Intracellular and extracellular calcium utilization during hypoxic vasoconstriction of cyclostome aortas

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HYPOTHETICAL PULMONARY VASOCONSTRICION (HPV) in mammalian pulmonary vascular smooth muscle is mediated by both endothelial and direct smooth muscle effects (4, 5, 11, 33), and it matches ventilation to perfusion by decreasing blood flow to underventilated alveoli. The calcium dependence of HPV in mammals has been well documented (15, 24). During HPV, a rise in free cytosolic calcium (Ca2+) accompanies a series of plasma membrane channel events that lead to full depolarization and vasoconstriction, although the order and relative importance of these events remain controversial (15, 23, 32, 34, 35).

Vasoconstriction appears to be prevalent in the respiratory circuits of most nonmammalian vertebrates during hypoxia (10, 12); however, the role of calcium and the intracellular mechanisms mediating these responses have not been examined. We have recently described for the first time a profound hypoxic vasoconstriction (HV) in postgill, but not pregill, systemic vessels from the New Zealand hagfish, the Pacific hagfish, and the sea lamprey (22). Although the physiological significance of this response in these animals is not presently known, we previously hypothesized that cyclostomes may be a useful model with which to study the intrinsic mechanisms underlying HPV (22).

The purpose of the present study was to characterize the role of calcium during HV in the primitive cyclostome model. We examined the utilization of stored intracellular calcium (Ca2+) and extracellular calcium (Ca2+) during the hypoxic response in postgill systemic arteries (dorsal aortas [DA]) from sea lamprey and New Zealand hagfish. Results indicate that in lamprey DA, 1) Ca2+ is mobilized during HV and the rise in [Ca2+]i during HV is temporally correlated with contractile force, 2) Ca2+ is not required for HV, 3) L-type calcium channels are not activated for Ca2+ influx during HV, 4) Ca2+ stores are used differently during HV than during adrenergic stimulation and are not easily depleted using standard pharmacological agents, and 5) Na+/Ca2+ exchange is present, but extracellular sodium (Na+) is not required for relaxation following HV. The hypoxic response in hagfish DA is somewhat different in that Ca2+ influx contributes significantly to the overall magnitude of HV, and Na+ is requisite for relaxation following HV.

MATERIALS AND METHODS

Animals. Sea lamprey (Petromyzon marinus, 130–450 g) were captured by the U.S. Geological Survey, Biological Resources Division, in Michigan during the spring-summer
spawning migration and airlifted to Notre Dame. At Notre Dame, they were housed in 500-liter rectangular tanks in aerated, flowing well water (15°C) and exposed to a 12:12-h light-dark photoperiod. They were not fed. Lamprey were anesthetized with benzocaine (ethyl-p-aminobenzonate; 1:5,000 w/vol), and the vessels were dissected out and placed in lamprey HEPES-buffered saline (LHBS) at 4°C.

New Zealand hagfish (Eptatretus cirrhatus, 800–2,100 g) were collected off Motunau Beach, New Zealand, and were transferred to Christchurch where they were held in aquariums containing running seawater (16°C). They were held at least 1 week before experimentation and were not fed during this period. Hagfish were anesthetized with benzocaine (1:5,000 w/vol), and vessel segments were dissected out, rinsed with a modified hagfish HEPES-buffered saline (HHBS), and stored in fresh HHBS at 4°C until use.

Vascular smooth muscle. DA from lamprey and hagfish were cut transaxially into 3- to 4-mm rings. Rings were hung on 280-μm stainless steel hooks and suspended in 20-ml water-jacketed (15°C) smooth muscle chambers (21) containing the appropriate saline. In experiments with lamprey, stainless steel hooks attached vascular rings to plastic diffusers inside the smooth muscle chambers. The diffusers were designed and fabricated in our lab to allow rapid mixing of chemicals and gasses while protecting the rings from direct gas contact, thereby reducing turbulence. The diffusers were attached to lids to minimize surface gas exchange with the atmosphere and facilitated the addition of drugs and bath changes. Hypoxia was administered by aerating the muscle chambers with 100% nitrogen gas (N₂) and normoxia was restored by aeration with room air. Tension was measured with Grass PT03C force-displacement transducers and recorded on either a computer-interfaced Gould 8000 series or Grass model 8TC polygraph. Data were collected electronically using Labtech Notebook data-collection software (Laboratory Technologies, Andover, MA).

In experiments with New Zealand hagfish DA, tension was measured with Ugo Basile (Comerio, Italy) isometric force transducers (model 7004), and the signals were amplified with Gould (Valley View, OH) transducer preamplifiers (model 1350). Signals were displayed on a Yokogawa LR4100E recorder (Yokogawa Electric, Tokyo, Japan) and recorded electronically with Labtech Notebook as described above. Diffusers were not available for these experiments. In all instances, polygraph sensitivities were set to detect changes as small as 5 mg.

Optimal resting tension for each of the different types of vessels used in this study was determined in preliminary experiments by measuring the magnitude of 80 mM KCl contractions over a range of resting tensions from 0 to 1.5 g. Optimal resting tension (500–750 mg) was subsequently applied to lamprey vessels for at least 30 min before experimentation. Hagfish aortas were equilibrated for 1 h before experimentation due to their slower response characteristics (22). Vessels were precontracted with either KCl (80–90 mM), the acetylcholine analog carbacholcholine chloride (carbachol, 10⁻⁵ M), or epinephrine (10⁻⁵ M) and washed three times or aerated with 100% N₂ for 15–20 min and returned to room air. Baseline tension was then reestablished for at least 30 min before further experimentation.

The effects of hypoxia on vessels pretreated with various agonists or drugs that have been shown to affect [Ca²⁺]ᵢ were tested in baths of the appropriate buffered saline, containing either 2 mM Ca²⁺ (lamprey), 5 mM Ca²⁺ (hagfish), or without Ca²⁺ but in the presence of the calcium-chelating agent EGTA (200 μM). Agonists and drugs were applied to lamprey vessels 15 min before hypoxia. The slower responding hagfish vessels (22) were treated for 1 h before hypoxia. In another series of experiments, helical strips were cut from lamprey DA and suspended in a tissue fluorometer system (see Fura 2-AM below) to simultaneously measure changes in intracellular [Ca²⁺]ᵢ and force.

Fura 2-AM. The experimental apparatus for measuring [Ca²⁺]ᵢ was similar to that described previously by Chen and Rembold (7). Helical DA strips from lamprey were stretched to optimal length and loaded with 5 μM fura 2-AM and 0.3 mM neostigmine in cold LHBS for 90 min. Fura 2 fluorescence is bright enough following this loading protocol to permit measurements at intracellular dye concentrations determined in rats to have no effect on calcium buffering or damping of calcium transients that might interfere with excitation-contraction coupling (7). Fluorophore leakage does not significantly contribute to the fluorescence measurements, because 1) bath perfusion rates of 1 ml/min diluted any leaking indicator, and 2) the illuminated area includes only a small volume of bath solution. The tissue was then mounted isometrically to a capacitive force transducer and bathed in 3-ml water-jacketed (15°C) smooth muscle chambers (21) containing 1.00 glucose, pH 7.8. All chemicals were purchased from Sigma Chemical (St. Louis, MO). Briefly, changing calcium concentrations can be correlated with the fluorescence ratio at 340/380 nm excitation after subtracting background fluorescence at each excitation wavelength. Background fluorescence was determined at the end of each experiment by lysing cells with 0.2 mM MnCl₂ solution, followed by 5 mM MnCl₂ solution to quench fura 2 fluorescence, then measuring autofluorescence at pertinent wavelengths (340- and 380-nm excitation, 525-nm emission). Data were used from experiments where fluorescence at 380 nm was at least 2.5 times as great as the background signal, and the 340- and 380-nm fluorescence signals changed in opposite direction in response to the agonist. Simultaneous changes in isometric force and 340/380 nm fluorescence ratios were recorded in response to 90 mM KCl (maximum response) and in response to hypoxia. Changes in both tension and fluorescence ratio at 340/380 nm to hypoxia were expressed as a percentage of the maximum response.

Chemicals. The composition of LHBS was as follows (in g/l): 8.74 NaCl, 0.22 KCl, 0.29 CaCl₂·2 H₂O, 0.14 MgSO₄·7 H₂O, 0.72 HEPES acid form, 1.8 HEPES sodium salt, and 0.9 glucose, pH 7.8. The composition of HHBS was as follows (in g/l): 27.70 NaCl, 0.60 KCl, 0.75 CaCl₂·2 H₂O, 0.75 MgSO₄·7 H₂O, 0.72 HEPES acid form, 1.82 HEPES sodium salt, and 1.00 glucose, pH 7.8. All chemicals were purchased from Sigma Chemical (St. Louis, MO).

Calculations. At the end of an experiment, the vessel was blotted on paper towel, weighed, and vessel tension was normalized to wet weight, i.e., milligrams of tension per grams wet weight. Because the hypoxic responses of individual vessels were reproducible (22), a vessel served as its own control and treatment effects were statistically examined by paired t-test or repeated-measures tests. Results are pre-

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sented as means ± SE. Student’s t-test and ANOVA were used for comparisons between vessels. The fiducial limit of significance was set at $P \leq 0.05$.

RESULTS

Sea lamprey. Contractile force and $[Ca^{2+}]_{c}$ increased simultaneously when DA were exposed to either 90 mM KCl or hypoxia (Fig. 1A). A temporal correlation between the active force generated by HV in lamprey DA and a rise in $[Ca^{2+}]_{c}$ was noted in all vessels treated. The active force, normalized as the ratio of the force generated by HV to the force generated by depolarization with 90 mM KCl, was linearly related to the change in intracellular calcium, normalized as the ratio of the calcium signal produced by HV to the calcium signal produced during 90 mM KCl (Fig. 1B).

The magnitude of HV was not significantly affected by either prior removal of Ca$^{2+}$ (105.4 ± 12.4% of control) or the restoration of Ca$^{2+}$ (2 mM) during hypoxia (118.2 ± 12.0% of control, $n=4$ fish; Fig. 2A). Substitution of Na$^{+}$ with sucrose (290 mM; Fig. 2B) produced a constriction in lamprey DA that was significantly stronger than the magnitude of the initial HV ($n=8$ fish), and tension was further enhanced by subsequent exposure to hypoxia. The magnitude of this HV in zero [Na$^{+}$]o was not significantly different from the previous control HV, and vessels relaxed to prehypoxic levels when aerated with room air. Removal of Na$^{+}$ in the absence of Ca$^{2+}$ did not produce a significant contraction; however, subsequent application of hypoxia produced a significantly stronger HV ($n=8$ fish) than a control HV in normal [Na$^{+}$]o, and vessel tension remained at 39.9 ± 2.2% of the HV when vessels were returned to normoxia (Fig. 2C). All vessels in Fig. 2, B and C, relaxed to baseline when [Na$^{+}$]o was restored.

Pretreatment of lamprey DA with the L-type Ca$^{2+}$ channel antagonist methoxyverapamil (D600; 10$^{-4}$ M) did not affect total tension during HV (Fig. 3), but addition of the L-type Ca$^{2+}$ channel agonist BAY K 8644 (1 µM) to the baths significantly enhanced HV to 122.6 ± 6.9% of control. Addition of the sarco/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) inhibitor cyclo-

Fig. 1. A: representative traces showing simultaneous measurement of fura 2 signal [cytosolic calcium concentration ([Ca$^{2+}]_{c}$); top trace] and force (bottom trace) during stimulation with 90 mM KCl or hypoxia (N2). B: correlation between active force (in g) and [Ca$^{2+}]_{c}$ in lamprey dorsal aortas (DA). The hypoxia tension (N2) and Ca$^{2+}$ responses are expressed relative to a maximal KCl (80 mM) response; $n$ = number of fish.

Fig. 2. Effects of extracellular calcium (Ca$^{2+}$) and extracellular sodium (Na$^{+}$) on hypoxic vasoconstriction (HV) in lamprey DA. A: HV in zero Ca$^{2+}$, is not different from control HV in normal Ca$^{2+}$. Restoration of Ca$^{2+}$ produces a slight but insignificant increase in tension. B: removal of Na$^{+}$ significantly increases tension compared with a control HV (N2). HV in the absence of Na$^{+}$ is unaffected. Return to normoxia (Air) relaxes vessel to prehypoxic level; however, full relaxation is not achieved until [Na$^{+}$]o is restored. C: in the absence of Ca$^{2+}$, removal of Na$^{+}$ produces a transient contraction that is weakly sustained. Under these conditions, HV is augmented, prehypoxic tension is not restored on return to air, and full relaxation is achieved only when [Na$^{+}$]o is restored.
HV was augmented an average of 20%, and these vessels also did not relax completely on return to normoxia (Fig. 3, inset).

The Ca\(^{2+}\) ionophore ionomycin (IO; 50 μM) and the ryanodine-sensitive receptor agonist ryanodine (RY; 50 μM) significantly reduced HV to 36.2 ± 6.7 and 60.3 ± 8.2% of their respective control responses in zero Ca\(^{2+}\) (Fig. 4). The SERCA pump inhibitor thapsigargin (TH; 5 μM) did not affect the magnitude of HV (Fig. 4). Addition of 5 mM caffeine in the absence of Ca\(^{2+}\) did not reduce HV in DA from two fish; however, caffeine added to vessels pretreated with TH (Fig. 4) significantly reduced HV to 27.4 ± 5.9% of control.

New Zealand hagfish. HV in hagfish was significantly reduced by 38.1 ± 4.8% of the previous control HV (n = 6 fish) in the absence of Ca\(^{2+}\). Subsequent restoration of [Ca\(^{2+}\)]\(_o\) to normal (5 mM) increased HV to 156.8 ± 15.6% of control (Fig. 6A).

The effect of replacing HHBS Na\(^+\) with an osmotically equivalent amount of sucrose (800 mM) is shown in Fig. 6B. Na\(^+\) removal produced a significant constriction that was further enhanced by hypoxic exposure (n = 4 fish). These vessels did not relax on return

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\text{Ca}^{2+}\text{ required for HV in lamprey DA was depleted more effectively by adrenergic stimulation than by repeated hypoxic exposure (Fig. 5). Vessels were repeatedly (8 times) exposed to 20-min bouts of hypoxia in Ca}^{2+}\text{-free baths before a significant reduction in HV was noted (Fig. 5A). Applying hypoxia to vessels stimulated with }10^{-5}\text{ M norepinephrine (NE) after 13 consecutive hypoxic exposures in the absence of }\text{Ca}^{2+}\text{ produced a hypoxic constriction as strong as the initial HV in normal }\text{Ca}^{2+}\text{, and a final hypoxic exposure following this NE stimulation produced a constriction as strong as the first HV in zero }\text{Ca}^{2+}\text{ (Fig. 5A). Repeated NE treatment (12 times) in zero }\text{Ca}^{2+}\text{ reduced HV to near baseline, yet hypoxic exposure during the 12th NE stimulation of vessels in zero }\text{Ca}^{2+}\text{ produced contractions as strong as the HV that followed the first NE treatment (Fig. 5B). HV was significantly reduced in unstimulated vessels when hypoxia followed a single NE treatment in Ca}^{2+}\text{, and HV following the final NE washout in vessels exposed to 11 NE treatments remained significantly lower than the first HV in zero }\text{Ca}^{2+}\text{ (Fig. 5B).}

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of Ca$^{2+}$ reduced by 44.9.

L-type Ca$^{2+}$ by treatment with ionomycin or RY, or by the addition
in lamprey DA was significantly reduced in zero Ca$^{2+}$ as 
assumed relaxation of hypoxia-stimulated vessels. However, these responses varied between the two cyclostomes. Results clearly show that, although the specifics of Ca$^{2+}$ use may be different in cyclostomes and mammals, an increase in [Ca$^{2+}$]c is a requisite for HV in cyclostomes as it is for HPV.

Ca$^{2+}$ dependence. The calmodulin-dependent crossbridge cycling between actin and myosin filaments that produces contraction in mammalian smooth muscle is mediated by an increase in [Ca$^{2+}$]c (1, 14), and an increase in [Ca$^{2+}$]c is, for the most part, temporally coupled with active tension during HPV (24). The present study shows for the first time that HV in lamprey is also a calcium-mediated process.

Contraction of lamprey DA was temporally coupled with [Ca$^{2+}$]c during stimulation with either 90 mM potassium chloride or hypoxia (Fig. 1). A similar rise in [Ca$^{2+}$]c accompanied HPV in rat pulmonary arteries, except that the rat response included a transient [Ca$^{2+}$]c-independent relaxation that separated the initial and sustained contractions (24). This difference between the lamprey and rat responses is likely due to additional factors modulating rat HPV that are not present in lamprey HV.

The attenuating effect of the Ca$^{2+}$ ionophore IO on the magnitude of HV in lamprey DA (Fig. 4) provides additional evidence that an increase in [Ca$^{2+}$]c was required for HV. Ionomycin caused a transient contraction in lamprey DA in zero Ca$^{2+}$ that was greater than a control HV. Presumably, this was due to release of Ca$^{2+}$ from the sarcoplasmic reticulum (SR), which has also been shown to occur in mammalian vascular smooth muscle (6). After the initial ionomycin spike, tension returned to near baseline within 10 min, suggesting that ionomycin also increased Ca$^{2+}$ efflux from the cell, partly by the action of IO as an ionophore and partly through IO activation of Na$^+$/Ca$^{2+}$ exchange (28).

Due to the limited availability of animals, the force-[Ca$^{2+}$]c relationship during HV in the hagfish was not examined in the present study. However, given the shared characteristics of lamprey and hagfish HV such as the strength, duration, endothelium independence, and reproducibility of HV (22), it seems reasonable to assume that an increase in [Ca$^{2+}$]c is likely involved during HV in hagfish DA as well.

Contributions of Ca$^{2+}$ and Ca$^{2+}$ during HV. Ca$^{2+}$ is required for HPV in most mammalian tissues (8, 9, 13, 16, 25, 34); however, the relative contributions of Ca$^{2+}$ and Ca$^{2+}$ vary among species. Rat (25) and fetal lamb pulmonary vascular smooth muscles (8) have been shown to be completely dependent on Ca$^{2+}$, and HPV was markedly decreased by removal of Ca$^{2+}$ in per-
fused ferret lungs (13). In isolated pulmonary arteries from the cat, Harder et al. (16) found that raising or lowering \([Ca^{2+}]_o\) produced concomitant changes in the magnitude of HPV, which could be abolished by \(Ca^{2+}\) blockade. \(Ca^{2+}\) release was shown to be an initial step in HPV of isolated rat pulmonary arteries (15) and of cultured pulmonary arterial smooth muscle cells (26). These studies also showed HPV to be ultimately dependent on \(Ca^{2+}\). Experimental conditions during the present lamprey and hagfish DA studies were nearly identical, excluding differences in saline composition. However, the \(Ca^{2+}\) and \(Ca^{2+}\) requirements between these species during HV were quite different.

Results of the current study show that \(Ca^{2+}\) is not requisite for HV in lamprey DA and that HV in these tissues is mediated primarily by \(Ca^{2+}\). The magnitude of HV in lamprey DA was not significantly affected by the removal (105% of control) or restoration (118% of control) of \(Ca^{2+}\) (Fig. 2A). These results are similar to those obtained by Jabr et al. (17), who concluded that HPV in small isolated canine pulmonary arteries is independent of \(L\)-type channels and perhaps via a age-dependent \(Ca^{2+}\) pathways of \(Ca^{2+}\) arteries, involves the voltage-independent capacitative \(Ca^{2+}\), which attenuates HPV (20). The second pathway, nels (34). \(L\)-type channels are sensitive to D600 blockade. \(Ca^{2+}\) or that CPA blocked the export of \(Ca^{2+}\) during HV or that CPA blocked the export of \(Ca^{2+}\) (Fig. 3). Treatment with the SERCA pump inhibitor CPA augmented HV in normal \([Ca^{2+}]_o\) (Fig. 3, inset), and this augmentation was not affected by the presence of D600 (Fig. 3). Additionally, CPA had no effect on HV in zero \(Ca^{2+}\) (Fig. 4). These results suggest that either the entry of \(Ca^{2+}\) independent of \(L\)-type channels was responsible, in part, for the augmented HV or that CPA blocked the export of \(Ca^{2+}\).

Hagfish DA used \(Ca^{2+}\) during HV (Fig. 6B). However, the inability of D600 to affect HV suggests that \(L\)-type \(Ca^{2+}\) channels are either not present or inactive and that an alternate pathway for \(Ca^{2+}\) influx is used in these fish. The full recovery of tension following restoration of \(Ca^{2+}\) even in the presence of D600 supports the latter conclusion. Two pathways of \(Ca^{2+}\) influx have been proposed to mediate the strength and duration of HPV. The first pathway was described in pulmonary vessels from adult sheep and involves voltage-dependent \(Ca^{2+}\) influx through \(L\)-type \(Ca^{2+}\) channels (34). \(L\)-type channels are sensitive to D600 blockade, which attenuates HPV (20). The second pathway, described by Robertson et al. (25) in rat pulmonary arteries, involves the voltage-independent capacitative \(Ca^{2+}\) entry (CCE) during HPV. A pathway that is independent of \(L\)-type channels and perhaps via a mechanism similar to CCE seems the most likely method for \(Ca^{2+}\) influx during HV in hagfish DA.

\(Ca^{2+}\) channels. Large-conductance \((L\)-type\) \(Ca^{2+}\) channels appeared to be present in lamprey DA, because HV was augmented by pretreatment with the \(L\)-type channel agonist BAY K 8644 (Fig. 3). However, \(L\)-type channels were either inactive or insignificant during HV in these vessels, because HV was independent of \(Ca^{2+}\) and was unaffected by D600 in the presence of 2 mM \(Ca^{2+}\) (Fig. 3). Treatment with the SERCA pump inhibitor CPA augmented HV in normal \([Ca^{2+}]_o\) (Fig. 3, inset), and this augmentation was not affected by the presence of D600 (Fig. 3). Additionally, CPA had no effect on HV in zero \(Ca^{2+}\) (Fig. 4). These results suggest that either the entry of \(Ca^{2+}\) independent of \(L\)-type channels was responsible, in part, for the augmented HV or that CPA blocked the export of \(Ca^{2+}\).

Removal of \(Ca^{2+}\) from hagfish DA reduced HV to only 62% of a control response. Thus it appears that HV in hagfish is more like HPV than is the lamprey HV in that it is at least partly dependent on the influx of \(Ca^{2+}\).

\(SR Ca^{2+}\) receptors. Two specific receptors mediate \(Ca^{2+}\) release from the SR in mammalian vascular smooth muscle; those sensitive to the plant alkaloid RY and those sensitive to inositol 1,4,5-trisphosphate (IP3). RY-sensitive receptors are opened by RY, \(Ca^{2+}\), or caffeine (19). IP3-sensitive receptors on the SR may be opened directly by \(Ca^{2+}\) (19). Both receptor types can occur in the same vascular smooth muscle (17). Calcium stores sensitive to IP3 and RY appear to be organized into spatially distinct compartments in pulmon-ary arteries but are conjoined in renal arteries, allowing differential, agonist-dependent release of calcium in the former but not in the latter (18). SR receptors in lamprey DA have not been previously characterized. Our experiments indicate that these vessels also possess more than one type and that they may be spatially or functionally distinct.

HV in lamprey DA appears to be at least partly dependent on an RY-sensitive receptor, because treatment with RY reduced the response by 40%. Neither of the SERCA pump inhibitors (TH and CPA) nor caffeine alone reduced HV in the absence of \(Ca^{2+}\). However, in the presence of TH, caffeine reduced HV by 80% (Fig. 4). This suggests that at least 20% of the RY-like receptors were activated by caffeine. Of the three RY receptor (RYR) isoforms that have been described, the RYR2 type is the most caffeine sensitive (19). Thus a RYR2-like receptor may be one of the caffeine-sensitive receptor types present in lamprey DA.

An IP3-sensitive receptor appears to be present in lamprey DA, based on the assumption that an NE contraction is mediated by IP3 in these vessels as it is in mammalian vascular smooth muscle (19). The apparent rise in \([Ca^{2+}]_i\), that accompanies the NE response and the fact that in zero \(Ca^{2+}\) the NE effect is rapidly diminished (Fig. 5B) support this conclusion. HV in lamprey DA may also be at least partly dependent on IP3-sensitive \(Ca^{2+}\) release because repeated NE treatments in zero \(Ca^{2+}\) effectively reduced HV (Fig. 5B).

\(Ca^{2+}\) cycling during HV. \(Ca^{2+}\) cycling during hypoxia in lamprey DA may involve the release and/or reuptake of \(Ca^{2+}\) by intracellular stores (hypoxia-dependent stores) that are spatially or functionally different from the \(Ca^{2+}\) stores used by other contractile agonists. \(Ca^{2+}\) appeared to be more efficiently recycled during HV than during adrenergic stimulation in these fish, because in zero \(Ca^{2+}\), repeated hypoxic exposures (8 times) were required compared with one NE treatment before a significant drop in HV was registered (Fig. 5, A and B). These results suggest that lamprey DA possess efficient mechanisms for uptake of \(Ca^{2+}\) into these hypoxia-dependent stores. Inhibition of \(Na^{+}\)/ \(Ca^{2+}\) exchange during hypoxia may enhance \(Ca^{2+}\) uptake into the hypoxia-dependent stores by reducing \(Ca^{2+}\) efflux.

Hypoxia does not affect \([Ca^{2+}]_i\), in mammalian pulmonary vascular smooth muscle unless a prior precon-ditioning stimulus is applied (3). This has been proposed to be due to increased \(Ca^{2+}\) sequestration during hypoxia, which effectively isolates \(Ca^{2+}\) from contract-
tile elements (28). Preconditioning with another agonist is thought to increase [Ca\(^{2+}\)]\(_i\), sufficiently to overcome sequestration, thus effecting a hypoxic response (30). No prestimulation of lamprey DA was required for HV. However, as we have described previously (22), HV was augmented in NE-pretreated lamprey vessels, implying that there is a conditioning effect in lamprey DA as well. In the present study, prestimulation with NE appeared to provide additional Ca\(^{2+}\) for HV in zero Ca\(^{2+}\), because HV was augmented in NE-treated vessels that appeared to be Ca\(^{2+}\) deplete, i.e., no longer responsive to NE or hypoxia (Fig. 5, A and B). This augmentation may be due to uptake of Ca\(^{2+}\) released from NE-activated stores into hypoxia-dependent stores. A similar Ca\(^{2+}\) transfer between distinct stores has been noted in cultured rat fetal aorta cells (29).

Na\(^{+}\)/Ca\(^{2+}\) exchange during HV. The present study suggests that lamprey DA possess an active Na\(^{+}\)/Ca\(^{2+}\) antiporter and that this exchange mechanism may be inhibited during HV. However, our results indicate that neither Ca\(^{2+}\) influx nor Na\(^{+}\)/Ca\(^{2+}\) exchange is important during HV or recovery from HV in these vessels.

The presence of a functional Na\(^{+}\)/Ca\(^{2+}\) antiporter in lamprey DA was suggested in the present study, because removal of Na\(^{+}\) in normal [Ca\(^{2+}\)]\(_o\) produced a significant contraction (Fig. 2B), whereas removal of Na\(^{+}\) in the absence of Ca\(^{2+}\) failed to increase tension (Fig. 2C). In mammalian vascular smooth muscle with a functional Na\(^{+}\)/Ca\(^{2+}\) antiporter, elimination of Na\(^{+}\) in the presence of normal [Ca\(^{2+}\)]\(_o\) removes the gradient for Na\(^{+}\) entry into the cell. Ca\(^{2+}\) extrusion from the cell is decreased, and the cell contracts (2). The lamprey results imply that an influx of Ca\(^{2+}\) mediates the contraction caused by Na\(^{+}\) removal and that, under resting conditions, tension remains stable through continual Na\(^{+}\)/Ca\(^{2+}\) antiporter activity. Removal of Ca\(^{2+}\) alone did not significantly lower tension in lamprey DA (Fig. 2C), which suggests that maintenance of basal tone in these vessels does not require Ca\(^{2+}\).

The Na\(^{+}\)/Ca\(^{2+}\) antiporter appears to have been inhibited in lamprey DA during hypoxia. This is indicated by the ability of lamprey DA to endure multiple hypoxic bouts in zero Ca\(^{2+}\) before HV is diminished (Fig. 5A) and may explain, in part, the apparent Ca\(^{2+}\) sparing during hypoxia compared with stimulation with NE in zero Ca\(^{2+}\) (Fig. 5, A and B). These results agree favorably with those obtained in mammals. Restoration of [Ca\(^{2+}\)]\(_i\) following the activation of mammalian vascular smooth muscle has been attributed, in part, to a Na\(^{+}\)/Ca\(^{2+}\) antiporter on the plasmaemmal membrane (36). Inhibition of this Na\(^{+}\)/Ca\(^{2+}\) exchange mechanism during hypoxia has been described in mammalian pulmonary arteries (31). Neither Ca\(^{2+}\) influx nor Na\(^{+}\)/Ca\(^{2+}\) exchange was important during HV in lamprey DA, because HV in the absence of Ca\(^{2+}\) or Na\(^{+}\) was not significantly different from a control HV (Fig. 2, A and B) and vessels relaxed to prehypoxia levels on return to normoxia. However, full relaxation was not achieved until [Na\(^{+}\)]\(_o\) was restored (Fig. 2B). These data imply that an increase in free [Ca\(^{2+}\)]\(_i\) resulted from inhibition of the Na\(^{+}\)/Ca\(^{2+}\) antiporter and that this Ca\(^{2+}\) was responsible for activating contractile elements distinct from those used during HV. Removal of Na\(^{+}\) in the absence of Ca\(^{2+}\) did not significantly increase tension, yet it increased HV and inhibited relaxation on return to normoxia (Fig. 2C). Therefore, Ca\(^{2+}\) released during HV under these conditions may also have reached contractile elements not normally activated during HV, perhaps due to the absence of both Ca\(^{2+}\) efflux and Ca\(^{2+}\) entry.

The presence of an Na\(^{+}\)/Ca\(^{2+}\) exchange mechanism was also indicated in hagfish DA in the present study. Removal of Na\(^{+}\) from [Ca\(^{2+}\)]\(_o\)-replete solutions constricted hagfish DA (Fig. 6B). Although lamprey and hagfish DA both constricted further to hypoxia, hagfish DA did not relax on return to normoxia until bath [Na\(^{+}\)]\(_o\) was restored. The failure of these vessels to relax shows that an Na\(^{+}\)/Ca\(^{2+}\) exchange mechanism is clearly a requisite for Ca\(^{2+}\) extrusion during HV in hagfish DA.

Perspectives

We have previously described HV in cyclostomes as the possible antecedent for HPV. The present study examined the utilization of Ca\(^{2+}\) during HV in two species of cyclostomes, and the results suggest that Ca\(^{2+}\) handling during HV in lamprey DA relies on many of the same intrinsic mechanisms that are used during HPV. Ca\(^{2+}\) handling during HV indicated by this study offers a mechanistic corollary for HPV in the simplest vertebrates and further supports our assumption that HPV has a long lineage in vascular smooth muscle.

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