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Gap junctions support the sustained phase of hypoxic pulmonary vasoconstriction by facilitating calcium sensitization

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Aims To determine the role of gap junctions (GJs) in hypoxic pulmonary vasoconstriction (HPV).

Methods and results Studies were performed in rat isolated intrapulmonary arteries (IPAs) mounted on a myograph and in anaesthetized rats. Hypoxia induced a biphasic HPV response in IPAs preconstricted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 3 μM) or 20 mM K^+. The GJ inhibitors 18β-glycyrrhetinic acid (18β-GA, 30 μM), heptanol (3.5 mM), or 2-aminoethoxydiphenyl borate (2-APB) (75 μM) had little effect on the transient Phase 1 of HPV, but abolished the sustained Phase 2 which is associated with Ca^{2+} sensitization. The voltage-dependent Ca^{2+} channel blocker diltiazem (10 μM) had no effect on HPV, and did not alter the inhibitory action of 18β-GA. Sustained HPV is enhanced by high glucose (15 mM) via potentiation of Ca^{2+} sensitization, in the presence of high glucose 18β-GA still abolished sustained HPV. Simultaneous measurement of tension and intracellular Ca^{2+} using Fura PE-3 demonstrated that whilst 18β-GA abolished tension development during sustained HPV, it did not affect the elevation of intracellular Ca^{2+}. Consistent with this, 18β-GA abolished hypoxia-induced phosphorylation of the Rho kinase target MYPT-1. In anaesthetized rats hypoxia caused a biphasic increase in systolic right ventricular pressure. Treatment with oral 18β-GA (25 mg/kg) abolished the sustained component of the hypoxic pressor response.

Conclusion These results imply that GJs are critically involved in the signalling pathways leading to Rho kinase-dependent Ca^{2+} sensitization during sustained HPV, but not elevation of intracellular Ca^{2+}, and may explain the dependence of the former on an intact endothelium.

Keywords Hypoxic pulmonary vasoconstriction • Pulmonary artery • Ca^{2+} sensitization • Gap junctions • 18β-glycyrrhetinic acid

1. Introduction

Hypoxic pulmonary vasoconstriction (HPV) optimizes pulmonary ventilation–perfusion matching in response to alveolar hypoxia, thereby diverting blood away from poorly ventilated regions of the lung. Despite many years of extensive research, the precise mechanisms underlying HPV remain incompletely resolved.[1]

In isolated intrapulmonary arteries (IPAs), and some perfused lung preparations, the contractile response to acute hypoxia is typically biphasic, with a rapid transient vasoconstriction (Phase 1) superimposed on a more slowly developing sustained contraction (Phase 2).[1–4] The mechanisms of these two phases differ. Phase 1 is associated with a transient elevation in intracellular Ca^{2+} ($[Ca^{2+}]_i$) involving store-operated Ca^{2+} entry (SOCE) and Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels (VDCC), whereas the sustained Phase 2 is critically dependent on ryanodine-sensitive Ca^{2+} stores and RhoA/Rho kinase-dependent Ca^{2+} sensitization.[1,3–6] Intriguingly, both Phase 2 of HPV and the associated Ca^{2+} sensitization have a yet unexplained strong dependence on an intact endothelium and glycolysis.[2,4,7–9]

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Gap-junction-mediated communication within and between the endothelium and smooth muscle cells (SMCs) is important for the control and co-ordination of normal vascular function. Gap junctions (GJs) are formed of adjacent connexons between cells each comprising six connexins (Cxs); the complete channel allows the passage of electrical current and small signalling molecules.[10–12] Vascular tissues including pulmonary artery express Cx37, Cx40, Cx43, and Cx45.[11,13] The endothelium couples to smooth muscle via myoendothelial GJs (MEGJs),[10,11] which are present in IPAs and probably play an important role in the integration of smooth muscle and endothelial function.[13–15]

Inhibition of GJs supresses HPV in perfused lungs,[16] and it has recently been proposed that the signal for HPV originates at the alveolocapillary level, from which it is propagated through the endothelium to upstream arterioles in a Cx40-dependent manner.[17] We have also reported that inhibition of glycolysis abolishes the hypoxia-induced depolarization of pulmonary artery endothelial cells,[9] suggesting that GJs might be involved in the transduction pathways underlying the glycolysis and endothelium-dependent sustained Phase 2 of HPV.

The aim of the present study was to test the hypothesis that GJs contribute to local signalling in HPV, in addition to any upstream signal propagation, and to examine their role in the development of the glycolysis- and endothelium-dependent sustained phase of HPV.

2. Methods

2.1 Animals and tissue isolation

This study conforms with the UK Home Office regulations and Directive 2010/63/EU of the European Parliament. Ethical approval was obtained from the relevant committees at King's College London and Institute of Pharmacology and Toxicology of National Academy of Medical Sciences of Ukraine. For isolated tissue studies, adult male Wistar rats (225–275 g) were killed by lethal overdose of pentobarbital (i.p.) and cervical dislocation. The heart and lungs were excised and placed into cold physiological saline solution (PSS; in mM: 118 NaCl, 24 $NaHCO_3$, 1 $MgSO_4$, 0.44 NaH_2PO_4, 4 KCl, 5.5 glucose, and 1.8 $CaCl_2$).

2.2 Tension and intracellular Ca^{2+} measurements

Small IPA (150–400 μm in diameter) were dissected free of connective tissue, mounted on a wire myograph (Danish Myo Technology A/S, Aarhus, Denmark) and bathed in PSS gassed with 5% CO_2, balance air (pH 7.4) at 37°C. Vessels were stretched and pre-conditioned by stimulation with repeated 2 min exposures to 80 mM K^+ PSS (KPSS, equimolar substitution for NaCl) as previously described.[2,6,7] Simultaneous measurement of IPA tension and intracellular Ca^{2+} ($[Ca^{2+}]_i$) was performed using IPA mounted on a modified myograph, and following loading with Fura PE-3-AM (4 μM) for 1 h at 37°C. The myograph was placed on an inverted stage fluorescence microscope (Zeiss UK Ltd) with Fluor objective combined with a double-excitation microfluorimeter (CairnResearch Ltd, Faversham, UK). Tension was recorded simultaneously with light emitted by the artery at 510 nm at excitation wavelengths 340 and 380 nm. The ratio of emission intensities ($R_{340/380}$) was taken as a measure of smooth muscle $[Ca^{2+}]_i$ as described previously.[6–8] Tension and $R_{340/380}$ were recorded using Acquisition Engine software (Cairn Research Ltd).

2.3 Experimental protocols for *in vitro* studies

As previously described,[6–8] IPAs were preconstricted with sufficient $PGF_{2\alpha}$ to produce tension equivalent to 10–15% of that produced by KPSS (typically 3 μM) in order to elicit a full contractile response to hypoxia. In some experiments equivalent pretone was induced with PSS containing 20–25 mM $[K^+]$. Hypoxia was induced by switching from 95% air/5% CO_2 to 5% CO_2/balance N_2, which we have shown to provide a PO_2 of 15–20 mmHg during hypoxia and 145–150 mmHg during normoxia in the myograph chamber.[3] Consecutive hypoxic challenges, separated by 60 min normoxia, demonstrated highly consistent HPV responses within the same IPA. Tension and changes in $[Ca^{2+}]_i$ ($R_{340/380}$) are expressed as percentages of the maximal responses to contraction to KPSS.

IPA incubated under the same conditions as above were snap frozen and protein extracted for western blot analysis of myosin phosphatase targeting protein (MYPT-1) phosphorylation as previously described.[6] Membranes were blocked for 1 h with 5% milk, probed with primary antibody overnight at 4°C (1:1000–1:5000 in 5% milk), followed by application of horseradish peroxidase-conjugated anti-IgG secondary antibody (Sigma, UK) for 1 h at room temperature (1:5000). Membranes were first probed with anti-phospho-MYPT-1 (thr-855; Upstate, UK), stripped for 1 h (Pierce stripping buffer), re-blocked, and re-probed with anti-pan MYPT-1 (Cell Signalling, UK). Data are expressed as ratio phospho/pan MYPT-1 as a percentage of in-gel controls.

2.4 *In vivo* studies

Experiments were conducted on adult male Wistar rats (230–300 g) divided into control and treatment groups. Plasma concentrations of 18β-GA in rats have been shown to fall rapidly after oral administration, but after ~12 h become relatively stable for up to 24 h.[18] Animals were therefore treated orally with 18β-GA (25 mg/kg) 20 h before experimentation. Surgical anaesthesia was induced by intraperitoneal injection of chloralose–urethane (1:10; 40 mg of urethane per 100 g body weight). Once deep anaesthesia was confirmed, tracheal intubation was performed. The left jugular vein and left common carotid artery were catheterized, and heparin (50 U per 100 g body weight) infused. Catheterization of the right ventricle was performed through the right jugular vein. Right ventricular and carotid artery pressures were recorded with ISOTEC pressure transducers (HSE, Germany) and Chart 5 Pro (ADInstruments Ltd, Australia). Animals were mechanically ventilated with a minute volume of 140 mL/min (Ugo Basile 7025 ventilator), and initial values of parameters recorded for ~25 min after stabilization. Hypoxia was then induced for 30 min by ventilation with 10% O_2 in N_2. Animals were euthanized at the end of the experiment by means of intravenous urethane (400 mg/100 g).

2.5 Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed using ANOVA with a Holm–Sidak *post hoc* test or Student's *t*-test as appropriate (Sigmaplot 12, Systat Software Inc., CA).

2.6. Chemicals

Diltiazem, carbachol, 18β-glycyrrhetinic acid (18β-GA), heptanol, 2-aminoethoxydiphenyl borate (2-APB), and Fura-PE-3/AM were obtained from Sigma-Aldrich UK. All drugs were dissolved in deionized water except 18β-GA, heptanol, and Fura PE-3 AM, which were dissolved in dimethyl sulphoxide (DMSO) to make stock solutions; following final dilution DMSO had no effect on its own.

3. Results

3.1 Effect of GJ inhibitors on HPV in isolated IPA

We examined the effects of three structurally unrelated GJ inhibitors, which are thought to act by different mechanisms, 18β-GA, heptanol, and 2-aminoethoxydiphenyl borate (2-APB)[19,20]; whilst 2-APB is commonly used as an inhibitor of SOCE,[21,22] it is also an effective blocker of GJ, with differential efficacy according to the Cx of which they are formed.[19] The synthetic^{37,43}Gap27 GJ inhibitor had inconsistent effects, possibly due to diffusional limitations, and was not pursued.

As previously described, hypoxia elicited a biphasic response in tension in IPA preconstricted with $PGF_{2\alpha}$ (*Figure 1*). The first phase consisted of a transient contraction, which peaked within 3–5 min of the onset of hypoxia, followed by a relaxation which reached a nadir within 10–15 min. This was followed by a more slowly developing and sustained contraction (Phase 2). Reoxygenation resulted in a rapid return of tension to the initial $PGF_{2\alpha}$-induced values, and on washing with PSS tension returned to the original baseline.

Preincubation with 18β-GA (30 μM) was without effect on Phase 1 of HPV, but strongly suppressed Phase 2 ($P < 0.001$; *Figure 1*). The effect of 18β-GA on HPV was independent of the means of preconstriction, as equivalent results were obtained in IPA preconstricted by depolarization with PSS containing 20 mM $[K^+]$ (*Figure 1*). Both heptanol (3.5 mM) and 2-APB (75 μM) also strongly suppressed Phase 2; although both caused a small reduction in Phase 1, this only reached significance for heptanol (*Figure 2*). A lower concentration of 2-APB (30 μM) reduced Phase 2 at 40 min by only 58 ± 6% ($n = 5$, $P < 0.01$).

Together these data suggest that GJ are involved in the sustained Phase 2 of HPV, but not the transient Phase 1.

3.2 Effect of 18β-GA on HPV following blockade of L-type Ca^{2+} channels

Blockade of GJs could potentially affect membrane potential in the smooth muscle. We therefore compared the control HPV response with that following incubation with the L-type VDCC blocker diltiazem (10 μM), and in combination with 18β-GA (30 μM) (*Figure 3*). As previously reported,[3] diltiazem alone had no significant effect on either phase of HPV in this preparation; the reduction in Phase 1 did not reach significance (control: 28.7 ± 2.6% KPSS; diltiazem: 23.4 ± 2.3% KPSS, $n = 7$, NS). However, addition of 18β-GA to diltiazem strongly suppressed the sustained Phase 2 of HPV (*Figure 3*). Whilst Phase 1 was now significantly reduced compared with control ($P < 0.05$), it was not significantly different from that with diltiazem alone (diltiazem + 18β-GA: 18.8 ± 2.7% KPSS, $n = 7$, NS). These results, which are essentially equivalent to those shown in *Figure 1*, demonstrate that the contribution of GJs to sustained HPV is independent of VDCC.

3.3 Effect of 18β-GA on intracellular Ca^{2+} concentration during HPV

Hypoxia elicited a biphasic response in $[Ca^{2+}]_i$ in IPA preconstricted with 3 μM $PGF_{2\alpha}$, with a transient increase in $[Ca^{2+}]_i$ that mirrored the Phase 1 transient increase in tension (*Figure 4*). However, as previously reported,[7,8,23] whereas tension gradually increased during Phase 2, $[Ca^{2+}]_i$ remained stable. Reoxygenation resulted in a rapid return of both tension and $[Ca^{2+}]_i$ to the initial $PGF_{2\alpha}$-induced values. Application of 18β-GA (30 μM) had no effect on hypoxia-induced elevation of $[Ca^{2+}]_i$ during either phase of HPV (*Figure 4*). This suggests that GJs

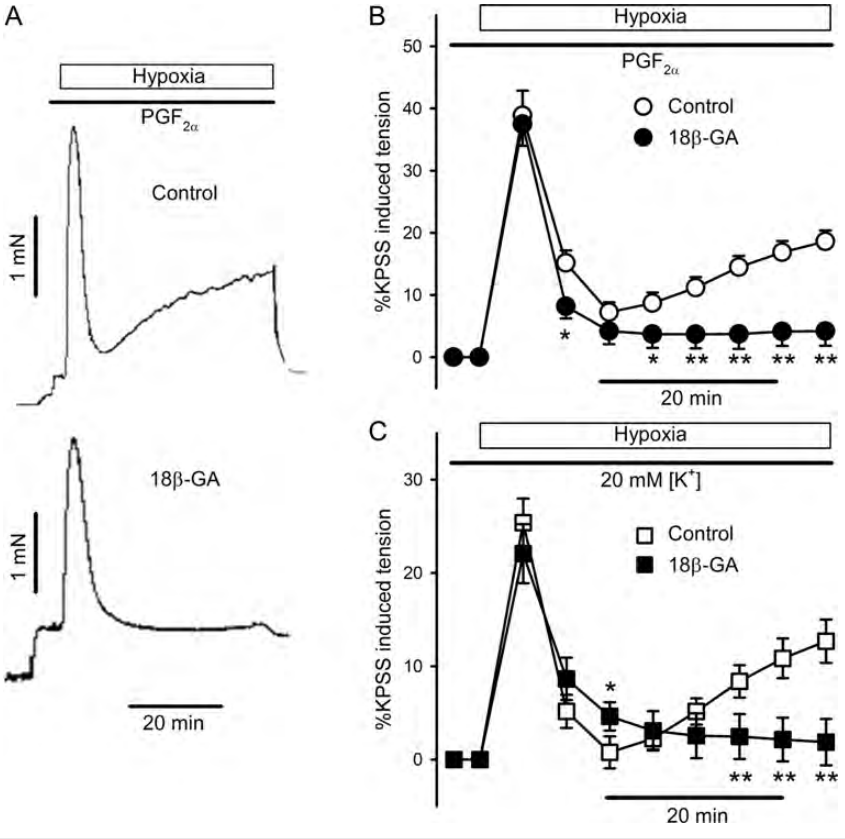

Figure 1 HPV in rat isolated IPA, and the effect of preincubation with 18β-GA (30 μM) on the response. (*A*) Example traces following preconstriction with 3 μM $PGF_{2\alpha}$ and the selective suppression of Phase 2 by 18β-GA; mean data are shown in (*B*) ($n = 10$). 18β-GA had the same effect in IPA preconstricted by depolarization with 20 mM $[K^+]$ (*C*, $n = 7$). Symbols represents the mean ± SE. *$P < 0.05$, **$P < 0.01$.

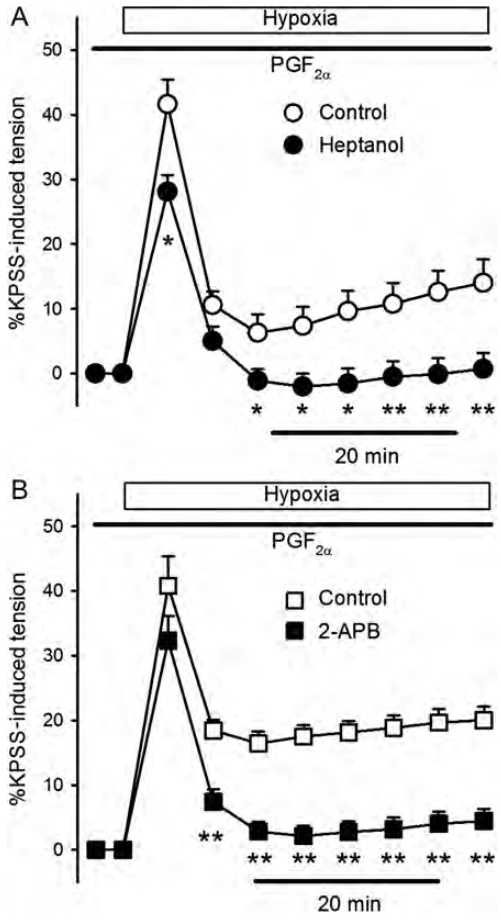

Figure 2 The effect of the GJ inhibitors heptanol (3.5 mM (*A*), $n = 6$) and 2-APB (75 μM (*B*), $n = 7$) on HPV in rat IPA preconstricted with 3 μM $PGF_{2\alpha}$. Symbols represents the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.001$.

are involved in the mechanisms leading to Ca^{2+} sensitization during sustained HPV, but not the elevation of $[Ca^{2+}]_i$.

3.4 Effect of 18β-GA on HPV in the presence of elevated glucose

We have previously shown that Phase 2 of HPV is selectively potentiated by an increase in extracellular glucose concentration, and suppressed or abolished by reduced glucose; this involves the mechanisms underlying the Phase 2-associated Ca^{2+} sensitization, as altering glucose had no effect on the hypoxia-induced elevation of $[Ca^{2+}]_i$.[7] As our results suggest that GJs are also involved in Ca^{2+} sensitization during Phase 2, we examined the effects of GJ inhibition on the high glucose-induced potentiation of Phase 2 of HPV. Increasing glucose concentration from 5 to 15 mM had no significant effect on basal tension, pretone elicited by $PGF_{2\alpha}$, or HPV Phase 1, but Phase 2 was significantly potentiated (*Figure 5*A). In the presence of 15 mM glucose, 18β-GA (30 μM) again had no effect on Phase 1 but abolished Phase 2 (*Figure 5*).

This suggests that the mechanisms underlying the potentiating action of high glucose on Phase 2 of HPV are not separate from those inhibited by block of GJs, and strengthens the hypothesis that Ca^{2+} sensitization during hypoxia requires functional GJs.

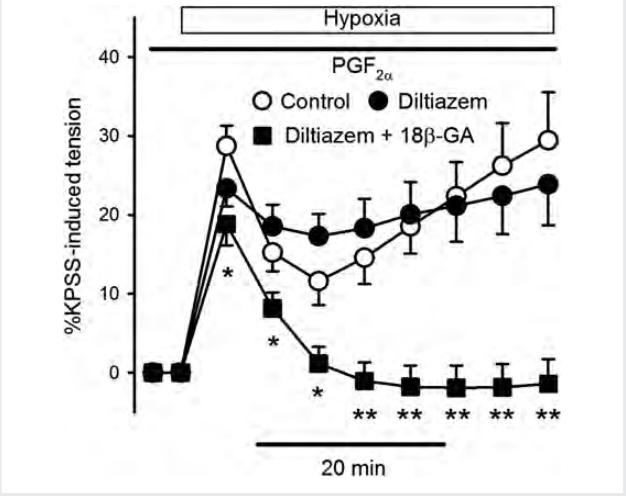

Figure 3 Blockade of VDCC with diltiazem (10 μM) had no significant effect on HPV in rat IPA preconstricted with 3 μM $PGF_{2\alpha}$ (filled circles); 18β-GA (30 μM) had almost identical effects in the presence of diltiazem as in its absence (*Figure 1*). Symbols represents the mean ± SE, $n = 7$. $^{*}P < 0.05$, $^{**}P < 0.001$.

3.5 Effect of 18β-GA on MYPT-1 phosphorylation during HPV

Ca^{2+} sensitization during sustained HPV depends on Rho kinase-dependent phosphorylation of MYPT-1.[5,6,26] We therefore examined the effect of 18β-GA on MYPT-1 phosphorylation in IPA preconstricted with $PGF_{2\alpha}$ as above and following 30 min hypoxia. In controls, hypoxia increased the ratio of phosphorylated to total MYPT-1 by ~30% compared with $PGF_{2\alpha}$ alone ($P < 0.05$, $n = 10$). This hypoxia-induced increase was however abolished following treatment with 18β-GA, although the response to preconstriction with $PGF_{2\alpha}$ alone was unaltered by 18β-GA ($n = 8–9$; *Figure 5*B). These results further suggest that blockade of GJs with 18β-GA impairs hypoxia-induced Rho kinase-mediated Ca^{2+} sensitization.

3.6 Effect of 18β-GA on right ventricular pressure *in vivo*

Basal systolic right ventricular pressure (sRVP) in untreated anaesthetized rats was 28.6 ± 1.1 mmHg and the mean systemic arterial pressure (MAP) was 101.5 ± 5.3 mmHg ($n = 7$). Induction of hypoxia resulted in a biphasic pressor response (*Figure 6*). sRVP rose to a peak of ~44% (12.6 ± 0.8 mmHg, $P < 0.001$) after 5 ± 1 min, then fell to a nadir of ~24% (7.2 ± 4.2 mmHg, $P < 0.001$ vs. peak) before increasing again to ~38% by 32 min (10.9 ± 0.8 mmHg, $P < 0.001$ vs. baseline and nadir) (*Figure 6*). Hypoxia caused a fall in MAP over the first 3 min to 64.8 ± 5.4 mmHg ($P < 0.001$), which thereafter remained constant throughout the period of hypoxic. All changes in haemodynamics reversed on re-oxygenation after ~5–10 min.

Following oral administration of 18β-GA (25 mg/kg) 20 h before the study, basal sRVP was reduced compared with controls, though this did not reach significance (26.0 ± 1.4 mmHg, $n = 5$, NS). Conversely there was significant elevation in MAP (120.5 ± 4.5 mmHg, $P < 0.05$). The initial elevation of sRVP following induction of hypoxia was reduced to 6.9 ± 4.3 mmHg, though this did not reach significance; however, the sustained component of the hypoxic pressor response was effectively

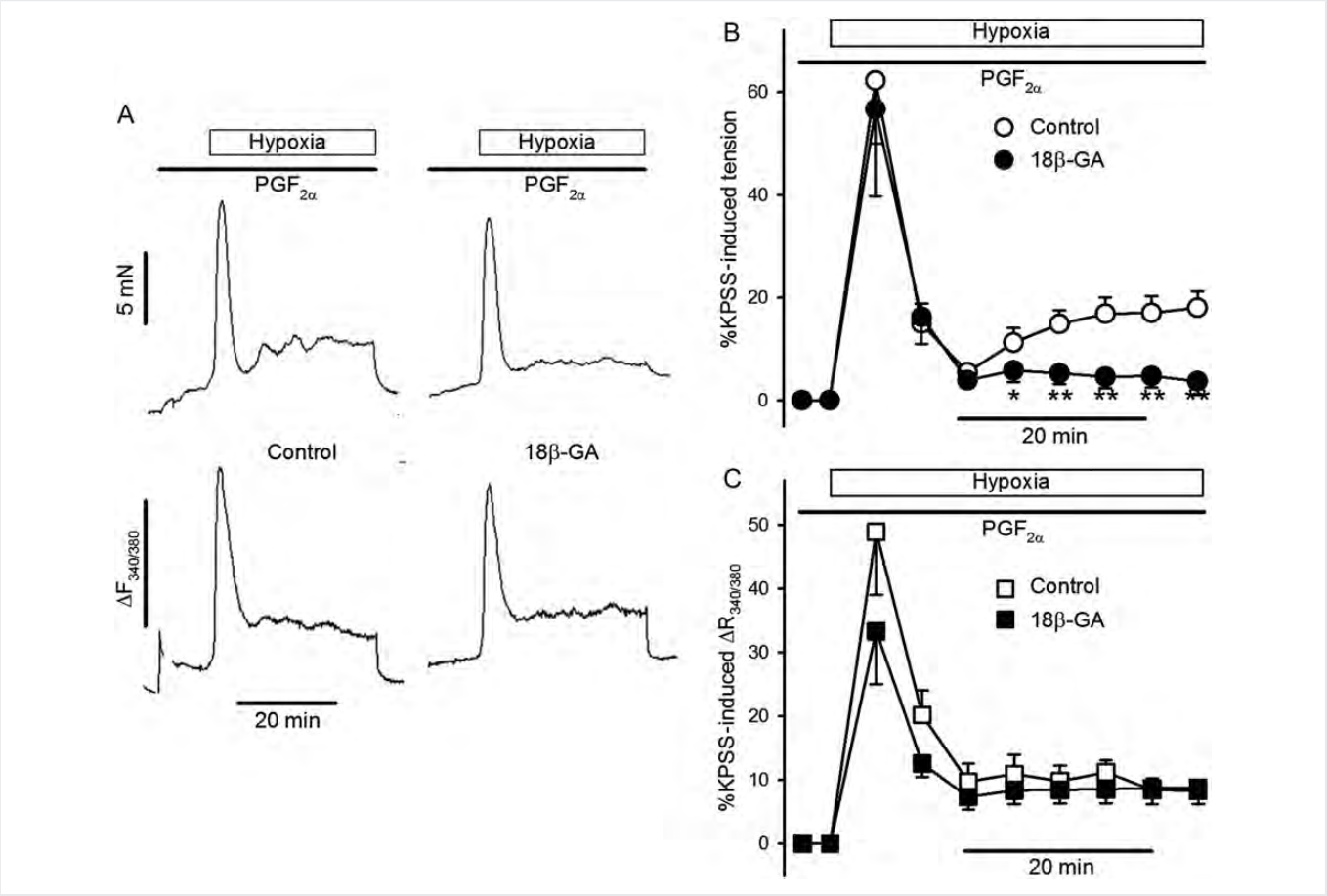

Figure 4 Simultaneous measurement of tension and $[Ca^{2+}]_i$ (expressed as $R_{340/380}$) during HPV in rat IPA preconstricted with 3 μM $PGF_{2\alpha}$ and following preincubation with 18β-GA (30 μM). 18β-GA abolished the rise in tension Phase 2, but had no effect on $[Ca^{2+}]_i$. Symbols represent the mean ± SE, $n = 7$. *$P < 0.05$, **$P < 0.01$.

abolished (32 min: 0.9 ± 1.9 mmHg, $P < 0.001$) (*Figure 6*). MAP during hypoxia showed no difference between control and treated animals (control: 64.8 ± 5.4 mmHg; 18β-GA: 67.0 ± 8.2 mmHg, NS).

4. Discussion

The key finding of this study is that the sustained Phase 2 of HPV, but not the transient Phase 1, is critically dependent on functioning GJs, and our evidence suggests a hitherto unrecognized role for GJs in hypoxia-induced Ca^{2+} sensitization. As this dependence was apparent in isolated IPA segments, it must be localized to the artery wall and independent of any longitudinal signal propagation from downstream regions of the pulmonary vascular tree.

The biphasic nature of HPV has been recognized for many years, and the initial Phase 1 constriction differs mechanistically from that during the sustained Phase 2.[1] Although both phases are associated with an elevation of smooth muscle $[Ca^{2+}]_i$, this is very small in Phase 2 and critically dependent on release from ryanodine-sensitive stores, with a reduced or absent dependence on Ca^{2+} entry.[3,4,25] In particular, and in contrast to Phase 1, tension development during Phase 2 is strongly dependent on the endothelium and Rho kinase-mediated Ca^{2+} sensitization.[4–6,8,23]

Whilst this biphasic response is most clearly demonstrated in isolated small IPA,[2,4,8,26] it is occasionally apparent in perfused lung preparations and *in vivo* where the hypoxic challenge is prolonged for more than 20 min.[1,27,28] However, the majority of studies on HPV, in whatever preparation, utilize shorter hypoxic challenges of up to 15 min, and are thus effectively only relevant to Phase 1.[1,25] Whilst the relative importance of the two phases for acute ventilation–perfusion matching is a subject of debate, the sustained Phase 2 is more relevant to Group 3 pulmonary hypertension with which it shares some common features, specifically a key role for Rho kinase-mediated Ca^{2+} sensitization.[24]

As our studies were designed to investigate a role for GJs in local signalling in the absence of signal propagation from remote regions of the pulmonary vasculature, the key experiments were methodologically constrained to small, endothelium-intact segments of IPA. This prevented a molecular approach as incubation of such preparations for sufficient time to allow transfection with, e.g. siRNAs causes significant changes in function (e.g. ref. 29 and own observations). It is also notoriously difficult to induce HPV in isolated mouse IPA, obviating use of GM models.[1] We therefore utilized a pharmacological approach with three GJ inhibitors that are believed to act via different mechanisms, 18β-GA, heptanol, and 2-APB. All three demonstrated a similar profile, with strong suppression of HPV Phase 2 and little or no effect on Phase 1 (*Figures 1* and *2*). Notably, the hypoxic pressor response *in vivo* also showed a biphasic response, and treatment with 18β-GA similarly abolished the sustained component of HPV but not the initial transient (*Figure 6*).

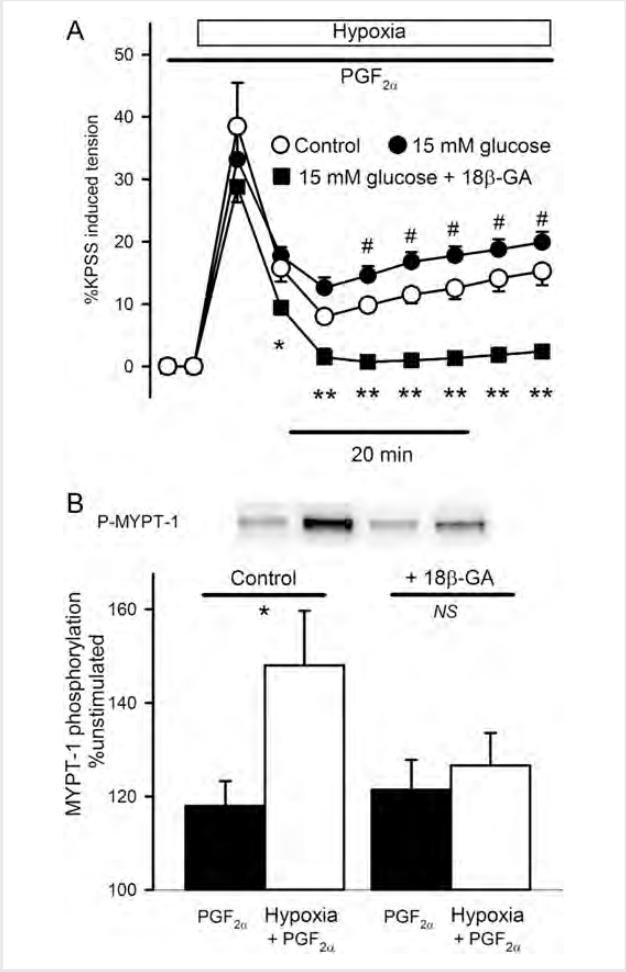

Figure 5 (*A*) Elevation of the glucose concentration from 5 to 15 mM potentiated Phase 2 of HPV in rat IPA preconstricted with 3 μM $PGF_{2\alpha}$ (filled circles, $n = 7$, $\#P < 0.05$). In the presence of 15 mM glucose 18β-GA still abolished Phase 2 (filled squares, $*P < 0.05$, $**P < 0.01$, comparison with high glucose alone). Symbols represent the mean ± SE; where error bars are not shown, they are smaller than the symbol. (*B*) In the absence of 18β-GA, MYPT-1 phosphorylation (expressed as % unstimulated) in IPA was increased by preconstriction with $PGF_{2\alpha}$ ($P < 0.05$) and further increased after 30 min hypoxia ($*P < 0.05$ $PGF_{2\alpha}$ vs. $PGF_{2\alpha}$ plus hypoxia; $n = 10$). In the presence of 18β-GA, the increase in MYPT-1 phosphorylation induced by preconstriction with $PGF_{2\alpha}$ was unaltered, but thereafter hypoxia had no further effect (NS, $PGF_{2\alpha}$ vs. $PGF_{2\alpha}$ plus hypoxia; $n = 8–9$).

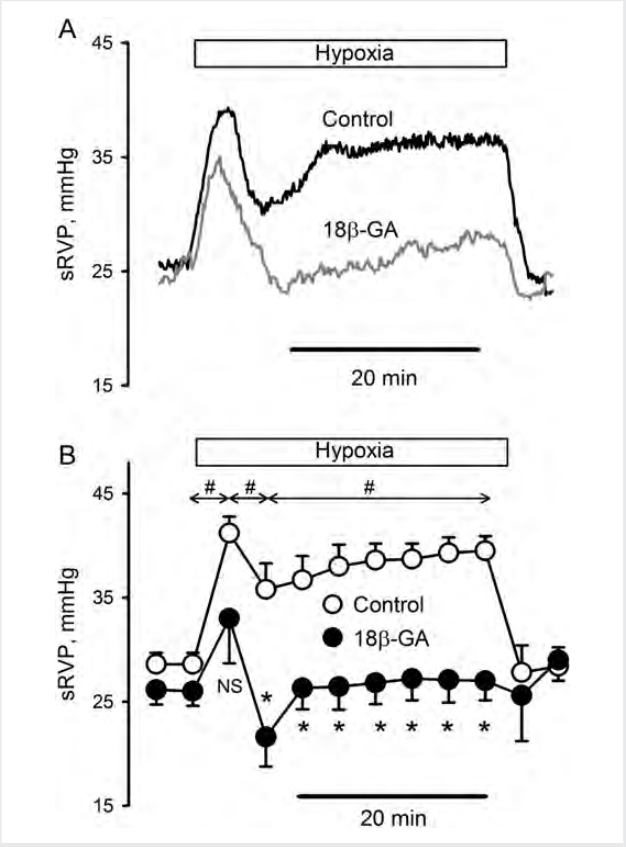

Figure 6 Hypoxic pressor response in anaesthetized rats *in vivo*, and following oral administration of 25 mg/kg 18β-GA. (*A*) Example traces from a control and a treated animal and (*B*) mean data. In control animals hypoxia elicited a rapid elevation in sRVP followed by a small but significant decline, after which sRVP rose more slowly towards a sustained plateau ($\#P < 0.01$ between points as shown, $n = 7$). This sustained component was abolished in 18β-GA-treated animals ($n = 5$). Symbols represent the mean ± SE, $*P < 0.01$ (control vs. treated). NS = not significant ($P > 0.05$).

18β-GA and heptanol are widely used as GJ inhibitors in vascular tissues.[19,30–32] 18β-GA is an triterpenoid saponin that disrupts GP plaques by affecting the structural integrity, distribution, and phosphorylation of connexons.[19,32] The long-chain alcohol heptanol causes selective disruption at the GJ–lipid interface by intercalating in the lipid bilayer and thus effectively gating GJ channels closed.[19,30–32] The mechanism of 2-APB is less clear, but it is recognized as a potent blocker of GJs with a much greater efficacy against Cx40 compared with Cx43.[19,20] It is commonly used as an inhibitor of IP_3 receptors (which do not play a major role in HPV[1]), and SOCE,[21,22] although notably it is reported that 75 μM 2-APB only inhibits SOCE in SMCs isolated from IPA by ~40%.[33]

Whilst SOCE has been implicated in HPV, the evidence is largely restricted to Phase 1 and studies in isolated PASMCs.[1,3,34] SOCE is highly sensitive to La^{3+}, and in IPA 1 μM La^{3+} abolished thapsigargin-induced SOCE and suppressed Phase 1 of HPV[3,22]; 10 μM La^{3+} also suppressed the acute hypoxic pressor response in perfused lungs.[34] However during Phase 2 of HPV in IPA, 1 μM La^{3+} was without effect on the elevation of either tension or $[Ca^{2+}]_i$, and La^{3+} only caused partial suppression at 100 μM, suggesting that SOCE plays a limited role during this phase.[3] Notably, at concentrations of 100 μM and above La^{3+} blocks Cx43-containing GJs.[35] In the light of this, and as the effect of 2-APB on HPV showed a similar profile to that of 18β-GA and heptanol with a strong and selective block of Phase 2, it is reasonable to suggest that its actions here are mediated by its established ability to block GJs.[19,20]

Inhibitors of GJs, including 18β-GA and Cx mimetic inhibitory peptides (Gap27), have been reported to elevate systemic blood pressure *in vivo*, as observed here. This has been ascribed to inhibition of EDHF-dependent vasorelaxation and/or interference with the renin–angiotensin system.[31,36] In contrast, GJ inhibitors *in vitro* generally induce vasorelaxation or attenuation of constriction, due to smooth muscle

hyperpolarization, inhibition of Ca^{2+} entry, and a consequent fall in $[Ca^{2+}]_i$.[31,32,37] As HPV has been associated with hypoxia-induced Ca^{2+} entry,[1,3,27] it is feasible that GJ inhibition could be suppressing Phase 2 in a similar fashion. However, as previously reported[3] block of voltage gated L-type Ca^{2+} channels had no effect on either phase of HPV in this preparation, and 18β-GA still selectively abolished Phase 2 (*Figure 5*). Moreover, whilst 18β-GA strongly inhibited tension development during Phase 2, it did not affect the hypoxia-induced elevation of $[Ca^{2+}]_i$ (*Figure 4*). This implies that inhibition of GJs is suppressing hypoxia-induced Ca^{2+} sensitization.

Phase 2 of HPV involves Rho kinase-mediated Ca^{2+} sensitization, which is endothelium- and glucose dependent.[1,5,7,8] Endothelial denudation or removal of glucose abolishes Phase 2 tension development, whilst high glucose potentiates it, in all cases without affecting the associated elevation of $[Ca^{2+}]_i$; notably, high glucose cannot restore HPV in the absence of endothelium.[5–8] If functioning GJs are required for hypoxia-induced Ca^{2+} sensitization, it would be predicted that high glucose would have no effect following GJ blockade with 18β-GA, and this is what was observed (*Figure 5A*). Moreover, 18β-GA abolished hypoxia-induced phosphorylation of MYPT-1, the target for Rho kinase and a key regulator of Ca^{2+} sensitivity (*Figure 5B*).

Our results are consistent with a hitherto unrecognized role for GJs in mediating hypoxia-induced and Rho kinase-dependent Ca^{2+} sensitization during HPV, which could underlie the so far unexplained dependence of Phase 2 in isolated arteries on an intact endothelium. It has been hypothesized that hypoxia causes release of an endothelium-derived constricting factor, but no such factor with the requisite characteristics has been positively identified; whilst endothelin 1 and others may play a role in HPV, possibly by providing the endogenous equivalent of pretone, they have been excluded as the mediator of Phase 2 Ca^{2+} sensitization.[1,38,39] MEGJs allow direct passage of small signalling molecules up to ~1.2 kDa.[12] Intriguingly, Gairhe *et al.*[40] have recently shown that serotonin synthesized in pulmonary vascular endothelial cells passes through MEGJs to modify smooth muscle differentiation, and binding of intracellular serotonin to RhoA (serotonylation) has been associated with the increased smooth muscle RhoA/Rho kinase activity observed in pulmonary hypertension.[41]

Blockade of MEGJs has been reported to inhibit contraction of IPA to exogenous serotonin and other agonists by preventing transfer of reactive oxygen species (ROS), most likely superoxide anion.[13] This is interesting because of strong evidence that ROS comprise the key mediator of HPV[1,24,42] and activate Rho kinase.[43,44] Diacylglycerol (DAG) is another small signalling molecule implicated in HPV. Although interest has focussed on its activation of TRPC6 channels during the acute phase,[27,45] inhibition of phosphatidylcholine-specific phospholipase C, potentially the source of DAG during hypoxia, is reported to selectively supress sustained HPV.[26,46]

GJs between endothelial cells allow longitudinal propagation of electrical signals along small vessels. A recent study by Wang *et al.*[17] suggests that retrograde propagation via Cx40-containing GJs between endothelial cells couples oxygen sensing in alveolar capillaries to contraction of upstream pulmonary arterioles, and may thus be a critical requirement for HPV. Such a mechanism is clearly not applicable to the short segments of IPA used in the current study. It also seems unlikely that Cx40 is critically involved in the responses we describe, as unlike Cx43 and most others, Cx40 is effectively completely blocked by 20 μM 2-APB,[20] whereas in our hands 30 μM 2-APB only caused partial inhibition of Phase 2. In any event, electrotonic coupling is unlikely to play a role in HPV of isolated IPA, as we have previously demonstrated that this is essentially unaltered following near-maximal depolarization with 80 mM $[K^+]$.[3] However, our studies do not rule out a role for longitudinal propagation in HPV in the intact lung, as proposed by Wang *et al.*[17]

In conclusion, our results suggest that GJs, probably MEGJs, play a critical and previously unsuspected role in sustained HPV by facilitating hypoxia-induced Ca^{2+} sensitization. This is independent of any role in longitudinal signal propagation as it is apparent in isolated IPA segments. Whilst the GJ inhibitors utilized also have non-junctional actions,[20,37] their disparate *modi operandi* yet identical actions on HPV, with little effect on Phase 1 but robust inhibition of Phase 2, strongly suggests that the latter is indeed due to block of GJs. The mechanism however remains unclear, but is likely to involve transfer of a small signalling molecule such as serotonin or superoxide between endothelium and smooth muscle rather than electrical coupling.

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