Role of Store-Operated Calcium Channels and Calcium Sensitization in Normoxic Contraction of the Ductus Arteriosus

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Background—At birth, the increase in oxygen causes contraction of the ductus arteriosus, thus diverting blood flow to the lungs. Although this contraction is modulated by substances such as endothelin and dilator prostaglandins, normoxic contraction is an intrinsic property of ductus smooth muscle. Normoxic inhibition of potassium channels causes membrane depolarization and calcium entry through L-type calcium channels. However, the studies reported here show that after inhibition of this pathway there is still substantial normoxic contraction, indicating the involvement of additional mechanisms.

Methods and Results—Using ductus ring experiments, calcium imaging, reverse-transcription polymerase chain reaction, Western blot, and cellular electrophysiology, we find that this depolarization-independent contraction is caused by release of calcium from the IP$_3$-sensitive store in the sarcoplasmic reticulum, by subsequent calcium entry through store-operated channels, and by increased calcium sensitization of actin-myosin filaments, involving Rho-kinase.

Conclusions—Much of the normoxic contraction of the ductus arteriosus at birth is related to calcium entry through store-operated channels, encoded by the transient receptor potential superfamily of genes, and to increased calcium sensitization. A clearer understanding of the mechanisms involved in normoxic contraction of the ductus will permit the development of better therapy to close the patent ductus arteriosus, which constitutes ≈10% of all congenital heart disease and is especially common in premature infants. (Circulation. 2006;114:1372-1379.)

Key Words: ductus arteriosus, patent heart defects, congenital ion channels oxygen vasoconstriction

In the fetus, oxygenated blood returning from the placenta is diverted away from the high-resistance pulmonary circulation through the ductus arteriosus (DA) to the aorta. At birth, an event that is associated with a rise in oxygen tension, pulmonary vascular resistance falls and the DA first contracts and then remodels.1 Normoxic contraction of the DA has been studied for 50 years.2 Continued patency of the DA in neonatal life is more common in the relative hypoxia of high altitude.3 Vasoactive substances such as prostaglandins and endothelin play an important role in the patency or contraction, respectively, of the DA, but normoxic contraction can be demonstrated in the absence of both.4,5 We have previously shown that normoxic contraction is explained partly by inhibition of voltage-gated K$^+$ (Kv) channels, membrane depolarization, and calcium entry through L-type Ca$^{2+}$ channels in the smooth muscle cells (SMCs) of the DA.6 The mechanism of this normoxic contraction is likely triggered by a change in the redox status of the DASMCs so that the cytosol or cell membrane is more oxidized during normoxia.7–9 However, in this article, we report that significant normoxic contraction of the DA can occur after Kv channels are blocked by 4-aminopyridine (4-AP). We hypothesize that this additional component of normoxic contraction is caused by calcium entry through store-operated channels (SOCs) and increased calcium sensitization. Failure of the DA to close may be associated with intracranial hemorrhage, pulmonary edema/hemorrhage, necrotizing enterocolitis, and broncho-pulmonary dysplasia.10 Because 70% of infants born before 28 weeks of gestation require either medical or surgical closure of a patent DA,11 understanding the mechanisms responsible for normoxic contraction is extremely important.

Methods

Tension Measurement in Isolated DA Rings

DA rings from term fetal rabbit pups were studied as previously described5 (details available in the online Data Supplement). Contractile responses on switching from hypoxia to normoxia or chang-
Oligonucleotide Sequences of the Primers Used for Reverse-Transcription Polymerase Chain Reaction

<table>
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<th>Standard Names</th>
<th>Fragment Size, bp</th>
<th>Sense</th>
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<th>Antisense</th>
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-oTRPC1 indicates rabbit TRPC1; mTRPC1, mouse TRPC1. All the other sequences are for rat TRPCs.

ING bath calcium from 0 to 2 mmol/L were recorded in the presence of drugs chosen to examine the role of different potassium and calcium channels.

Calcium Imaging
Dual-excitation imaging with Fura2 was used to measure the cytosolic Ca\(^{2+}\) response to changes in oxygen tension in freshly dispersed DASMcs as previously described.\(^8\) TRPC1 antibody (Alomone Laboratories, Jerusalem, Israel) binds amino acids 557 to 571 on the S5 pore-forming region of TRPC1 on the extracellular domain and inhibits Ca\(^{2+}\) entry via this channel.\(^12\)‐\(^14\) DASMcs were exposed to anti-TRPC1 (all in 1:200), TRPC1 peptide/anti-TRPC1 mixture (1 µg: 1 µg), or saline for 1 hour and then loaded with Fura2-AM.

Cell Electrophysiology
Conventional whole-cell patch-clamp studies were performed on freshly isolated DASMcs as previously described.\(^8\) Two voltage-clamp protocols were used in current studies. One used continuous current recording while holding the cells at a membrane potential of –80 mV during an intervention. The second protocol held the cell membrane potential at 0 mV, and currents were then evoked by 400-ms pulses in 20-mV steps from –120 to 60 mV at 10-second intervals.

Reverse-Transcription Polymerase Chain Reaction
RNA was extracted from DA after endothelium and adventitia had been removed. After reverse transcription (RT), cDNA samples were amplified and densitometry was used to quantify the polymerase chain reaction (PCR) product relative to β-actin (details online). Oligonucleotide primers used to amplify TRPC1-C7 in the DASMcs are shown in the Table.

Western Blot
Polyclonal primary antibodies (TRPC1, TRPC3, TRPC4, TRPC5: Alomone) were used to identify these channel proteins in the membranes of fetal rabbit DA, fetal rat brain, and rat brain. The specificity of the antibody for the intended antigen was confirmed in competitive experiments in which incubation with an excess of the relevant antigen neutralized the antibody. Western blots

Figure 1. A, Representative tension tracing showing the effect of 1 µmol/L nifedipine (NIF) on normoxia-induced DA ring contraction. Outside the shaded box, the ring is hypoxic. Nifedipine 1 µmol/L completely inhibits 80 mmol/L KCl (K\(^{+}\))–induced contraction but inhibits only part of the subsequent normoxia-induced contraction. B, Summary of DA ring data: 1 µmol/L nifedipine (n = 15) partly inhibits normoxia-induced contraction. The K\(_{ATP}\) channel blocker TEA (10 mmol/L; n = 5), TASK channel blocker anandamide (10 µmol/L; n = 3), T-type calcium channel blocker mibefradil (4 µmol/L; n = 5), Cl\(^{-}\) channel antagonist niflumic acid (50 µmol/L; n = 3), and Na\(^{+}\)–Ca\(^{2+}\) exchanger blocker KB-R 7943 (10 µmol/L; n = 5) have little or no effect compared with the earlier normoxic contraction in the same DA ring. C, Representative tension tracing showing that 2 control periods of acute normoxia result in similar contractions; a subsequent exposure to 30 µmol/L CPA causes contraction and reduces the next normoxic contraction, so the sum of the 2 is equal to the control normoxic contractions (n = 7). D, Summary of DA ring data showing that omitting bath calcium (n = 5), 30 µmol/L 2-APB (n = 8), and 10 µmol/L SKF 96365 (n = 6) inhibits normoxic contraction of DA rings, whereas the TRPV channel blocker ruthenium red (10 µmol/L; n = 6) has no effect. **P < 0.01 vs control.
were performed using protein samples pooled from DA tissue taken from 20 fetal rabbits (see Data Supplement).

Statistical Analysis
Data are expressed as mean±SEM. In all figures, the SEM is indicated when it exceeds the symbol size. The effects of drugs on current, intracellular Ca\(^{2+}\) levels, and DA tension were assessed by separate factorial ANOVAs with post hoc analysis (Fisher’s least-significant-difference test). The specific factors, drugs, and drug levels are given in the figures and figure legends. Values of \(P<0.05\) were considered significant.

All studies were approved by the Institutional Animal Care and Use Committee of the Minneapolis Veterans Affairs (VA) Medical Center and conform to current National Institutes of Health and American Physiology Society guidelines for the use and care of laboratory animals.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results
In DA rings, switching from a hypoxic to a normoxic bath solution causes reproducible contraction. KCl 80 mmol/L added to the bath increases ring tension by 66±6% of the normoxic contraction (n=24; Figure 1A). The KCI contraction is completely ablated by 1 μmol/L nifedipine (−102±5%, n=14). However, the same dose of nifedipine leaves a residual normoxic contraction 48±7% of control (Figure 1B). The K\(_v\) blocker 4-AP (10 mmol/L) increases tension by 50±13%, and a superimposed normoxic contraction is still 76±10% of the control normoxic contraction.

These observations suggest that part of the normoxic contraction may occur through a mechanism other than inhibition of K\(_v\) channels, membrane depolarization, and calcium entry through L-type channels. Blockers of K\(_{ca}\) channels, tetraethylammonium (TEA; 10 mmol/L); TASK channels, anandamide (10 μmol/L); T-type calcium channels, mibefradil (4 μmol/L); and chloride channels, niflumic acid (50 μmol/L), and Na\(^+\)-Ca\(^{2+}\) exchanger KB-R 7943 (10 μmol/L) did not alter normoxic contraction of the DA rings (Figure 1B).

Cyclopiazonic acid (CPA), which inhibits SR Ca\(^{2+}\) adenosine triphosphatase and depletes the calcium store by preventing calcium uptake, causes a contraction of 1079±123 mg (n=8) (Figure 1C). The superimposed dose of nifedipine to normoxia is 37±7% of the control contraction to normoxia. Omission of calcium from the ring bath or addition of the SOC blockers 2-APB (30 μmol/L) or SKF96365 (10 μmol/L) decreases normoxic DA contraction to 19±5%, 34±8%, and 44±6%, respectively (Figure 1D), suggesting that a substantial part of the normoxic contraction is related to SOC calcium influx. A role for other mechanisms appears less likely because the following agents had no effect: 10 μmol/L ruthenium red (blocker for TRPV family), 96±6% of control (n=6) (Figure 1D); 200 nmol/L menthol (TRPM8 agonist), 103±1% of control (n=3); 3 μmol/L BIS-1 (protein kinase C blocker), 98±3% of control (n=5); and 100 nmol/L H89 (PKA blocker) 92±4% of control (n=5).

When calcium stores in the SR are depleted by thapsigargin (TG; 100 nmol/L), L-type calcium channels are blocked by nifedipine, and the bath solution contains no calcium, switching the DA ring from hypoxia to normoxia causes only a small contraction (Figure 2A). When 2 mmol/L calcium is introduced during hypoxia, there is a contraction, and then switching to normoxia stimulates a further marked contraction, presumably related to influx of calcium through SOCs, although increased calcium sensitization cannot be excluded. In DA rings treated with nifedipine (1 μmol/L), switching the bath calcium from 0 to 2 mmol/L causes a much greater contraction under normoxic conditions than when the same switch is made under hypoxic conditions (\(P<0.001\); Figure 2B). This was true whether the normoxic or the hypoxic exposure was made first. Thus, in this experiment, normoxia appears to facilitate calcium release and/or calcium entry through the SOCs. Concordant with this observation, measurement of cytosolic calcium in DASMCs in the presence of nifedipine (1 μmol/L) shows that the calcium level is higher in normoxia (Figure 3A).

The presence of SOCs is illustrated by the rise in calcium on switching from 0 to 2 mmol/L calcium in the presence of CPA and nifedipine during normoxia (Figure 3B). Using TRPC1 antibody significantly reduces the calcium rise on switching from 0 to 2 mmol/L calcium, suggesting that TRPC1 may play an important role in the SOCs of DASMCs (Figure 3B). The TRPC subfamily of transient receptor potential (TRP) channels is thought to be responsible for store-operated calcium influx. mRNA for TRPC1, TRPC3, TRPC4, and TRPC6 is identified in DA by reverse transcription polymerase chain reaction (Figure 4A). Both rabbit and mouse TRPC1 primers were used. In addition, TRPC1 and TRPC4 proteins were detected in DA by Western blot (Figure 4B). The physiological function of SOCs was confirmed in
patch-clamp experiments with DASMCs. The introduction of CPA or normoxia elicits an inward current when the membrane potential of the SMC is clamped at $-80\, \text{mV}$ (Figure 5A). Similarly, when the calcium current is isolated by inhibition of chloride and potassium currents, both CPA and normoxia markedly enhance the current across a range of membrane potentials (Figure 5B and 5C).

Calcium sensitization is a critical mechanism in the modulation of vascular tone. Therefore, we examined the role of calcium sensitization in normoxic contraction in DA rings. The role of calcium sensitization in normoxic contraction was studied in DA rings. The Rho-kinase inhibitor Y-27632 (3 $\mu\text{mol/L}$) reduces normoxic DA ring tone below baseline, whether given during or before the introduction of normoxia.

With washout of Y-27632, normoxic contraction recovers very rapidly (Figure 6A and 6B). Fasudil, a structurally dissimilar inhibitor of Rho-kinase, also reduces normoxic DA tone (Figure 6B). In the case of SOC-related contraction induced by switching from 0 to 2 mmol/L calcium in the presence of CPA and nifedipine during hypoxia, Y-27632 again reduces DA ring tone to below the baseline, whether given before the calcium switch or during the contraction (Figure 6C). After pretreatment, the 2-mmol/L calcium contraction is greatly reduced ($P<0.001$) (Figure 6C). In KCl-induced contraction, Y-27632 blocks 74±12% when given during the contraction. After pretreatment and the subsequent lowered baseline tension, the KCl-induced contraction also is reduced ($P<0.05$) (Figure 6D). In control experiments, the Rho-kinase inhibitor Y-27632 (3 $\mu\text{mol/L}$) has no effect on $K^+$ current over the range of $-70$ to $50\, \text{mV}$ or on membrane potential ($n=6$ for both; online Figure I).

**Discussion**

Continued patency of the DA is a common cardiac anomaly in the newborn, especially in premature infants. It can lead to high flow through the lungs, heart failure, and failure to thrive. On the other hand, in the case of pulmonary atresia, continued patency of the DA may be the most important source of blood flow to the lungs, permitting survival until surgical intervention is possible. Consequently, a better understanding of the mechanisms by which normoxia causes contraction of the DA has major clinical implications. In the uterus, dilator prostaglandins are thought to play a role in keeping the ductus open, and after birth, endothelin helps to cause contraction. However, normoxic contraction of the human ductus can be demonstrated in the presence of...
prostaglandin and nitric oxide synthesis inhibitors and endothelin blockers, suggesting that these vasoactive substances are not essential for the onset of normoxic contraction. Although normoxic contraction is caused in part by the inhibition of K^+ channels, membrane depolarization, and entry of calcium through L-type calcium channels, the present studies show a significant normoxic contraction in the presence of the Kv blocker 4-AP and the KCa blocker TEA, indicating that another mechanism may be involved. This mechanism does not appear to involve KCa channels, TASK channels, T-type voltage-gated calcium channels, chloride channels, or the Na^+–Ca^{2+} exchanger, as judged by the lack of effect of the blockers shown in Figure 1B. Use of zero calcium outside the cell eliminates most of the normoxic DA ring contraction (Figure 1D), indicating that the contraction depends largely on the influx of extracellular calcium. The concept that SOCs permit calcium entry after depletion of intracellular stores was developed by Casteels and Droogmans. The SOC current is thought to pass through TRP channels. Release of calcium from the SR can stimulate DA contraction, as shown by the observations that CPA, which releases SR calcium, increases cytosolic calcium (Figure 3B) and induces contraction of DA ring (Figure 1C). Inhibition of calcium release from the IP_3-sensitive store in the SR by 2-APB or SKF96365 reduces normoxic DA contraction by >50% (Figure 1D). Previously, 2-APB has been shown to reduce the rise in DASMC calcium caused by switching from hypoxia to normoxia, but this experiment was performed in cultured DASMCs and the control normoxic calcium increase was 12% above baseline. In the present study, freshly dispersed DASMCs showed a 78% increase in calcium above baseline in response to normoxia, illustrating a significant difference between these models. Ryanodine has no effect on the increase in calcium stimulated by normoxia or on normoxic contraction. In addition, 8-Bromo-cyclic adenosine diphosphate ribose has no effect on normoxia DA contraction (1 to 100 μmol/L; data not shown), indicating that cADPR-dependent SR Ca^{2+} release is not involved. These observations suggest that calcium release from the SR by normoxia comes from the IP_3- rather than the ryanodine- and cADPR-sensitive stores.

If release from the SR contributes to normoxic contraction, what is the evidence for calcium entry through SOC? The presence in the DA of at least TRPC1 and TRPC4 is indicated by the finding of mRNA and protein by reverse transcription polymerase chain reaction and Western blot (Figure 4). TRPC1 forms part of the SOCs in pulmonary artery SMCs, and TRPC1 mRNA is expressed in mouse, rabbit, and human resistance pulmonary arteries. Similarly, TRPC4 has been detected in rat pulmonary artery smooth muscle. However, these channels have not previously been described in the DA. In terms of function, the involvement of SOCs is indicated by the observation that, in the presence of nifedipine to inhibit influx through the L-type calcium channel and
TG to deplete the IP₃ calcium store, a switch from 0 to 2 mmol/L calcium in the DA ring tissue bath during hypoxia results in an increase in tone. This indicates calcium entry through the SOC. Further contraction on switching from hypoxia to normoxia (Figure 2A) suggests that normoxia facilitates calcium entry through SOCs because the SR is already depleted by TG; therefore, the oxygen level should not affect the calcium release from the SR. The observation that changing the ring bath from 0 to 2 mmol/L calcium under hypoxic conditions in the presence of nifedipine causes only a small increase in tone indicates that normoxia induces Ca²⁺ current and hypoxia inhibits it (n=15). During both B and C, the extracellular solution contains (in mmol/L) 120 aspartic acid, 5 Ca(OH)₂, 10 HEPES, and 0.5 of 3,4-diaminopyridine (pH 7.4 adjusted with tetraethylammonium hydroxide); the pipette solution contains (in mmol/L) 130 N-methyl-D-glucamine, 10 HEPES, 1.15 ethyleneglycotetraacetic acid, 1 Ca(OH)₂, 2 Mg adenosine triphosphate, 1 N-phenylanthranilic acid, and 0.1 of 5-nitro-2-(3-phenylpropylaminono) benzoic acid. [Ca²⁺]ᵢ is estimated as 100 nmol/L (pH 7.2, adjusted with methane sulfonic acid).

The sensitivity of actin-myosin to any particular level of cytosolic calcium is an important determinant of vascular tone. Contraction is initiated when myosin light chain is phosphorylated. Dephosphorylation by myosin phosphatase causes relaxation. The myosin phosphatase can be inhibited through phosphorylation by Rho-kinases; thus, Rho-kinases may increase contraction, even at a fixed calcium concentration. Inhibition of Rho-kinases with agents such as Y-27632 or fasudil may cause vasodilatation and indicate whether calcium sensitization is playing a significant role. Y-27632 and fasudil both ablate the normoxic contraction of the DA.
regardless of whether they are given before normoxic contraction (pretreatment) or during contraction (posttreatment) (Figure 6A and 6B). These agents also ablate DA contraction caused by other stimuli (Figure 6C and 6D), indicating that the source of calcium-initiating contraction is immaterial. The efficacy of these agents, if they are specific for Rho-kinase, highlights the importance of the calcium sensitization mechanism in the DA. In terms of specificity, Y-27632 has no effect on whole-cell K^+ current or membrane potential. In summary, the novel finding in these experiments is the observation of SOC current in DASMCs that is markedly enhanced by normoxia and correlates with the functional studies and the finding of TRPC channels in the DA. These results, together with new observations on calcium sensitization, introduce mechanisms that can be pharmacologically manipulated to help close or open the DA as clinically indicated.

**Acknowledgment**

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**Disclosures**

None.

**References**

In 1628, William Harvey described the role of the ductus arteriosus in the fetus, saying that as the lungs are in “a state of inaction,” nature propels blood by the ductus arteriosus into the aorta. Although he reported the function of the ductus arteriosus and its closure after birth, the mechanisms responsible for contraction of the ductus in response to an increase in oxygen are still being debated. At term, the neonatal ductus shows strong normoxic contraction, but premature neonates have a high incidence of persistent patency of the ductus. In a current study of premature infants with respiratory failure, 75% had symptomatic patent ductus arteriosus requiring mechanical or surgical closure. Continued patency results in a reversed shunt with a high flow of blood from the aorta to the pulmonary arteries. This may lead to failure to thrive, heart failure, and pulmonary hypertension. We have previously reported that an increase in oxygen tension inhibits an outward potassium current in the smooth muscle cells of the ductus wall, leading to membrane depolarization and calcium entry through L-type calcium channels. This increase in cytosolic calcium causes contraction. Here, we describe 2 additional mechanisms that contribute to normoxic contraction. One is the entry of calcium through store-operated channels. The other is calcium sensitization, which means that the interaction of actin and myosin is prolonged at any level of cytosolic calcium. A better understanding of the mechanisms responsible for normoxic contraction of the ductus will lead to more effective treatment of the patent ductus.