Developmental Absence of the O_2 Sensitivity of L-Type Calcium Channels in Preterm Ductus Arteriosus Smooth Muscle Cells Impairs O_2 Constriction Contributing to Patent Ductus Arteriosus

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ABSTRACT: Patent ductus arteriosus (PDA) complicates the hospital course of premature infants. Impaired oxygen (O_2)-induced vasoconstriction in preterm ductus arteriosus (DA) contributes to PDA and results, in part, from decreased function/expression of O_2-sensitive, voltage-gated potassium channels (Kv) in DA smooth muscle cells (DASMCs). This paradigm suggests that activation of the voltage-sensitive L-type calcium channels (Ca_L), which increases cytosolic calcium ($[Ca^{2+}]_i$), is a passive consequence of membrane depolarization. However, effective Kv gene transfer only partially matures O_2 responsiveness in preterm DA. Thus, we hypothesized that Ca_L are directly O_2 sensitive and that immaturity of Ca_L function in preterm DA contributes to impaired O_2 constriction. We show that preterm rabbit DA rings have reduced O_2- and 4-aminopyridine (Kv blocker)–induced constriction. Preterm rabbit DASMCs have reduced O_2-induced whole-cell calcium current (I_{Ca}) and $[Ca^{2+}]_i$. BAY K8644, a Ca_L activator, increased O_2 constriction, I_{Ca}, and $[Ca^{2+}]_i$ in preterm DASMCs to levels seen at term but had no effect on human and rabbit term DA. Preterm rabbit DAs have decreased γ and increased α subunit protein expression. We conclude that the Ca_L in term rabbit and human DASMCs is directly O_2 sensitive. Functional immaturity of Ca_L O_2 sensitivity contributes to impaired O_2 constriction in premature DA and can be reversed by BAY K8644. (*Pediatr Res* 63: 176–181, 2008)

In the hypoxic environment *in utero*, the ductus arteriosus (DA), a vital fetal artery that connects the pulmonary artery to the aorta, is widely patent and shunts more than half of the right heart's cardiac output away from the nonventilated lung into the umbilicoplacental circulation, where gas exchange takes place (1). Within minutes of birth, the increased P_{O_2} constricts the DA (2) and simultaneously dilates the pulmonary circulation (3). The response of the *term* DA to oxygen (O_2) rarely fails; however, in humans, approximately 50% of *preterm* DAs do not close, despite adequate oxygenation (4).

Failure of DA closure after birth complicates the hospital course of preterm infants. PDA is associated with an increased incidence of chronic lung disease, intraventricular hemorrhage, and necrotizing enterocolitis (4). Both medical and surgical interventions to close the DA, although usually effective, are associated with additional morbidity (5).

The crucial role of endothelium-derived relaxing and constricting factors in regulating DA tone is well established (6). However, O_2 constricts the DA in the absence of endothelium (7), suggesting that the core of the O_2-sensing mechanism is intrinsic to the DA smooth muscle cell (DASMC). Potassium (K^+) channels in the vascular smooth muscle cells (SMCs) regulate vascular tone through modulation of the membrane potential (E_M) (8). Closure of K^+ channels leads to vasoconstriction by depolarizing E_M. Depolarization opens voltage-gated L-type calcium (Ca^{2+}) channels (Ca_L), thereby increasing influx of extracellular Ca^{2+}. In term rabbit (9) and human (10) DAs, O_2-induced constriction is initiated by the inhibition of O_2 and 4-aminopyridine (4-AP)–sensitive voltage-gated, K^+ channels (Kv), including Kv1.5 and Kv2.1. Preterm rabbit DAs have reduced O_2 constriction due, in part, to decreased function and expression of O_2-sensitive Kv (11). Kv1.5 or Kv2.1 gene transfer partially (50%) "rescues" the developmental deficiency, conferring O_2 responsiveness to preterm rabbit DAs and human DAs (11). However, O_2 responsiveness is not completely restored, suggesting additional mechanisms contribute to O_2-induced constriction in the DASMCs. In resistance pulmonary artery SMC, another cell from the specialized O_2-sensing system (12), the Ca_L have intrinsic O_2 sensitivity in addition to their response to Kv inhibition–induced depolarization of E_M (13). We suggest that DASMC Ca_L are more active participants in DA constriction to O_2 than was previously recognized, responding not just to membrane depolarization but directly to increased P_{O_2}. We tested the hypothesis that the Ca_L are intrinsically O_2 sensitive in rabbit and human DASMCs. We also tested the hypothesis that

Abbreviations: 4-AP, 4-aminopyridine; $[Ca^{2+}]_i$, cytosolic calcium; Ca_L, L-type Ca^{2+} channels; **DA,** ductus arteriosus; E_M, membrane potential; I_{Ca}, whole-cell calcium current; **Kv,** voltage-gated K^+ channels; **SMC,** smooth muscle cell

reduced expression and/or function of these channels contributes to impaired O$_2$ constriction in preterm rabbit DA.

METHODS

All procedures were approved by the Animal Welfare and the Human studies committees of the University of Alberta. All investigators had access to the data and take responsibility for its integrity.

Rabbit DAs. New Zealand White rabbits ($n = 50$) were delivered by cesarean section at gestational d 26 (preterm) or 30 (term), as previously described (11,14). The endothelium-intact DA was used within 5 min of harvest and was maintained hypoxic (pH $= 7.40 \pm 0.08$, Po$_2$ $= 31 \pm 1$ mm Hg) until Po$_2$ was intentionally increased.

Rabbit DASMCs. DASMCs were obtained by enzymatic digestion as described (11,14).

Human DASMCs. Human DAs were obtained from hypoplastic left heart term infants during the Norwood procedure, and the SMCs were isolated by enzymatic digestion, as described (11,14).

Tension measurements in isolated preterm and term rabbit DA rings. Isolated rabbit DAs were placed in an organ bath and equilibrated in hypoxic Krebs solution (Po$_2$ $= 31 \pm 1$ mm Hg to mimic *in utero* conditions), at the experimentally derived optimal resting tension values of 400 mg (preterm) and 800 mg (term), as previously described (11,14). To investigate the contribution of maturational differences in Ca$_L$ function to diminished O$_2$ constriction, the response of preterm and term DA rings to increased Po$_2$ was compared in the presence and absence of the Ca$_L$ opener BAY K8644 (10^{-6}M) or the Ca$_L$ inhibitor nifedipine (10^{-6}M). The responsiveness to K$^+$ channel inhibitors Kv inhibitor 4-AP and iberiotoxin (IBTX), a highly specific inhibitor of large conductance calcium-sensitive K$^+$ channels (BK$_{Ca}$) was also compared.

Whole cell patch clamp. The effect of Po$_2$ on whole-cell Ca^{2+} current (I$_{Ca}$) and E$_M$ were measured in freshly dispersed preterm and term rabbit DASMCs and human DASMCs, using voltage and current clamp protocols, as previously described (11,15) and detailed in the online supplement. Ca^{2+} channel current recordings were obtained using the whole-cell configuration. Barium (20 mM) was used as a charge carrier because its favorable conduction by the Ca$_L$ increases current amplitude.

The current-voltage relationship was obtained using a voltage-step protocol (from -60 to $+50$ mV, duration of 250 ms in 10-mV increments in hypoxic, Po$_2$ $= 40$ mm Hg, perfusate). The effects of increasing O$_2$ (Po$_2$ $= 165$ mm Hg) and the Ca$_L$ agonist BAY K8644 on I$_{Ca}$ were studied.

Intracellular calcium [Ca^{2+}]$_i$ measurements. Ca^{2+} was measured using a spectrofluorometer (Photon Technology International, Birmingham, NJ). Cells were incubated with fura 2-AM (10^{-6} M) and pluronic (8×10^{-7} M) (Molecular Probes, Eugene, OR) for 20 min in 4% O$_2$. The plates were then washed with hypoxic Hanks' balanced salt solution and incubated in a 4% O$_2$ incubator for an additional 20 min, with or without BAY K8644 (1 μM) (Sigma Chemical Co.-Aldrich, St. Louis, MO). Plates were placed on the stage of an inverted microscope and perfused with a warmed normoxic Hanks' solution (32–33°C). Background fluorescence was recorded from each dish of cells and subtracted before calculation of the 340- to 380-nm ratio. Emission was measured at 510 nm. Duration of each measurement was 1200 s.

Immunoblotting. DAs were flash frozen in liquid N$_2$ and homogenized in buffer containing an antiprotease cocktail (Sigma Chemical Co.) and run on 7.5%–10% gels. Specific antibodies against Ca$_L$ subunits α1c and γ2 were purchased from US Biologic (Cedarlane Laboratories Ltd., Hornby, Ontario) and Sigma Chemical Co.-Aldrich, respectively. Expression was quantified using densitometry and expressed as the percentage of the loaded protein density, measured using the Ponceau stain.

Statistics. Values are expressed as means $\pm$ SEM. All sample sizes are listed in the figures. Intergroup comparisons were performed with a *t* test or factorial repeated-measures analysis of variance, as appropriate. Fisher's probable least significant differences test was used for *post hoc* comparisons. A *p* value <0.05 was considered statistically significant.

RESULTS

Decreased O$_2$-induced constriction in preterm rabbit DA is restored by a Ca$_L$ opener. In both term and preterm DAs, O$_2$-induced constriction increased in proportion to Po$_2$ (Fig. 1A, B). O$_2$-induced constriction was significantly weaker in preterm DAs (Fig. 1A, B). Pretreatment with the Ca$_L$ opener BAY K8644 enhanced O$_2$-induced constriction in preterm DAs to a magnitude similar to that achieved in term DAs (Fig. 1A, B). In contrast, BAY K8644 had no effect on O$_2$-induced constriction in term DAs. BAY K8644 had no effect on phenylephrine-induced constriction in preterm and term DAs (data nor shown). The Ca^{2+} channel inhibitor nifedipine blunted O$_2$ constriction in both preterm and term DAs (by 60% in 20% O$_2$), suggesting a crucial role of extracellular calcium influx through the Ca$_L$ in O$_2$-induced constriction in this acute phase of O$_2$-induced DA constriction (Fig. 1A, B).

Opening Ca$_L$ does not affect Kv inhibition–induced constriction in rabbit DA. In both term and preterm DAs, the Kv blocker 4-AP (1, 5, and 10 mM) caused a dose-dependent constriction (Fig. 2A). Kv inhibition was weaker in preterm than in term DAs. Pretreatment with BAY K8644 had no effect on 4-AP–induced constriction in preterm or term DA
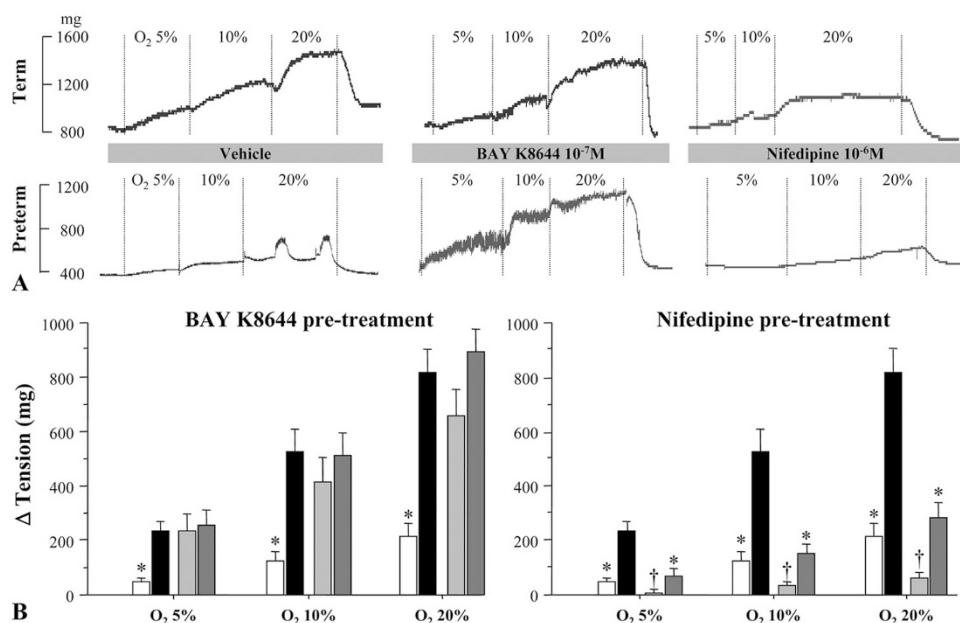

Figure 1. Reduced O$_2$-induced constriction in preterm DA rings is overcome by Ca$_L$ activation. Representative tracings (*A*) and mean data (*B*) showing decreased O$_2$-induced constriction in preterm *vs* term DA. In preterm DAs, pretreatment with the Ca$_L$ opener BAY K8644 significantly increases O$_2$-induced constriction to a magnitude similar to that of term DA ($n = 10$–15; *$p < 0.05$). BAY K8644 does not increase O$_2$-induced constriction in term DA ($n = 10$–15, *$p < 0.05$ *vs* term vehicle; †$p < 0.05$ *vs* preterm vehicle. Preterm vehicle (*white columns*), term vehicle (*black columns*), preterm BAY K8644 (*light gray columns*), term BAY K8644 (*dark gray columns*). The Ca$_L$ inhibitor nifedipine reduces O$_2$-induced constriction in preterm and term DA by 60% (preterm nifedipine (*light gray columns*), term nifedipine (*dark gray columns*).
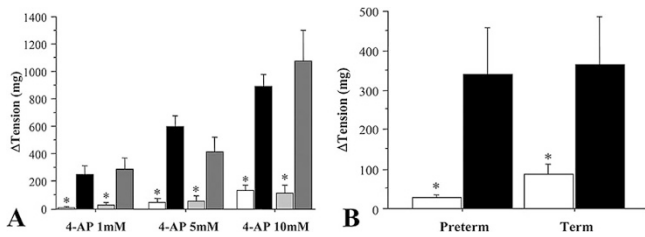
Figure 2. Effect of Ca_L activation on K^+ channel blockade in preterm and term DASMCs. (*A*) In both term and preterm DAs, 4-AP causes a dose-dependent constriction. Kv inhibition is weaker in preterm than in term DAs, consistent with Kv immaturity. Pretreatment with BAY K8644 has no effect on 4-AP–induced constriction in either preterm or term DA ($n = 10–15$, *$p <$ 0.05, preterm vehicle (*white columns*), preterm BAY K8644 (*light gray columns*), term vehicle (*black columns*), term BAY K8644 (*dark gray columns*).(*B*) Pretreatment of preterm and term DA with BAY K8644 significantly enhances BK_{Ca} inhibition–induced constriction with IBTX ($n = 10–15$; *$p < 0.05$; vehicle (*white columns*), BAY K8644 (*black columns*).

(Fig. 2*A*). Conversely, pretreatment of preterm and term DA with BAY K8644 significantly enhanced BK_{Ca}-induced constriction, although this constriction was very small compared with that induced by either increased Po_2 or 4-AP (Fig. 2*B*).

E_M is unchanged by the Ca_L opener BAY K8644. In hypoxia ($Po_2 = 40$ mm Hg), preterm rabbit DASMCs are more depolarized than term DASMCs (Fig. 3*A*). As expected, BAY K8644 had no effect on E_M in either term or preterm DA.

Compared with hypoxic term DASMCs, hypoxic preterm DASMCs have decreased I_{Ca} (Fig. 3*B*). BAY K8644 increases I_{Ca} in hypoxic term and preterm rabbit DASMCs. In hypoxic preterm DASMCs, BAY K8644 enhances I_{Ca} to levels similar to those in term DASMCs. BAY K8644 also increased I_{Ca} in term DASMCs (Fig. 3*B*). Although the time to maximal increase in I_{Ca} caused by BAY K8644 was the same in term and preterm DASMCs, the percentage of increase in I_{Ca} was significantly greater in preterm DASMCs (Fig. 3*C*).
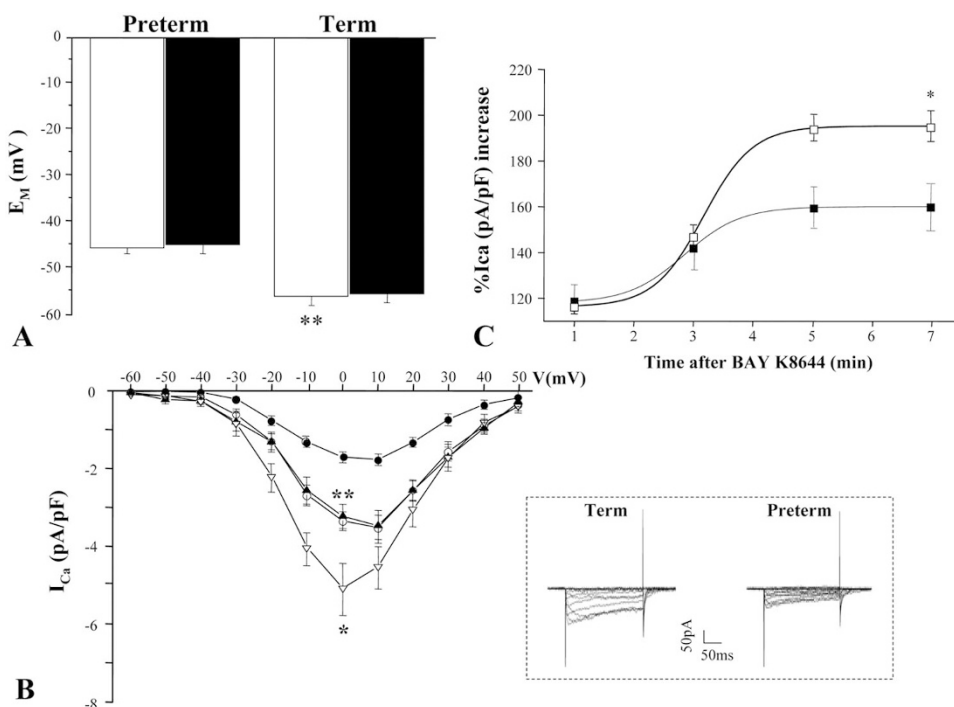
$DASMC$ Ca_L are O_2 sensitive and this sensitivity is absent in preterm rabbit DASMCs. In term rabbit DAs, O_2 significantly increased I_{Ca} (Fig. 4*A*). This effect is evident beginning at E_M -25 mV (Fig. 4*A*). In contrast, O_2 did not alter I_{Ca} in preterm rabbit DASMCs (Fig. 4*B*). BAY K8644 increased I_{Ca} in preterm rabbit DASMCs. The increase in I_{Ca} caused by BAY K8644 was the same whether it was given in normoxia or hypoxia (Fig. 4*B*). In term human DASMCs, O_2 significantly increased I_{Ca} (Fig. 4*C*). This increase was largely eliminated by the Ca_L blocker nicardipine (Fig. 4*C*).

Oxygen elicits less increase in $[Ca^{2+}]_i$ in preterm versus term DASMCs. $[Ca^{2+}]_i$ increased less in preterm *versus* term DASMCs upon 20 min of exposure to increased Po_2 (Fig. 5). BAY K8644 significantly enhanced $[Ca^{2+}]_i$ after O_2 exposure in preterm DASMCs, bringing it to levels similar to those achieved without BAY K8644 in term DA. BAY K8644 had no additional effect on $[Ca^{2+}]_i$ in term DA beyond that achieved by increased Po_2.

Expression of Ca_L α subunit is increased but γ subunit is decreased in preterm DA. Immunoblotting showed increased $\alpha1c$ subunit expression in preterm DAs in pooled specimens derived from four term and four preterm DAs (Fig. 6). In contrast, expression of the Ca_L's γ_2 subunit was significantly decreased in preterm compared with term DAs.

DISCUSSION

Vasoconstriction of the DA in response to increased Po_2 is a robust response that is a major determinant of functional closure of the DA, a crucial step in the transition of the fetal circulation at birth. It is not surprising that a response so essential to the neonate's postnatal adaptation is mediated by multiple, complementary mechanisms. The preterm rabbit DAs (at 26 d of gestation) offers an ideal model in which to

Figure 3. I_{Ca} is reduced in preterm DASMCs but can be increased by BAY K8644. (*A*) In hypoxia ($Po_2 = 40$ mm Hg), preterm rabbit DASMCs are more depolarized than term DASMCs ($n = 7$, **$p <$ 0.01). BAY K8644 has no effect on E_M in either term or preterm DA (vehicle, *white columns*; BAY K8644, *black columns*). (*B*) Compared with term DASMCs ($n = 16$, solid triangle), preterm DASMCs ($n = 19$, solid circle) have decreased I_{Ca}. BAY K8644 significantly increases I_{Ca} in preterm DASMCs (*open circles*) to term (*open triangles*) values. BAY K8644 also increases I_{Ca} in term DASMCs (*$p < 0.05$, **$p < 0.01$). (*C*) Time to maximal increase in I_{Ca} caused by BAY K8644 is the same in term (ET_{50} 2.89 min, *solid squares*) and preterm DASMCs (open squares, ET_{50} 3.19 min, *$p < 0.05$, $n =$ 10). However, the percentage of increase in I_{Ca} is significantly greater in preterm DASMCs.
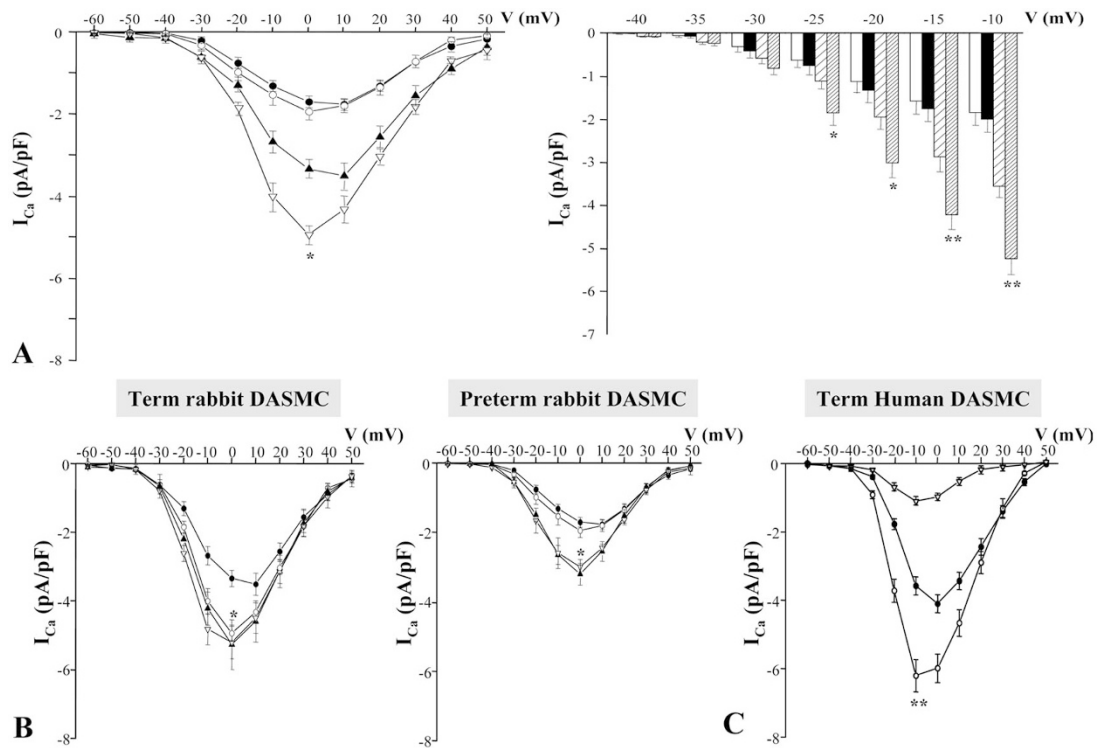
Figure 4. DASMC Ca$_L$ are O$_2$ sensitive, and this sensitivity is absent in preterm DASMCs. (*A*) O$_2$ increases I$_{Ca}$ in term (*open triangles*), but not preterm DASMCs (*open circles*). *Solid circles* and *solid triangles* represent term and preterm DASMCs in hypoxia, respectively. The bar graph highlights the effects at physiologic membrane potentials ($n = 6$–7 per group, *$p < 0.05$, **$p < 0.01$). *Open columns*, preterm hypoxia; *solid columns*, preterm normoxia; *hatched columns*, term hypoxia; *cross-hatched columns*, term normoxia. (*B*) BAY K8644 significantly increases I$_{Ca}$ in hypoxic term and preterm DASMCs but does not increase I$_{Ca}$ during normoxia in term DASMCs ($n = 7$–12, *$p < 0.05$). *Solid circles*, hypoxia; *open circles*, normoxia; *solid triangles*, hypoxia + BAY K8644; *open triangles*, normoxia + BAY K8644. (*C*) Ca$_L$ in human term DASMCs also display O$_2$ sensitivity. The O$_2$-induced increase in I$_{Ca}$ is abolished by the Ca$_L$ blocker nicardipine ($n = 6$, **$p < 0.01$). *Solid circles*, normoxia; *open circles*, hypoxia; *open triangles*, normoxia + nicardipine.
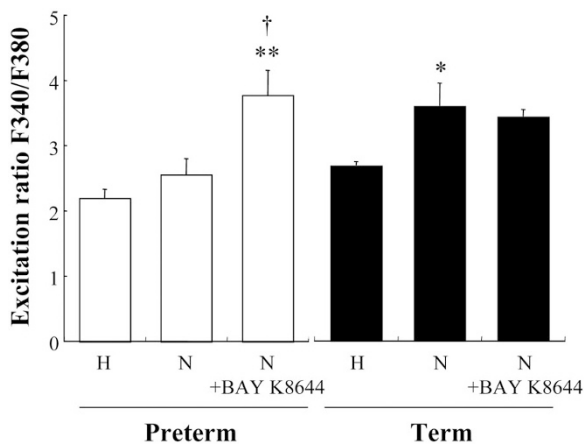

Figure 5. Decreased [Ca²⁺]$_i$ in preterm DASMCs can be enhanced by BAY K8644. O$_2$ causes a smaller increase in [Ca²⁺]$_i$ in preterm DASMCs as compared with term DASMCs. BAY K8644 significantly enhances the Po$_2$-induced increases in [Ca²⁺]$_i$ in preterm, but not in term DASMCs (*$p < 0.05$ vs hypoxia (H), **$p < 0.01$ vs hypoxia, †$p < 0.05$ vs normoxia (N), $n = 5$ per group).

Figure 6. Preterm DAs have conserved expression of the pore-forming α subunit of Ca$_L$ but express less of the Ca$_L$ γ2 subunit. (*A*) Immunoblot showing increased protein expression of the putative O$_2$-sensing α1c subunit in preterm compared with term rabbit DAs. (*B*). The γ2 subunit in preterm rabbit DAs is decreased compared with term DAs ($n = 4$ per group, *$p < 0.05$).

explore the mechanisms that must mature to permit DA constriction and closure at term (11). Patency is favored in preterm DA by several mechanisms, including (relative to term DA) increased production of vasodilator prostanoids (6), reduced production of endothelin (6), decreased expression and function of O$_2$-sensitive Kv (6,11) and more recently decreased rho-kinase activity (16,17). The current investiga-
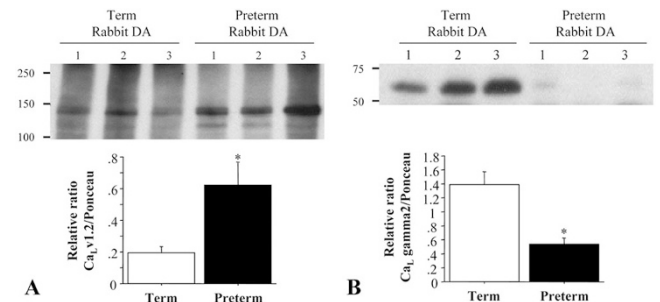
tion identifies a new, developmentally regulated mechanism of O$_2$ sensitivity that is absent in preterm DA and that contributes to O$_2$-induced constriction in term DAs. We demonstrate for the first time that the Ca$_L$ in term rabbit and human DASMCs are intrinsically O$_2$ sensitive and that impaired O$_2$ constriction in preterm DAs results, in part, from decreased Ca$_L$ activation by increases in Po$_2$ that mimic birth (40 to 100 mm Hg) (Fig. 7). The immaturity of the Ca$_L$ is evident both physiologically and electrophysiologically. In DA rings from premature rabbits, O$_2$ constriction is reduced but can be restored to term levels by administration of BAY K8644, which increases the opening of Ca$_L$ (Fig. 1).
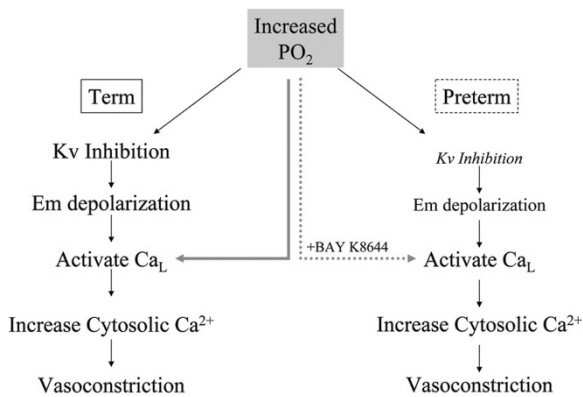
Figure 7. Proposed mechanism for impaired Ca_L-mediated O_2 constriction. (*Left*) Previous work showed that term DAs respond with constriction to O_2, in part due to intact function/expression of the O_2-sensitive Kv, which results in depolarization and Ca_L activation. (*Right*) In addition to a reduced Kv-E_M mechanism in preterm DAs, new data show that O_2 directly activates Ca_L in term DAs (*arrow*) and that this mechanism is lacking in preterm DAs, but can be restored by BAY K8644. This explains why a Ca_L opener (BAY K8644) increases O_2 constriction in preterm DAs to a magnitude similar to that in term DAs.

The observation that pharmacological activation of Ca_L with BAY K8644 rapidly restores O_2-induced constriction in preterm DA to the same magnitude as that observed in term DA and likewise increases I_{Ca} and $[Ca^{2+}]_i$ suggests that Ca_L are present in preterm DA, but somehow are not activated in response to O_2 alone. Moreover, this response to BAY K8644 seems relatively specific for the effects of increased O_2. BAY K8644 does not enhance the constrictor response to 4-AP (Fig. 2) or phenylephrine (data not shown). The fact that BAY K8644 does not enhance constriction or increase I_{Ca} in term DA may reflect the relative hyperpolarization of term DASMCs (Fig. 3*A*). BAY K8644 has little effect on vascular tone unless there is depolarization and our findings in hypoxic term DAs are consistent with BAY K8644's lack of effect on the normoxic pulmonary vascular resistance in normal lungs (18). Moreover, BAY K8644 had no effect on tension or calcium influx in rabbit aorta when they were maximally activated by high K^+ depolarization, whereas it enhanced norepinephrine constriction and calcium influx (19). Likewise, BAY K8644 had no effect on 4-AP constriction in DA. This suggests that in oxygenated term DA, maximal depolarization has occurred, reducing the possibility of an additive effect on Ca_L opening. BAY K8644 exerts its effects (enhancing cardiac contractility and increasing vascular tone) by increasing the open probability of the calcium channels evoking a shift of the open-probability curve to more negative E_M (20). In term DASMCs, adequate O_2-sensitive Kv ensure that increased Po_2 achieves maximal depolarization and Ca_L activation *via* a voltage-dependent mechanism. Perhaps BAY K8644 selectively enhances O_2 constriction in preterm DAs because they have an immature O_2-sensitive Kv channel mechanism. We now show that oxygen's ability to activate the Ca_L and increase calcium influx relies in part on the intrinsic O_2-sensitivity of the Ca_L. This mechanism of Ca_L activation is dificient in preterm DASMC and this deficiency can be overcome by administration of the Ca_L opener BAY K8644. In preterm DASMC BAY K8644 enhances Ca_L opening and

shifts the activation threshold, allowing it to occur at relatively more physiological (negative) membrane potentials (Fig 4*A*).

On an electrophysiological level, the use of the patch-clamp technique allowed us to examine the effects of Po_2 on I_{Ca}, independent on changes in E_M caused by Kv inhibition. These studies show that the premature DASMC has reduced I_{Ca}, but that this is restored to term levels with the addition of BAY K8644 (Fig. 4*B*). Even more importantly, whereas an increase in Po_2 increases I_{Ca} in term DASMCs, there is no effect on preterm I_{Ca} (Fig. 4*B*). Thus, much like Kv, the function of which is reduced in preterm DAs (11), the Ca_L's function is also reduced. However, unlike the Kv channel, whose expression is decreased (11), expression of the $\alpha1c$ subunit, the putative O_2-sensing subunit of Ca_L, is paradoxically increased in premature DASMC in the face of reduced Ca_2 function (Fig. 6).

More recently, we identified a unique O_2 sensor in the DA comprised of a sensor, the proximal electron transport chain of the mitochondria, that produces a diffusible mediator (H_2O_2) that inhibits redox-sensitive Kv promoting constriction (7,9,10,15,21) (Fig. 7). Reduced expression and function of putative O_2-sensing Kv1.5 and Kv2.1 in preterm DAs appear to contribute to DA patency in preterm newborn rabbits. Kv1.5 or Kv2.1 overexpression in preterm rabbit DAs does not restore O_2 response to term DA levels (rather it reaches 50% of term constriction) (11). Kv gene therapy confers 300–400 mg of constriction to the ductus exposed to oxygen *versus* 800 mg in the normal term ductus without gene therapy. In contrast, BAY K8644 fully restores O_2 sensitivity in preterm DAs (Fig. 1*B*). With BAY K8644 pretreatment, there was no difference between O_2-induced constriction in preterm DAs (light gray columns) *versus* untreated term DAs (black columns). This suggests that Ca_L are present but functionally immature in preterm DAs.

O_2 sensitivity of K^+ channels have been described in various specialized, O_2-sensing tissues such as chemoreceptors in the carotid body, the pulmonary SMCs, neuroepithelial bodies, the placenta, and adrenal chromaffin cells (12). More recently, Ca_L in vascular SMCs (13,22,23) and carotid body chemoreceptor cells (24) have been shown to be O_2 sensitive. Whereas hypoxia reversibly inhibits Ca_L in SMCs from systemic arteries and proximal pulmonary arteries [vessels where hypoxia causes vasodilatation (22)], hypoxia activates Ca_L in resistance pulmonary artery SMCs (25), suggesting that rather than solely responding to Kv-determined changes in E_M, Ca_L actively contribute to hypoxic pulmonary vasoconstriction. Here we demonstrate a similar phenomenon: Ca_L in DASMCs are directly O_2 sensitive in that increased Po_2 rapidly and reversibly increases I_{Ca} in freshly isolated term rabbit DASMCs (Fig. 3). The activation of Ca_L increases $[Ca^{2+}]_i$, leading to DA constriction (Fig. 5). The fact that the O_2 sensitivity of the channel is deficient in preterm DAs and can be restored by BAY K8644 (which also restores O_2 constriction in premature DA rings) suggests that there are two nonadditive means of activating maximally the Ca_L: depolarization (which requires Kv inhibition) and activation of Ca_L by O_2 (which is enhanced by BAY K8644).

In both term and preterm DAs approximately two thirds of O_2 constriction depends on calcium influx *via* the Ca_L and thus is inhibited by a Ca_L blocker, consistent with previous

reports (9,16,17). The remaining one third of constriction appears to reflect calcium sensitization (*i.e.* rho-kinase activation) and persists in the absence of extracellular calcium and inhibition of sarcoplasmic reticulum calcium release (17). The rho-kinase pathway also displays functional immaturity in preterm DAs (17).

Ca$_L$ are composed of a central pore-forming, voltage-sensing α_1 subunit and auxiliary subunits including various isoforms of β, δ, and γ (26–28). α1c is the putative O$_2$-sensing subunit, and work by Fearon *et al.* (29,30) indicate that auxiliary subunits are not necessary for O$_2$ sensing in the human cardiac Ca$_L$. Several splice variants of the α1 subunits exist. The rat DA predominantly expresses α1C and α1G subunits (31). The α1C subunit is highly expressed in the DA's neointimal cushion (31), where proliferating and migrating SMCs are abundant. This localization is consistent, with such channels contributing to both functional and anatomical closure of the DA, although mechanistic studies are lacking.

To our knowledge, the role of auxiliary Ca$_L$ subunits in the vasculature is unknown. Term rabbit DAs strongly express the γ2 subunit, whereas preterm rabbit DA express very little of the γ2 subunit (Fig. 6). We speculate that the γ2 subunit is somehow important in conferring O$_2$ sensitivity and/or BAY K8644 responsiveness to Ca$_L$ in term DASMCs. Further studies will be required to evaluate the putative role of Ca$_L$ subunits in the diminished O$_2$ sensitivity of I$_{Ca}$ in preterm DASMCs.

Maturational changes in DASMC electrophysiology are not the only factors favoring patency of the preterm DA; the endothelium has a major modulatory role. O$_2$ responsiveness of the term DA is reinforced by reduced synthesis and reduced responsiveness to endothelium-derived vasodilating prostaglandins (32,33) and perhaps by increased endothelin-1 production (10,34–36).

In conclusion, we demonstrate that Ca$_L$ in DASMCs are O$_2$ sensitive and that impaired O$_2$ constriction in preterm DA results in part from decreased O$_2$ activation of Ca$_L$. Reduced O$_2$ constriction in preterm rabbit DA can be overcome by enhancing/prolonging Ca$_L$ activation with BAY K8644. O$_2$-induced activation of Ca$_L$ in the DASMCs is a novel additional mechanism contributing to DA closure (Fig. 7). With the growing interest in developing selective Ca$_L$ blockers and activators for a variety of diseases associated with Ca^{2+} channelopathies (including fertility, neuronal growth, bone formation, and epilepsy) (28), modulation of DA patency can be added to this list of potential therapeutic targets.

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