Hypoxic Modulation of Ca\textsuperscript{2+} Signaling in Human Venous Endothelial Cells

**MULTIPLE ROLES FOR REACTIVE OXYGEN SPECIES***

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Parvinder K. Aley†, Karen E. Porter‡, John P. Boyle§, Paul J. Kemp¶, and Chris Peers††

From the ‡School of Medicine, University of Leeds, Leeds LS2 9JT and ¶School of Biosciences, University of Cardiff, Cardiff CF10 3US, United Kingdom

The effects of hypoxia (pO\textsubscript{2} \textasciitilde 25 mm Hg) on Ca\textsuperscript{2+} signaling stimulated by extracellular ATP in human saphenous vein endothelial cells were investigated using fluorimetric recordings from Fura-2 loaded cells. In the absence of extracellular Ca\textsuperscript{2+}, ATP-evoked rises of cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{c}) because of mobilization from the endoplasmic reticulum (ER). These responses were reduced by prior exposure to hypoxia but potentiated during hypoxia. Hypoxia itself liberated Ca\textsuperscript{2+} from the ER, but unlike the effects of ATP this effect was not inhibited by blockade of the inositol trisphosphate receptor. By contrast, ryanodine blocked the effects of hypoxia but not those of ATP. Antioxidants abolished the effects of hypoxia but potentiated the effects of ATP. Inhibition of NADPH oxidase also augmented ATP-evoked responses but was without effect on hypoxia-evoked rises of [Ca\textsuperscript{2+}]\textsubscript{c}. However, either uncoupling mitochondrial electron transport or inhibiting complex I markedly suppressed the actions of hypoxia yet exerted only small inhibitory effects on ATP-evoked rises of [Ca\textsuperscript{2+}]\textsubscript{c}. Both hypoxia and ATP were able to activate capacitative Ca\textsuperscript{2+} entry. Our results indicate that hypoxia regulates intracellular Ca\textsuperscript{2+} signaling via two distinct pathways. First, it modulates agonist-evoked liberation of Ca\textsuperscript{2+} from the ER primarily through regulation of reactive oxygen species generation from NADPH oxidase. Second, it liberates Ca\textsuperscript{2+} from the ER via ryanodine receptors, an effect requiring mitochondrial reactive oxygen species generation. These findings suggest that local O\textsubscript{2} tension is a major determinant of Ca\textsuperscript{2+} signaling in the vascular endothelium, a finding that is likely to be of both physiological and pathophysiological importance.

The vascular endothelium plays a central role in the control of vascular function, exerting important influences on vital functions as diverse as coagulation, inflammation, vessel permeability, angiogenesis, and vascular tone (reviewed by Refs. 1–4). Many of these functions, such as production of vasoactive agents (5, 6), rely on regulated changes of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}). As in other non-excitable cells, Ca\textsuperscript{2+} homeostasis in endothelial cells involves uptake and release of Ca\textsuperscript{2+} into intracellular organelles (particularly the endoplasmic reticulum (ER)) as well as controlled influx from the extracellular environment (1, 2, 7–10). This process of Ca\textsuperscript{2+} influx is linked to depletion of intracellular stores such that store depletion triggers capacitative Ca\textsuperscript{2+} entry (7, 11–13), which has been linked specifically to the activation of nitric oxide synthase (6).

Although the vascular endothelium can be considered a synctium, it clearly experiences different environments in different regions of the vasculature. The most striking difference is between arterial and venous environments; clearly, venous endothelial cells experience an environment which, as compared with those of the arterial vessels, is of much lower pressure and is also relatively hypoxic and hypercapnic. Of these parameters, we have focused on the effects of hypoxia on Ca\textsuperscript{2+} signaling in venous endothelial cells. This is a poorly studied area that deserves investigation for several reasons. First, most in vitro studies of Ca\textsuperscript{2+} signaling in endothelial (and other) cells have been conducted using perfusate equilibrated with room air (\textasciitilde 150 mm Hg), which is hypoxic even for the arterial endothelium. Second, where any effects of hypoxia have been studied, they have usually been studied in combination with other altered parameters such as glucose removal (to mimic ischemia/reperfusion conditions) and/or have been studied over very prolonged time courses (14–16). Third, whereas the effects of acute hypoxia on ion channel function have been studied in depth in a variety of cell types (17–19), their actions on other ion flux mechanisms, such as those responsible for Ca\textsuperscript{2+} homeostasis in non-excitable cells, are poorly understood. Finally, although local venous O\textsubscript{2} levels clearly influence physiological functions (such as release of nitric oxide (20)), hypoxia may be important in the development of specific vascular disease states, such as varicose. It has been proposed that hypoxic “activation” of endothelial cells (which can occur via blood stasis, particularly in leg veins) requires mobilization of Ca\textsuperscript{2+} and initiates a cascade of events leading to varicose formation (21, 22). Thus, we have investigated the effects of acute hypoxia on Ca\textsuperscript{2+} homeostasis in primary cultures of human saphenous vein endothelial cells and compared responses to those of the well characterized Ca\textsuperscript{2+} mobilizing agent, extracellular ATP (23).

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‡ To whom correspondence should be addressed: School of Medicine, University of Leeds, Leeds LS2 9JT, UK. Tel.: 113-343-4174; Fax: 113-343-4803; E-mail: c.s.peers@leeds.ac.uk.

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1 The abbreviations used are: ER, endoplasmic reticulum; IP\textsubscript{3}, inositol trisphosphate; 2-APB, 2-aminophosphonoylphosphoryl borate; RyR, ryanodine receptor; cADPR, cyclic ADP-ribose; ROS, reactive oxygen species; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid; TEMPO, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl; CCE, capacitative Ca\textsuperscript{2+} entry; TRP, transient receptor potential; TRPC, canonical TRP.
Hypoxia and Endothelial Ca\(^{2+}\) Signaling

**MATERIALS AND METHODS**

**Isolation and Culture of Saphenous Vein Endothelial Cells**—The isolation of primary cultures of saphenous vein endothelial cells was adapted from methods described previously (24). Saphenous vein samples were collected from patients undergoing coronary bypass grafting following local ethical permission and informed, written patient consent. Tissue from a total of 36 patients was used, 10 female (28%) and 26 male (72%). The age range was 44–79, the median age was 67, and the mean age was 65.7 ± 1.5 years. All patients were undergoing elective coronary artery bypass surgery, and any patients with potentially confounding conditions (e.g., diabetes) were excluded.

Individual samples ranging from 10–50 mm in length were opened longitudinally and pinned, lumen uppermost, onto silicone elastomer-coated 60-mm Petri dishes using A1 Minuten pins. The tissue sample was then incubated in 1 ml of Type II collagenase (Worthington) dissolved in Medium 199 (37 °C, 15 min). The collagenase solution was collected along with 2–10 ml of wash solution (minimal essential medium supplemented with 5% fetal calf serum and 1% antibiotic/antimycotic), which was used to detach any residual endothelial cells from the tissue. The suspension was centrifuged for 6 min at 600 × g, the supernatant was removed, and the pellet was resuspended in 25 ml of wash solution and recentrifuged. The supernatant was once again removed, and the final pellet was resuspended in 4 ml of complete endothelial culture medium (M199) supplemented with 20% fetal calf serum, 1% penicillin-streptomycin, 1% glucose, 1 mM HEPES (Invitrogen), 5 units/ml heparin (Leo Laboratories), endothelial growth factor (15 μg/ml), and pyruvate (1 μM, Sigma-Aldrich, Poole, Dorset, UK). This mixture was then plated into a 25 cm\(^2\) flask and maintained in a humidified incubator at 37 °C (95% air, 5% CO\(_2\)). 2 days following plating, cells received a full medium change to remove non-adherent cells. Culture medium was then half changed every 2–3 days, resulting in a confluent flask within 2–3 weeks. This was designated passage 0; cells were subcultured using trypsin and used for experiments up to passage 3.

**Measurement of \([\text{Ca}^{2+}]_i\)**—Cells were plated onto glass coverslips in 24-well culture plates and grown to ~80% confluence. Coverslips onto which cells had grown were incubated in 2 ml of culture medium containing 4μM Fura-2AM (Molecular Probes, Cambridge, UK) for 40 min, at 37 °C, washed, and then left to de-esterify for 15 min in control solution. Fragments of coverslips were then transferred into an 80-μl recording chamber mounted on the stage of an inverted microscope where cells were continuously perfused under gravity at a rate of ~5 ml/min. Control perfusate was composed of: 135 mM NaCl, 5 mM KCl, 1.2 mM MgSO\(_4\), 2.5 mM CaCl\(_2\), 5 mM HEPES, and 10 mM glucose (pH 7.4, osmolarity adjusted to 290 mOsm/l by 0.2 M sucrose). This solution was bubbled continuously with N\(_2\) for at least 30 min prior to perfusion of cells, which produced no shift in pH. \(P_{\text{O}_2}\) was measured (at the cell) using a polarized carbon fiber microelectrode (25) and ranged from 20–25 mm Hg. Ca\(^{2+}\)-free solution (replaced with 1 mM EGTA) was then used to perfuse the cell and ranged from 20–25 mm Hg. Ca\(^{2+}\)-free perfusate (used in all experiments except part of those in Fig. 6) contained 1 mM EGTA and no added Ca\(^{2+}\). This was determined dextrorotationally using an Improvision monochromator-based imaging system (Openlab Image Processing & Vision Company Ltd, Coventry, UK) with alternating excitation 340 and 380 nm (0.2 Hz), emission 510 nm. Regions of interest were used to restrict data collection to individual cells. All imaging was controlled by Improvision software, including Openlab 2.2.5. Drugs and agonists were applied to cells via the gravity-fed perfusion system, and all experiments were conducted at 21–24 °C. Fluorescence signals were calibrated according to the formula of Ref. 26. Changes in \([\text{Ca}^{2+}]_i\) were calculated by determining the rise of \([\text{Ca}^{2+}]_i\), relative to basal levels measured immediately before that particular experimental maneuver. Where relevant, results are expressed as means ± S.E. together with example traces, and statistical comparisons were made using unpaired Student’s t tests. All mean data were obtained from the number of individual cells indicated. In each case these were collected from cells of at least 3 (and usually 4–6) different patients.

**RESULTS**

**Hypoxia Mobilizes Intracellular Ca\(^{2+}\) and Modulates ATP-evoked Ca\(^{2+}\) Signaling**—Agonists evoke rises of \([\text{Ca}^{2+}]_i\), in non-excitable cells via Ca\(^{2+}\) mobilization from intracellular stores and Ca\(^{2+}\) influx. To delineate these pathways, we examined the ability of ATP to evoke rises of \([\text{Ca}^{2+}]_i\), while cells were perfused with a Ca\(^{2+}\)-free solution (replaced with 1 mM EGTA) to prevent Ca\(^{2+}\) influx. Fig. 1A shows an example transient rise of \([\text{Ca}^{2+}]_i\), caused by the application of 10 μM ATP, a well-defined mobilizer of Ca\(^{2+}\), in endothelial cells, acting via P2Y nucleotide receptors to generate inositol triphosphate (IP\(_3\)) (23). Exposure of cells to hypoxia (\(P_{\text{O}_2} < 25\text{mmHg}\)) caused a small but discernible, transient rise of \([\text{Ca}^{2+}]_i\), (Fig. 1B) in 96 of 113 cells (85%) examined. Whenever cells were exposed to acute hypoxia followed by ATP (10 μM), B, as in A, except the cell was first exposed to a hypoxic solution (\(P_{\text{O}_2} < 25\text{mmHg}\)) before application of 10 μM ATP. Inset shows a magnified region indicated by the transient rise of \([\text{Ca}^{2+}]_i\), caused by hypoxia. C, as in B, except that hypoxia was applied after the cell was exposed to 10 μM ATP. D, as in B, except that ATP was applied during continued exposure of cell to hypoxia. In each panel (A–D), the period of application of ATP is indicated by open bars, and exposure to hypoxia is indicated by solid bars. Ca\(^{2+}\)-free perfusate was present throughout. Scale bars apply to all main panels. E, bar graph showing mean peak responses under the experimental conditions illustrated in A–D. Each bar is the mean ± S.E. of data taken from 29–100 cells. Significant differences are indicated by p values above bars.

**Fig. 1. Hypoxia stimulates Ca\(^{2+}\) release from an intracellular, ATP-sensitive pool.** A, example recording of \([\text{Ca}^{2+}]_i\) from an endothelial cell during perfusion with a Ca\(^{2+}\)-free solution. Note the transient rise evoked by ATP (10 μM), B, as in A, except the cell was first exposed to a hypoxic solution (\(P_{\text{O}_2} < 25\text{mmHg}\)) before application of 10 μM ATP. Inset shows a magnified region indicated by dashed lines to illustrate the transient rise of \([\text{Ca}^{2+}]_i\), caused by hypoxia. C, as in B, except that hypoxia was applied after the cell was exposed to 10 μM ATP. D, as in B, except that ATP was applied during continued exposure of cell to hypoxia. In each panel (A–D), the period of application of ATP is indicated by open bars, and exposure to hypoxia is indicated by solid bars. Ca\(^{2+}\)-free perfusate was present throughout. Scale bars apply to all main panels. E, bar graph showing mean peak responses under the experimental conditions illustrated in A–D. Each bar is the mean ± S.E. of data taken from 29–100 cells. Significant differences are indicated by p values above bars.
signaling with Ca^{2+}-free solution (e.g. Fig. 2A). We also investigated the effects of 2-aminoethoxydiphenyl borate (2-APB), originally considered an antagonist of IP_3 receptors (27) but now recognized as a modulator of Ca^{2+} signaling via additional mechanisms (e.g. Ref. 28). Bath application of 500 μM 2-APB in Ca^{2+}-free perfusate caused a rise of [Ca^{2+}], but consistent with its ability to inhibit IP_3 receptors, it fully blocked rises of [Ca^{2+}], evoked by ATP (Fig. 2, B and C). In contrast, hypoxia was still able to evoke rises of [Ca^{2+}], because it appeared additive with the 2-APB responses (Fig. 2, B and C). Thus, hypoxia appeared to mobilize Ca^{2+} from the endothelial ER via a mechanism that did not involve IP_3 receptors. The involvement of ryosine receptors (RyRs) was investigated by exposing cells to either ATP or hypoxia in the continued presence of 100 μM ryanodine. As illustrated in Fig. 2C (upper trace), ATP-evoked rises (i.e., responses to ATP) were unaffected (representative of 12 cells), but hypoxic responses were significantly attenuated (Fig. 2, C (lower trace) and E). Responses to hypoxia were also markedly suppressed by pre-exposure for 20 min to the cyclic ADP-ribose (cADPR) antagonist, 8-Br-cADPR (29, 30) or 8-Br-cADPR (30 μM, Fig. 2D (upper trace) and E) or by pre-exposure for 30 min to nicotinamide, which prevents cADPR formation (30). Collectively, these data indicate that hypoxia evokes Ca^{2+} release from the ER via cADPR-mediated activation of RyRs.

Reactive Oxygen Species (ROS) Differentially Modulate Ca^{2+} Signaling Evoked by Hypoxia and ATP—ROS modulate Ca^{2+} signaling in endothelial cells (31, 32). Much evidence suggests that mitochondria provide one important intracellular source of ROS, and recent evidence suggests that ROS production is modulated by hypoxia (33, 34). Therefore we explored the possibility of ROS acting as a signal between mitochondria and the ER by investigating first the ability of antioxidants to modulate Ca^{2+} signaling in endothelial cells. To do this, we employed two mechanistically distinct antioxidants: trolox (6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid, a cell-permeable, water-soluble derivative of vitamin E (see Ref. 35)) and TEMPO (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), applied together with catalase. TEMPO catalyzes the conversion of superoxide to H_2O_2, which is then removed by catalase (see Ref. 36). Cells were pretreated with 500 μM trolox for 30 min or 500 μM TEMPO plus 250 units/ml catalase for 45 min before exposure to either hypoxia or ATP. The antioxidants were also present during recordings. Trolox itself had no effect on basal [Ca^{2+}], but TEMPO/catalase caused a small, significant increase (p < 0.01) of ~30% (data not shown). Fig. 3A (the mean data are plotted in Fig. 3C) indicates that ATP-evoked rises of [Ca^{2+}], measured in Ca^{2+}-free perfusate, were significantly enhanced in the presence of antioxidants. However, hypoxia-evoked rises (again measured in Ca^{2+}-free perfusate) were completely inhibited, and indeed small decreases of [Ca^{2+}], were evident (Fig. 3B and D). These data strongly suggest an
important role for ROS in endothelial Ca^{2+} signaling during both normoxia and hypoxia and further support the idea that the mechanisms underlying ATP- and hypoxia-evoked release of Ca^{2+} from the ER are distinct.

Distinct Sources of ROS Differentially Modulate Ca^{2+} Signaling—ROS are produced at various distinct sites within cells, and of these mitochondria and NADPH oxidase are of particular importance in endothelial cells (31, 32, 37). To investigate NADPH oxidase as a ROS source, we examined the effects of hypoxia and ATP during inhibition of the oxidase by two distinct pharmacological inhibitors. Again, these experiments were conducted during perfusion of cells with Ca^{2+}-free solution. Both phenylarsine oxide (2 mM) and diphenylene iodonium (5 μM) significantly augmented the magnitude of Ca^{2+} transients evoked by ATP (Fig. 4, A and B; mean data plotted in E). In contrast, neither agent significantly altered rises of Ca^{2+} evoked by hypoxia (Fig. 4, B and D; mean data plotted in F). It is also noteworthy that both agents produced a small but sustained rise of basal Ca^{2+} levels (seen most clearly in Fig. 4, C and D).

To investigate a role for mitochondria in mediating Ca^{2+} signaling, we exposed cells either to the mitochondrial uncoupler FCCP (10 μM applied with 2.5 μg/ml oligomycin to prevent ATP consumption by the F_{0}/F_{1} ATP synthase) or to the complex I inhibitor, rotenone (2 mM), which inhibits hypoxia-evoked increases in ROS production in umbilical vein endothelial cells (38). Both agents caused transient rises of [Ca^{2+}] by loss of Ca^{2+} from the mitochondria (Fig. 5, A–D). Subsequent exposure to ATP evoked rises of [Ca^{2+}], that were slightly smaller than those observed when mitochondria were functional (Fig. 5, A, B, and E). In contrast, the rises of [Ca^{2+}], evoked by hypoxia were strikingly suppressed (Fig. 5, C, D, and F). In particular FCCP/oligomycin inhibited hypoxic responses by ~75% (Fig. 5, C and F).

**Hypoxia Stimulates Capacitative Ca^{2+} Entry—**Store depletion mediated (or capacitative) Ca^{2+} entry (CCE) is an important route of Ca^{2+} entry in non-excitable cells. In our endothelial cells, re-admission of Ca^{2+} to the perfusate (without prior discharge of intracellular stores) had no effect on [Ca^{2+}], (e.g., Fig. 6A), but when we first exposed cells to thapsigargin (in Ca^{2+}-free solution) to deplete intracellular stores as completely as possible and then re-admitted Ca^{2+}(2.5 mM) to the perfusate also triggered a Gd^{3+} (Fig. 6B) and La^{3+} (Fig. 6C) in a concentration-dependent manner indicative of CCE. When stores were initially depleted by application of 10 μM ATP, subsequent addition of Ca^{2+} to the perfusate also triggered a Gd^{3+}- and La^{3+}-sensitive influx of Ca^{2+} (Fig. 6D), and the same was true when stores were initially depleted by hypoxia (Fig. 6E). Interestingly, when comparing the magnitude of capacitative Ca^{2+} entry evoked by these different maneuvers, thapsigargin-evoked store depletion produced the largest CCE, and this was also the most sensitive to Gd^{3+}, which suppressed the influx by ~86% at 1 mM (Fig. 6F). ATP- and hypoxia-evoked CCE responses were remarkably similar in terms of magnitude and Gd^{3+} sensitivity (1 mM causing 40% inhibition of ATP-evoked CCE and 44% inhibition of hypoxia-evoked CCE, Fig. 6E). La^{3+} was clearly more potent, reducing thapsigargin-evoked CCE by 88%, ATP-evoked CCE by 86%, and hypoxia-evoked CCE by 96% (Fig. 6F).

**DISCUSSION**

The present study demonstrates that local O_{2} levels have marked effects on Ca^{2+} signaling in vascular endothelial cells and modulate Ca^{2+} signaling initiated by extracellular ATP. We have defined two distinct pathways by which hypoxia regulates Ca^{2+} release from the ER. First is the mitochondrial...
pathway in which hypoxia evokes an increased production of ROS at the mitochondrion to trigger release of Ca\(^{2+}\) from the ER via RyRs (Fig. 7, mitochondrial regulation). Second is the oxidase pathway, in which substrate limited reduction of ROS levels during hypoxia relieves tonic inhibitory influences of oxidase-derived ROS on IP\(_3\)-dependent Ca\(^{2+}\) release from the ER (Fig. 7, oxidase regulation). Note also that there is cross talk between these two regulatory pathways; specifically hypoxia-induced mitochondrial ROS production augments agonist-evoked Ca\(^{2+}\) release.

Because mitochondrial ROS have been shown to increase during hypoxia in a variety of cell types (34), we explored their possible role in mediating Ca\(^{2+}\) signaling by investigating the effects of two mechanistically distinct antioxidants (Fig. 3). Surprisingly, both agents had opposing effects on rises of [Ca\(^{2+}\)]\(_{i}\), evoked by ATP as compared with hypoxia. These observations further strengthen our hypothesis that ATP and hypoxia mobilize Ca\(^{2+}\) from the ER via separate mechanisms but also raise the question of how ROS inhibition could exert such diverse effects. To explore this, we attempted to inhibit selectively ROS production from two separate sources, NADPH oxidase and mitochondria. Our findings strongly suggest the ROS derived from these two sources can exert very different effects on Ca\(^{2+}\) signaling. During normoxia, NADPH oxidase produces ROS, causing a tonic suppression of ATP-evoked rises of [Ca\(^{2+}\)]\(_{i}\), (Fig. 4), and pharmacological inhibition of the oxidase potentiated ATP-evoked rises of [Ca\(^{2+}\)]\(_{i}\), (Fig. 7). Thus, pharmacological inhibition of the oxidase pathway in which hypoxia evokes capacitative Ca\(^{2+}\) entry (28, 40), its application here clearly distinguishes between the ability of hypoxia and ATP to raise [Ca\(^{2+}\)]\(_{i}\). Furthermore, exposure of cells to ryanodine fully prevented hypoxic rises of [Ca\(^{2+}\)]\(_{i}\), yet did not significantly alter responses to ATP. Thus hypoxia mobilized Ca\(^{2+}\) from the ATP-sensitive pool via activation of RyRs. That 8-Br-cADPR and nicotinamide prevented the effects of hypoxia strongly suggests that RyR Ca\(^{2+}\) release was mediated by cADPR (29, 30).

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that ATP-evoked signals are potentiated during hypoxia (Fig. 1, D and E) despite the fact that hypoxia causes partial depletion of the ATP-sensitive pool of Ca$^{2+}$ (Fig. 1B).

Hypoxia also stimulated Ca$^{2+}$ release from the ER, but the mechanism appears quite distinct (Fig. 7, mitochondrial regulation pathway); antioxidants fully prevented rises of [Ca$^{2+}$], evoked by hypoxia (Fig. 3), indicating that ROS are required for this effect but not from NADPH oxidase (Fig. 4). Instead, the primary source of ROS mediating the effects of hypoxia appears to be mitochondria. Numerous studies indicate that hypoxia increases mitochondrial ROS production (34), and ROS can be generated at different sites along the respiratory chain (38, 41, 42). The mechanism is unknown, but we have found hypoxia depolarizes mitochondria. In umbilical vein endothelial cells, ROS generated at complex I appear to be functionally important because rotenone prevents ROS-mediated interleukin-6 production (38). Finally, mitochondria-derived ROS may also cause a small stimulatory effect on ATP-mediated signaling (Fig. 7, cross talk pathway) because both rotenone and FCCP effected small decreases in ATP-evoked signals (Fig. 5E). However, this effect is normally masked by the inhibitory effect of NADPH oxidase-derived ROS because antioxidants (which would presumably buffer all ROS, regardless of source) potentiated ATP-evoked rises of [Ca$^{2+}$].

Results presented in Fig. 6 indicate an additional, important effect of hypoxia on Ca$^{2+}$ homeostasis. Thus, store depletion by hypoxia clearly activated a Ca$^{2+}$ influx pathway because addition of Ca$^{2+}$ to the perfusate following discharge of Ca$^{2+}$ stores either by thapsigargin, ATP, or hypoxia led to a rise of [Ca$^{2+}$]. Regardless of the method of store depletion, the subsequent Ca$^{2+}$ influx pathway was clearly sensitive to Gd$^{3+}$ and La$^{3+}$. However, there were differences between the influx pathway(s) activated by thapsigargin pretreatment and those activated by ATP or hypoxia. Thus, the thapsigargin-activated Ca$^{2+}$ influx was much greater in magnitude than that activated by ATP or hypoxia and was inhibited >85% by 1 mM Gd$^{3+}$. In contrast, the magnitudes of ATP- and hypoxia-evoked influxes were extremely similar to each other (despite these agents discharging the magnitudes of ATP- and hypoxia-evoked influxes were ex-

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