Hypoxia inhibits vasoconstriction induced by metabotropic Ca\(^{2+}\) channel-induced Ca\(^{2+}\) release in mammalian coronary arteries

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Aims We have previously described in rat basilar arterial myocytes that in the absence of extracellular Ca\(^{2+}\) influx, activation of L-type Ca\(^{2+}\) channels stimulates a metabotropic cascade leading to Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) and contraction [a calcium channel-induced Ca\(^{2+}\) release (CCICR) mechanism]. On the other hand, it is known that hypoxia reduces Ca\(^{2+}\) channel activity in coronary myocytes. In the present study, we have investigated whether CCICR is present in coronary arterial myocytes and whether arterial ring contraction induced by CCICR can be inhibited by hypoxia.

Methods and results Isometric force, arterial diameter, cytosolic [Ca\(^{2+}\)] and electrical activity were recorded on mammalian (porcine, rat, and human) coronary artery preparations (dispersed myocytes, arterial rings, and intact arterial segments). In the absence of extracellular Ca\(^{2+}\), Ca\(^{2+}\) channel activation increased cytosolic [Ca\(^{2+}\)] in isolated myocytes and contracted arterial rings. This contraction was suppressed by antagonists of L-type Ca\(^{2+}\) channels and by inhibiting Ca\(^{2+}\) release from the SR.

Hypoxia induced dilatation of coronary arterial rings pre-contracted by activation of Ca\(^{2+}\) channels in the absence of extracellular Ca\(^{2+}\). This effect was present although KATP channels and Rho kinase were blocked by glibenclamide and Y27632, respectively.

Conclusion We show that Ca\(^{2+}\) channel activation can induce metabotropic coronary arterial ring contraction in the absence of extracellular Ca\(^{2+}\) and that this CCICR mechanism is inhibited by hypoxia. Thus, besides reduction of Ca\(^{2+}\) entry through Ca\(^{2+}\) channels, hypoxia seems to induce coronary vasorelaxation by inhibition of metabotropic CCICR.

1. Introduction

In most vascular beds, including the coronary circulation, reducing arterial oxygen tension (PO\(_2\)) produces vasodilation. Hypoxic coronary vasodilatation is of critical physiological importance because it contributes to adjust the amount of oxygen supplied to the working heart to its metabolic needs;\(^{1,2}\) however, the underlying mechanisms remain poorly understood. There are several routes by which a reduction in PO\(_2\) could cause coronary arteries to dilate. The first one involves release of vasodilator substances, such as nitric oxide or prostaglandins, from endothelial cells lining the arteries or the production of vasoactive agents by cardiac muscle.\(^{3-5}\) Alternatively, it has been suggested the existence of oxygen sensing mechanisms in the arterial myocyte that in hypoxia would modulate the cell signalling pathways mediating excitation–contraction coupling. In this regard, it is known that in coronary myocytes, reduction of intracellular ATP level in hypoxia results in the opening of ATP-regulated K\(^+\) channels (K\(_{ATP}\)), leading secondarily to membrane hyperpolarization, closure of voltage-dependent Ca\(^{2+}\) channels and relaxation.\(^{6,7}\) Apart from the effects on K\(^+\) channels, hypoxia can inhibit the activity of Ca\(^{2+}\) channels in arterial myocytes.\(^{8-13}\) Moreover, it has been shown that low PO\(_2\) reduces calcium influx from extracellular medium\(^{14,15}\) and modulates intracellular Ca\(^{2+}\) stores.\(^{16-18}\) We previously described that in endothelium-denuded porcine coronary arterial rings, contraction induced with high [K\(^+\)] was inhibited by lowering PO\(_2\) in the presence of glibenclamide, excluding the participation of K\(_{ATP}\) channels in this vasorelaxing effect of hypoxia. In isolated human coronary smooth muscle cells, low PO\(_2\) decreased L-type calcium current and reduced cytosolic Ca\(^{2+}\), suggesting that, as in other systemic arteries, voltage-gated Ca\(^{2+}\) channels in

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coronary myocytes are under control of O2 tension. On the other hand, we have reported in rat basilar arterial myocytes a novel metabotropic role of L-type Ca2+ channels that in the absence of extracellular Ca2+ or any transmembrane Ca2+ influx, mediate the coupling of channel activation to Ca2+ release from the sarcoplasmic reticulum (SR). This new form of excitation–contraction coupling, denoted as calcium channel-induced Ca2+ release (CCICR), depends on the conformational change of the Ca2+ channels and the downstream activation of the G protein-PLC biochemical cascade, leading to synthesis of InsP3 and vasoconstriction. It has been suggested that CCICR could contribute to pathophysiological conditions presenting persistent vasoconstriction, since CCICR can be elicited by chemical agents (like ATP) or small depolarizations without the actual opening of Ca2+ channels. In this paper, we report our investigations on the presence of CCICR in mammalian (rat, porcine, and human) coronary arterial circulation and its contribution to the hypoxia-induced vasodilatation characteristic of this vascular bed.

2. Methods
2.1 Preparation of arterial rings and dispersed myocytes
Experiments were performed on arterial rings and isolated myocytes from epicardial (anterior descendent) human and porcine coronary arteries. Porcine arteries were obtained from anesthetized (sodium pentobarbital, 50 mg/kg, i.v.) animals of weight 25 kg (40 pigs and 150 arterial rings) after exsanguinations and cardiotomy. Human arteries were dissected from hearts of three patients (33, 61, and 47 years old) subjected to orthotopic heart transplantation that had suffered heart failure owing to dilated cardiomyopathy of ischaemic origin (stages II–IV of the New York Heart Association). Experiments on arterial rings were done as indicated in a previous report from our laboratory. In most experiments, exposure to hypoxia was done by switching the bubbling of the solution in the chamber from 95% O2 and 5% CO2 to 95% N2 and 5% CO2. A low PO2 value (~20 mmHg) was obtained in less than 1 min. In some experiments (see figure legends), hypoxia was obtained by switching from a solution equilibrated with 2% O2 (normoxic solution) to another equilibrated with either N2 or 6% O2 (hypoxic solutions). Experiments were performed at 37°C.

To prepare dispersed coronary arterial myocytes, the adventitia was carefully removed and the arterial segments cut into 1–2 mm pieces and placed in a Petri dish with 5 mL salt solution (see below) to which 3–5 mg/mL papain (Sigma), 2–3 mg/mL collagenase (type IA; Sigma), and 1–2 mg/mL bovine serum albumin (fraction V; Sigma) had been added. The tissue was stored for ~4 h at 7°C and afterwards placed for 15 min in a shaker at 37°C. When the first myocytes appeared dispersed in the solution, the tissue was transferred to fresh salt solution containing 2% O2 (normoxic solution) to another equilibrated with either N2 or 6% O2 (hypoxic solutions). Experiments were performed at 37°C. To monitor the video camera image and have its fluorescence intensity recorded. Arterial diameter and [Ca2+]i signals were digitized at 2 Hz and analysed using IonWizard edge-detection software (IonOptix Corporation).

2.2 Cytosolic [Ca2+] measurements and patch clamp recordings
Cytosolic [Ca2+] was estimated in intact and dialysed patch clamped cells. In the first case, myocytes were incubated in standard external solution (see below) with 2.5 μM Fura 2-AM added for 15 min at room temperature. In dialysed cells, Fura 2 potassium salt (50 μM) was added to the patch pipette solution (see below). Experiments were done as indicated in previous reports from our laboratory. Voltage-clamp recordings were performed using the whole cell configuration of the patch clamp technique. When cytosolic [Ca2+] and membrane currents were recorded simultaneously, the signals were digitized at a sampling interval of 500 ms. Before digitization, membrane currents obtained from the EPC7 patch clamp amplifier were filtered at 3 kHz. All the experiments were performed at room temperature (~22°C).

2.3 Simultaneous measurement of intracellular [Ca2+] and arterial diameter
Experiments in intact arteries were done using rat coronary arteries incubated for 30 min in salt solution containing pluronic acid (0.02%) and subsequently for 1 h in the same solution containing 4 μM Fura-2 AM at room temperature. An artery was cannulated at each end in a temperature-controlled perfusion chamber (Living Systems Instrumentation), pressurized to 60 mmHg, and perfused with standard Krebs solution (5 mL/min; 37°C) for 40 min to allow stabilization. An optical-based measuring system (Myocam, IonOptix Corporation) coupled to an inverted microscope (Axiovert 35 Zeiss), was used for combined monitoring of Ca2+ concentration and outer arterial diameter. The Fura-2 loaded artery was alternately excited at 340 or 380 nm and Fura-2 emitted fluorescence was derived to a photomultiplier tube by using a 5850DCXR nm dichroic mirror. [Ca2+] was monitored by the 340/380 ratio of the fluorescence intensities measured at 510 nm. Fluorescence was background-corrected. By restricting the transmitted light to longer wavelengths (using a deep red filter in the microscope condenser), we could simultaneously monitor the Ca2+ fluorescence intensity recorded. Arterial diameter and [Ca2+] signals were digitized at 2 Hz and analysed using IonWizard edge-detection software (IonOptix Corporation).

This investigation on animal tissue samples conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and the investigation conforms with the principles outlined in the Declaration of Helsinki. Permission for the use of ex vivo human material was granted by the Institutional Review Board.

2.4 Solutions
Standard Krebs solution (mM): NaCl 118.5, KCl 4.7, CaCl2 2.5, NaHCO3 25, MgSO4 1.2, KH2PO4 1.2, glucose 5.

Salt solution (mM): NaCl 125, KCl 5.36, KH2PO4 0.44, Na2HPO4 0.34, NaHCO3 5, sucrose 1.45, glucose 10, Hapes 10, pH 7.4.

Standard external solution (mM): NaCl 140, KCl 2.7, CaCl2 2.5, MgCl2 1, Hapes 10, pH 7.4.

Patch pipette solution (mM): Kglutamate 83, KCl 47, MgCl2 1, Hapes 10, ATP-Mg 4, Fura 2-potassium salt 50 μM, pH 7.2.

Unless otherwise noted, all the drugs used were added to these solutions at the indicated concentration. In experiments on arterial rings, the high [K+] solution was obtained by adding KCl from a 2 M stock solution. In the case of pressurized whole artery experiments, the high [K+] was obtained by equimolar substitution of Na+ with K+. Quantiatively, similar results were obtained with the two solutions. The 0 mM Ca2+ solution (OCa) was obtained by substitution of Ca2+ with Mg2+ and by adding either 0.2–1 mM (arterial rings) or 2 mM (isolated myocytes) EGTA.

2.5 Statistical analysis
Data are expressed as mean ± SE and the statistical significance was estimated using the Student’s t test or Mann–Whitney U-test when n > 3 or n = 3, respectively. Values of P < 0.05 were considered significant.
3. Results

3.1 Ca\(^{2+}\) release and metabotropic-induced contraction mediated by Ca\(^{2+}\) channel activation in calcium-free external solution

Microfluorimetric measurements in undialysed coronary myocytes bathed in 0Ca medium showed that in several cases (in four of seven experiments) exposure of the cells to the isosmotic depolarizing solution induced a clear rise of cytosolic \([\text{Ca}^{2+}]\) (Figure 1A). The magnitude of these increases in \([\text{Ca}^{2+}]\) was \(59 \pm 13\%\) (\(P = 0.02, n = 4\)) with respect to the changes in \([\text{Ca}^{2+}]\) observed in the presence of extracellular \(\text{Ca}^{2+}\). Subsequent experiments were done to determine whether CCICR could evoke contraction of coronary arterial rings. We used a 40 mM K\(^+\) (40K) external solution to induce a relatively mild depolarization. It has been shown before that this concentration of extracellular K\(^+\) depolarizes isolated patch clamped rat arterial myocytes to \(-25.4 \pm 5.2\ mV\) (\(n = 5\)).\(^{20}\) In the absence of extracellular \(\text{Ca}^{2+}\), pig coronary arterial rings exposed to a hypertonic 40K depolarizing solutions underwent a gradual rise in isometric force, which reached a magnitude of \(3.9 \pm 0.4\ g, n = 9\) within 10 min. Although this effect could be due to both membrane depolarization and exposure to a hyperosmolar...
solution, the isometric force obtained in the same high [K+] solution without Ca2+ was decreased to 83% of the contraction induced in the presence of Ca2+ (Figure 1B and E). In human coronary arterial rings, application of the same high [K+] depolarizing solution in the absence of external Ca2+ elicited an increase on isometric force of 3.7 ± 0.3 g (n = 3; data not shown). Figure 1B also shows that after contraction of the ring by 40K in 0Ca, subsequent application of FPL 64176 (FPL), a benzoylpyrrole Ca2+ channel agonist23,24 that can by itself elicit CCICR,20 failed to induce contraction, suggesting that previous vasoconstriction induced by 40K was mediated by Ca2+ release from intracellular stores. The contraction evoked by the hypertonic 40K in the absence of extracellular Ca2+ was also present in vessels denuded of endothelium, which was evidenced by the lack of vasorelaxing effect of adenosine diphosphate an endothelium-dependent inducer of nitric oxide production (Figure 1C).4 Endothelium removal had no effect on 40K-induced vessel contractility, as contraction was 5.4 ± 0.6 and 4.7 ± 0.25 g (P = 0.2, n = 9) in the presence and absence of endothelium, respectively. Similar results were obtained when in 0Ca, FPL was used to activate Ca2+ channels to avoid membrane depolarization-dependent InsP3 formation unrelated to Ca2+ channel activation.25–27 We have shown before in dispersed arterial myocytes that CCICR is independent of extracellular Na+ (which could leak through Ca2+ channels made unselective by removal of extracellular Ca2+) and that FPL does not induce depolarization of dialysed myocytes.29 Moreover, we have tested that strong FPL-induced contractions can be evoked in arterial rings bathed in low extracellular Na+ concentration (see Supplementary material online, Figure S1). In the absence of extracellular Ca2+, FPL-induced contraction of arterial rings reached 5.09 ± 0.31 g (n = 56) within 12–14 min. This magnitude represents 86% of the contraction induced by FPL in the presence of extracellular Ca2+ (Figure 1D and E). Vasoconstriction was not present during a second exposure to FPL, suggesting that intracellular Ca2+ stores had been depleted by the first FPL application (Figure 1D).

We also did experiments in intact rat coronary arteries (Figure 2). Ca2+ channel antagonists inhibit contraction induced by Ca2+ channel activation in the absence of extracellular Ca2+ in porcine and human arterial rings. (A and D) Contraction induced by exposure to hypertonic 40K solution was inhibited by D600 (25 μM) (90 ± 1% inhibition, P < 0.001). (B–D) Contraction induced by FPL (1 μM) was inhibited by diltiazem (10 μM) (93 ± 1% inhibition, P < 0.001) and nifedipine (5 μM) (95 ± 2% inhibition, P < 0.001) in porcine coronary arterial rings. Similar effect was recorded when nifedipine (95 ± 1% inhibition, n = 3, P < 0.001) and diltiazem (96 ± 0.5% inhibition, n = 3, P < 0.001) were applied to human coronary arterial rings (E and F). Note in (E) the lack of significant effect of ACh after washing nifedipine. All experiments were done in the absence of extracellular Ca2+ (0Ca2+ plus 200 μM EGTA added).

![Porcine coronary artery ring](image)

![Human coronary artery ring](image)
where FPL-induced contraction and changes in the intracellular calcium concentration can be simultaneously monitored. Figure 1F shows that FPL induced a significant transient increase of fluorescence ([Ca$^{2+}$]) in the absence of extracellular Ca$^{2+}$, which was accompanied of a significant reduction in arterial diameter. Thus, the data indicate that FPL-induced contraction requires elevation of cytosolic Ca$^{2+}$. Both the latency and time course of K and FPL induced ring contractions were quite variable, possibly due to uncontrolled experimental parameters (e.g. degree of contractile filaments sensitization, drug diffusion, quality of the preparation, etc.). In the case of FPL (0.2 mM EGTA solution), transient (Figure 1D) and relatively sustained (Figures 2 and 3) responses were observed. However, in all rings studied, the FPL-induced contractile responses were transient (139 ± 7 s; n = 3) when 1 mM EGTA was added to the OCa solution (see Supplementary material online, Figure S2).

In coronary porcine arterial rings, contraction evoked by either hypertonic 40K or FPL was inhibited by D-600, diltiazem, and nifedipine, different families of Ca$^{2+}$ channel antagonists (Figure 2A–D). FPL application in the absence of extracellular Ca$^{2+}$ to human coronary arterial rings also induced an increase of isometric force that after 10 min reached the average value of 2.5 ± 0.3 g (n = 8) and was suppressed by blockade of Ca$^{2+}$ channels with nifedipine and diltiazem (Figure 2E and F). The lack of significant effect of acetylcholine (Ach) on isometric force in myocytes pre-treated with FPL (Figure 2E) suggests that intracellular Ca$^{2+}$ stores had been emptied by the previous exposure to the Ca$^{2+}$ channel agonist. Besides in porcine and human arterial rings, we also tested the effect of Ca$^{2+}$ channel antagonists on the FPL or 70K-induced contraction and cytosolic [Ca$^{2+}$] rise in intact rat coronary arteries with identical qualitative results (Supplementary material online, Figures S3 and S4). These findings indicate that in coronary arteries bathed in a Ca$^{2+}$-free solution, either membrane depolarization or application of L-type Ca$^{2+}$ channel pharmacological agonists trigger contraction, possibly due to the activation of Ca$^{2+}$ channels and subsequent Ca$^{2+}$ release from intracellular stores. This CCICR-mediated contraction is almost abolished in the presence of Ca$^{2+}$-channel antagonists.

Because CCICR is mediated by InsP$_3$ synthesis, activation of InsP$_3$ and ryanodine receptors, and Ca$^{2+}$ release from the SR, experiments were designed to study the role of this metabolic pathway in the vasoconstriction induced by Ca$^{2+}$ channel activation in the absence of extracellular Ca$^{2+}$. We have used 2-aminoetoxidifenil borato (2APB) and tetracaine, membrane permeable blockers of InsP$_3$ and ryandine receptors respectively,28,29 because these agents block CCICR in myocytes isolated from rat basilar artery.20 Figure 3A–C shows that FPL-induced contraction on pig coronary arterial rings was significantly inhibited by 2APB and tetracaine, suggesting that Ca$^{2+}$ channel activation induced by FPL can trigger InsP$_3$ synthesis and Ca$^{2+}$ release mediate by InsP$_3$ and ryanodine receptors activation, as described in other VSMCs.20,22 Since at high concentrations, 2APB may also inhibit Ca$^{2+}$ entry via store-operated channels (SOC), we tested the effect of diethylstilbestrol (DES) an inhibitor of SOC channels.30 DES (1–0.5 μM) (I$^{50}$=260 nM) did not significantly reduce contraction induced by FPL in the absence of extracellular Ca$^{2+}$ (13.5 ± 2% reduction, n = 3, P = 0.6), suggesting that the reduction of isometric force by 2APB is dependent on InsP$_3$ receptor blockade.

3.2 Hypoxia reduces isometric force induced by CCICR in coronary arterial rings

Ca$^{2+}$ channels in vascular smooth muscle are modulated by vasoactive agents, and we have reported that in basilar arteries extracellular ATP produces facilitation of Ca$^{2+}$ channel activation in isolated myocytes.21 In contrast, hypoxia inhibits Ca$^{2+}$ currents in rabbit mesenteric myocytes as well as in pig and human coronary artery VSMCs, and reduces the magnitude of isometric contractions induced by high [K$^+$] solution in the presence of extracellular Ca$^{2+}$.5,19 Because ATP potentiates CCICR-dependent vasoconstriction in basilar arterial rings,21 we explored whether CCICR contributes to the hypoxia-induced vasodilatation characteristic of the coronary arterial circulation. In voltage-clamped myocytes isolated from pig coronary arteries, sustained membrane depolarization to 10 mV from −50 mV caused an increase in [Ca$^{2+}$] that was reversibly inhibited by hypoxia (70 ± 28% inhibition, n = 3, P < 0.05), thus further supporting the view that low oxygen

Figure 3 Vasoconstriction induced by FPL in porcine coronary arterial rings is mediated by Ca$^{2+}$ release from the sarcoplasmic reticulum. (A and C) Application of 2APB (50 μM) and (B and C) tetracaine (50 μM), inhibitors of InsP$_3$, and ryanodine-mediated Ca$^{2+}$ release from intracellular stores respectively, relaxed coronary arterial rings. 2APB (80 ± 5% inhibition, P = 0.001) and tetracaine (80 ± 7% inhibition, P < 0.001). All experiments were done in the absence of extracellular Ca$^{2+}$ (OCa$^{2+}$ plus 200 μM EGTA added).
tension leads to inhibition of voltage-dependent calcium currents and \(\text{Ca}^{2+}\) influx from extracellular medium (Figure 4A).\textsuperscript{13,19} Qualitatively similar results were obtained in intact vessels where cytosolic \([\text{Ca}^{2+}]\) and arterial diameter were measured simultaneously. In 4/5 rat coronary arteries exposed to isosmotic high \([K^+]\) solution, hypoxia induced a significant reduction of cytosolic \([\text{Ca}^{2+}]\) accompanied of a significant increase of arterial diameter (Figure 4B). To test for the effect of hypoxia on CCICR, coronary arterial rings were exposed to \(0\text{Ca}\) solutions with FPL added to induce activation of \(\text{Ca}^{2+}\) channels (Figure 1). We used mainly this pharmacological approach because the effect of hypoxia on contraction induced by \(\text{Ca}^{2+}\) channels agonists has been poorly studied and to elude the direct effect of membrane depolarization on InsP\(_3\) synthesis.\textsuperscript{25–27} As shown in Figure 4C, the increase of isometric force induced by FPL in endothelium-denuded arteries was reversibly inhibited by hypoxia. This effect was unaltered in the presence of glibenclamide, used to block \(K_{\text{ATP}}\) channels (Figure 4D). The effect of hypoxia on FPL-evoked contraction was also observed in arterial rings with intact endothelium (Figure 5A and B) and was independent on the order of application. In these experimental conditions, hypoxia applied for 8 min relaxed \(\approx\)80% arterial rings pre-contracted with FPL. In the presence of glibenclamide, hypoxia inhibited FPL-induced contraction by \(\approx\)62% (Figure 5C). The same effects of hypoxia were observed when human coronary arterial rings were subjected to a FPL treatment; hypoxia applied for 8 min reduced isometric force to 67.6 ± 5% (\(P = 0.04\), \(n = 3\)).

Although it has been reported that hypoxia can interfere with internal \(\text{Ca}^{2+}\) stores,\textsuperscript{16–18} this mechanism does not seem to operate in coronary myocytes since \(\text{Ca}^{2+}\) release induced by ryanodine receptor activation with caffeine is not affected by hypoxia.\textsuperscript{19} Moreover, we have also tested whether lowering oxygen tension does alter the contraction of coronary arterial rings elicited by ACh, a response independent of \(\text{Ca}^{2+}\) channel activation and mediated by InsP\(_3\) synthesis and activation of the InsP\(_3\) receptors.\textsuperscript{31,32} In rings bathed in \(0\text{Ca}^2+\) solution, ACh-induced vasoconstriction was not significantly altered by hypoxia (Figure 5D and E). These results further strengthen the view that the relaxing effect of hypoxia in the coronary circulation is exerted through inhibition of \(\text{Ca}^{2+}\) channel activity.

### 3.3 Hypoxia-induced vasorelaxation is not mediated by Rho kinase modulation

Although previous\textsuperscript{19} and current results suggest that hypoxia-induced coronary vasorelaxation is mediated by \(\text{Ca}^{2+}\) channel inhibition, other groups have reported that both \(\text{Ca}^{2+}\)-dependent and \(\text{Ca}^{2+}\)-independent mechanisms
can be involved in hypoxic vasodilatation of the porcine coronary artery.\textsuperscript{33,34} A potential target of the Ca\textsuperscript{2+}-independent O\textsubscript{2} sensing mechanism is the GTP-mediated RhoA/Rho kinase (ROK) pathway. We have tested the effect of hypoxia on isometric force induced by FPL in the presence of Y27632, a selective ROK inhibitor, to suppress the vasoconstriction mediated by RhoA/Rho kinase activation.\textsuperscript{35} Figure 6A shows that application of FPL induced a reproducible contraction which was reduced in the presence of Y27632 and that this response was reversibly inhibited by hypoxia. A summary of the effect of ROK and hypoxia on PFL-induced vasoconstriction is illustrated in Figure 6B.

As experiments to test the effect of hypoxia on arterial tension were done in preparations bathed in a control solution bubbled with non-physiological 95\% O\textsubscript{2} (subsequently exposed to a hypoxic solution bubbled with N\textsubscript{2}), we performed additional experiments with control normoxic solutions equilibrated with 21\% O\textsubscript{2} and exposed to less drastic hypoxia (6\% O\textsubscript{2}). Application of moderate hypoxia (6\% O\textsubscript{2}) from a control level of 21\% O\textsubscript{2}, resulted in a significant decrease of the contraction induced by FPL (39\% relaxation, \( n = 5 \), \( P = 0.02 \)). The reduction of isometric force induced by moderate hypoxia was not significantly affected by Y27632 (46\% inhibition, \( n = 5 \), \( P = 0.78 \)) (Figure 6C).

Because the inhibitory effect of Y27632 could be unspecific, for example by blocking Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels, we have measured cytosolic [Ca\textsuperscript{2+}] in isolated pig coronary myocytes in the presence of the ROK inhibitor. The rise of cytosolic [Ca\textsuperscript{2+}] induced by 70K was not significantly affected upon exposure to Y27632 (Figure 6D). Although it has not been studied in detail, a significant inhibitory effect of hypoxia on depolarization-induced contraction (51\% inhibition, \( n = 3 \), \( P = 0.04 \)) also was present in the presence of chelerythrine (5 \( \mu \text{M} \)), a PKC inhibitor. Together, these results strongly support the view that vasorelaxation induced by hypoxia is mediated through inhibition of the metabotropic action of Ca\textsuperscript{2+} channels and that the sensitization mechanisms do not seem to have a major part on this action.

Regardless of the effect of hypoxia, contraction evoked by FPL was clearly reduced by Y27632 (\( \approx 50\% \)), thus suggesting that a component of isometric force induced by the Ca\textsuperscript{2+} agonist depends on ROK activation (Figure 6A). The interaction between FPL and Y27632 was also studied in an intact vessel preparation (rat coronary artery) with simultaneous monitoring of contraction and cytosolic Ca\textsuperscript{2+} concentration. The rise of cytosolic [Ca\textsuperscript{2+}] induced by FPL was not significantly affected upon exposure to 5 \( \mu \text{M} \) Y27632, whereas this agent (possibly by inhibiting sensitization of the contractile filaments) induced a marked decrease in PFL-evoked vasoconstriction (Figure 6E).

4. Discussion

The main observations described in the present study are: (i) L-type Ca\textsuperscript{2+} channel activation, induced by either high [K\textsuperscript{+}] solution or Ca\textsuperscript{2+} channel agonist, evokes vasoconstriction of coronary arteries without Ca\textsuperscript{2+} influx from the extracellular medium. (ii) Ca\textsuperscript{2+} channel-induced contractility in the absence of extracellular Ca\textsuperscript{2+} requires functional SR receptors, thus suggesting the existence of CCICR in the coronary arteries. (iii) CCICR is markedly inhibited by low oxygen
tension. Therefore, hypoxia facilitates vasorelaxation by inhibiting both the ionotropic and metabotropic actions of voltage-dependent Ca\(^{2+}\) channels in vascular smooth muscle cells.

In this paper, we demonstrate that contraction mediated by Ca\(^{2+}\) channel activation through a metabotropic pathway (CCICR), described in basilar artery,\(^{20}\) is also present in coronary artery. We used in our experiments both iso and hyperosmolar high [K\(^{+}\)] solutions to open Ca\(^{2+}\) channels with similar qualitative results. However, most of the data in this paper are based on Ca\(^{2+}\) channel activation with FPL, a Ca\(^{2+}\) channel agonist that facilitates Ca\(^{2+}\) channel activation without changing the membrane potential.\(^{20,21}\) This pharmacological approach eludes the direct regulation of InsP\(_3\) production induced by changing membrane potential with high [K\(^{+}\)] solution.\(^{25,27}\) Several lines

Figure 6  Hypoxic relaxation is preserved in Y27632-treated coronary arteries. (A and B) Y27632 (5 μM), an inhibitor of Rho kinase, reduces isometric force induced by FPL in porcine arterial ring (P < 0.001). Hypoxia (switching from a solution equilibrated with 95% O\(_2\) to another equilibrated with N\(_2\)) inhibits isometric force induced by FPL in the presence of the Rho kinase inhibitor (P = 0.004). (C) Intermediate hypoxia (switching from a solution equilibrated with 21% O\(_2\) to another equilibrated with 6% O\(_2\)) induces a significant inhibition of isometric force induced by FPL in the presence of Y27632. (D) Rise of cytosolic [Ca\(^{2+}\)] in an isolated coronary arterial myocyte induced by 70K (247 ± 48 nM, n = 4) and lack of significant effect of Y27632 (5 μM) on cytosolic [Ca\(^{2+}\)] (218 ± 56 nM, n = 4, P = 0.25). (E) Simultaneously, measurement of cytosolic Ca\(^{2+}\) concentration and arterial diameter in an intact rat coronary artery. FPL induced cytosolic [Ca\(^{2+}\)] increase (16 ± 0.05%) was unaltered in the presence of Y27632 (5 μM) (15 ± 0.05%, n = 5, P = 0.7). In contrast, contraction induced by FPL (15 ± 3%) was significantly reduced by Y27632 (7 ± 3.5%, n = 5, P = 0.04). The arrow indicates exposure to Y27632 (5 μM) for 15 min prior FPL application. Asterisk indicates statistically significant difference (P < 0.05).
of evidence suggest that vasoconstriction induced in coronary arteries by FPL is mediated by CCICR. (i) Contraction induced by FPL is present in the absence of extracellular Ca\(^{2+}\), thus discounting the possibility that isometric force is mediated by Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism.\(^{36}\) (ii) In the absence of extracellular Ca\(^{2+}\), arterial ring contraction triggered by FPL is inhibited by several Ca\(^{2+}\) channel antagonists (nifedipine, diltiazem, and D600). (iii) In the absence of extracellular Ca\(^{2+}\), a second exposure to FPL fails to evoke contraction indicating that intracellular Ca\(^{2+}\) stores had been emptied by the first application of the agonist. (iv) Application of 2APB and ryanodine, inhibitors of InsP\(_3\), and ryanodine receptors in smooth muscle, respectively,\(^{28,29}\) suppress the vasoconstriction evoked by FPL. These results indicate that in the coronary artery, Ca\(^{2+}\) channel activation mediates not only the well-known ionotropic Ca\(^{2+}\) influx from extracellular medium, but additionally can induce an important vasoconstriction through a metabotropic pathway by activating Ca\(^{2+}\) release from SR.

Although the effect of hypoxia on the ionotropic action of voltage-dependent Ca\(^{2+}\) channels has been widely studied, the possible effect of hypoxia on the metabotropic pathway mediated by Ca\(^{2+}\) channels (CCICR) has remained unexplored. In this paper, we show that the contraction induced by Ca\(^{2+}\) channel activation, in the absence of extracellular Ca\(^{2+}\), is markedly inhibited by low oxygen tension, thus suggesting that hypoxia modulates CCICR. The mechanisms underlying hypoxic coronary vasorelaxation, although studied by numerous groups, are still under debate. Hypoxic vasodilatation of porcine coronary arteries has been postulated to depend on both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent mechanisms.\(^{33}\) The Ca\(^{2+}\)-dependent mechanism relies on either transmembrane Ca\(^{2+}\) influx or Ca\(^{2+}\) release from SR. The vasorelaxing effect of low O\(_2\) tension described in this paper has been recorded in the absence of extracellular Ca\(^{2+}\). This experimental approach eludes the vasorelaxation induced by hypoxia by the inhibition of Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels described in coronary artery\(^{19}\) and in other vascular territories.\(^{8-14,37}\) On the other hand, because contraction induced by CCICR is mediated by Ca\(^{2+}\) release from intracellular stores and it has been described that hypoxia can modulate SR in vascular smooth muscles,\(^{16-18}\) we have tested whether hypoxia regulates intracellular stores in coronary artery. Our data indicate that low O\(_2\) tension has no effect on contraction induced by ACh, a drug that produces vasoconstriction through agonist-mediated synthesis of InsP\(_3\). Similarly, we have previously shown that caffeine-mediated ryanodine receptor activation is unaffected by hypoxia.\(^{19}\) Therefore, it seems that the inhibitory effect of hypoxia in coronary artery vasoconstriction is not mediated by the direct suppression of Ca\(^{2+}\) release from intracellular stores. A second pathway that can mediate hypoxic vasodilatation is the Ca\(^{2+}\)-independent mechanism, which involves a change in the Ca\(^{2+}\) sensitivity of the contractile apparatus of the cell.\(^{33,37,38}\) The GTP-mediated, Rho-kinase-coupled pathway has been proposed as Ca\(^{2+}\)-independent O\(_2\) sensing mechanism.\(^{34,39}\) In our experimental conditions, hypoxia-induced relaxation of coronary artery was accompanied of reduction of Ca\(^{2+}\) concentration and was preserved in arteries pre-contracted with FPL and pretreated with Y27632, a selective inhibitor of Rho kinase. So, although the participation of Rho-kinase and other Ca\(^{2+}\)-sensitization mechanisms cannot be totally excluded, our data suggest that the hypoxic vasorelaxation in intact porcine coronary artery is mainly mediated by a Ca\(^{2+}\)-dependent mechanism.

In conclusion, our results suggest that, besides its well-known inhibitory role on the ionotropic action of Ca\(^{2+}\) channels, hypoxia also facilitates coronary vasorelaxation by inhibiting the metabotropic pathway initiated by activation of voltage-dependent Ca\(^{2+}\) channels, leading to InsP\(_3\) synthesis and Ca\(^{2+}\) release from the SR. As CCICR can be activated even by moderate membrane depolarization or chemical agents,\(^{20,21}\) these mechanisms could contribute to the pathophysiology of coronary vasospasms.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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