Oxygen-sensitive calcium channels in vascular smooth muscle and their possible role in hypoxic arterial relaxation

(O₂ sensing/hypoxia/cytosolic Ca²⁺ oscillations)

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ABSTRACT We have investigated the modifications of cytosolic [Ca²⁺] and the activity of Ca²⁺ channels in freshly dispersed arterial myocytes to test whether lowering O₂ tension (PO₂) directly influences Ca²⁺ homeostasis in these cells. Unclamped cells loaded with fura-2 AM exhibit oscillations of cytosolic Ca²⁺ whose frequency depends on extracellular Ca²⁺ influx. Switching from a PO₂ of 150 to 20 mmHg leads to a reversible attenuation of the Ca²⁺ oscillations. In voltage-clamped cells, hypoxia reversibly reduces the influx of Ca²⁺ through voltage-dependent channels, which can account for the inhibition of the Ca²⁺ oscillations. Low PO₂ selectively inhibits L-type Ca²⁺ channel activity, whereas the current mediated by T-type channels is unaltered by hypoxia. The effect of low PO₂ on the L-type channels is markedly voltage dependent, being more apparent with moderate depolarizations. These findings demonstrate the existence of O₂-sensitive, voltage-dependent, Ca²⁺ channels in vascular smooth muscle that may critically contribute to the local regulation of circulation.

Oxygen tension (PO₂) has been recognized for decades as an important factor in the local regulation of vascular tone in vivo (1), and it is known that hypoxia causes relaxation of systemic arteries in vitro (2–4). Nevertheless, the mechanisms underlying this physiological response to low PO₂ remain largely unknown. Based on studies done in organ bath preparations, it is believed that oxygen influences vascular resistance by directly interfering with the rise of cytosolic Ca²⁺ required for contraction of smooth muscle cells (4–7); nevertheless, very little is understood about oxygen-regulated processes in arterial myocytes. It has been suggested that dilatation of the coronary artery (8, 9), and perhaps other arteries, in response to extreme hypoxia might be mediated by myocyte hyperpolarization in response to the opening of ATP-regulated K⁺ channels. However, it seems unlikely that this is the only underlying mechanism because the sensitivity to hypoxia in most arteries occurs over a physiologic range of PO₂ without a compromise of energy metabolism (3). Since in the past few years O₂-regulated ion channels have been demonstrated to participate in a number of cellular functions (10), we hypothesized that vascular tone could be also regulated by direct modulation of voltage-gated Ca²⁺ channels by PO₂. Here, we show the reversible inhibition of the macroscopic Ca²⁺ current of arterial myocytes in response to hypoxia, which can explain the suppression of the cytosolic [Ca²⁺] oscillations in the same cells by low PO₂. These data demonstrate the existence of O₂-regulated Ca²⁺ channels in vascular smooth muscle and suggest that they might participate in hypoxic arterial relaxation.

METHODS Experiments were performed on enzymatically dispersed smooth muscle cells from the celiac and femoral arteries of adult rabbits. In brief, arteries were removed, placed in cold (4°C) Hanks’ balanced salt solution, and opened longitudinally. Their outer and inner surfaces were cleaned of the adventitia and endothelium, respectively. Pieces of artery (~1 mm³) were placed into 5 ml of Hanks’ solution to which 7 mg of papain, 5 mg of collagenase (Sigma, type IA), and 3.5 mg of bovine serum albumin (Sigma, fraction V) had been added. The tissue was then stored in this enzyme solution for 1–6 hr at 6°C. In preparation for the dissociation of smooth muscle cells, the tissue and enzyme solution were placed at 37°C for 15–25 min with low stirring. Upon detecting the first free cells, the tissue was then transferred to fresh Hanks’ solution containing bovine serum albumin (10 mg/50 ml) and mechanically dissociated through the fire-polished tip of a glass pipette. Cells were plated on pieces of poly(L-lysine)-coated glass coverslips. For experiments, a coverslip was placed in a recording chamber of ~0.2 ml with continuous flow of solution. External solutions were equilibrated with either air (PO₂ ≈ 150 mmHg) or mixtures of N₂ and air to obtain the desired O₂ concentrations. PO₂ in the chamber was monitored with an O₂-sensitive electrode (11). Cytosolic [Ca²⁺] was estimated in unclamped myocytes loaded with fura-2 acetoxymethyl ester. Experiments were performed on an inverted microscope with standard optical components and equipped for epifluorescence and dual-wavelength photometry (12, 13). Calibration of the fluorescence signals in terms of [Ca²⁺] was performed in vitro as described (14). Macroporous calcium currents were recorded in isolation using the whole-cell configuration of the patch-clamp technique (15, 16) after blockade of the voltage-dependent K⁺ channels. Although Na⁺ channels are practically absent in arterial myocytes, some experiments were also performed with tetrodotoxin (0.2 μM) added to the external solution. The holding potential was either ~80 or ~70 mV. Ba²⁺ was used as charge carrier instead of Ca²⁺ to favor the flow of current through the Ca²⁺ channels, and ATP was added to the internal solution to prevent the wash-out of the channels. The composition of the recording solutions is given in the figure legends. Capacity current transients were well fitted by single exponential functions with average time constants of 120 ± 45 μs (n = 24) and 113 ± 30 μs (n = 10) (mean ± SD) for celiac and femoral myocytes, respectively. This indicates that despite the large size of the cells (~100 μm in length and 6–8 μm in diameter), we had a reasonably fast voltage-clamp and, thus, we could directly monitor tail currents. Voltage-clamp speed was favored by ballistic charge of membrane capacitance and the use of low-resistance electrodes (between 1 and 3 MΩ) (16). Due to the relatively small size of the currents, series resistance was not systematically compensated. Analog current signals were low-pass filtered*.

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(cutoff frequency between 3 and 10 kHz), digitized at a sample interval of 20 or 50 μs, and stored on computer for analysis. The experiments were conducted at room temperature (22–25°C).

RESULTS

The effect of hypoxia on single arterial smooth muscle cells was first studied by monitoring the modifications of cytosolic [Ca2+] in response to changes in PO2 with double-wavelength microfluorimetry. Fura-2-loaded myocytes stimulated with a purinergic agonist (ATP) generated rhythmical oscillations of cytosolic [Ca2+]. In all myocytes exhibiting regular Ca2+ oscillations that were then subjected to the complete experimental protocol (n = 8), exposure to hypoxia (switching from a bath solution equilibrated with a PO2 of 150 to one of 20 mmHg) elicited a marked reversible reduction in the amplitude and frequency of the Ca2+ spikes. Hypoxic treatment eventually resulted in the complete suppression of the Ca2+ oscillations. A representative example of this cellular response to low PO2 is shown in Fig. 1A, which also includes the signal from an O2-sensing electrode placed in the vicinity of the cell. In those myocytes that had a relatively high resting [Ca2+] (above 50 nM), the inhibition of the oscillations by hypoxia was accompanied by a decrease in resting cytosolic Ca2+ levels. These observations indicate that low PO2 may exert its relaxing action by decreasing intracellular [Ca2+], which is the variable that determines contraction in vascular smooth muscle (17–20). Although largely due to Ca2+ release from intracellular stores (21, 22), Ca2+ oscillations in excitable (23) and nonexcitable (24) cells require, and are facilitated by, transmembrane Ca2+ influx. In accord with this idea, depolarization of myocytes with 60 mM external K+ elicits an increase in the frequency of the Ca2+ oscillations preceding a maintained elevation of cytosolic [Ca2+] (Fig. 1B). An opposite effect (abolishment of the Ca2+ spikes and decrease of resting cytosolic Ca2+) was observed after briefly removing external Ca2+ (Fig. 1C) or blockade of voltage-gated Ca2+ channels with nifedipine (Fig. 1D).

The hypoxic suppression of Ca2+ oscillations could be caused by various O2-dependent cellular processes. Refilling of stores previously depleted with caffeine was not prevented by hypoxia. Furthermore, hypoxia did not affect the release of Ca2+ from internal stores evoked by either caffeine (10 mM) or norepinephrine (3 μM). Therefore, one of the actions of low PO2 might be to inhibit Ca2+ influx through voltage-dependent channels. This was directly tested by recording the current through Ca2+ channels in whole-cell patch-clamped myocytes. Current sweeps generated in response to step depolarizations to +10 mV in a celiac myocyte exposed to normoxic (control and recovery) and hypoxic external solutions are shown in Fig. 2A. The recordings demonstrate an ≈40% reversible reduction in current amplitude upon exposure to low PO2. The relationship between current amplitude and PO2 is illustrated in Fig. 2B, where the values of peak current (dots) elicited by depolarizing pulses delivered at different PO2 levels are plotted. The graphs indicate that the reversible hypoxic inhibition of current amplitude occurs roughly with the time course of bath exchange and that it is particularly apparent at PO2 levels below 70 or 80 mmHg. The time course of the inhibition of the calcium current by low PO2 was similar to that of nifedipine block (not shown). For comparison, reversible hypoxic inhibition of Ca2+ channel activity in a femoral myocyte is illustrated in Fig. 2C. Similar qualitative results have been obtained in all cells studied so far (n = 33). It has been shown that vascular smooth muscle cells contain two major kinetically

![Fig. 1. Oscillations of cytosolic [Ca2+] in arterial myocytes and effect of changes in ambient oxygen tension (PO2).](image-url)
distinct populations of Ca\(^{2+}\) channels (L and T type) (25, 26) that, as in other cell types (16, 27), can be identified by their deactivation time courses as slow- (T) and fast- (L) deactivating channels. In many femoral and celiac myocytes we detect two components of the tails. The slowly deactivating component of the tail current, which is resistant to nifedipine (Fig. 3A) and inactivated by a small depolarizing prepulse (Fig. 3B), was unaffected by hypoxia (Fig. 3C) in all cells tested (see also Figs. 2C and 4A). These observations indicate that the effect of low PO\(_2\) is selective for the dihydropyridine-sensitive, fast-deactivating (L-type), channels, whereas the slow-deactivating (T-type) Ca\(^{2+}\) channel population is unaffected by changes in PO\(_2\).

A remarkable characteristic of the regulatory action of oxygen on Ca\(^{2+}\) channel activity is its strong voltage dependence. Fig. 4A shows a family of calcium currents generated by depolarizations from -80 mV to the indicated membrane potentials. Current traces recorded in a low PO\(_2\) solution (H) are compared with those obtained at normal PO\(_2\) (C). Recovery from hypoxia was almost perfect and an example is shown at +10 mV (trace R). Low PO\(_2\) produced an inhibition of current amplitude that was larger with moderate depolarization. With stronger depolarization the effect of hypoxia was almost negligible. In celiac myocytes, the average inhibition of current amplitude by hypoxia (PO\(_2\) ≈ 20 mmHg) was 43.17% ± 13.8% (mean ± SD, n = 12) of the control value at 0 mV, but only 2.08% ± 7% (n = 8) at +20 mV. In femoral myocytes, these values were 40% ± 27% (n = 7) at 0 mV and 10% ± 13% (n = 6) at +20 mV. The voltage dependence of the hypoxic inhibition of Ca\(^{2+}\) channel activity is also clearly evident in Fig. 4B, where we have plotted the average current–voltage relationship under normoxic (filled symbols) and hypoxic (open symbols) conditions obtained with measurements from four cells.

**DISCUSSION**

Our results indicate that exposure to low PO\(_2\) leads to inhibition of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels in arterial myocytes. This is selective since T-type Ca\(^{2+}\) channels appear to be unaffected. The effect of O\(_2\) tension on the Ca\(^{2+}\) channels is fast, is completely reversible, and occurs with PO\(_2\) levels within the physiological range. This phenomenon explains the decrease of cytosolic Ca\(^{2+}\) upon exposure to low PO\(_2\) and, thus, it is conceivable that it contributes to the hypoxic relaxation of systemic arteries. In agreement with previous work by other authors (28, 29), we have not detected so far any modulatory effect of PO\(_2\) on the macroscopic K\(^+\) currents of systemic myocytes. Pharmacological studies done in isolated perfused hearts have suggested that hypoxic vasodilatation of the coronary arteries could be a consequence of the decrease of intracellular ATP and the subsequent hyperpolarization caused by the opening of ATP-sensitive (K\(_{ATP}\)) K\(^+\) channels (8, 9). Activation of K\(_{ATP}\) channels in response to hypoxia most likely requires maintained exposure to extreme low PO\(_2\) (8, 30). Thus, although possibly important, this process may act on a slower, more protracted, time scale than the acute response of Ca\(^{2+}\) channels described here, occurring over a full range of PO\(_2\) values and surely critical for an immediate arterial relaxation in response to hypoxia.

The Ca\(^{2+}\) channels selectively regulated by PO\(_2\) are of the dihydropyridine-sensitive, L-type, which are broadly distributed in vascular smooth muscle and are known to be activated by norepinephrine and other vasoactive agents (20, 26, 31). In mesenteric artery myocytes, the membrane potential–force

**Fig. 2.** Inhibition of voltage-gated Ca\(^{2+}\) channels by low PO\(_2\). (A) Macroscopic calcium currents recorded from a myocyte dispersed from the celiac artery during 15-ms step depolarizations to +10 mV from a holding potential of -80 mV. Exposure to hypoxia (switching from an external solution equilibrated with PO\(_2\) ≈ 150 mmHg to another with PO\(_2\) ≈ 20 mmHg; records 2 and 3) induces an inhibition in current amplitude. Reversibility is illustrated by the recovery trace. (B) Parallel time courses of the changes of PO\(_2\) in the chamber and the reduction in current amplitude. The records shown in A are indicated by the corresponding number. Current amplitudes were measured immediately before the end of the depolarizing pulses. (C) Reversible reduction of calcium current amplitude by low PO\(_2\) (20 mmHg) in a myocyte dispersed from the femoral artery. Depolarizing pulses to +10 mV (15 ms) were applied from a potential of -80 mV. Note that a slow component of the tail current was unaffected by low PO\(_2\). The recording solutions contained (in mM) the following: External [140 NaCl, 2.7 KCl, 10 BaCl\(_2\), and 10 Hepes (pH 7.4)]. Internal (solution in the patch pipette and inside the cell) [100 CsCl, 25 CsF, 2 MgCl\(_2\), 10 Hepes, 10 EGTA, 5 bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate, and 4 MgATP (pH 7.3)].
FIG. 3. Separation of fast- and slowly deactivating components in the Ca\(^{2+}\) tail currents and selective inhibition of the fast component by low PO\(_2\). (A) Superposition of current traces recorded in a celiac myocyte during 15-ms depolarizations to +10 mV from -80 mV in the control solution and after addition of 0.2 \(\mu\)M nifedipine (upper panel). The tail currents in the two experimental conditions are shown at an expanded time scale in the middle and bottom panels. As in other cell types (16, 27), the tail currents have fast and slow components that most likely represent the L-type (fast-deactivating) and T-type (slow-deactivating) Ca\(^{2+}\) channels described in arterial smooth muscle cells (25, 26). Nifedipine almost completely abolished the fast component of the tail but left unaltered the slow component. This is shown by the similar amplitude of single exponential functions that in the two experimental conditions were fitted to the slow component of the tail. The exponential functions were extrapolated to the onset of repolarization (indicated by downward arrows). (B) Superposition of current traces obtained from a femoral myocyte during 10-ms depolarizations to +10 mV from the indicated holding potentials (HP) (upper traces). The middle and bottom panels show the exponential functions fitted to the slow component of the tail at the two holding potentials. Note that a small maintained depolarization (from -80 to -50 mV) leads to >80% reduction of the slow component in the tail, indicating inactivation of the T-type Ca\(^{2+}\) channels. The fast component of the tail was almost unaltered. (C) Superposition of current traces in a celiac myocyte generated during 15-ms step depolarizations to +10 mV from -80 mV in a myocyte exposed to normoxic (C, control), PO\(_2\) = 150 mmHg) and hypoxic (H, PO\(_2\) = 20 mmHg) solutions (upper panel). As in A, the recordings in the middle and bottom panels show that the slow component of the tail currents was unaffected by low PO\(_2\) and that the reduction of current amplitude is due to selective inhibition of the fast component of the tail current.

relation almost perfectly matches with the voltage dependence of Ca\(^{2+}\) channel open probability \(p_{\text{open}}\) (20). Thus, it has been suggested that this channel type is a major regulator of smooth muscle tension and hence of arterial tone (20, 28, 31).

FIG. 4. Voltage dependence of the inhibitory effect of low PO\(_2\) on the Ca\(^{2+}\) channels. (A) Current traces recorded during 15-ms step depolarizations from -80 mV to the indicated membrane potentials. Sweeps recorded from the same celiac myocyte in normoxic (C, PO\(_2\) = 150 mmHg) and hypoxic (H, PO\(_2\) = 20 mmHg) solutions are superimposed. Note that the effect of low PO\(_2\) is larger with moderate depolarizations. (B) Average current–voltage relation in normoxic (filled symbols) and hypoxic (open symbols) solutions. Current amplitudes were measured in four celiac myocytes before the end of 15-ms depolarizing pulses. Vertical bars are the standard error of the mean.
Interestingly, the inhibition of the calcium current by low $P_{O_2}$ is more pronounced at potentials between $-30$ and $0$ mV (see Fig. 4), which is the range at which the $P_{O_2}$-voltage relationship of the channels is very steep (20, 31). Therefore, in partially depolarized myocytes low $P_{O_2}$ would be expected to have a major influence on channel $P_{O_2}$. In good agreement with this idea, early studies had already shown that the sensitivity of systemic arteries to oxygen is more obvious when testing is carried out on preconditioned samples using low to moderate concentrations of agonist (5).

In conclusion, we have found in arterial smooth muscle a type of $Ca^{2+}$ channel modulation that may participate in hypoxic arterial dilatation. The properties of the oxygen-sensitive $Ca^{2+}$ channels make them well suited for having a major physiological role in the fast adaptation of regional arterial resistance to the degree of blood oxygenation. The existence of oxygen-sensitive $K^+$ channels in various tissues has been previously described (10) but oxygen-sensitive $Ca^{2+}$ channels are without precedent in the literature. Besides celiac and femoral myocytes (representative examples of visceral and skeletal muscle arteries), we have observed a similar regulatory action of $P_{O_2}$ in muscle cells dispersed from rat and rabbit mesenteric arteries as well as from the main trunk of the rabbit pulmonary artery. This suggests that $O_2$-regulated $Ca^{2+}$ channels are distributed throughout the circulatory system and in different species. This channel type might be involved in some cardiocirculatory disturbances such as hypertension.

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