Metabolic inhibition with cyanide induces calcium release in pulmonary artery myocytes and *Xenopus* oocytes

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We examined the effects of metabolic inhibition on intracellular Ca²⁺ release in single pulmonary arterial smooth muscle cells (PASMCs). Severe metabolic inhibition with cyanide (CN, 10 mM) increased intracellular calcium concentration ([Ca²⁺]i) and activated Ca²⁺-activated Cl⁻ currents (I_CaCa) in PASMCs, responses that were greatly inhibited by BAPTA-AM or caffeine. Mild metabolic inhibition with CN (1 mM) increased spontaneous transient inward currents and Ca²⁺ sparks in PASMCs. In *Xenopus* oocytes, CN also induced Ca²⁺ release and activated I_CaCa, and these responses were inhibited by thapsigargin and cyclopiazonic acid to deplete sarcoplasmic reticulum (SR) Ca²⁺, whereas neither heparin nor anti-inositol 1,4,5-trisphosphate receptor (IP₃R) antibodies affected CN responses. In both PASMCs and oocytes, CN-evoked Ca²⁺ release was inhibited by carbonyl cyanide m-chlorophenylhydrazone (CCCP) and oligomycin or CCCP and thapsigargin. Whereas hypoxic stimuli resulted in Ca²⁺ release in pulmonary but not mesenteric artery myocytes, CN induced release in both cell types. We conclude that metabolic inhibition with CN increases [Ca²⁺]i in both pulmonary and systemic artery myocytes by stimulating Ca²⁺ release from the SR and mitochondria.

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established finding that hypoxia exposure induces Ca\(^{2+}\) release in myocytes from pulmonary but not systemic arteries (11, 12, 28), led us to question whether Ca\(^{2+}\) release from intracellular stores after metabolic inhibition is a unique response in PASMCs. Here, we sought to examine the cellular mechanisms underlying CN-induced Ca\(^{2+}\) release in freshly isolated PASMCs and *Xenopus* oocytes to determine whether CN-induced Ca\(^{2+}\) release is a response unique to PASMCs and to compare CN and hypoxia-induced Ca\(^{2+}\) release in pulmonary and systemic arterial myocytes.

**METHODS**

**Cell preparation.** Single smooth muscle cells of rat resistance (external diameter <300 mm) pulmonary arteries were isolated as described previously (32). Briefly, female or male Sprague-Dawley rats were euthanized by intraperitoneal injection of sodium pentobarbital (150 mg/kg) under approved animal care and use protocols. The heart and lungs were rapidly removed en bloc and placed in normal physiological saline solution (PSS). After the connective tissues and endothelium were removed, resistance pulmonary arteries were cut into small pieces (1 × 10 mm). The tissue was incubated in nominally Ca\(^{2+}\)-free PSS (1.5 ml) containing 2 mg papain (Worthington) and 0.2 mg dithioerythritol (Sigma) for 20 min (37°C), then in nominally Ca\(^{2+}\)-free PSS containing 0.5 mg type H collagenase (Sigma), 1.0 mg type F collagenase (Sigma), and 100 μM Ca\(^{2+}\) for 10–15 min (37°C), and finally in ice cold nominally Ca\(^{2+}\)-free PSS for 10–15 min. Single cells were harvested by gentle trituration and then stored on ice for use up to 8 h.

Freshly isolated rat mesenteric artery smooth muscle cells were prepared using the same procedure as described above.

*Xenopus* oocytes were prepared as described previously (31). Adult female *Xenopus* laevis were euthanized by the anesthetic aminobenzoic acid ethyl ester. Ovarian fragments were gently teased out with forceps, and oocytes were defolliculated in Ca\(^{2+}\)-free Barth’s medium containing collagenase (20 mg/ml) at a temperature of 19°C. Stage V or VI *Xenopus* oocytes were selected for experiments.

**Membrane current recording.** Whole cell membrane currents in single PASMCs were measured by the nystatin-perforated patch-clamp technique (34) using a patch-clamp amplifier (EPC-9; Heka Electronics, Germany). When filled with intracellular solution, patch pipettes for the perforated patch-clamp experiments had a resistance of 2–3 MΩ. When electrical access was detected, cells were clamped at a holding potential of −55 mV. Membrane capacitance and series resistance were continuously monitored and compensated, and experiments were initiated after a decrease in the access resistance to below 40 MΩ. Voltage-command protocols were generated by the EPC-9 system (Heka Electronics, Germany). Data were recorded on a Macintosh computer and VHS tape for off-line analysis.

Whole cell membrane currents in *Xenopus* oocytes were measured by the two-electrode voltage-clamp technique (31). Recording electrodes had a resistance of 0.5–1.5 MΩ. After impalement, oocytes were clamped at −60 mV and continuously perfused with bath solution (19°C). Current and voltage signals were recorded on a computer for off-line analysis.

The bath solution used for pulmonary artery myocytes was (in mM) 125 NaCl, 5 KCl, 1 MgSO\(_4\), 10 HEPES, 1.8 CaCl\(_2\), and 10 glucose (pH 7.4). The pipette solution contained (in mM) 130 CsCl, 5 MgCl\(_2\), 3 EGTA, 1 CaCl\(_2\), and 10 HEPES (pH 7.3). The standard oocyte extracellular solution contained (in mM) 115 NaCl, 2.8 KCl, 1.8 CaCl\(_2\), 1.8 MgCl\(_2\), and 10 HEPES (pH 7.2). To test the ion selectivity of CN-activated currents, 92% of extracellular NaCl was replaced with an equal concentration of NaI, Na-isethionate, Tris·Cl, or NMDGCl.

**Measurement of whole cell [Ca\(^{2+}\)].** Measurements of spatially averaged Ca\(^{2+}\) fluorescence in pulmonary and mesenteric arterial myocytes were made by a dual excitation wavelength fluorescence method as described previously (33), using the IonOptix fluorescence photometric system (Milton, MA). Cells were loaded with 4 μM fura 4-AM (Molecular Probes, OR) for 30 min at 35°C. Experiments were initiated after 20 min of perfusion to wash out extracellular fura 2-AM and to allow the conversion of intracellular dye into its nonester form. The dye was excited at 340 and 380 nm wavelengths (Xenon 75 W arc lamp), and the emission fluorescence at 510 nm was detected by a photomultiplier tube. Photobleaching was minimized by the use of neutral density filters and by shuttering excitation light between sampling periods. Background fluorescence was determined by removing the cell from the field after the experiment.

**Confocal laser scanning microscopic imaging of Ca\(^{2+}\) sparks.** Localized Ca\(^{2+}\) release events (Ca\(^{2+}\) sparks) were measured as described previously (5) using a high-speed confocal laser scanning microscopic system (Zeiss LSM510, Germany) coupled to an inverted microscope (Zeiss Axert 300). Single myocytes were loaded with fluo-4-AM (5 μM) (Molecular Probes) for 30 min at 35°C. After 20 min of bath perfusion to wash out extracellular fluo-4-AM and to allow the conversion of intracellular dye into its nonester form, the dye fluorescence was excited with 488 nm light emitted from a Krypton/Argon laser and detected by a confocal laser scanning head. High bandwidth time profiles of fluorescence intensity were obtained using line scanning mode.

**Hypoxia.** Hypoxic responses were achieved by switching the perfusate solution from a bath solution equilibrated with 20% O\(_2\) and 5% CO\(_2\) and balanced with N\(_2\) (normoxic) to an equilibrated with 5% CO\(_2\) and balanced with various O\(_2\)/N\(_2\) mixtures, as described previously (32). The oxygen tension of the solution was continuously monitored by means of an oxygen electrode (OXEL-1, WPI). The bath Po\(_2\) was ≥140 and 10–20 Torr in the normoxic and hypoxic solutions, respectively. Under normoxic and hypoxic conditions, pH values were the same (7.4). To avoid atmospheric O\(_2\) reequilibration with the hypoxic bath solution, mineral oil was placed on top of the recording chamber.

**Reagents.** CN (Mallinckrodt, Phillipsburg, NJ) was freshly prepared just before experiments and applied to individual cells through a puffer pipette connected to a Picospritzer pressure ejection device (Parker Instrumentation, Fairfield, NJ). Caffeine, CCCP, heparin, norepinephrine, nystatin, and oligomycin were obtained from Sigma (St. Louis, MO); fura 2-AM and fluo-4-AM were from Molecular Probes; and antibody against inositol 1,4,5-triphosphate receptors (anti-IP\(_3\)R antibody), cyclopiazonic acid, and thapsigargin were from Calbiochem (La Jolla, CA).

**Statistics.** Data were expressed as means ± SE of n cells investigated. Student’s t-test was used for determining the significance of differences between two groups, whereas one-way ANOVA was used for multiple comparisons. P < 0.05 was accepted as the level of statistical significance.

**RESULTS**

Metabolic inhibition with CN induces an increase in [Ca\(^{2+}\)], and activates I\(\text{Cl(Ca)}\) in PASMC cells. Freshly isolated PASMCs were loaded with fura 2-AM and...
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tage clamped at −55 mV using the perforated patch-clamp technique. The intracellular solution contained Cs+ ions in place of K+ ions to block outward potassium currents, and myocytes were preexposed to nisoldipine (5 μM) for 5 min before CN exposure to prevent potential influx of Ca2+ through voltage-dependent Ca2+ channels during metabolic inhibition (30). Under these conditions, application of CN (10 mM) induced an increase in spatially averaged whole cell [Ca2+]i and an inward current that mirrored the kinetics of the rise in [Ca2+]i. A typical example of these experiments is shown at left in Fig. 1A, in which both Ca2+ and current signals activated over a period of several seconds and decayed over tens of seconds. The current, previously shown to be a Ca2+-activated Cl− current [ICl(Ca)] (30), activated with a slight delay after the rise in [Ca2+]i, and was sustained as long as CN exposure was maintained, unlike the more rapidly inactivating [Ca2+]i transient observed after Ca2+ release by engagement of G protein-coupled receptors (34). In a total of six myocytes tested, [Ca2+]i was increased from a resting level of 114 ± 27 nM to a peak of 749 ± 33 nM, whereas ICl(Ca) had a mean amplitude of 551 ± 42 pA (Fig. 1B), similar to the values reported in our previous study (30).

CN-induced Ca2+ release may occur through ryanodine receptors in pulmonary artery myocytes. The CN-induced [Ca2+]i increase and ICl(Ca) were blocked by buffering intracellular Ca2+ with BAPTA. As shown at middle in Fig. 1A, in a pulmonary artery myocyte pretreated with BAPTA-AM (50 μM) for 30 min, application of CN failed to induce either an increase in [Ca2+]i or ICl(Ca). We obtained similar results in four other myocytes tested (Fig. 1B). Prior application of the ryanodine receptor (RyR) activator caffeine markedly decreased, but did not abolish, CN-induced Ca2+ and current responses, consistent with a previous study (30). As shown at right in Fig. 1A, application of CN only induced a small increase in [Ca2+]i and ICl(Ca) in a cell pretreated with caffeine (10 mM) for 5 min. The effects of caffeine on CN Ca2+ and current responses are summarized in Fig. 1B. The CN-induced [Ca2+]i increase and ICl(Ca) were reduced by 72 and 77%, respectively. These results suggest that RyRs may be potential targets for metabolic inhibition.

In agreement with this notion, exposure of voltage-clamped myocytes to a lower concentration of CN (1 mM) increased the frequency and amplitude of spontaneous transient inward currents (STICs). An example of these experiments is shown at left in Fig. 2A. In a total of six cells tested, the frequency and amplitude of STICs were increased from 0.27 ± 0.04 to 0.51 ± 0.06 Hz and from 43 ± 10 to 71 ± 22 pA (P < 0.05), respectively. Moreover, in electrically quiescent cells, CN (1 mM) induced transient inward currents that showed similar characteristics to STICs (n = 5). In both cases, no significant changes in global [Ca2+]i were observed. By contrast, using a high-speed confocal imaging system (Zeiss LSM510), we have found that exposure of CN (1 mM) significantly increased the amplitude and frequency of localized Ca2+ release events (Ca2+ sparks) in PASMCs (Fig. 2B). Previous studies have shown that STICs occur due to the simultaneous opening of many Ca2+-activated Cl− channels, which are caused by Ca2+ sparks generated by the opening of RyRs in airway, cerebral artery, and blad-

Fig. 1. Cyanide (CN) triggers Ca2+ release and activates Ca2+-activated Cl− currents [ICl(Ca)] in pulmonary artery smooth muscle cells (PASMCs). A: application of CN (10 mM) induced a sustained intracellular calcium concentration ([Ca2+]i) increase (top) and ICl(Ca) (bottom) in a control cell (left), failed to induce either Ca2+ or current response in another cell preincubated with BAPTA-AM (50 μM) for 30 min (middle), and induced a much smaller [Ca2+]i increase and ICl(Ca) in a third cell pretreated with caffeine (10 mM) for 5 min (right). All cells were loaded with fura 2-AM and voltage clamped at −55 mV using the perforated patch-clamp method. Cesium intracellular solution was used to block potassium currents. Nisoldipine was added in the bath solution to block voltage-dependent Ca2+ channels. B: graphs summarize the effects of BAPTA and caffeine on CN-induced Ca2+ and current responses. Numbers in parenthesis indicate the number of cells tested. *P < 0.05 vs. CN alone (control).
der smooth muscle cells (5, 23, 42). Therefore, metabolic inhibition after CN exposure may result in the opening of RyRs, thereby inducing Ca\(^{2+}\)/H\(_{\text{11001}}\) release from the SR.

RyRs and IP\(_{3}\)Rs are functionally coupled to the same SR in PASMCs. Experiments indicate a substantial functional overlap between SR Ca\(^{2+}\)/H\(_{\text{11001}}\) stores expressing RyRs and IP\(_{3}\)Rs (1, 15, 16, 20, 35), suggesting that IP\(_{3}\)Rs might also be involved in the Ca\(^{2+}\)/H\(_{\text{11001}}\) response after metabolic inhibition. To test this hypothesis, experiments were performed in which pulmonary artery myocytes were first exposed to the \(/H_{9251}\)-adrenergic receptor agonist norepinephrine and then to caffeine. As shown in Fig. 3A, norepinephrine (300 \(\mu M\)) induced an increase in \([\text{Ca}^{2+}]_{\text{i}}\) and \(I_{\text{Cl(Ca)}}\) in a cell. In the continued presence of norepinephrine, however, application of caffeine (10 mM) failed to evoke further \([\text{Ca}^{2+}]_{\text{i}}\) increase or \(I_{\text{Cl(Ca)}}\) in the same cell. After washout of norepinephrine, caffeine induced typical Ca\(^{2+}\) and current responses. Similar observations were obtained in six similar experiments. Consistent with this result, after the depletion of SR Ca\(^{2+}\) with caffeine (10 mM), norepinephrine (300 \(\mu M\)) was no longer able to induce an increase in \([\text{Ca}^{2+}]_{\text{i}}\), whereas, after washout of caffeine, norepinephrine triggered a normal Ca\(^{2+}\) release (Fig. 3B). We observed similar results in a total of seven cells. These experiments indicate that IP\(_{3}\)Rs and RyRs are functionally coupled to the same SR in pulmonary artery myocytes, complicating the interpretation of the role of RyRs in CN-induced Ca\(^{2+}\) release.

CN induces the SR Ca\(^{2+}\) release through IP\(_{3}\)Rs in Xenopus oocytes. Because IP\(_{3}\)Rs, but not RyRs, are expressed in the SR of Xenopus oocytes (24), we sought to use these cells as a simplified system to define the role of IP\(_{3}\)Rs in Ca\(^{2+}\) release after metabolic inhibition. To determine whether CN induced Ca\(^{2+}\) release in oocytes, cells were voltage clamped at \(-60\ \text{mV}\) using the two-electrode voltage-clamp technique and bathed in nominally Ca\(^{2+}\)-free solution to prevent Ca\(^{2+}\) influx. Under these conditions, exposure of Xenopus oocytes to CN (10 mM) induced a sustained current similar to that observed in pulmonary artery myocytes (Fig. 4). The CN current was blocked in oocytes preloaded with a Ca\(^{2+}\) buffer; as shown in Fig. 4A, incubation of oocytes with BAPTA-AM (50 \(\mu M\)) for 4 h almost completely blocked CN-induced currents in 6 cells tested.
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Fig. 3. Ryanodine receptors (RyRs) and inositol 1,3,5-trisphosphate receptors (IP\textsubscript{3}Rs) are functionally coupled to the same sarcoplasmic reticulum in pulmonary artery myocytes. A: norepinephrine (300 \mu M) induces an increase in [Ca\textsuperscript{2+}], and I\textsubscript{Cl(Ca)} in a myocyte. In the continued presence of norepinephrine, however, application of caffeine (10 mM) failed to induce a further Ca\textsuperscript{2+} release and I\textsubscript{Cl(Ca)}. After washout of norepinephrine, caffeine-induced Ca\textsuperscript{2+} and current responses were restored. The cell was voltage clamped at −55 mV, and cesium intracellular solution was used. B: Application of caffeine (10 mM) caused an increase [Ca\textsuperscript{2+}], and blocked subsequent norepinephrine (300 \mu M)-induced Ca\textsuperscript{2+} response in a non-voltage-clamped cell. After washout of caffeine, norepinephrine evoked a normal Ca\textsuperscript{2+} release in the same cell.

Replacement of 92% external NaCl with NaI or Na-

isethionate (n = 6) shifted the reversal potential, whereas similar replacement with NMGCl or Tris-Cl (n = 5) did not, indicating anion selectivity of the CN-induced current (Fig. 4C). Moreover, niflumic acid, a chloride channel blocker, blocked CN-induced currents (Fig. 4B). Collectively, these results indicate that CN induces Ca\textsuperscript{2+} release from intracellular stores, activating I\textsubscript{Cl(Ca)}.

Using I\textsubscript{Cl(Ca)} as an indicator of Ca\textsuperscript{2+} release, we next sought to determine whether the CN-induced Ca\textsuperscript{2+} release was associated with release of Ca\textsuperscript{2+} from the endoplasmic reticulum (ER) of Xenopus oocytes. As an example shown in Fig. 5A, depletion of ER Ca\textsuperscript{2+} with thapsigargin (1 \mu M) for 4 h markedly inhibited CN-induced current. The effect of thapsigargin on CN-induced currents is summarized in Fig. 5B. The mean current amplitude was 1.84 ± 0.25 \mu A in six control cells and 0.69 ± 0.16 \mu A in six cells treated with thapsigargin (P < 0.05). Similar inhibition of CN-induced currents was observed in experiments in which oocytes were incubated with both thapsigargin and cyclopiazonic acid (10 \mu M for 4 h) (Fig. 5B). These data indicate that metabolic inhibition results in the release of Ca\textsuperscript{2+} from ER Ca\textsuperscript{2+} stores in Xenopus oocytes. The expression of a single type of ER Ca\textsuperscript{2+} release channel in this system suggests that CN exposure may result in the gating of IP\textsubscript{3}R calcium release channels.

Next, we sought to examine whether inhibition of IP\textsubscript{3}Rs with heparin, a prototypical IP\textsubscript{3}R antagonist, could block CN-induced Ca\textsuperscript{2+} release in oocytes. Figure 6A shows an example of these experiments in which preinjection of heparin at a final concentration of 10 mg/ml for 10 min, previously shown to block IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release in oocytes (31), did not affect CN-induced current. As summarized in Fig. 6B, the mean amplitude of CN currents was 1.32 ± 0.28 \mu A in a total of eight cells tested, which was not significantly different from that in control oocytes (1.40 ± 0.31 \mu A, n = 18). Because dialysis of anti-IP\textsubscript{3}R antibody (5 \mu g/ml) through the patch pipette prevents neurotransmitter-induced Ca\textsuperscript{2+} release in tracheal smooth muscle cells (21), we sought to further test whether inhibition of IP\textsubscript{3}Rs by preinjection of this antibody was able to block CN response in oocytes. Similar to heparin, preinjection of anti-IP\textsubscript{3}R antibody (5 \mu g/ml) for 10 min did not produce any significant effect on CN-induced currents (Fig. 6B). Therefore, metabolic inhibition with CN may directly affect the channel domain, rather than the binding domain of IP\textsubscript{3}Rs on the ER of Xenopus oocytes.

CN induces Ca\textsuperscript{2+} release from mitochondria in Xenopus oocytes and pulmonary artery myocytes. As shown in Fig. 5, depletion of ER Ca\textsuperscript{2+} did not completely block the rise in [Ca\textsuperscript{2+}], after exposure to cyanine in Xenopus oocytes recorded in Ca\textsuperscript{2+}-free bath solution. We reasoned that a second source of intracellular Ca\textsuperscript{2+}, possibly arising from the mitochondria, might also contribute to the CN response. To test this possibility, oocytes were first exposed to CCCP, which disrupts mitochondrial transmembrane potential and depletes mitochondrial Ca\textsuperscript{2+}, and then to CN. As shown in Fig. 7, CN induced a smaller I\textsubscript{Cl(Ca)} in oocytes pretreated with CCCP (50 \mu M) for 10 min than in control oocytes. To ensure the complete depletion of mitochondrial Ca\textsuperscript{2+}, oocytes were exposed to CCCP and oligomycin (10 \mu M for 10 min), which inhibits mitochondrial ATP synthesis, was simultaneously applied to oocytes. Under these conditions, similar inhibition of CN-induced Ca\textsuperscript{2+} release was observed; the mean current was 2.44 ± 0.28 \mu A in control oocytes (n = 6), 1.46 ± 0.21 \mu A in CCCP-pretreated oocytes (n = 6), and 1.48 ± 0.13 \mu A in CCCP/oligomycin-pretreated oocytes (n = 7). Thus mitochondrial Ca\textsuperscript{2+} release likely contributes to CN-induced intracellular Ca\textsuperscript{2+} rise.

To further examine the contributions of ER and mitochondrial Ca\textsuperscript{2+} release in the CN-induced Ca\textsuperscript{2+} response in oocytes, cells were exposed to both thapsigargin and CCCP to deplete ER and mitochondrial Ca\textsuperscript{2+} and then to CN. Figure 8A shows an example of
such an experiment; application of CN failed to induce any chloride current in an oocyte pretreated with thapsigargin (1 μM) for 4 h and CCCP (50 μM) for 10 min, indicating no Ca\(^{2+}\) release. Similar results were observed from a total of six oocytes tested (Fig. 8B).

Collectively, these data suggest that CN-induced Ca\(^{2+}\) release results from both ER and mitochondria in X. laevis oocytes.

We conducted similar experiments in PASMCs to explore whether mitochondrial Ca\(^{2+}\) also contributes to CN-induced Ca\(^{2+}\) release. Fura 2-AM-loaded cells were treated with CCCP (50 μM) for 5 min to deplete mitochondrial Ca\(^{2+}\). Nisoldipine (5 μM) was added to the bath solution to prevent potential influx of Ca\(^{2+}\) during exposure of CN. An example of these experiments is shown in Fig. 9A. Application of CN induced a smaller [Ca\(^{2+}\)]\(_i\) rise than that in control cells. Similar results were obtained from eight other identical experiments. The mean values of CN-induced [Ca\(^{2+}\)]\(_i\) rise were 617 ± 31 nM in eight control cells and 439 ± 24 nM in nine CCCP-treated cells (P < 0.05) (Fig. 9B), suggesting that mitochondrial Ca\(^{2+}\) release contributes to CN-induced Ca\(^{2+}\) release in pulmonary artery myocytes.

Moreover, as with X. laevis oocytes, combined exposure to thapsigargin (1 μM) and CCCP (50 μM) for 5 min completely ablated CN-induced increases in [Ca\(^{2+}\)]\(_i\). These data suggest that metabolic inhibition with CN releases Ca\(^{2+}\) from the SR and mitochondria in pulmonary artery myocytes.

CN, unlike hypoxia, induces Ca\(^{2+}\) release in systemic (mesenteric) artery smooth muscle cells. Although CN has been widely used to examine hypoxic cellular responses, this metabolic inhibitor may produce different effects than hypoxia (14). Thus we sought to examine whether CN exposure also induced Ca\(^{2+}\) release in freshly isolated systemic (mesenteric) artery smooth muscle cells. As an example of these experiments shown in Fig. 10A, application of CN (10 mM) induced an increase in [Ca\(^{2+}\)]\(_i\) in mesenteric artery myocytes. In a total of seven cells tested, the mean [Ca\(^{2+}\)]\(_i\) increase was 658 ± 28 nM. By contrast, hypoxia exposure did not induce an increase in [Ca\(^{2+}\)]\(_i\) in mesenteric artery smooth muscle cells. Figure 10B shows a typical example of these experiments. However, in the same cell, application of norepinephrine evoked a typical Ca\(^{2+}\) response. Similar results were obtained from five other myocytes. As shown in Fig. 10C, hypoxia induced
an increase in $[Ca^{2+}]_{i}$ in PASMCs. These results are consistent with previous reports that hypoxic $[Ca^{2+}]_{i}$ increase has not been observed in myocytes from other types of systemic arteries such as celiac, cerebral, coronary, and femoral artery (11, 12, 28). Therefore, metabolic inhibition with CN induces $Ca^{2+}$ responses in both pulmonary and systemic (mesenteric) artery smooth muscle cells, whereas hypoxia-induced $Ca^{2+}$...
release is unique to PASMCs but not systemic artery myocytes.

DISCUSSION

Inhibition of cellular metabolism by exposure to CN or glucose analogs results in an increase in [Ca\(^{2+}\)]\(_i\) in PASMCs in a manner similar to that observed for hypoxic exposure. Although intracellular Ca\(^{2+}\) release appears to make an important contribution to the increase in [Ca\(^{2+}\)]\(_i\) during metabolic inhibition (4, 30, 41), the underlying mechanisms are not fully understood. In an effort to more fully understand this process, we have exposed single cells to CN and attempted to identify the sources and mechanisms of intracellular Ca\(^{2+}\) release. Application of low concentrations of CN increased the frequency and amplitude of spontaneous inward currents or induced typical STICs in PASMCs, suggesting the gating of RyRs, whose activity is known to underlie these currents (5, 23, 42). These findings are similar to previous studies of hypoxic Ca\(^{2+}\) release that occurs through RyRs (7–9, 13, 17, 22, 28, 39).

Because the activity of voltage-dependent channels is an important mechanism for the refilling of intracellular Ca\(^{2+}\) stores, it is possible that activation of depolarizing Ca\(^{2+}\)-activated Cl\(^-\) currents due to Ca\(^{2+}\) release during metabolic inhibition and hypoxia may not only cause Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels but may also induce more Ca\(^{2+}\) to be released from the SR.

Based on the finding that the [Ca\(^{2+}\)]\(_i\) rise during exposure of 2-deoxy-D-glucose is blocked by the SR Ca\(^{2+}\) pump inhibitor cyclopiazonic acid in cultured PASMCs, it has been suggested that SR Ca\(^{2+}\) release after metabolic inhibition is mediated by IP\(_3\)Rs (4). However, our data indicate that the functional coupling of IP\(_3\)Rs and RyRs to SR Ca\(^{2+}\) stores overlaps

![Fig. 9. Mitochondrial Ca\(^{2+}\) contributes to CN Ca\(^{2+}\) release in PASMCs. A: application of CN (10 mM) induced an increase [Ca\(^{2+}\)]\(_i\) in a control myocyte (left) and a smaller increase in [Ca\(^{2+}\)]\(_i\) in another cell pretreated with CCCP (50 \(\mu\)M) for 5 min (middle) and failed to induce any Ca\(^{2+}\) response in a third myocyte pretreated with thapsigargin (1 \(\mu\)M) and CCCP (50 \(\mu\)M) for 5 min (right). B: graphs summarize the effects of CCCP alone and CCCP plus thapsigargin on CN-induced [Ca\(^{2+}\)]\(_i\) increase. * \(P < 0.05\) vs. control.](http://ajpcell.physiology.org/)

![Fig. 10. CN, unlike hypoxia, also induces Ca\(^{2+}\) release in systemic (mesenteric) artery smooth muscle cells. A: CN (10 mM) induced an increase [Ca\(^{2+}\)]\(_i\) in a freshly isolated mesenteric artery smooth muscle cell. B: hypoxia exposure failed to induce [Ca\(^{2+}\)]\(_i\) increase in a mesenteric artery myocyte. However, application of norepinephrine (NE, 100 \(\mu\)M) evoked a typical Ca\(^{2+}\) response in the same cell. C: in a pulmonary artery myocyte, hypoxia induced an increase in [Ca\(^{2+}\)]\(_i\).](http://ajpcell.physiology.org/)
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substantially, because stimulation of α-adrenergic receptors with norepinephrine failed to induce CA2+ release in cells pretreated with caffeine to deplete SR CA2+ and vice versa (Fig. 3). Similar findings have been obtained in other smooth muscle cells (1, 15, 16, 20, 35). To further address this question, we sought to use Xenopus oocytes as a simplified model system to determine the role of IP3Rs in CN-induced CA2+ release, because Xenopus oocytes express IP3Rs but not RyRs (24). Similar to PASMCs, exposure of Xenopus oocytes to CN activated IC(Ca), a commonly used assay system for CA2+ release in oocytes (Fig. 4). CN-induced CA2+ release in oocytes was greatly inhibited by the prior depletion of SR CA2+ with thapsigargin and/or cyclopiazonic acid in the absence of extracellular CA2+ (Fig. 5). Taken together, these results further indicate that CA2+ release after metabolic inhibition is associated with IP3Rs, which is similar to the findings obtained in cerebellar Purkinje cells (18). However, it is worth noting that inhibition of IP3Rs by preinjection of hep- arin, a prototypical IP3R antagonist that has been shown to block IP3-mediated CA2+ release in oocytes (31), did not affect CN-induced CA2+ release (Fig. 6). Similarly, preinjection of anti-IP3R antibody that inhibits CA2+ release after stimulation of muscarinic receptors in tracheal smooth muscle cells (21) was also without effect on CN response. The data suggest that CA2+ release after metabolic inhibition with CN may occur by directly affecting the channel domain of IP3R CA2+ release channels.

In the absence of extracellular CA2+ influx, the de- pletion of SR CA2+ with the SR CA2+ pump inhibitors thapsigargin and cyclopiazonic acid cannot completely prevent CA2+ release after metabolic inhibition in Xenopus oocytes (Fig. 5). This result, together with the view that mitochondria play an important role in maintaining intracellular CA2+ homeostasis under physiologic and pathophysiologic conditions, suggests that mitochondrial CA2+ release may be involved in the CN-induced CA2+ response. We tested this possibility by examining whether the depletion of mitochondrial CA2+ could inhibit CN response. As shown in Fig. 7, pretreatment of Xenopus oocytes with CCCP and oligomycin significantly blocked CN-induced CA2+ release. Similarly, CCCP also blocked CN response in pulmonary artery myocytes (Fig. 9). Moreover, after depletion of both SR and mitochondrial CA2+ with thapsigargin and CCCP, application of CN failed to induce CA2+ release in both Xenopus oocytes (Fig. 8) and PASMCs (Fig. 9). These experiments suggest that mitochondrial CA2+ release makes a contribution, although smaller than that of the SR, to a rise of [CA2+] during metabolic inhibition.

CCCP collapses mitochondrial membrane potential, and oligomycin inhibits mitochondrial ATP synthesis by direct blockade of the FoF1 ATPase. Despite uncoupling and inhibition of phosphorylation, CA2+ release by CN was only seen partially inhibited. Similar observations have been made in dorsal root ganglia and adrenal chromaffin cells, in which, in the presence of CCCP and oligomycin, CN is still able to cause cytosolic CA2+ mobilization (3, 10). These findings, together with the fact that CN inhibits mitochondrial cytochrome c oxidase, suggest that CN effects may occur through complicated signaling pathways rather than through the direct inhibition of ATP production. Consistent with this view, recent studies have shown that the mitochondrial proximal electron transport chain (ETC) inhibitors such as rotenone and myxothiazol inhibit generation of reactive oxygen species (ROS) and block HPV, whereas the distal ETC inhibitors antimycin A and CN increase generation of ROS and mimic/potentiate HPV in PASMCs (2, 19, 37).

Metabolic inhibitors such as CN have been widely used to examine hypoxic cellular responses in a variety of cell types, including PASMCs. Indeed, metabolic inhibition after CN exposure mimics hypoxia in many aspects of cellular responses. For example, CN, similar to hypoxia, induces an increase in [CA2+] in freshly isolated PASMCs (30) and vasoconstriction in isolated lungs, pulmonary artery strips, and PASMCs (2, 25, 26, 37). However, we found that CN exposure resulted in CA2+ release in both pulmonary and systemic (mesen- teric) artery myocytes (Figs. 1 and 10A). Consistent with our findings, a recent study has shown that hypoxia hyperpolarizes, whereas CN depolarizes, membrane potential, possibly by affecting inward Na+ and outward K+ currents in Drosophila neurons (14). In contrast to CN, hypoxia induces an increase [CA2+] in only in pulmonary and not systemic artery smooth muscle cells (Fig. 10B). Similarly, hypoxia [CA2+] increase has not been seen in smooth muscle cells from celiac, cerebral, coronary, or femoral arteries (11, 12, 28). Furthermore, hypoxia has been shown to inhibit outward K+ currents in PASMCs but not in mesenteric artery myocytes (40). Collectively, these data indicate that metabolic inhibition with CN may produce different cellular effects from hypoxia.

It has been suggested that mitochondria are likely implicated in O2 sensing, by which hypoxia increases generation of ROS through the ETC, mediating HPV (19, 37). However, other reports have shown that metabolic inhibition with CN also increases the generation of ROS in isolated lungs and cultured PASMCs (2, 37). In addition, our data indicate that CN, unlike hypoxia, induces CA2+ release in both pulmonary and systemic (mesenteric) artery smooth muscle cells. Therefore, additional experiments are needed to further verify the view that mitochondria serve as the oxygen sensor and ROS as the hypoxic signaling molecule in PASMCs.

In summary, the present study has demonstrated that metabolic inhibition with a high concentration of CN results in intracellular CA2+ release and activation of IC(Ca) in both PASMCs and Xenopus oocytes. Exposure to a low concentration of CN induces transient inward CI− currents and increases the frequency and amplitude of STICs, as well as increases the amplitude and frequency of localized CA2+ release events (CA2+ sparks). CA2+ release after metabolic inhibition results from both SR and mitochondria, although the former makes a greater contribution. Both RyRs and IP3Rs may be targets for metabolic inhibition and hypoxia.
CA\textsuperscript{2+} RELEASE BY METABOLIC INHIBITION WITH CYANIDE

Unlike hypoxia, CN also induces Ca\textsuperscript{2+} release in systemic (mesenteric) artery myocytes.

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