island, NY) for up to 20 passages. A portion of detached cells from a culture of more than five passages was transferred to a tube containing normal Tyrode (NT) solution at room temperature for patch-clamp experiments and aliquoted into microtubes for pretreatment with Tempol or staurosporine.

**Superoxide measurement using HPLC.** 2-Hydroxy-Mito-ethidium (2-OH-Mito-ethidium), the superoxide-specific product of MitoSOX (Mito-2-hydroxyethidium, MitoSOX Red; Thermo Fischer Scientific) was measured using a previously-described HPLC method (3, 55). Increasing concentrations of 2-OH-Mito-ethidium were loaded onto the column to generate a standard curve. Confluent A7r5 cells in 6-well plates were treated with 5 \( \mu \)M MitoSOX for 1 h and rotenone (10 \( \mu \)M) for 3 min. Wells that were treated with MitoSOX but not with rotenone served as controls. Cells were washed with PBS, and 100% acetaminol (HPLC grade; Sigma-Aldrich, St. Louis, MO) was added. Following this, cells were scraped from the 6-well plates and collected in 1-ml Eppendorf tubes, incubated at ~20°C for 1 h, and centrifuged at 11,000 rpm for 10 min at 4°C. The resulting pellets were assayed for protein concentration using the Bradford method. 2-OH-Mito-ethidium in supernatant was quantified using an HPLC system with an FP-1520 fluorescence detector (JASCO Analytical Instruments, Easton, MD) and a Beckman Ultrasphere column (C18) (5 \( \mu \), 250 \times 4.6 mm; Thermo Fischer Scientific). HPLC data were normalized by protein content.

**Solutions and drugs.** NT solution contained the following (in mM): NaCl, 135; KCl, 5.4; CaCl\(_2\), 1.8; MgCl\(_2\), 1; HEPES, 5; and glucose, 5.5; pH was adjusted to 7.4 using NaOH. The Ba\(^{2+}\)-solution contained the following (in mM): NaCl, 108; TEACl, 20; CsCl, 5.4; BaCl\(_2\), 10; MgCl\(_2\), 1; HEPES, 5; and glucose 5.5; pH was adjusted to 7.4 using NaOH. To obtain the background current for subtraction, 30 \( \mu \)M CdCl\(_2\) in a Ba\(^{2+}\)-solution was prepared. The pipette solution contained the following (in mM) C\(_5\)-aspartate, 115; TEACl, 20; MgCl\(_2\), 1; BAPTA, 5; Mg-ATP, 3; GTP, 0.2; and HEPES, 10; pH was adjusted to 7.2 using CsOH. All chemicals used to make solutions, and rotenone, \( \text{H}_2\text{O}_2 \), DTT, Tempol, and staurosporine were from Sigma-Aldrich.

**Patch-clamp.** A drop of cell suspension was placed in a chamber on a wide mechanical stage with XY control. Cells attached to the glass bottom were observed with an upright microscope (BX51; Olympus, Tokyo, Japan) using a \( \times 10 \) objective and \( \times 40 \) water-immersion objective with a long working distance. Solutions flowed continuously to the chamber by gravity with a flow rate of 2–4 ml/min during patch-clamp and were removed using an adapted U-tube. The first solution used was NT. After establishing patch-clamp, the NT solution was exchanged to the Ba\(^{2+}\)-solution. Solution exchange was conducted by manually switching the inlet tube to another solution container (time 0 for the exchange, indicated with an arrow in figures). It took ~30 s for the new solution to enter the chamber. Recording was performed near the entry point of the solution in the chamber. To minimize mechanical issues related to flow, the \( \times 40 \) objective was used only to select the target cell, position the patch pipette, and examine cells at the end of the experiment. Patch-clamp was conducted under the \( \times 10 \) objective. Patch pipettes were prepared from hard glass capillary tubing containing a glass filament using a micro pipette puller (P-97; Sutter Instrument, Novato, CA). Pipette resistance was relatively high (15–25 M\( \Omega \)) when filled with pipette solution. Following establishment of a gig-ohm seal by gentle suction, the membrane was ruptured using the Zap function of the amplifier. Junction potential was adjusted before establishment of the seal, and experimental drift was estimated after each experiment by removing the cell from the pipette in NT solution. Changes in junction potential were within ±2 mV. The voltage-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA) was driven by Clampex 9 software via a digital interface (Digidata 1332A; Molecular Devices). Currents were filtered at 2 kHz using the amplifier’s low-pass eight-pole Bessel filter. Membrane capacitance was estimated using the built-in program in Clampex. The average membrane capacitance was 66 pF. \( I_{\text{Ca,L}} \) for \( I-V \) was obtained by applying 500-ms depolarization steps in 10-mV increments at 0.2 Hz from ~40 to 50 mV, starting from a holding potential (HP) of ~80 mV. Test depolarization steps were preceded by a 50- or 70-ms prepulse to detect and inactivate the T-type Ca\(^{2+}\) channel current, which appeared infrequently and with relatively small amplitude. About 50 s were necessary to obtain one \( I-V \) and the series was repeated at approximately 2-min intervals. Following the application of rotenone and other agents, \( I-V \) was obtained starting after 1 min of perfusion. Quasi-steady-state inactivation curves (\( I_{\text{Ca,L}} \) for various \( I-V \) was generated using a gapped double-pulse protocol at 0.1 Hz. Two seconds of prepulse to potentials between ~100 and 30 mV in 10-mV increments from an HP of ~80 mV were followed by a 50- or 70-ms step to ~40 mV, then a 500-ms test pulse to 0 mV. In the presence of rotenone, \( I_{\text{Ca,L}} \) was obtained following a 5-min perfusion. Time lapse change in \( I_{\text{Ca,L}} \) was obtained with repetitive application of 500-ms pulses to ~15 mV (unless otherwise noted) preceded by a short prepulse at a rate of 1/20 s from a holding potential of ~80 mV. \( I_{\text{Ca,L}} \) was quantified after subtracting the current obtained in the presence of 30 \( \mu \)M Cd\(^{2+}\). Current traces shown in all figures display subtracted currents in which capacitive transients have sometimes been truncated for illustrative purposes. The effect of Tempol was examined after pretreatment with 1 mM Tempol at room temperature for periods longer than 40 min. In several experiments, additional overnight exposure at 4°C was performed to equilibrate mitochondria with Tempol. Patch-clamp experiments were conducted at room temperature.

**Data analysis.** \( I_{\text{Ca,L}} \) was analyzed using low-pass filtering with a cutoff frequency of 1 kHz with Clampfit v9 or v10 software (Molecular Devices). Statistical analysis was performed using Prism software (v6; GraphPad Software, San Diego, CA). Igor Pro (v6; Wave metrics, Portland, OR) was used for curve fitting and illustration. Subtraction of Cd\(^{2+}\)-resistant current was performed using the Table function of Igor Pro and Microsoft Excel. The \( I-V \) relationship of \( I_{\text{Ca,L}} \) was obtained by calculating means ± SE. \( I-V \) curves were obtained by
Rotenone induces a dose-dependent increase in ICa,L. ICa,L was identified from its voltage dependence and marked increase by 100 nM (−) Bay-K 8644 in preliminary experiments. The run-down of ICa,L was small in the present recordings with a thin pipette containing 5 mM BAPTA [cf. (38)]. To study time- and dose-dependent modulation of ICa,L by rotenone, various concentrations of rotenone (10 nM to 10 μM) were applied during repetitive depolarization to −15 mV at 20-s intervals (Fig. 2). Representative records at various concentrations show that ICa,L amplitude was clearly increased by 100 nM (Fig. 2c), 1 μM (Fig. 2d), and 10 μM (Fig. 2e) rotenone and that the increase rapidly decays during pulses, except at 100 nM. A plot of the time-lapse change in amplitude of ICa,L shows that the change mediated by 10 nM rotenone was negligible, 100 nM induced a rapid and transient increase, 1 μM produced a larger and more sustained increase, and that the largest increase was produced by 10 μM (Fig. 2A). The changes induced by 100 nM and 10 nM rotenone were different, with statistical significance only at 1 min, whereas the differences between 1 μM or 10 μM and 10 nM were highly significant, starting from 40 s until 5 min. The greatest increase was caused by 10 μM rotenone (22.1 ± 3.4%, n = 18) at 80 s, and the increase gradually decreased to 9% after 5 min. In the presence of rotenone, the amplitude at the end of the pulse (I500) initially increased only slightly at 100 nM, and gradually decreased in the presence of rotenone in a dose-dependent manner by 9% at 10 nM, 11% at 100 nM and 1 μM, and 19% at 10 μM. I500 in the presence of 10 μM and 10 nM rotenone differed with statistical significance (P < 0.05) after 3 min (Fig. 2B). Peak amplitude of ICa,L elicited by repetitive depo-

Fig. 2. Rotenone-induced dose- and time-dependent increase in ICa,L. a: test pulse, b–e: typical current traces with superposition of control (black) and trace with maximal increase of ICa,L (gray). b: 10 nM, c: 100 nM, d: 1 μM, e: 10 μM. A: Change in peak amplitude of ICa,L. B: change in ICa,L amplitude at 500 ms (I500) before and during application of various concentrations of rotenone (Rot). Test pulse to −15 mV was applied at 20-s intervals. Values shown are means ± SE of the ratio to the control amplitude obtained before switching to rotenone. n = 11, 10 nM; 12, 100 nM; 13, 1 μM; and 18, 10 μM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with the values obtained in the presence of 10 nM rotenone; #P < 0.05 compared with 1 μM rotenone.
larization pulses to 0 mV was significantly increased by 16.4 ± 2.2% \((n = 22)\) with 10 \(\mu M\) rotenone (data not shown).

**Effect of rotenone on current voltage relationship of \(I_{\text{Ca,L}}\).** After the maximal amplitude of \(I_{\text{Ca,L}}\) was stabilized, two control \(I-V\) measurements were recorded and 10 \(\mu M\) rotenone was introduced. The recording of test series in the presence of rotenone started 1 min after changing the solution. The difference between the two control \(I-V\) measurements (Con 1 and Con 2) was negligible (Fig. 3, b and c). Rotenone produced an acute increase in \(I_{\text{Ca,L}}\) at each potential between −30 and 10 mV (Fig. 3, A and B). The relative increase was greater at a small depolarization of −30 and −20 mV compared with that at a large depolarization. The larger increase at negative voltages is also apparent when comparing the current traces of −20 mV (Fig. 3c vs. Fig. 3d). Fitting of \(I-V\) curves using the Boltzmann equation showed that rotenone increased macroscopic conductance \((G_{\text{max}})\) by \(13.0\%\) \((P < 0.01)\) and induced a 2.6 mV leftward shift of \(V_{0.5}\) from −19.6 ± 1.3 mV (SD) to −22.2 ± 1.4 mV (SD, \(P < 0.001\)) in Figure 3A. Similar percentage increases in \(G_{\text{max}}\) and a leftward shift of \(V_{0.5}\) were also obtained in \(I-V\) measurements of normalized amplitude (Fig. 3B).

**Effect of DTT and \(H_2O_2\) on rotenone-induced increase in \(I_{\text{Ca,L}}\).** To examine the role of thiol-oxidation, thiol-glutathionylation, and thiol-nitrosylation in the rotenone-induced increase in \(I_{\text{Ca,L}}\), the effect of DTT was examined. DTT \((2 \text{ mM})\) applied during repetitive depolarization to −15 mV increased peak amplitude of \(I_{\text{Ca,L}}\) by 17.9 ± 2.5% \((n = 13, \text{Fig. 4A})\). Thereafter, 10 \(\mu M\) rotenone applied in the presence of DTT increased \(I_{\text{Ca,L}}\) by 18% in 1–2 min, resulting in an overall increase of 29.4 ± 4.6%. When the application of rotenone and DTT was conducted in a reverse order (Fig. 4B), DTT resulted in an \(I_{\text{Ca,L}}\) increase to a similar extent as in Figure 4A. In the \(I-V\) relationship, DTT produced a significant increase in \(I_{\text{Ca,L}}\) at voltages between −10 mV and 20 mV (Fig. 5), increased the \(G_{\text{max}}\) by 18.1%, and shifted \(V_{0.5}\) slightly in a positive direction by 0.9 mV. DTT did not induce significant changes in \(I_{500}\). Rotenone added in the presence of DTT resulted in a significant increase in \(I_{\text{Ca,L}}\) in both peak amplitude and \(I_{500}\) at potentials between −20 mV and 0 mV, increased \(G_{\text{max}}\) by 12.6%, and slightly shifted \(V_{0.5}\) to a hyperpolarizing direction by 1.5 mV.

The ineffectiveness of DTT in influencing the rotenone-induced increase in \(I_{\text{Ca,L}}\) suggests that \(H_2O_2\) is not involved in this process because it targets oxidation-sensitive amino acid residues, and the \(H_2O_2\)-induced increase in \(I_{\text{Ca,L}}\) due to CaMKII-dependent protein phosphorylation is suppressed by DTT (41). Rotenone was applied in the presence of \(H_2O_2\) to further clarify the involvement of \(H_2O_2\) in this process. Externally applied \(H_2O_2\) \((0.3 \text{ mM})\) inhibited \(I_{\text{Ca,L}}\) with a gradual onset, decreasing peak amplitude by 13.1 ± 3.6% \((\text{means ± SE, } n = 12)\) and \(I_{500}\) by 43.7 ± 4.5% in 5 min (Fig. 6A). Following inhibition, 10 \(\mu M\) rotenone in the presence of \(H_2O_2\) produced a rapid increase in \(I_{\text{Ca,L}}\) by 19% \((\text{Fig. 6B})\), which gradually declined. The subsequent application of DTT produced a large (Fig. 6C) and variable increase of 26% in peak amplitude. In the \(I-V\) relationship, 0.3 mM \(H_2O_2\) produced a comparable inhibition of \(I_{\text{Ca,L}}\) and \(I_{500}\) at voltages between −20 mV and 30 mV with statistical significance \((P < 0.001)\) (Fig. 7). Fitting \(I-V\) curves with the Boltzmann equation revealed an \(H_2O_2\)-induced decrease of 19% in \(G_{\text{max}}\) \((P < 0.0001)\) without a significant shift in \(V_{0.5}\). In the presence of \(H_2O_2\), 10 \(\mu M\) rotenone resulted in an \(I_{\text{Ca,L}}\) increase at negative potentials between −30 and −10 mV with statistical significance, increased \(G_{\text{max}}\) by 12%
Gmax (and 40 mV with statistical significance, with a 12% increase in period at 4°C; temperature, with or without an additional 15- to 20-h exposure activity (48). Cells were treated with 1 mM Tempol for periods membrane-permeable SOD mimetic of weak catalase-like ac-

calculated in the presence of Tempol. In the presence of Tempol, I_{Ca,L} was considerably suppressed by Tempol. In the presence of Tempol, rotenone shifted V_{0.5} from T2, -21.8 ± 1.2 mV to T + Rot, -23.4 ± 1.3 mV (P < 0.05).

Effect of staurosporine on I_{Ca,L} and rotenone-induced increase in I_{Ca,L}. I_{Ca,L} increase by a β-adrenergic agonist through PKA-dependent channel phosphorylation is associated with a negative shift of I-V in rabbit ventricular myocytes (43). To determine the involvement of phosphorylation in the superoxide-induced increase in I_{Ca,L}, we examined the effect of staurosporine (S), a broad-spectrum protein kinase inhibitor (31). Because I_{Ca,L} is modulated by the coordinated activity of protein kinases and protein phosphatases, S inhibits I_{Ca,L} in the presence of active phosphatases in mice ventricular myocytes (15). To obtain a steady-state effect of S, cells were pretreated with 100 nM S for 40 – 450 min (average 165 min). I_{Ca,L} was inhibited by the sustained application of 100 nM S, with a decrease of the maximal density in I-V from 4.21 ± 0.30 pA/pF (control, means ± SE, n = 40) to 2.67 ± 0.40 pA/pF (S, n =

(P < 0.01) and shifted V_{0.5} by 2.9 mV (P < 0.001) in a negative direction. In the presence of H_{2}O_{2} and rotenone, DTT led to a further increase in I_{Ca,L} at voltages between -10 mV and 40 mV with statistical significance, with a 12% increase in G_{max} (P < 0.05) and a V_{0.5} shift in a positive direction by 1.8 mV (P < 0.05). The decrease in I_{SO} induced by H_{2}O_{2} was not significantly affected by rotenone or by DTT.

Reduction of rotenone-induced increase in I_{Ca,L} by Tempol.

To determine the involvement of superoxide in a rotenone-induced increase in I_{Ca,L}, cells were treated with Tempol (T), a membrane-permeable SOD mimetic of weak catalase-like activity (48). Cells were treated with 1 mM Tempol for periods of time greater than 40 min (usually 4 to 6 h) at room temperature, with or without an additional 15- to 20-h exposure period at 4°C; I_{Ca,L} was then measured in the presence of Tempol. Rotenone (10 μM) applied during repetitive depolarization increased I_{Ca,L} amplitude transiently by 5.2% at its maximum (Fig. 8A). This rotenone-induced increase was sig-

Fig. 4. Time-dependent changes in I_{Ca,L} induced by DTT and rotenone. A: effect of 2 mM DTT on basal I_{Ca,L} and on 10 μM rotenone (Rot)-induced modulation of I_{Ca,L}. Test pulse to −15 mV was applied at 20-s intervals. Values shown are means ± SE from 13 cells plotted vs. time. a: superposed traces of control (black) in the presence of DTT (dark gray) and DTT + 10 μM Rot (gray). B: effect of 10 μM rotenone (shown in Fig. 1) and 2 mM DTT in the presence of rotenone. Values shown are means ± SE, n = 18, a and b: typical traces [a, control (black) after 1 min in the presence of rotenone (dark gray) and after 5 min (gray); b, after 5 min in the presence of rotenone (black) and in the presence of rotenone and DTT (dark gray)].

Fig. 5. Effect of DTT and rotenone (Rot) on I-V relationship of I_{Ca,L}. a–d: typical current traces (a: Con 1; b: Con 2; c: 2 mM DTT; d: 2 mM DTT + 10 μM Rot recorded with a 2-min interval). Traces are shown in black from -40 to 0 mV, in gray from 10 to 50 mV, and by a thick line at -20 mV. In I-V, normalized amplitude by the maximal amplitude of control (values shown are means ± SE, n = 10) is plotted vs. voltage. The curves of Con 2 and Con 1, obtained 1–2 min before Con 2, almost overlap. Statistical comparison was performed between values obtained before and after the exchange. *P < 0.05, ****P < 0.0001 for the exchange from control to DTT. #P < 0.01, ###P < 0.001 for that from DTT to DTT + Rot. I-V was fitted using the Boltzmann equation. V_{0.5} (mV): Con 1, -19.0 ± 1.4; Con 2, -18.9 ± 1.4; 2 mM DTT, -17.8 ± 1.3; 2 mM DTT + 10 μM Rot, -19.3 ± 1.3, n = 10.
that $S_2$ decreased slightly compared with $S_1$, and that $P$ increased it at large positive potentials. Rotenone decreased at voltages between $12$, $P$ decreased in $G_{\text{max}}$ from $81.5 \pm 4.4$ pS/pF ($S_1$, $P < 0.001$) and $V_{0.5}$ of activation was shifted by $S$ in a negative direction from control by $-19.6 \pm 1.2$ mV to $-22.3 \pm 1.4$ mV ($S_1$, $P < 0.001$). Figure 9, $a$–$c$, is a representative record showing that 10 µM rotenone had little effect on $I_{\text{Ca,L}}$ in the presence of $S$. An $I$–$V$ relationship of normalized amplitude (Fig. 9B) shows that $S_2$ decreased slightly compared with $S_1$, and that 10 µM rotenone applied after $S_2$ did not affect the peak amplitude of $I_{\text{Ca,L}}$ at the negative membrane potentials but slightly decreased it at large positive potentials. Rotenone decreased $I_{500}$ at voltages between $10$ and $20$ mV.

**Effect of rotenone on steady-state inactivation and window current of $I_{\text{Ca,L}}$.** The rotenone-induced increase in $I_{\text{Ca,L}}$ gradually decreased along with $I_{500}$ over $5$ min (Fig. 2). These findings suggest that rotenone augments the voltage-dependent inactivation of $I_{\text{Ca,L}}$. We examined the effect of rotenone on a quasi-steady state inactivation curve ($f_{\varphi}$-$V$). In Figure 10, control $f_{\varphi}$-$V$ preceded control $I$–$V$ before the application of rotenone, and $f_{\varphi}$-$V$ in the presence of rotenone was obtained after $5$–$10$ min treatment with rotenone. $f_{\varphi}$-$V$ was shifted by $10$ µM rotenone in a hyperpolarizing direction. Fitting of $f_{\varphi}$-$V$ using the Boltzmann equation showed a change in $V_{0.5}$ from $-33.0$ mV in the control to $-38.4$ mV in the presence of $12$, $P < 0.05$) (Fig. 9A). Compared with control, $S$ led to a decrease in $G_{\text{max}}$ from $81.5 \pm 6.7$ to $51.8 \pm 4.4$ pS/pF ($S_1$, $P < 0.001$) and $V_{0.5}$ of activation was shifted by $S$ in a negative direction from control by $-19.6 \pm 1.2$ mV to $-22.3 \pm 1.4$ mV ($S_1$, $P < 0.001$). Figure 9, $a$–$c$, is a representative record showing that 10 µM rotenone had little effect on $I_{\text{Ca,L}}$ in the presence of $S$. An $I$–$V$ relationship of normalized amplitude (Fig. 9B) shows that $S_2$ decreased slightly compared with $S_1$, and that 10 µM rotenone applied after $S_2$ did not affect the peak amplitude of $I_{\text{Ca,L}}$ at the negative membrane potentials but slightly decreased it at large positive potentials. Rotenone decreased $I_{500}$ at voltages between $10$ and $20$ mV.

**Effect of rotenone on steady-state inactivation and window current of $I_{\text{Ca,L}}$.** The rotenone-induced increase in $I_{\text{Ca,L}}$ gradually decreased along with $I_{500}$ over $5$ min (Fig. 2). These findings suggest that rotenone augments the voltage-dependent inactivation of $I_{\text{Ca,L}}$. We examined the effect of rotenone on a quasi-steady state inactivation curve ($f_{\varphi}$-$V$). In Figure 10, control $f_{\varphi}$-$V$ preceded control $I$–$V$ before the application of rotenone, and $f_{\varphi}$-$V$ in the presence of rotenone was obtained after $5$–$10$ min treatment with rotenone. $f_{\varphi}$-$V$ was shifted by $10$ µM rotenone in a hyperpolarizing direction. Fitting of $f_{\varphi}$-$V$ using the Boltzmann equation showed a change in $V_{0.5}$ from $-33.0$ mV in the control to $-38.4$ mV in the presence of $12$, $P < 0.05$) (Fig. 9A). Compared with control, $S$ led to a decrease in $G_{\text{max}}$ from $81.5 \pm 6.7$ to $51.8 \pm 4.4$ pS/pF ($S_1$, $P < 0.001$) and $V_{0.5}$ of activation was shifted by $S$ in a negative direction from control by $-19.6 \pm 1.2$ mV to $-22.3 \pm 1.4$ mV ($S_1$, $P < 0.001$). Figure 9, $a$–$c$, is a representative record showing that 10 µM rotenone had little effect on $I_{\text{Ca,L}}$ in the presence of $S$. An $I$–$V$ relationship of normalized amplitude (Fig. 9B) shows that $S_2$ decreased slightly compared with $S_1$, and that 10 µM rotenone applied after $S_2$ did not affect the peak amplitude of $I_{\text{Ca,L}}$ at the negative membrane potentials but slightly decreased it at large positive potentials. Rotenone decreased $I_{500}$ at voltages between $10$ and $20$ mV. 
rotenone, and a rotenone-mediated decrease in c0, a voltage-independent constant, from 0.11 to 0.06 (Fig. 10, a, b, and A). From the I-V curve shown in Figure 3A and the f-v-V relationships, the window current (I_{WD}) was calculated as the product of the simulated I-V and f_{v-V} curves at the voltage range between −40 and 0 mV [Fig. 10B (34)]. In the control, the I_{WD} density was maximal at a voltage between −20 and −10 mV. Rotenone shifted the peak of I_{WD} to −20 mV and increased the density of I_{WD} by 20–30% at a voltage range between −30 mV and −20 mV (Fig. 10B, Rot 1). The shift of f_{v-V} to a hyperpolarizing direction in the presence of rotenone decreased window I_{WD} at all voltage ranges, a decrease more prominent at voltages more positive than −20 mV. Because I_{Ca,L} decreases over time in the presence of rotenone (Fig. 2), the actual decrease in I_{WD} at 5 min was expected to be more extensive than the curve shown as Rot 2 in Figure 10B.

**DISCUSSION**

Rotenone augmented mitochondrial superoxide release estimated by mitochondrial-specific MitoSOX products using HPLC in A7r5 cells (Fig. 1), as shown previously by the
increase in MitoSOX fluorescence in bovine coronary ASMCs (16). In this study we found that rotenone resulted in an acute increase in \( I_{\text{Ca,L}} \). BAPTA (5 mM) in the pipette solution separated the superoxide-dependent increase in \( I_{\text{Ca,L}} \) from the increase produced by \( \text{H}_2\text{O}_2 \) mediated by CaMKII in rat ventricular myocytes (41) and PKC\( \alpha \) in rat cerebral artery ASMCs (5, 12). Furthermore, \( I_{\text{Ca,L}} \) was increased by rotenone in the presence of \( \text{H}_2\text{O}_2 \), which induced weak inhibition of \( I_{\text{Ca,L}} \). In addition, a rotenone-induced increase occurred in the presence of DTT, which suppresses the \( \text{H}_2\text{O}_2 \)-induced increase in \( I_{\text{Ca,L}} \) in rat ventricular myocytes (41). The inhibition of rotenone-induced \( I_{\text{Ca,L}} \) increase by Tempol, a membrane-permeable SOD mimetic (48), further supports the notion that superoxide can increase \( I_{\text{Ca,L}} \) prior to its dismutation to \( \text{H}_2\text{O}_2 \).

The percentage of \( \text{O}_2 \) converted to superoxide in normal oxidative metabolism of the MRC is known to be \(-2\%\) (11, 44). Complexes I and III of the MRC are major mitochondrial superoxide release sites, into the matrix, and into the matrix and intermembrane space, respectively (7), although complex II and other proteins also contribute to the release (7, 17). Rotenone is a complex I inhibitor and augments superoxide release in neurons (44), ASMCs from bovine coronary artery (16), rat renal artery (29), and A7r5 cells (Fig. 1). Superoxide released from complex I is dismutated to \( \text{H}_2\text{O}_2 \) by MnSOD in the matrix and by Cu/ZnSOD in the cytoplasm and intermembrane space (44). Superoxide in the matrix must traverse the inner and outer mitochondrial membranes to reach the cytoplasm (Fig. 11). We hypothesize that the superoxide efflux is facilitated by physiological openings in the mitochondrial permeability transition pores composed of the c-subunit ring of FI50 ATP synthase in the inner mitochondrial membrane (1) and VDACs in the outer mitochondrial membrane, which is inhibited by 4'-disothiocyanato-2,2'-disulfonic acid stilbene (DIDS) (19). It has been shown that rotenone augments the release of superoxide from the matrix to the outside of mitochondria isolated from mice skeletal muscle, and that the release is largely enhanced by knockdown of the MnSOD gene and suppressed by DIDS (28). Because a population of mitochondria is located close to the \( \text{Ca}_V \) channel complex in rat cerebral ASMCs (13) and \( \text{Ca}_V \) is mechanically connected via cytoskeletal proteins to VDACs in the outer membrane of mitochondria in guinea pig ventricular myocytes (45), it seems possible that superoxide could reach targets outside of the mitochondria and modulate \( \text{Ca}_V \) function.

The suppression of the rotenone-induced \( I_{\text{Ca,L}} \) increase by Tempol indicates that superoxide is involved in this process.

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**Fig. 10.** Effect of rotenone on steady-state inactivation and window current of \( I_{\text{Ca,L}} \). \( a \) and \( b \) show typical current traces during test pulse to 0 mV after conditioning steps shown to the left (\( c \): control; \( h \): in the presence of 10 \( \mu \)M rotenone). \( A \): quasi-steady-state inactivation curves (\( f_a-V \)) in control and after 5- to 10-min application of 10 \( \mu \)M rotenone; values shown are means \( \pm \) SE, \( n = 17 \). Curves were obtained by fitting with the Boltzmann equation. \( V_{1/2} \) for 50% reduction of the voltage-dependent component was \(-33.0 \text{ mV} \) for control and \(-38.4 \text{ mV} \) for rotenone. \( B \): effect of 10 \( \mu \)M rotenone on window \( I_{\text{Ca,L}} \) (\( I_{\text{WD}} \)) obtained as the product of the fitting curve of \( I-V \) of the peak amplitude in Fig. 3A and of \( f_a-V \) in Fig. 10A. Control \( I_{\text{WD}} \) represents the product of control \( I-V \) and control \( f_a-V \); \( I_{\text{WD}} \) of Rot 1 and Rot 2 were obtained as the product of \( I-V \) obtained from 1-2 min in the presence of rotenone and control \( f_a-V \) and the product of the same \( I-V \) and \( f_a-V \) obtained after a 5-min application of rotenone, respectively.

**Fig. 11.** Model for the superoxide-induced increase in \( I_{\text{Ca,L}} \) in A7r5 aortic smooth muscle cells. Rotenone stimulates superoxide (\( \text{O}_2^{\cdot -} \)) release from complex I in the inner mitochondrial membrane to the matrix. Superoxide diffuses out of the matrix to the cytoplasm through the inner mitochondrial membrane, intermembrane space (IMS), and outer mitochondrial membrane. Superoxide is partly dismutated to \( \text{H}_2\text{O}_2 \) by SOD in the matrix, IMS, and in the cytoplasm. \( \text{H}_2\text{O}_2 \) activates PKC\( \alpha \) and increases \( I_{\text{Ca,L}} \). However, DTT prevents thiol-oxidation by \( \text{H}_2\text{O}_2 \) and prevents PKC\( \alpha \) activation, and BAPTA from the pipette solution decreases cytoplasmic [\( \text{Ca}^{2+} \)] and inhibits the PKC\( \alpha \) activation. Superoxide binds nitric oxide (NO) and generates peroxynitrite (ONOO\(^{-} \)), which can increase \( I_{\text{Ca,L}} \). However, DTT inhibits ONOO\(^{-} \)–induced thiol-nitrosylation and inhibits the increase. NOS-derived NO activates guanylate cyclase (GC) and generates cGMP, which activates PKG, resulting in inhibition of \( I_{\text{Ca,L}} \). Reduction of NO by superoxide-downregulates PKG and increases \( I_{\text{Ca,L}} \). Tempol decreases the concentration of superoxide. Staurosporine inhibits PKC\( \alpha \) and PKG and eventually inhibits the superoxide-induced increase in \( I_{\text{Ca,L}} \).
Although further study is necessary, the Tempol-induced increase in $I_{\text{Ca,L}}$ and shift of $I-V$ to a negative direction could be explained by the direct action of Tempol on the Cav1.2 channel, since Tempol increases the $I_{\text{Ca,L}}$ current by direct action on the BK$_{\text{Ca}}$ α subunit in ASMSCs from rat mesenteric artery (53). $I_{\text{Ca,L}}$ is closely regulated by protein phosphorylation, even under basal conditions, through the balanced activity of protein kinases and protein phosphatases in cardiac myocytes and smooth muscle cells (20, 21, 24, 32). Staurosporine, originally isolated from the culture medium of Actinomycetes, exhibits broad-spectrum protein kinase inhibitor activities (31, 36). The pIC$_{50}$ of staurosporine against protein kinases ranges from 8 to 9 for most PKC, PKA, CaMKII, and tyrosine kinases (31). Staurosporine decreases $I_{\text{Ca,L}}$ in the presence of phosphatase activity in mouse ventricular myocytes (15). Here, staurosporine (100 nM) progressively inhibited $I_{\text{Ca,L}}$ and suppressed the rotenone-induced $I_{\text{Ca,L}}$ increase (Fig. 9). This finding indicates that protein phosphorylation supports basal $I_{\text{Ca,L}}$ in A7r5 cells and that superoxide modifies protein kinase-dependent modulation of $I_{\text{Ca,L}}$. The staurosporine-induced suppression of the increase in $I_{\text{Ca,L}}$ rules out any direct interaction of superoxide or rotenone with Cav1.2 channels from the mechanism of the rotenone-induced increase in $I_{\text{Ca,L}}$.

Thiol residues of channel proteins or their modulatory proteins are oxidized or nitrosylated in the initial step of ROS-induced regulation of protein function (22, 50, 51). H$_2$O$_2$-induced inhibition of $I_{\text{Ca,L}}$ is likely due to protein thiol oxidation because it is reversible by DTT (cf. Figs. 6 and 7). NO synthase (NOS) is expressed in ASMSCs and in endothelial cells (6). Because superoxide rapidly binds NO to produce peroxynitrite (ONOO$^-$), it has dual roles in the presence of NO as a scavenger of NO and as a precursor of ONOO$^-$ (Fig. 11). DTT inhibits ONOO$^-$-induced increases of high K+-induced contraction in guinea pig gall bladder smooth muscle strips (2). ONOO$^-$ binds with thiol residues of proteins to result in S-nitrosylation and S-nitrosoglutathionylation of proteins (10). Extracellular application of S-nitrocysteine and S-nitrosoglutathione (GSNO) augments $I_{\text{Ca,L}}$ in ferret ventricular myocytes (10), while GSNO induces inhibition of $I_{\text{Ca,L}}$ in Cav1.2b-expressing HEK293 cells (37). These GSNO-induced $I_{\text{Ca,L}}$ changes are inhibited by DTT (10, 37) (Fig. 11). Moreover, protein S-nitrosylation in the diabetic rat heart is reduced by DTT (35). Because DTT had little effect on the superoxide-induced increase in $I_{\text{Ca,L}}$, the contribution of thiol oxidation, S-nitrosylation, and S-nitrosoglutathionylation to the increase in $I_{\text{Ca,L}}$ could be ruled out. H$_2$O$_2$ transfers two electrons during oxidation while superoxide transfers one (49), suggesting that difference in the chemical reactivity may explain the lower sensitivity of superoxide-induced modification to DTT.

NO activates guanylate cyclase (GC) to generate cGMP, which in turn activates PKG. Application of 8-BrcGMP, a membrane-permeable cGMP analog, inhibits $I_{\text{Ca,L}}$ in A7r5 cells (27) and the PKG inhibitor Rp-8-Br-PET-cGMPS rapidly induces a moderate increase in $I_{\text{Ca,L}}$ in rabbit portal vein smooth muscle cells (39). Staurosporine-induced inhibition of PKG could abolish NO-induced inhibition prior to the application of rotenone, thus eliminating the NO-scavenging effect of superoxide to increase $I_{\text{Ca,L}}$. The hypothetical cGMP-PKG-mediated mechanism of rotenone-induced $I_{\text{Ca,L}}$ increase (shown in Fig. 11) proceeds as follows:

$\text{Rotenone} \rightarrow \text{complex I superoxide release} \uparrow \rightarrow \text{matrix superoxide} \uparrow \rightarrow \text{cytoplasmic superoxide} \uparrow \rightarrow \text{cytoplasmic NO} \downarrow \rightarrow \text{GC activity} \downarrow \rightarrow \text{cGMP} \downarrow \rightarrow \text{PKG} \downarrow \rightarrow \text{I}_{\text{Ca,L}} \uparrow$.

The notion that a superoxide-induced leftward shift in the $I-V$ relationship by the decrease of NO is seemingly not compatible with the absence of a detectable $I-V$ shift in 8-BrcGMP-induced inhibition of $I_{\text{Ca,L}}$ in rabbit portal vein smooth muscle cells (23), and an NO donor-induced inhibition of $I_{\text{Ca,L}}$ in rat vestibular hair cells (4). The sustained treatment by staurosporine shifted the $V_{0.5}$ to a negative direction, however. If $V_{0.5}$ contributed by basal PKG activation is more positive than in its absence, suppression of PKG by superoxide or staurosporine could shift $V_{0.5}$ to a negative direction. Superoxide generated by the xanthine/xanthine oxidase system augments the activity of purified PKC isolated from rat brain, and the activation is associated with a marked depletion of Zn$^{2+}$, thereby, superoxide-induced activation of PKC was partially (about 30%) suppressed by DTT (26). Direct modification of such regulatory proteins by superoxide may contribute to the mechanism of superoxide-induced increase in $I_{\text{Ca,L}}$.

The superoxide-induced transient $I_{\text{Ca,L}}$ increase associated with a shift in the $I-V$ relationship in a hyperpolarizing direction increased window $I_{\text{Ca,L}}$ close to the resting potential. Increased $I_{\text{WD}}$ is expected to transiently augment the contraction of ASMCs. However, the effect of superoxide on [Ca$^{2+}$]i, increase of ASMCs is overlapped with that of H$_2$O$_2$ and ROS-induced modulation of potassium current. Sustained pre-treatment by rotenone did not affect high-K$^+$-induced contraction of bovine coronary artery rings (16), and rotenone-induced ROS production results in relaxation of rat renal arterial rings by increasing voltage-dependent K$^+$ current, while it induces constriction of rat pulmonary artery by decreasing the K$^+$ current (29). The effect of modulation of $I_{\text{Ca,L}}$ by mitochondrial ROS on arterial contraction is considered to be variable among ASMSCs from different vascular beds and this may originate from the difference in mechanisms influenced by superoxide and/or hydrogen peroxide.

**Limitation of the present study.** Staurosporine is a broad-spectrum protein kinase inhibitor. Although suppression by staurosporine of the superoxide-induced increase in $I_{\text{Ca,L}}$ suggests the involvement of one or more protein kinases, no responsible protein kinase was identified.

In conclusion, the complex I inhibitor rotenone resulted in stimulating superoxide release in mitochondria and increasing $I_{\text{Ca,L}}$ recorded with a BAPTA-containing pipette associated with a shift in $I-V$ to a hyperpolarizing direction. Because the increase was suppressed by the SOD mimetic Tempol, and was not affected by H$_2$O$_2$ or DTT, it was concluded that superoxide (but not H$_2$O$_2$) was responsible for this increase. From its occurrence in the presence of DTT, the involvement of peroxynitrite or S-nitrosylation was excluded from the mechanism. Staurosporine suppression of the increase in $I_{\text{Ca,L}}$ indicates that protein kinase is involved in this mechanism. Although further experiments are necessary to confirm the NO-scavenging effect of superoxide as the mechanism of $I_{\text{Ca,L}}$ increase, with other mechanisms possible, this study has provided novel evidence of mitochondrial superoxide-mediated increase in $I_{\text{Ca,L}}$. 

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SUPEROXIDE-INDUCED $I_{Ca,L}$ INCREASE

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AUTHOR CONTRIBUTIONS
R.O. and S.A.G. conception and design of research; R.O., V.D., A.L., D.P., M.S.W., and S.A.G. approved final version of manuscript. D.P. performed experiments; R.O. and A.L. analyzed data; R.O., M.S.W., and S.A.G. interpreted results of experiments; R.O. prepared figures; R.O. drafted manuscript; R.O., M.S.W., and S.A.G. edited and revised manuscript; R.O., V.D., A.L., D.P., M.S.W., and S.A.G. approved final version of manuscript.

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