Effects of Hypoxia on Isometric Force, Intracellular Ca\(^{2+}\), pH, and Energetics in Porcine Coronary Artery

Shunichi Shimizu, Peggy Sue Bowman, George Thorne III, Richard J. Paul

Abstract—When exposed to hypoxic conditions, coronary arteries dilate, which is an important protective response. Although vessel sensitivity to oxygen is well documented, the mechanisms are not known with certainty. To further characterize the mechanisms of oxygen sensing in the coronary artery, we tested the major classes of hypotheses by measuring the effects of hypoxia on energetics, [Ca\(^{2+}\)], K\(^+\) channel function, and pH. Hypoxia relaxes porcine coronary arteries stimulated with either KCl or U46619. The extent of relaxation is dependent on both the degree and kind of stimulation. [Ca\(^{2+}\)] was measured in endothelium-denuded arteries using fura 2-AM and ratiometric fluorescent techniques. At lower stimulus levels, hypoxia decreased both force and [Ca\(^{2+}\)]. Inhibitor studies suggest that K\(_\text{Ca}\) and K\(_\text{ATP}\) channels are not involved in the hypoxic relaxation, whereas K\(_\text{V}\) channels may play a minor role, if any. Despite the hypoxia-mediated decrease in force, [Ca\(^{2+}\)], was unchanged or increased at high levels of stimulation. Despite a marked increase in lactate content, pH (measured with the ratiometric fluorescent dye BCECF) was also little affected by hypoxia. Measurement of the phosphagen and metabolite profile of freeze-clamped arteries with analytical isotachophoresis indicated that hypoxia increased lactate content by 4-fold and decreased phosphocreatine to 60% of control. However, neither ATP nor Pi was affected by hypoxia. Interestingly, additional stimulation under hypoxia increased force but not ATP utilization, as estimated from measurements of anaerobic lactate production. Thus, surprisingly, the economy of force maintenance is increased under hypoxia. In porcine coronary artery, both Ca\(^{2+}\)-dependent and, importantly, Ca\(^{2+}\)-independent mechanisms are involved in hypoxic vasodilatation. For the latter, mechanisms involving either ATP, [Ca\(^{2+}\)], pH, or Pi cannot be invoked. This novel oxygen sensing mechanism involves a decreased Ca\(^{2+}\) sensitivity. (Circ Res. 2000;86:862-870.)

Key Words: coronary arteries ■ hypoxia ■ pH ■ Ca\(^{2+}\) ■ metabolism

The sensitivity of vascular contractility to P\(_O\_2\) has been an important subject among physiologists for more than 100 years.\(^1\)\(^-\)\(^3\) Although there is little question that P\(_O\_2\) affects vascular reactivity, the mechanisms have been the subject of much investigation. Given that pulmonary vessels contract, but most systemic vessels relax when exposed to hypoxia, the mechanisms are likely to be diverse. Hypoxia elicits a relaxation in coronary arteries that may be a physiologically protective response. Many theories have been put forth; however, they can be roughly divided into those involving energy limitation and those involving interruption of excitation-contraction coupling.

The first class involves some form of limitation on ATP synthesis. Relaxation is attributable to the inability of cellular ATP production under hypoxia to support the actin-myosin ATPase activity and hence contractile activity. This could be simply because of an inherently low glycolytic capacity for ATP synthesis under hypoxic conditions, or the capacity could be compromised; for example, acidic pH associated with hypoxia could inhibit glycolysis. It is also possible that regulatory sites for oxidative or anaerobic metabolism, such as phosphofructokinase, may be oxygen sensing modulatory elements.\(^4\) We have reported that an energy limitation mechanism is likely to underlie hypoxic relaxation observed in guinea pig taenia coli\(^5\)\(^,\)\(^6\); however, the available evidence does not support an energy limitation mechanism for the oxygen sensitivity of vascular smooth muscle. Pittman and Duling\(^7\) reported that contractility was depressed only when the smooth muscle cells in the core of a hypoxic artery became anoxic, suggesting that vascular sensitivity to P\(_O\_2\) could involve an energy limitation. However, because P\(_O\_2\) for arterioles would be unlikely to reach these low levels, a direct role for oxygen in situ could not be supported. There is also evidence that vascular reactivity of both large and small arteries is depressed for P\(_O\_2\) in the 20 to 100 torr range,\(^2\)\(^,\)\(^8\) which is higher than the range generally associated with inhibition of mitochondrial oxidative phosphorylation. Evidence for an oxygen sensing mechanism in vessels independent of mitochondrial metabolism was also provided by Coburn,\(^7\) who reported that even after inhibition of respiration by cyanide, contractility in rabbit aorta could still be depressed by hypoxia. More recent studies\(^4\)\(^,\)\(^10\) indicate that...
hypoxic relaxation is not simply a function of energy stores, and oxidative metabolism-contraction coupling is regulated by energy delivery to a reaction- or reactions-controlling muscle force.

An alternative class of hypotheses involves oxygen sensing mechanisms that would modulate the cell signaling pathways involved in excitation-contraction coupling, including mechanisms in which [Ca\(^{2+}\)] is the regulated parameter, for example by oxygen-dependent or ATP-dependent changes in Ca\(^{2+}\) permeability.\(^{11,12}\) A hypothesis of this class with considerable experimental support is that lowered [Ca\(^{2+}\)], concomitant with hyperpolarization is attributable to activation of K\(^{+}\) channels.\(^{13,14}\) Alternatively, evidence for signaling changes at constant [Ca\(^{2+}\)], is accumulating.\(^{3,5}\) Mechanisms involving altered Ca\(^{2+}\) sensitivity of the contractile apparatus, such as those potentially associated with pH changes with hypoxia, would be in this category. These categories are not necessarily mutually exclusive.

Although vessel sensitivity to oxygen is well documented, the mechanisms are not known with certainty. To further characterize the mechanisms of oxygen sensing in the coronary artery, we investigated the effects of hypoxia on energetics and [Ca\(^{2+}\)], the central second messenger for control of vascular smooth muscle contractility. In addition, we studied the roles of K\(^{+}\) channels and pH, major factors known to modulate contractility. In these coronary arteries, both Ca\(^{2+}\)-dependent and -independent mechanisms underlie hypoxic vasodilatation.

Materials and Methods

Preparation of Arterial Rings

Porcine hearts obtained shortly after slaughter were rinsed of blood and placed in a cold (4°C) bicarbonate-buffered physiological salt solution (PSS). The distal portions of the left anterior descending coronary artery were dissected, cleaned of fat and connective tissue, and cut into 5-mm segments with a wall thickness between 300 and 500 µm. The segments were everted and deendothelialized by rubbing gently on filter paper.\(^{15}\)

Isometric force measurements were previously described.\(^{16,17}\) Hypoxia was operationally defined as aeration with 95% N\(_2\)/5% CO\(_2\), which decreases the bath P\(_O_2\) to <1%, as we have reported.\(^{18}\)

Intracellular Ca\(^{2+}\) measurements were made with the fluorescent probe fura 2-AM in an apparatus that permitted simultaneous measurements of force and [Ca\(^{2+}\)], as previously described.\(^{19,20}\) In some experiments, the rings were mounted isometrically on a wire loop that was fitted into a spectrofluorometer cuvette; no differences were noted in the [Ca\(^{2+}\)] values, and the data were averaged.

[Ca\(^{2+}\)]\(^{+}\) Calibration

The fluorescent intensity at 340-nm excitation was divided by fluorescent intensity at 380 nm, and this ratio was used as an index of [Ca\(^{2+}\)]. For statistical analysis, the ratio was assigned values of 0% for resting muscle and 100% for tissue stimulated with 40 mmol/L KCl. This protocol was chosen as a general routine over standard procedures previously reported in detail.\(^{15}\)

Results

Effects of Hypoxia on Isometric Force

Figure 1 shows a typical isometric force response to hypoxia, as previously described.\(^{19,20}\) In some experiments, the rings were mounted isometrically on a wire loop that was fitted into a spectrofluorometer cuvette; no differences were noted in the [Ca\(^{2+}\)] values, and the data were averaged.

High-energy phosphagens, metabolites, and lactate production measurements have been previously reported in detail.\(^{22}\) Briefly, artery rings were frozen in liquid N\(_2\), pulverized, and mixed with frozen extraction solution (1 vol methanol and 1 vol 2 mmol/L EDTA) at liquid N\(_2\) temperature. An LKB analytical isotachophoresis apparatus was used to measure negatively charged metabolites in the thawed solution. Lactate production was measured using standard procedures previously reported in detail.\(^{15}\)

Solutions

The PSS contained (in mmol/L) NaCl 122, KCl 4.73, NaHCO\(_3\) 15, MgCl\(_2\) 1.19, EDTA 0.02, KH\(_2\)PO\(_4\) 1.19, glucose 11.10, and CaCl\(_2\) 2.50. When aerated with 95% O\(_2\)/5% CO\(_2\), the pH of the PSS was 7.3 to 7.4 at 37°C.

Analysis of Data

The values given are mean±SEM. N values for sample size represent the number of hearts from which arteries were taken. Student’s test for paired data and standard ANOVA with Bonferroni discrimination for multiple groups were used: a significance level of P<0.05 was chosen for rejection of the null hypothesis.

An expanded Materials and Methods section is available online at http://www.circresaha.org.
basal tone, which has been reported in deendothelialized porcine coronary arteries to be $\approx 20\%$ of the maximum KCl contracture. Addition of 29 mmol/L KCl under hypoxia elicited a markedly diminished contraction, with maximum force similar to that obtained in the steady state after imposition of hypoxia on a control KCl contracture, as in Figure 2A.

Interestingly, increasing the level of stimulation can override the inhibitory effects of hypoxia. The dependence of the inhibitory effects of hypoxia on isometric force on the level of stimulus is shown in Figure 3. Cumulative concentration-response curves for KCl (Figure 3A) and receptor-mediated stimulation using U46619, a thromboxane A$_2$ analog (Figure 3B), were generated under aerobic and hypoxic conditions. Both stimuli elicit similar levels of maximum isometric force; however, hypoxia clearly has a more pronounced inhibitory effect on the U46619 response. The extent of the inhibition by hypoxia was strongly dependent on the concentration of the stimuli used. For example, at 29 mmol/L KCl, which elicits $\approx 80\%$ of the maximum force, hypoxia reduces this response to $\approx 20\%$. However, increasing KCl to 80 mmol/L increases isometric force under hypoxia to $\approx 75\%$ of that under oxygenated conditions. If metabolism were limited by hypoxia, this would be expected to set an absolute limit to force production independent of stimulus intensity. Thus, the effects of stimulus intensity on the inhibition of isometric force by hypoxia argue against an energy limitation hypothesis based on a limited anaerobic metabolic capacity.

In addition to energy limitation or mechanisms involving the mitochondrial respiratory electron transport chain$^{29}$ as the sensing element, a number of potential sites for oxygen sensing have been proposed. They include the Na$^{+}$ pump,$^1$ eicosanoid pathways,$^{30,31}$ alterations in plasma membrane electrical properties,$^{32,33}$ Ca$^{2+}$ permeability,$^{11,12}$ hydrogen peroxide formation,$^{34}$ potentially oxygen free radicals,$^{35–37}$ cGMP,$^{38}$ and more recently, ATP-dependent K$^{+}$ channels$^{13}$ and the inositol phosphate pathway. To delimit the potential mechanisms of oxygen sensing in porcine coronary artery, we also screened a number of these potential pathways using similar pharmacological approaches. We have previously reported$^{17}$ the effects of inhibitors, including ouabain, aminotriazole, superoxide dismutase, catalase, indomethacin, and propranolol, on the response to hypoxia. Although some minor modulatory effects were seen, as might be anticipated, on the whole these agonists had little effect on hypoxic relaxation.

To add to this database, we further tested the effects of chelerythrine (3 $\mu$mol/L), an inhibitor of the C-kinase pathway, and LY83583 (3 $\mu$mol/L), an inhibitor of the G-kinase pathway. Table 1 shows that neither inhibitor had significant effects on the force generated by 29 mmol/L KCl or the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Isometric Force, %</th>
<th>Hypoxic Relaxation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Chelerythrine (3 $\mu$mol/L)</td>
<td>95.3±2.9</td>
<td>108.5±2.7</td>
</tr>
<tr>
<td>LY83583 (3 $\mu$mol/L)</td>
<td>104.2±8.7</td>
<td>104.0±11.0</td>
</tr>
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</table>

Artery rings were contracted with KCl (29 mmol/L). One member of a paired set (experimental) was treated for 30 minutes with the drug; the other served as a control, and then arteries were exposed to hypoxia. Values are mean±SEM; n=4.
Effects of Hypoxia on [Ca\(^{2+}\)]

Central to most theories of hypoxic vasodilation are mechanisms for decreasing [Ca\(^{2+}\)]. We studied the effects of hypoxia on [Ca\(^{2+}\)], using the ratiometric fluorescent dye fura 2. Figure 4 shows representative recordings of the effects of hypoxia on [Ca\(^{2+}\)], at high (Figures 4A and 4C) and low (Figures 4B and 4D) stimulation intensity. At 40 mmol/L KCl, the 340/380 ratio rapidly increases and then is characterized by a small, slow decline. A decline in [Ca\(^{2+}\)] is less steep with 20 mmol/L KCl than with 40 mmol/L KCl. The effects of hypoxia with 20 mmol/L KCl are considerably different than those with 40 mmol/L KCl. Impression of hypoxia elicits a rapid and steady decline of [Ca\(^{2+}\)] despite maintained force is characteristic of smooth muscle. After imposition of hypoxia, [Ca\(^{2+}\)] slightly increases (during which time isometric force is decreasing) and then resumes a slow decline. After 30 minutes of hypoxia, [Ca\(^{2+}\)] is indistinguishable from the level just before the hypoxia intervention. Reoxygenation elicits a small rapid decrease in [Ca\(^{2+}\)], followed by a return to the original small, slow decrease. The rise of [Ca\(^{2+}\)] is less steep with 20 mmol/L KCl than with 40 mmol/L KCl. The effects of hypoxia with 20 mmol/L KCl are considerably different than those with 40 mmol/L KCl. Impression of hypoxia elicits a rapid and steady decline of 25%, which is reversed on reoxygenation. Average values for these experiments are graphically summarized in Figure 5.

Ishida and Honda reported that [Ca\(^{2+}\)] was not affected by hypoxia when stimulated with KCl, whereas hypoxia decreased [Ca\(^{2+}\)], for receptor-mediated stimulation in guinea pig aorta. Thus, we measured the effects of hypoxia on [Ca\(^{2+}\)], for U46619 contractures. Typical experiments are shown in Figure 4, and the average values are graphically summarized in Figure 5. The general results are similar to those with KCl stimulation. At 1 μmol/L U46619, hypoxia increased [Ca\(^{2+}\)] by 23% but force decreased by 50%. At 0.1 μmol/L U46619, hypoxia decreased both force (≈80%) and [Ca\(^{2+}\)] (≈50%).

Role of K\(^{+}\) Channels in the Ca\(^{2+}\)-Dependent Hypoxic Vasodilatation

Activation of K\(^{+}\) channels is one of the more extensively studied hypotheses for hypoxic vasodilatation. This hypothesis involves K\(^{+}\) channel activation, hyperpolarization, and consequent closing of voltage-dependent Ca\(^{2+}\) channels. Because [Ca\(^{2+}\)] was decreased by hypoxia at low levels of stimulation, the potential for K\(^{+}\) channel involvement was suggested. We used a pharmacological approach to identify whether K\(^{+}\) channels were involved in the low-stimulus hypoxic vasodilatation. Arteries were contracted with U46619 (0.1 μmol/L) and treated with K\(^{+}\) channel inhibitors before exposure to hypoxia. As with any pharmacological approach, the specificity of K\(^{+}\) channel inhibitors is open to interpretation. We made the following operational assignments in our investigation based on the literature and our recent studies on K\(^{+}\) channels in porcine coronary artery:

- K\(_{\text{Ca}}\), channel inhibition, tetraethylammonium (TEA) (1 mmol/L), apamin (1 μmol/L), and charybdoxin (0.1 μmol/L); K\(_{\text{V}}\) channel inhibition, 4-aminopyridine (4-AP) (1 mmol/L); K\(_{\text{ATP}}\) channel inhibition, glibenclamide (10 μmol/L); K\(_{\text{Ca}}\), and K\(_{\text{V}}\), TEA (10 mmol/L), K\(_{\text{IR}}\) and K\(_{\text{ATP}}\), BaCl\(_2\) (10 μmol/L); non-specific, tetrabutylammonium (TBA) (5 mmol/L). The effects of these K\(^{+}\) channel inhibitors on isometric force in response to U46619 and subsequent hypoxia are summarized in Table 2. Inhibition of K\(_{\text{ATP}}\) and K\(_{\text{Ca}}\) channels was associated with only small effects. This is somewhat surprising because the liter-
TABLE 2. Effects of K⁺ Channel Inhibitors on Hypoxic Vasorelaxation in U46619 Contractures in Porcine Coronary Artery

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Isometric Force* (0.1 μmol/L U46619)</th>
<th>Hypoxia-Induced Relaxation (10 mmol/L KCI)</th>
</tr>
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<tbody>
<tr>
<td>Untreated (n=6)</td>
<td>100.1±1.7</td>
<td>99.2±4.3</td>
</tr>
<tr>
<td>TBA (n=8)</td>
<td>98.1±3.5</td>
<td>57.3±7.4†</td>
</tr>
<tr>
<td>TEA (n=4)</td>
<td>104.1±3.0</td>
<td>106.7±11.2</td>
</tr>
<tr>
<td>(1 mmol/L)</td>
<td>108.0±1.8†</td>
<td>88.3±7.3</td>
</tr>
<tr>
<td>(3 mmol/L)</td>
<td>117.4±3.5†</td>
<td>85.4±4.9</td>
</tr>
<tr>
<td>(10 mmol/L)</td>
<td>Charybdotoxin (n=4)</td>
<td>103.2±3.2</td>
</tr>
<tr>
<td>(0.1 μmol/L)</td>
<td>104.6±4.7</td>
<td>84.1±2.1†</td>
</tr>
<tr>
<td>Apamin (n=6)</td>
<td>(1 μmol/L)</td>
<td>103.0±6.7</td>
</tr>
<tr>
<td>4-AP (n=6)</td>
<td>(1 mmol/L)</td>
<td>118.2±3.9†</td>
</tr>
<tr>
<td>(10 μmol/L)</td>
<td>Glibenclamide (n=10)</td>
<td>94.5±1.8†</td>
</tr>
<tr>
<td>BaCl₂ (n=5)</td>
<td>(10 μmol/L)</td>
<td>96.3±3.6</td>
</tr>
</tbody>
</table>

*After a steady-state response to U46619 was achieved, addition of K⁺ channel inhibitors immediately increased isometric force, which then decreased to a steady state. This column gives these steady-state values just before hypoxia. Values are mean±SEM, expressed as a percentage of control responses to U46619 or the subsequent response to hypoxia. **P<0.05 vs paired control responses.

Mechanisms of Ca²⁺-Independent Hypoxic Vasodilatation

The data in Figure 5 demonstrate that at high levels of stimulation, hypoxia decreases force with a little change or increase in [Ca²⁺]. To further demonstrate a Ca²⁺-independent relaxation, we attempted to maximize [Ca²⁺], by exposure of the arteries to KCl (40 mmol/L), added ionomycin (1 μmol/L), a Ca²⁺ ionophore, and finally added U46619 (1 μmol/L) to promote sarcoplasmic reticulum Ca²⁺ release. Data from these experiments, graphically summarized in Figure 6, show that isometric force under normoxic conditions increased to 150% of a KCl contracture by the cumulative treatment with ionomycin and U46619. Even under these conditions to fix [Ca²⁺], at maximal levels, hypoxia was still able to elicit a substantial relaxation. In light of this Ca²⁺-independent relaxation to hypoxia, we investigated pH, and energy metabolism, 2 alternative mechanisms proposed for hypoxic vasodilatation.5,6

Effects of Hypoxia on pH₅

Hypoxia is known to increase both lactate content (see below) and lactate production in porcine coronary artery.44 Moreover, we have reported that acidic pH₅ is associated with an inhibition of force in coronary arteries.21 Thus, it is plausible that the inhibition of force by hypoxia could be attributed to an acidification of pH. We measured the effects of hypoxia on pH using the ratiometric fluorescent dye BCECF, shown in Figure 7. As previously reported,21 KCl addition had only minor effects on pH despite the known increase in lactate production.45 Similarly, imposition of hypoxia did not acidify, as might be anticipated, because of the significant increase in lactate production (see below). The trend with hypoxia is alkalization, although the differences were not statistically significant. Because alkalization was shown to increase force in previous studies,21 the effects of hypoxia on pH₅ are unlikely to play any role in the observed Ca²⁺-independent relaxation.
Effects of Hypoxia on Phosphagen Content and Metabolite Profile

To gain more insight into the mechanisms of hypoxic vasorelaxation, we measured the phosphagen content and metabolite profile in freeze-clamped coronary arteries using isotachophoresis. The results are graphically summarized in Figure 8. As previously reported for carotid artery, stimulation of maximum isometric force with KCl was associated with little change in high-energy phosphagens or other metabolites. Hypoxia, as might be expected, was associated with a 3-fold increase in lactate content. The only other measured change was a decrease of ~50% in phosphocreatine (PCr) content. Significantly, there was no change in ATP or Pi.

As shown in Figure 9A, addition of U46619 (1 µmol/L) reverses the hypoxic relaxation of a KCl (29 mmol/L) contracture. This large increase in force under hypoxic conditions was not associated with a proportional increase in [Ca^{2+}]. The order of stimulation was not important, because adding KCl reversed the effects of hypoxia on a U46619 contracture (88.7 ± 5.5% of normoxic force, n=4, not significant). These data would again support the hypothesis that metabolic ATP production is not limiting, for presumably there is additional ATP mobilized to support the higher level of isometric force. In general, ATP utilization is proportional to the level of isometric force.

Figure 8. Effects of hypoxia on high-energy phosphagens (ATP and PCr), Pi, and lactate content of porcine coronary artery. Bar graph shows mean ± SEM; n=12. Open bars, Unstimulated, oxygenated conditions. Shaded bars, KCl stimulation, oxygenated conditions. Filled bars, KCl stimulation, hypoxia. *P<0.001.

Figure 9. Effects of addition of U46619 (1.0 µmol/L) to a KCl (29 mmol/L) contracture under hypoxia for a representative artery, isometric force (A), and [Ca^{2+}] (B). Under hypoxia, addition of U46619 to a KCl contracture was associated with a significant increase in isometric force, without a proportionate increase in [Ca^{2+}].

Figure 10. Statistical summary of the effects on isometric force, rate of lactate production (J_{lac}), and high-energy phosphagens (ATP and PCr) of addition of U46619 (0.1 µmol/L) to a KCl (29 mmol/L) contracture under hypoxia. Light shaded bars, Mean values with KCl stimulation. Dark shaded bars, Mean values with KCl+U46619. Bar graph shows mean ± SEM; all paired differences were significant at P<0.05; n=8 to 11. Despite the maintenance of higher forces with addition of U46619 than with KCl alone, J_{lac} was lower, indicating an increase in economy under hypoxic conditions.

It is possible that the increase in force with additional stimulation under hypoxia could be accomplished by increasing the force per ATP utilization (economy) rather than increasing ATP utilization at a constant economy. Thus, from an energetics viewpoint, it is of particular interest to know what, if any, changes in metabolism were associated with the increase in force observed when U46619 was added to the KCl contracture under hypoxic conditions (Figure 10).

Under these hypoxic conditions (~1% O_2), anaerobic glycolysis is virtually the only source for production of ATP. The rate of lactate production (J_{lac}) was equal to that measured in the bath, and no significant differences in tissue lactate content were found. Under hypoxia with KCl stimulation (Figure 10), J_{lac} was ~4 times the rate of oxygen consumption. This would suggest that the capacity for anaerobic glycolysis is adequate to stoichiometrically replace the ATP attributable to oxidative metabolism. After the addition of U46619 under hypoxia, force increased and remained elevated for the entire 60-minute measurement period at levels similar to the original force in the presence of KCl under aerobic conditions. J_{lac} did not increase with U46619 treat-
Hypoxia inhibits force development and relaxes force in precontracted porcine coronary artery. This is likely a physiologically protective response to increase blood flow under stress conditions. Our understanding of the mechanisms is complicated by our incomplete knowledge of the regulation of smooth muscle. It is generally accepted that Ca\(^{2+}\) is the major second messenger, acting via binding to calmodulin and subsequent activation of myosin light chain kinase. There is evidence suggesting a role for modulatory mechanisms, including those linked to the thin filament, such as caldesmon and calponin. In addition, there is a growing body of evidence suggesting that the Ca\(^{2+}\) sensitivity of the contractile system is also a parameter subject to modulation. Metabolism is also known to be closely linked to contractile function in smooth muscle; however, its role as a regulatory mechanism is less well-defined.

A most striking observation in these studies is that the relaxation or inhibition of force associated with hypoxia could be ascribed to at least two different mechanisms. At low stimulus levels, the inhibition of force was accompanied by a decrease in [Ca\(^{2+}\)]. At high stimulus levels, hypoxia decreased force in the presence of constant or increasing [Ca\(^{2+}\)]. In our conditions, hypoxia (bubbling with N\(_2\)) was such that bath PO\(_2\) was <1%, and the tissue PO\(_2\) was likely below that for mitochondrial oxidative phosphorylation throughout. In physiological terms, this is severe hypoxia, but it does enable one to set defined conditions for the tissue as a whole, which is useful to separate Ca\(^{2+}\)-dependent and -independent mechanisms. The dependence of these mechanisms on the PO\(_2\) is currently under investigation and will be important to understanding tissue oxygen sensing, which can be observed at considerably higher PO\(_2\).

The decrease in [Ca\(^{2+}\)] at low stimulus levels was not affected by inhibitors of K\(_{ATP}\) or K\(_{Ca}\) channels. There was a moderate inhibition (~15%) of the hypoxic relaxation associated with inhibition of K\(_{Ca}\) channels. These results are consistent with a recent study from our laboratory indicating that K\(_{Ca}\) channels are the predominant K\(^{+}\) channels in porcine coronary artery. Interpretation of these data, however, is not straightforward, because the specificity of K\(_{Ca}\) channel inhibitors is not absolute. In addition, 4-AP and TEA (but not TBA) also increased the force (~20%) before exposure to hypoxia, which could also account for some of the reduction in the relaxation to hypoxia.

A direct effect of hypoxia on Ca\(^{2+}\) channels or Ca\(^{2+}\) uptake or extrusion systems, such as enhanced Na\(^+-\)Ca\(^{2+}\) exchange, cannot be ruled out. These arteries are able to relax to hypoxia after inhibition of the sarcoplasmic reticulum Ca\(^{2+}\) pump with cyclopiazonic acid (G.T. and R.J.P., unpublished observations, 1999). Thus, a major role for the sarcoplasmic reticulum in hypoxic relaxation is unlikely in these arteries.

The Ca\(^{2+}\)-independent relaxation to hypoxia was observed for both receptor-mediated stimulation and depolarization, as long as the intensity was high. A Ca\(^{2+}\)-independent pathway has been suggested for several smooth muscles. The Ca\(^{2+}\)-independent effects of hypoxia are by definition a decrease in Ca\(^{2+}\) sensitivity; ie, a lower force maintained under hypoxic conditions was not associated with a change in [Ca\(^{2+}\)].

In a related set of experiments, we observed that although hypoxia inhibited force, increasing stimulation could override...
this inhibition. As shown in Figure 9, addition of U46619 to KCl-stimulated arteries under hypoxia (or vice versa) restored force to near initial levels. No major changes in high-energy phosphagens from those of hypoxia alone were measured (Figure 10). Thus, changes in ATP levels do not seem to be a major signaling pathway.

To further investigate the effects of hypoxia on energetics, we measured the rate of ATP production by arterial lactate production in parallel experiments of similar design. Because changes in high-energy phosphagens were negligible, the ATP production is equivalent to utilization. Interestingly, whereas U46619 increased force under hypoxia, there was no parallel increase in Jac (Figure 10). Thus, hypoxia seems to affect the coupling between force production and ATP utilization. This may simply mean that ATP was diverted to the myosin ATPase from other processes. It is also possible that the crossbridge cycle might be modified under hypoxic conditions. Because PCR was decreased by hypoxia, the calculated free ATP levels would increase, assuming that the creatine kinase reaction was in equilibrium.\(^56\) Thus, it is plausible that changes in [ADP] may underlie hypoxic relaxation. One might anticipate that increasing ADP could affect the crossbridge detachment rate, which may also underlie the change in energetics. Changes in [ADP] are known to alter the crossbridge cycle rate in smooth muscle, but the effective concentration ranges seem to be too high\(^59\) or too low\(^60\) based on the calculated [ADP] in vascular smooth muscle.

It is also plausible that changes in myosin regulatory light chain phosphorylation (MLC-P) may be the regulated step for hypoxic relaxation. Coburn et al\(^6\) reported that hypoxia decreased MLC-P in receptor-mediated contractures but was less affected in KCl contractures in rabbit aorta. Because ATP was not unaffected, presumably myosin light chain kinase activity would not be the site for the Ca\(^{2+}\)-independent oxygen sensing. There is evidence that myosin phosphatase may be a site for MLC-P regulation,\(^61\) which may involve a G protein and C kinase. Within the scope of limited pharmacological data, our negative results with chelerythrine lessen the likelihood for the involvement of C kinase in the mechanism of hypoxic relaxation. However, oxygen sensing at the level of MLC-P remains to be tested.

In conclusion, hypoxic vasorelaxation in porcine coronary arteries involves both Ca\(^{2+}\)-dependent and -independent mechanisms. The hypoxic vasodilatation cannot be attributed to changes in pH, Pio, or ATP. Under hypoxia, Ca\(^{2+}\) sensitivity is decreased but the force per ATP seems to be increased. Large coronary arteries can play a significant role in the regulation of coronary circulation.\(^62\) The relaxation induced by hypoxia in coronary arteries likely plays a protective role in coronary ischemia. Our results rule out many of the current theories for hypoxic relaxation, but the sites for this important oxygen sensing mechanism remain to be elucidated.

Acknowledgments

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References


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