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Prevention of a hypoxic $Ca^{2+}{}_i$ response by SERCA inhibitors in cerebral arterioles

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1 The aim of the study was to investigate the mechanism of a novel effect of hypoxia on intracellular Ca^{2+} signalling in rabbit cerebral arteriolar smooth muscle cells, an effect that was resistant to the L-type Ca^{2+} channel antagonist methoxyverapamil (D600).

2 $[Ca^{2+}]_i$ of smooth muscle cells in intact arteriolar fragments was measured using the Ca^{2+}-indicator dye fura-PE3. Hypoxia (PO_2 10–20 mmHg) lowered basal $[Ca^{2+}]_i$ but did not inhibit Ca^{2+} entry pathways measured by Mn^{2+}-quenching of fura-PE3.

3 The effect of hypoxia was completely prevented by thapsigargin or cyclopiazonic acid, selective inhibitors of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA). Since these inhibitors do not block Ca^{2+} extrusion or uptake *via* the plasma membrane, the data indicate that the effect of hypoxia depends on a functional sarcoplasmic reticulum.

4 Because actions of nitric oxide (NO) on vascular smooth muscle are also prevented by SERCA inhibitors it was explored whether the effect of hypoxia occurred *via* modulation of endogenous NO release. Residual NOS-I and NOS-III were detected by immunostaining, and there were NO-dependent effects of NOS inhibitors on $Ca^{2+}{}_i$-signalling. Nevertheless, inhibition of endogenous NO production did not prevent the effect of hypoxia on $[Ca^{2+}]_i$.

5 The experiments reveal a novel nitric oxide-independent effect of hypoxia that is prevented by SERCA inhibitors.

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Keywords: Hypoxia; nitric oxide; cerebral arteriole; smooth muscle; calcium signalling

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CPA, cyclopiazonic acid; Cy3, cyanine 3; D600, methoxyverapamil; EDHF, endothelium-derived hyperpolarizing factor; L-NAME, Nω-nitro-L-arginine methyl ester; Mn^{2+}, manganese; N_2, nitrogen; ROI, region of interest; RT, room temperature; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; α-SMA, α-smooth muscle actin; TG, thapsigargin; VIP, vasoactive intestinal peptide

Introduction

A major protective function of cerebral arterioles is to dilate in response to hypoxia (Kontos *et al*., 1978; Pearce, 1995). The oxygen-sensitivity of arterioles enables increased blood flow to neurones requiring extra oxygen because of physiological or seizure-induced increases in excitability, or to neurones in danger of damage due to low oxygen tensions associated with cardiac or respiratory arrest or occlusion of upstream cerebral arteries.

There are multiple mechanisms underlying the vasodilatory responses to local and systemic hypoxia and these may be broadly divided into indirect effects and direct effects on the vascular smooth muscle cells (Marshall, 1999). Direct effects are suggested because, for example, endothelium-denuded vessels relax in response to hypoxia *in vitro* (Vinall & Simeone, 1986; Pearce *et al*., 1989; Pearce *et al*., 1992), as do arterioles separated from the parenchyma (Jackson & Duling, 1983). Direct effects include inhibition of L-type voltage-gated Ca^{2+} channels and activation of ATP-sensitive K^+ channels (Pearce *et al*., 1992; Franco-Obregon & Lopez-barneo, 1996; Herrera & Walker, 1998; Faraci & Heistad, 1998). The effect on K^+ channels is thought to lower the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of vascular smooth muscle cells because it causes hyperpolarization, which reduces voltage-dependent Ca^{2+}-entry. Thus both direct effects centre around modulation of voltage-gated Ca^{2+} channels. It is less clear whether hypoxia has other effects on Ca^{2+}-handling in vascular smooth muscle. There is some evidence for direct relaxant effects that are independent of voltage-gated Ca^{2+} channels and ATP-sensitive K^+ channels (Herrera & Walker, 1998) and effects on contractile proteins and Ca^{2+}-handling by the sarcoplasmic reticulum have been suggested (Taggart & Wray, 1998). In pulmonary arteries, which have a dominant hypoxic vasoconstrictor response, hypoxia modulates store-operated Ca^{2+} channels in the smooth muscle layer (Robertson *et al*., 2000). Voltage-gated Ca^{2+} channels play a prominent role in arteriolar function, but there are also vasoconstrictor and intracellular Ca^{2+} responses in cerebral arterioles that are resistant to classical Ca^{2+} antagonists (Pierre *et al*., 1999; Guibert & Beech, 1999), suggesting a need for cerebral vessels to have mechanisms for hypoxic vasodilatation that are independent of voltage-gated Ca^{2+} channels.

Nitric oxide (NO) is widely believed to play a pivotal role in the regulation of cerebral blood flow and be involved in responses of the brain to hypoxia and ischaemia (Iadecola *et al*., 1994; Bolanos & Almeida, 1999). Like hypoxia, NO acts

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via multiple effectors including voltage-gated Ca^{2+} channels and K^+ channels; indeed, several of the resulting effects are similar for hypoxia and NO (Beech, 1997; Cohen *et al*., 1999; Beech *et al*., 2001). The similarities might result in part because responses to hypoxia are mediated by NO (Wingrove & O'Farrell, 1999). For example, in the vasculature, hypoxia is known to stimulate EDHF release and NO contributes to hypoxic vasodilation in forearm resistance vessels in humans (Pohl & Busse, 1989; Blitzer *et al*., 1996). In cat cerebral arterioles less than 100 μm in diameter, inhibition of NO synthase (NOS) attenuated hypoxic vasodilatation without having a direct vasoconstrictor effect (Ishimura *et al*., 1996). *In vivo* experiments on the rat show NOS inhibition attenuates hypoxic cerebral vasodilatation (Reid *et al*., 1995). The NOS-I isoform may be particularly important for the cerebrovascular effect because tetrodotoxin (an inhibitor of nerve-mediated effects) and 7-NINA (a preferential NOS-I inhibitor) attenuate hypoxic vasodilatation in piglet pial arterioles (Wilderman & Armstead, 1997; 1998]. NOS-III and the inducible NOS-II can also be expressed in blood vessels and may be involved in hypoxic responses (e.g. Blitzer *et al*., 1996). Therefore, it is important to explore if hypoxic responses are mediated by nitric oxide.

In this study we investigated the effects of hypoxia on cellular Ca^{2+}-handling by pial arteriolar smooth muscle cells. The initial aim was to explore if there are effects of hypoxia other than those mediated by voltage-gated Ca^{2+} channels. We observed an effect that resembled a SERCA-dependent response found also for NO (Cohen *et al*., 1999) and tested if the effect of hypoxia was explained by modulation of endogenous NO release.

Methods

Male rabbits (1.0–1.5 kg) were killed by an intravenous overdose of 70 mg.kg^{-1} sodium pentobarbital in accordance with the Code of Practice as set out by The U.K. Animals Scientific Procedures Act 1986. The brain was removed and placed in ice-cold Hanks solution bubbled with 100% O_2. Pieces of pial membrane were removed and incubated with 0.032 mg.ml^{-1} pronase (Type E, Sigma) and 0.2 mg.ml^{-1} collagenase (Type 1A, Sigma) in Hanks solution at 37°C for 10 min. The mixture was then kept at 4°C for 15 min and agitated before washing in enzyme-free Hanks solution and centrifugation at 1000 r.p.m. for 1 min. Arterioles (external diameter 20–40 μm) were stored on glass coverslips at 4°C in Hanks solution and used within 10 h.

Arterioles were incubated in 1 μM of the fura-2-related ratiometric Ca^{2+} indicator dye fura-PE3-AM (Vorndran *et al*., 1995) for 60 min at 23°C in the standard bath solution. In some experiments this fura-PE3-AM incubation included cyclopiazonic acid, thapsigargin, a NOS inhibitor, or a NO donor. The arterioles were then viewed on the stage of an inverted epifluorescence microscope (Nikon, Japan). The source of excitation light was a xenon arc lamp and excitation wavelengths were selected by a monochromator (Till Photonics, Planegg, Germany). Light was transmitted to the microscope *via* a quartz fibre-optic guide and reflected by a dichroic mirror (Omega Optical, Glen Spectra Ltd, Stanmore, U.K.). Digital images were sampled at 14-bit resolution by a C4880-82 camera (Hamamatsu Photonics K. K., Japan). Fura-PE3 was excited alternately at 345 or 355 nm and 380 nm. Ratios of the resulting images (e.g. 345/380) were produced every 20 s. Three regions of interest (ROIs) used to restrict data collection to smooth muscle cells (see example in Figure 1A, and Guibert & Beech, 1999) were averaged for each experiment. Imaging was controlled by Openlab 2 software (Image Processing and Vision Company Ltd, Coventry, U.K.).

Normoxia was generated by gassing solutions with air, and hypoxia by gassing solutions with 100% N_2 for at least 30 min. In all experiments, PO_2 was measured within about 50 μm of each arteriole by using a polarized (−0.8 V) carbon fibre electrode (Dagan Corporation, Minneapolis, U.S.A.) (Mojet *et al*., 1997). When N_2 or hypoxia is indicated, PO_2 achieved adjacent to the arteriole was 10–20 mmHg. The carbon fibre was calibrated by perfusing with bath solution bubbled with air (PO_2 150 mmHg), and bath solution bubbled with 100% N_2 and containing 0.5 mM of $Na_2S_2O_4$ (PO_2 0 mmHg). The carbon fibre signal was captured on-line *via* an A-D converter (Picolog software, Pico Technology Ltd, Cambridge, U.K.). All tubing in the perfusion system was gas impermeant (Tygon tubing, BDH, Atherstone, U.K.). All the experiments were performed at about 23°C, and substances were applied to arterioles by completely exchanging the solution in the recording chamber. Solution exchange occurred in <1 min and the perfusion rate was about 2 ml.min^{-1}.

A decrease in PO_2 shifts the fura-2 Ca^{2+}-calibration curve upward and to the left such that the [Ca^{2+}] (340/380 ratio) value in hypoxic conditions is overestimated by 15–20% (Stevens *et al*., 1994a,b). Such a change will make inhibitory effects of hypoxia on $[Ca^{2+}]_i$ appear smaller than they really are. Correction for the effect was not made.

For immunofluorescence staining a suspension of arterioles was isolated as described, centrifuged at 1000 r.p.m. for 2 min, and the supernatant replaced with PB 2% paraformaldehyde for 30 min. Fixed arterioles were placed on coverslips and incubated for 5 min in methanol at −20°C. Slides were then rinsed three times for 5 min in PBS 0.1% triton X at room temperature (RT) and then incubated for 1 h at RT in blocking medium (1% BSA). Incubation with primary antibodies (anti-NOS antibodies diluted to 1 : 250 and anti-VIP antibody diluted to 1 : 2500) occurred overnight at 4°C. Slides were then washed three times for 20 min in PBS at RT before incubation for 1 h at RT in species-specific secondary antibody conjugated either to FITC or cyanine 3 (Cy3). Slides were then rinsed three times for 20 min in PBS and incubated for 1 h at RT in mouse anti-α-smooth muscle actin-Cy3 (anti-α-SMA-Cy3; diluted to 1 : 200–500). Slides were washed again three times for 20 min in PBS at RT and mounted with Vectashield (Vector Laboratories). In each case, negative controls were performed by omitting the primary antibody from the protocol given above. Non-specific binding in these experiments was not significant. Images were captured with the system also used for Ca^{2+} imaging. The excitation wavelength was 490 nm for FITC and 520 nm for Cy3.

All results are expressed as means ± s.e.mean and n indicates the number of arterioles used in Ca^{2+} -measurement experiments. Groups of data were compared using Student's unpaired t-test and statistical significance was concluded if $P<0.05$. For immunofluorescence data, n is given as '$=x$, y',

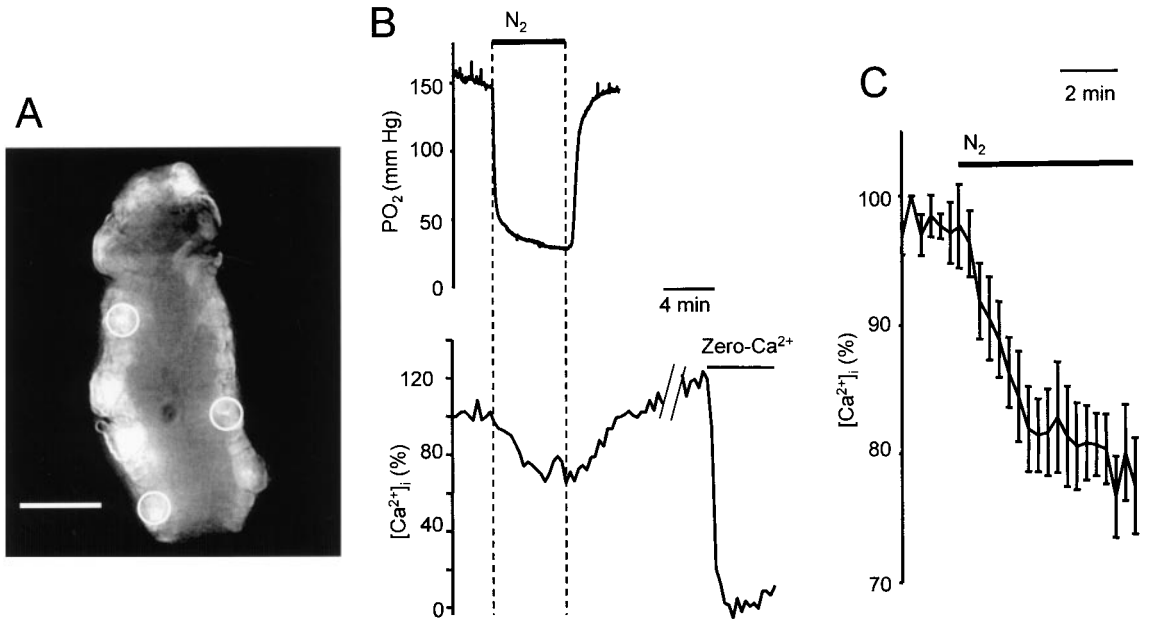

Figure 1 Hypoxia lowers $[Ca^{2+}]_i$ in rabbit pial arteriole smooth muscle cells. (A) Fura-PE3 loading of an enzymatically isolated pial arteriole fragment. The dye was excited by 380 nm light and emission collected at 510 nm. The superimposed white circles are example 'regions of interest' (ROIs) used for data collection from smooth muscle cells. The scale bar is 10 μm. (B) Carbon fibre O_2 (upper trace) and smooth muscle fura-PE3 Ca^{2+} (lower trace) signals measured simultaneously. Ca^{2+} concentration is represented as the fura-PE3 ratio expressed as a percentage relative to the zero level when bath 1.5 mM Ca^{2+} was replaced with 0.4 mM EGTA (zero-Ca^{2+}). Bubbling the bath solution with nitrogen (N_2) lowered PO_2 from 150 to 25 mmHg and reduced the intracellular Ca^{2+} concentration by about 30%. The break in the trace is 8.3 min. (C) Mean$\pm$s.e.mean ($n=7$) fura-PE3 data for experiments as described in (B). The mean minimum PO_2 while bubbling with N_2 was 19.8$\pm$2.2 mmHg.

where x is the number of microscope slides (tissue samples) and y is the number of animals. Data were processed using Origin 6.0 software (Microcal Inc, U.S.A.).

Hanks solution contained (mM): NaCl 137; KCl 5.4; $CaCl_2$ 0.01; NaH_2PO_4 0.34; K_2HPO_4 0.44; D-glucose 8; HEPES, 5. Standard bath solution contained (mM): NaCl 130; KCl 5; D-glucose 8; HEPES, 10; $MgCl_2$ 1.2; $CaCl_2$ 1.5; D600 0.01. Ca^{2+}-free bath solution was prepared by omitting 1.5 mM $CaCl_2$ from the standard bath solution, and in some experiments (as indicated) including 0.4 mM EGTA. In Mn^{2+} bath solutions, 1.5 mM $CaCl_2$ was replaced with 0.5 mM $MnCl_2$. The final pH of all the solutions was titrated to pH 7.4 using NaOH. Levcromakalim (10 μM) was included in some Ca^{2+}-measurement experiments in an effort to enhance passive Ca^{2+} or Mn^{2+} entry, but no difference was detected and the data have been aggregated.

General salts were from BDH, Sigma or Aldrich. EGTA, HEPES, protease (type E), collagenase (type 1A), thapsigargin (TG), manganese (Mn^{2+}), methoxyverapamil (D600), Nω-nitro-L-arginine methyl ester (L-NAME), tetrodotoxin, L-arginine, and methoxyverapamil (D600) were from Sigma. DEA/NONOate and fura-PE3 AM were from Calbiochem. L-s-methyl-thio-citrulline was from Tocris. Anti-NOS I, II and III antibodies (mouse monoclonal antibodies) were from Transduction Laboratories, anti-vasoactive intestinal peptide (anti-VIP, a polyclonal antibody generated in sheep) was from Chemicon, and anti-α-SMA-Cy3 (a mouse monoclonal antibody) and all secondary antibodies were from Sigma. Cyclopiazonic acid (CPA) was from Affiniti (U.K.). Levcromakalim was a gift from SB Pharmaceuticals. Fura-PE3, TG, CPA and levcromakalim were prepared as stock solutions in 100% DMSO such that the final DMSO concentration was $\leqslant$0.1%. Other substances were prepared directly in salt solution or in distilled water.

Results

Intracellular Ca^{2+} signals were sampled from smooth muscle cells within arteriolar fragments that were loaded with fura-PE3 Ca^{2+} indicator dye (Figure 1A). The aim of using isolated vessel fragments was to reduce the possibility that effects depended on cells other than smooth muscle cells while avoiding complete and potentially damaging isolation of smooth muscle cells from the vessels. Endothelial cells were not evident in Ca^{2+}-imaging experiments on short (Figure 1A) or long (data not shown) arteriolar fragments. To focus the study on mechanisms that were independent of L-type voltage-gated Ca^{2+} channels all recordings were in the presence of 10 μM D600, which abolishes voltage-dependent Ca^{2+}-entry in these arterioles (Guibert & Beech, 1999).

To study the effect of hypoxia, bath PO_2 was lowered from 150 to below 50 mmHg. This procedure caused a reversible reduction in the resting Ca^{2+} level ($[Ca^{2+}]_i$) in smooth muscle cells in arteriolar fragments (Figure 1B). The response to hypoxia was about 20% of the effect of completely removing Ca^{2+} from the bath solution (Figure 1C). To test if the reduction in $[Ca^{2+}]_i$ occurred because there was inhibition of Ca^{2+}-entry we used Mn^{2+}-quenching of fura-PE3 as an index of the activity of Mn^{2+}-permeable Ca^{2+} entry pathways. Hypoxia did not inhibit Mn^{2+} entry (Figure 2). After correction for hypoxia-induced inhibition of spontaneous

quenching (Figure 2B) it appeared that hypoxia slightly potentiated Mn^{2+} entry, but this effect was not statistically significant (Figure 2C). To test if lowering of $[Ca^{2+}]_i$ by hypoxia depended on intracellular Ca^{2+} stores, arterioles were exposed to thapsigargin or CPA, potent and specific inhibitors of SERCA that deplete sarcoplasmic reticulum Ca^{2+} content (Guibert & Beech, 1999; Thastrup *et al*., 1990; Luo *et al*., 2000). These agents produced striking inhibition of the effect of hypoxia (Figure 3A,B) without significantly affecting steady-state $[Ca^{2+}]_i$ ($P>0.2$, $n=7-11$). Mn^{2+}-quenching of fura-PE3 was also investigated in the presence of thapsigargin, but again there was no inhibitory effect of hypoxia (Figure 3C).

SERCA inhibitors have also been shown to prevent the action of NO on $[Ca^{2+}]_i$ in vascular smooth muscle (Cohen *et al*., 1999) and hypoxic vasodilation involves NO release (see Introduction). Therefore, it was investigated if the effect of hypoxia involved the modulation of endogenous NO release. If NO was involved, NOS isoforms must be present in the isolated arteriole preparation. NOS-II could not be detected in any experiments ($n=12$, 4) (data not shown) and NOS-I and NOS-III were not present in arteriolar smooth muscle cells (Fig 4A,B). However, NOS-I and NOS-III were detected in some arteriole fragments, especially those that were long and presumably less digested by enzymes. Punctate NOS-I staining was detected (Figure 4C). If this is NOS-I in nerve terminals or varicosities, it might be expected to co-localize with other transmitter substances such as vasoactive intestinal peptide (VIP), as occurs in major cerebral arteries (Gonzalez *et al*., 1997). We tested this idea and found partial co-localization of NOS-I and VIP immunoreactivity ($n=5$, 4) (Figure 4D). NOS-I staining occurred in all 11 vessel fragments observed but was notably very sparse in those fragments that were short in length (e.g. Figure 4E). NOS-III was also detected, but only in a small fraction of vessel fragments (7 out of 31). In the examples when NOS-III staining was evident it was in endothelial cells (Figure 4F). The isolated arterioles lack the longitudinally oriented endothelial cells when the fragments are sufficiently short (Cheong *et al*., 2001). The image in Figure 4F has been selected to show a vessel that was partly fragmented, with endothelial cells stretched between the two main blocks of smooth muscle. Thus, although arteriolar fragments appear from fura-PE3 images to be purely smooth muscle (i.e. other cellular structures do not take-up and/or de-esterify the fura-PE3 AM), residual NOS may be present in nerve terminals or endothelial cells not completely removed by the enzymatic treatment.

To test if residual NOS produced functionally important NO release, smooth muscle Ca^{2+}-signals were compared with and without L-NAME treatment to inhibit NOS activity. The dependence of $[Ca^{2+}]_i$ on external Ca^{2+} was significantly increased by L-NAME (Figure 5A). Competition with L-arginine or exogenous NO from DEA/NONOate (Morley & Keefer, 1993) prevented the effect of L-NAME (Figure 5B). NOS-I seemed the most likely source of NO because s-methyl-L-thiocitrulline (Narayanan *et al*., 1995) mimicked the effect of L-NAME (Figure 5C). Thus, endogenous NO was released and tonically suppressed $[Ca^{2+}]_i$, rather like hypoxia. Using this information we could be sure that 0.3 mM L-NAME was sufficient to block endogenous NO production and thus test if the endogenous NO had a role in the effect of hypoxia. The effect of hypoxia on $[Ca^{2+}]_i$ persisted following pre-incubation with, and the continued presence of, 0.3 mM L-NAME (e.g. Figure 5D). The mean$\pm$s.e.mean hypoxia-induced reduction in fura-PE3 ratio was 0.0143 ± 0.003 ($n=16$), not significantly different ($P=0.32$) from the control response of 0.009 ± 0.001 ($n=7$). In nine of the L-NAME experiments a supplement with 0.5 μM tetrodotoxin was included to inhibit neuronal activity but this had no effect and all data were aggregated. Therefore, a SERCA dependent effect of hypoxia exists in the arterioles and it is independent of nitric oxide.

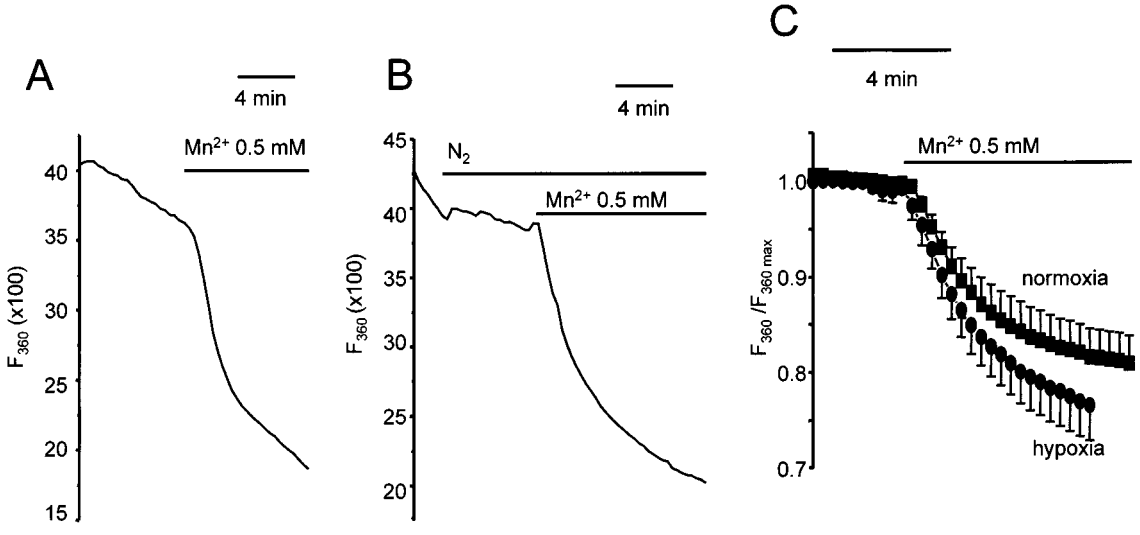

Figure 2 Hypoxia does not inhibit Ca^{2+}-influx mechanisms. (A) Effect of 0.5 mM Mn^{2+} (Ca^{2+}- and EGTA-free bath solution) on fura-PE3 excited at 360 nm in normoxic bath solution. Fura-PE3 fluorescence is given in arbitrary units. (B) As for (A) except hypoxia was achieved prior to application of Mn^{2+}. (C) Mean$\pm$s.e.mean data for experiments of the type described in (A) and (B) ($n=6$ normoxia, and $n=5$ hypoxia). Fura-PE3 signals were normalized and background (pre-Mn^{2+}) quenching rates were subtracted.

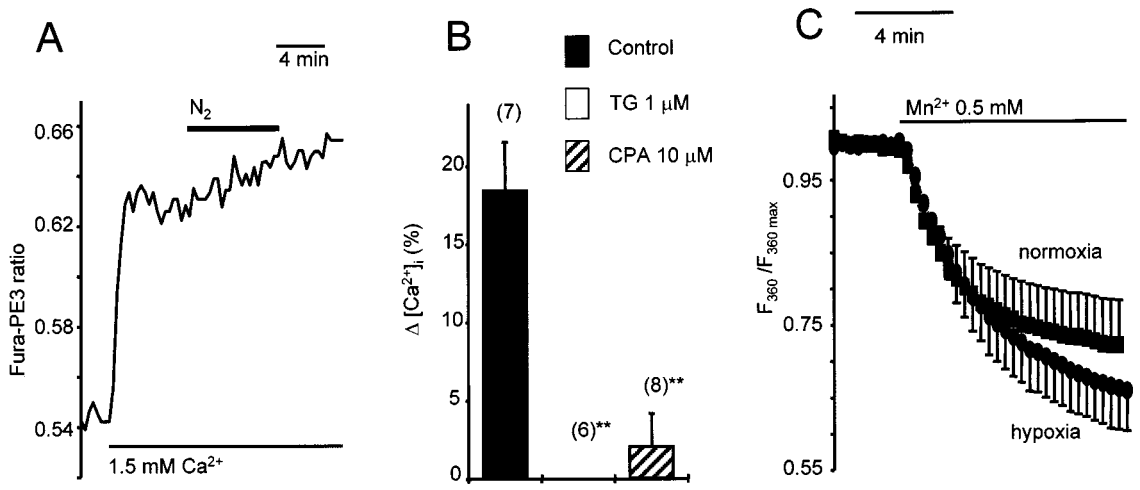

Figure 3 SERCA inhibitors prevent the effect of hypoxia. (A) The arteriole was pretreated with thapsigargin (TG, 1 μM) and then exposed to Ca^{2+}-free (0.4 mM EGTA) solution before 1.5 mM Ca^{2+} was reintroduced to the bath. Hypoxia (9.5 mmHg) did not affect $[Ca^{2+}]_i$. (B) Mean $\pm$ s.e.mean hypoxia-induced change (reduction) in fura-PE3 ratio expressed as a percentage relative to the response to Ca^{2+}-free bath solution (see Figure 1). Control arterioles were compared with arterioles pretreated with 1 μM TG or 10 μM cyclopiazonic acid (CPA) during the 1-h loading protocol with fura-PE3. The n values for the data sets are given in parentheses. CPA was also present during recordings. $^{**}P<0.01$. The minimum mean $\pm$ s.e.mean PO_2 values for TG and CPA groups were 18.4 $\pm$ 2.5 and 19.8 $\pm$ 1.5 mmHg, compared with 19.8 $\pm$ 2.2 mmHg in the controls ($n=7$). (C) As for Figure 2C except arterioles were pretreated for 1 h with 1 μM TG ($n=7$ normoxia, and $n=6$ hypoxia).

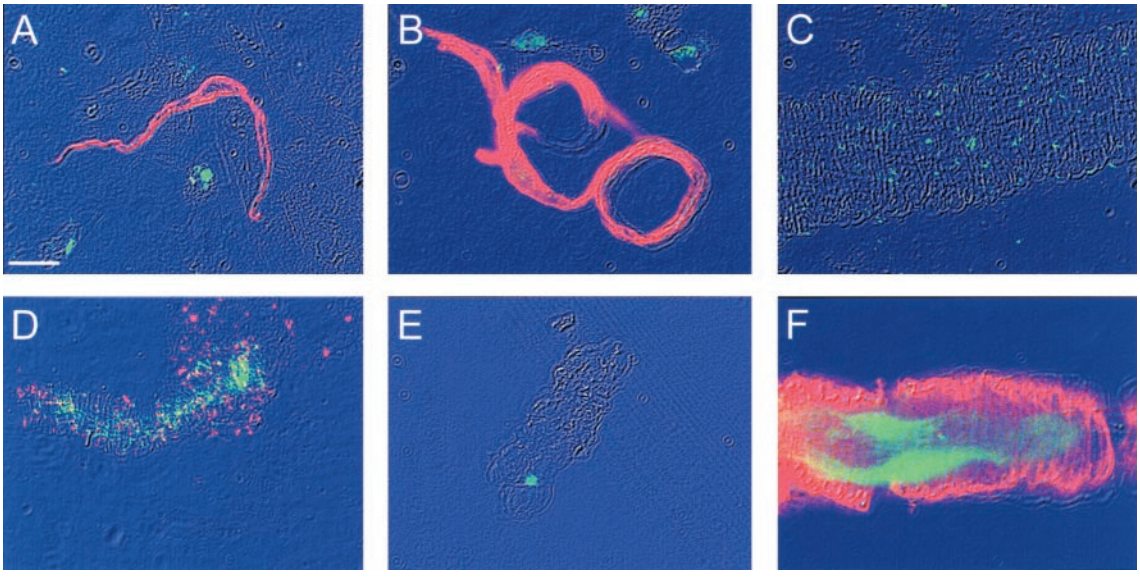

Figure 4 Detection of NOS-I and NOS-III in isolated rabbit pial arteriole fragments and smooth muscle cells. Each fluorescence image is overlaid on the bright field image (blue). (A) Isolated arteriolar smooth muscle cells double labelled with anti-α-SMA-Cy3 (red) and anti-NOS-I (green). (B) Isolated arteriolar smooth muscle cells double labelled with anti-α-SMA-Cy3 (red) and anti-NOS-III (green). (C) A long arteriolar fragment labelled with anti-NOS-I (green). (D) A long arteriolar fragment double labelled with anti-VIP (red) and anti-NOS-I (green). Yellow indicates co-localization of VIP and NOS-I. (E) A short arteriolar fragment labelled for NOS-I (green). (F) Partially fragmented long arteriole double labelled with anti-α-SMA-Cy3 (red) and anti-NOS-III (green). The scale bar in (A) is 10 μm and applies to all panels.

Discussion

The results of this study reveal a novel effect of hypoxia on $[Ca^{2+}]_i$ in arteriolar smooth muscle cells that it is independent of Ca^{2+}-entry through L-type voltage-gated Ca^{2+} channels and independent of nitric oxide release. Strikingly, the effect of hypoxia was abolished by two chemically distinct inhibitors of SERCA, suggesting a dependence on Ca^{2+}-handling by the sarcoplasmic reticulum.

In the normal cerebral cortex of rat or rabbit, arterioles of 25–40 μm are surrounded by PO_2 70–80 mmHg (Ivanov *et al.*, 1982; Vovenko, 1999). Physiological or pathological PO_2 levels around these vessels during hypoxia or ischaemia are unknown, but PO_2 levels around distal capillaries and venules are not less

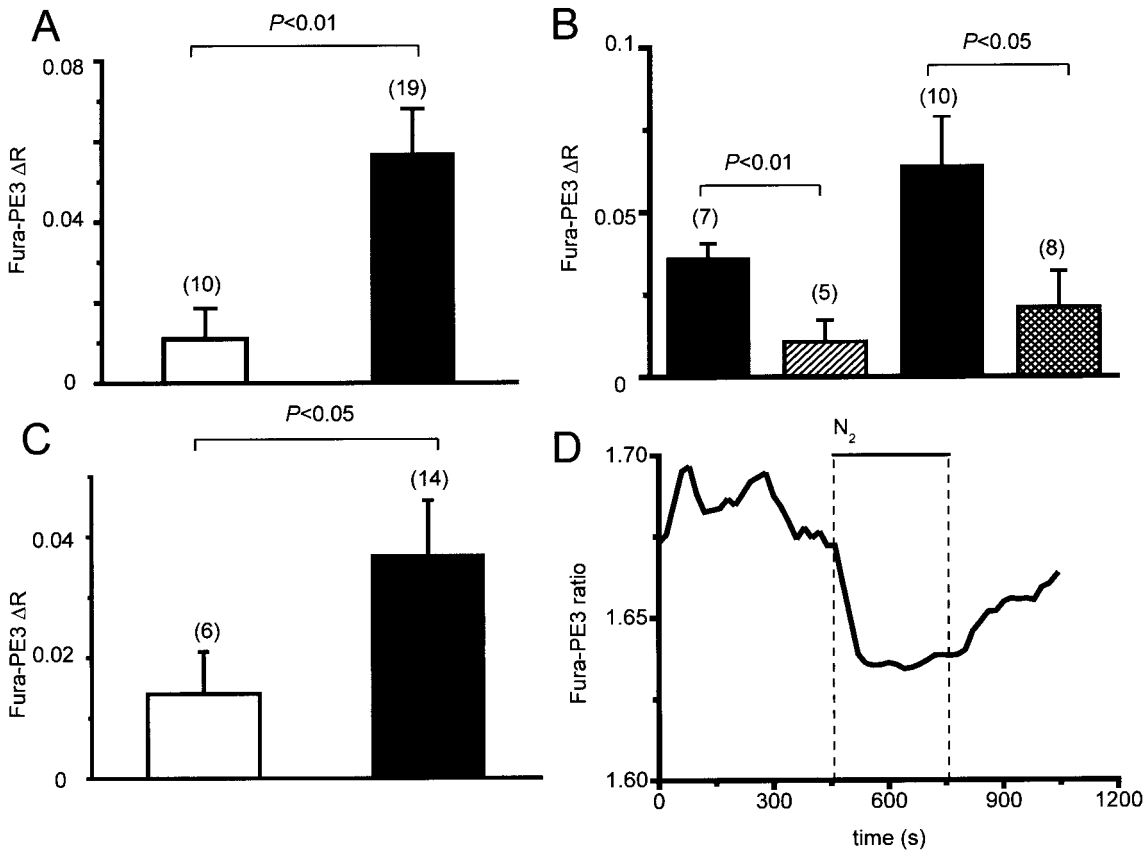

Figure 5 Endogenous NO regulates $[Ca^{2+}]_i$ but does not play a role in the action of hypoxia. In each panel, $Ca^{2+}{}_i$ signals are expressed as changes in fura-PE3 ratio, mean ± s.e.mean values are given, and *n* values are indicated in parentheses next to the error bars. NOS inhibitors or NO donors were also included during the 1-h fura-PE3 loading protocol. (A) $Ca^{2+}{}_i$ signal observed on changing from Ca^{2+}-free to Ca^{2+}-containing bath solution compared alternately for control conditions (open) and in the presence of 0.3 mM L-NAME (black). (B) $Ca^{2+}{}_i$ signal observed on changing from Ca^{2+}-free to Ca^{2+}-containing bath solution and compared alternately for two conditions: On the left for 0.3 mM L-NAME only (black) compared with 0.3 mM L-NAME plus 1 mM L-arginine (single hatch). On the right for 0.3 mM L-NAME only (black) compared with 0.3 mM L-NAME plus 0.3 mM DEA/NONOate (double hatched). (C) $Ca^{2+}{}_i$ signal observed on changing from Ca^{2+}-free to Ca^{2+}-containing bath solution compared alternately for control conditions (open) and in the presence of 1 μM s-methyl-L-thiocitrulline (black). (D) In the presence of $Ca^{2+}{}_o$, the effect of hypoxia (minimum PO_2 9.3 mmHg) on $[Ca^{2+}]_i$ in the presence of 0.3 mM L-NAME in the bath.

than 20–30 mmHg (Ivanov *et al*., 1982; Vovenko, 1999). The effects of hypoxia observed in our experiments occurred as PO_2 was lowered from 150 mmHg to a minimum of about 18 mmHg and thus in a range that is relevant to *in vivo* situations.

Voltage-gated Ca^{2+} channels are inhibited by hypoxia (Pearce *et al*., 1992; Franco-Obregon & Lopez-Barneo, 1996) but other types of Ca^{2+} channel do not seem to show a similar sensitivity. Ca^{2+}-influx pathways analysed by Mn^{2+}-quenching of fura-PE3 were not inhibited by hypoxia, whether or not Ca^{2+} stores were depleted. Mn^{2+}-entry appeared instead to be slightly stimulated. This effect may be explained if the cells were hyperpolarized by hypoxia or if there was a direct stimulatory rather than inhibitory effect of hypoxia on Ca^{2+} channels, as suggested recently for TRP channels (Agam *et al*., 2000). Either effect could also explain the slight stimulatory action of hypoxia on Ca^{2+} signals when SERCA was inhibited (Figure 3A). It is not ruled out that a stimulatory effect due to hyperpolarization masked a direct inhibitory effect of hypoxia on Ca^{2+} entry. Nevertheless, if there was such an effect it does not explain the ability of hypoxia to lower $[Ca^{2+}]_i$ and would presumably have little functional significance. Mn^{2+}-quench experiments do not exclude that hypoxia inhibits a Mn^{2+}-impermeable Ca^{2+}-entry pathway. However, our experiments do exclude the possibility that hypoxia inhibited a Mn^{2+}-impermeable Ca^{2+}-entry pathway because hypoxia had no effect in the presence of SERCA inhibitors, which do not block plasma membrane Ca^{2+} transport mechanisms.

Two chemically distinct inhibitors of SERCA prevented the negative effect of hypoxia on $[Ca^{2+}]_i$. These inhibitors do not block Ca^{2+} extrusion or mitochondrial uptake processes (Thastrup *et al*., 1990; O'Donnell & Owen, 1994) and Ca^{2+} pathways are enhanced rather than inhibited (e.g. Figure 2 *c.f.* Figure 3). Thus, an effect of hypoxia on SERCA, or an associated protein, is indicated. Such a stimulatory effect might seem unlikely if hypoxia causes ATP levels to fall. However, ATP levels do not fall substantially in acute hypoxia (Faraci & Heistad, 1998) and thus other, stimulatory, responses can dominate. In future studies it may be worthwhile to investigate several possible mechanisms, including: (1) SERCA is inhibited by reactive oxygen species such as hydrogen peroxide (Grover *et al*., 1992) and thus lowering hydrogen peroxide levels associated with hypoxia might enhance SERCA activity. (2) Hypoxia-evoked increases in cyclic GMP or AMP levels (Folbergrova *et al*., 1981;

Ashwal *et al*., 1988) could stimulate SERCA (Raeymaekers *et al*., 1988). It is also possible that hypoxia lowered $[Ca^{2+}]_i$ by inhibiting Ca^{2+} leak from the sarcoplasmic reticulum, without an effect on SERCA. Nevertheless, block by SERCA inhibitors strongly suggests that the action of hypoxia depends on Ca^{2+}-handling by the sarcoplasmic reticulum.

Endogenous NO was investigated in this study because of parallels between the actions of hypoxia and NO, and evidence that hypoxic vasodilatation is mediated by NO release. Although our final conclusion is that NO does not mediate the action of hypoxia, significant observations arose specifically relating to NO and NOS localization. First, endogenous NO inhibited the Ca^{2+}-reapplication signal in the presence of D600, which might be explained if the NO-dependent stimulation of SERCA that has been described for aortic myocytes (Cohen *et al*., 1999) also occurs in arteriolar smooth muscle and is relevant to endogenous as well as exogenous sources of NO. Second, we reveal the pial arteriole cellular localization of the two constitutively expressed NOS isoforms, NOS-I and NOS-III (also referred to as nNOS and eNOS). Although NOS-I and NOS-III are often associated with neuronal tissue and vascular endothelium respectively, there is evidence for NOS-I in smooth muscle of rat carotid artery, sarcoplasmic reticulum from rabbit cardiomyocytes and skeletal muscle (Nakane *et al*., 1993; Boulanger *et al*., 1998; Xu *et al*., 1999) and for NOS-III in cardiac myocytes, intestinal smooth muscle and corpus cavernosum (Feron *et al*., 1996; Teng *et al*., 1998; Bloch *et al*., 1998). However, in rabbit pial arterioles, NOS-I and NOS-III were absent from the smooth muscle cells. Instead, staining was associated with other structures – adventitial nerve ending-like structures for NOS-I (some being associated with VIP) and endothelial cells for NOS-III. A third isoform, NOS-II (also referred to as inducible NOS or iNOS) was not detected.

The observation that NOS-I and NOS-III are not expressed in arteriolar smooth muscle cells but can still be associated with enzymatically isolated arterioles suggests that non-smooth muscle structures sometimes exist in the arteriolar preparation. Although NO does not mediate the effect of hypoxia on $[Ca^{2+}]_i$ in arteriolar smooth muscle, we cannot exclude that other intermediate factors released from the other structures were involved in the action of hypoxia. Hypoxia releases several factors from the parenchyma and endothelium, including hydrogen and potassium ions, prostaglandins, adenosine or excitatory amino acids (Pearce, 1995; Berne *et al*., 1974; Wei *et al*., 1980; Pelligrino *et al*., 1995). Nevertheless, fura-PE3 dye loading was only associated with the smooth muscle cells and localization of data collection to regions of interest helped ensure that Ca^{2+} signals originated only from smooth muscle cells.

The complexities of arteriolar responses to hypoxia continue to unfold. In this study we reveal an additional action of hypoxia on $[Ca^{2+}]_i$ in arteriolar smooth muscle cells that was prevented by SERCA inhibitors. The effect is similar to that reported for NO on aortic myocytes (Cohen *et al*., 1999) and adds to the list of common mechanisms modulated by lowered O_2 and raised NO concentrations, which also includes inhibition of voltage-gated Ca^{2+} channels and activation of several types of K^+ channel (reviewed in Beech, 1997; Beech *et al*., 2001). Nevertheless, despite this similarity and evidence that effects of hypoxia can be mediated by NO, in this instance hypoxia did not act by modulating endogenous NO release.

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