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Effect of pH on trout blood vessels and gill vascular resistance

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Summary

pH is recognized as a modulator of vascular smooth muscle (VSM) tone in mammalian vessels, but little is known about its effects on fish VSM. We investigated the effects of extracellular and intracellular pH (pH_o and pH_i, respectively) on isolated vessels from steelhead and rainbow trout (*Oncorhynchus mykiss*, Skamania and Kamloops strains, respectively) and of pH_o on perfused gills from rainbow trout. In otherwise unstimulated (resting) efferent branchial (EBA) and coeliaco-mesenteric arteries (CMA), anterior cardinal veins (ACV) and perfused gills, increasing pH_o from 6.8 to 8.8–9.0 produced a dose-dependent contraction or increase in gill resistance (R_{GILL}) with an estimated half-maximal response of 8.0–8.2. pH_o interactions with other contractile stimuli were agonist specific; more force was developed at low pH_o in ligand-mediated (arginine vasotocin) contractions, whereas depolarization-mediated (40–80 mmol l^{-1} KCl) contractions were greatest at high pH_o. Increasing pH_i by application of 40 mmol l^{-1} NH_4Cl produced sustained contraction in afferent branchial arteries (ABA) suggesting that these vessels could not readily restore pH_i. NH_4Cl application only transiently contracted EBA and CMA in Hepes buffer, whereas it produced a slight, but prolonged, relaxation of EBA and CMA in Cortland buffer. The buffer effect was due to the presence of Hepes and in this environment EBA and CMA appeared to readily restore pH_i. Increasing pH_i in KCl-contracted EBA in Hepes produced an additional contraction, whereas ligand-contracted (thromboxane A_2 analog, U-46619) EBA relaxed. Reducing pH_i (NH_4Cl washout) transiently contracted resting EBA and CMA in both Hepes and Cortland buffer. NH_4Cl washout produced an additional, transient contraction of both KCl- and U-46619-contracted EBA in Hepes. EBA contractions produced by increased pH_i depend primarily on intracellular Ca^{2+}, whereas both intracellular and extracellular Ca^{2+} contributed to the response to decreased pH_i. Three cycles of perfusate acidification (pH_o 7.8 to 6.2 and back to 7.8) reproducibly halved, then restored R_{GILL} with no adverse effects, indicating that this was not a pathophysiological response. These studies show that the general effects of pH on VSM are phylogenetically conserved from fish to mammals but even within a species there are vessel-specific differences. Furthermore, as fish are exposed to substantial fluctuations in environmental (and therefore plasma) pH, the obligatory response of fish VSM to these changes may have substantial impact on cardiovascular homeostasis.

Key words: pH, fish, rainbow trout, *Oncorhynchus mykiss*, vessels, gills, vascular tone.

Introduction

Intracellular and extracellular pH (pH_i and pH_o, respectively) have considerable impact on tone and reactivity of mammalian vascular smooth muscle (VSM). In general, a decrease in either pH_i or pH_o relaxes blood vessels, whereas an increase in pH_i or pH_o contracts them (reviewed by Aalkjær and Poston, 1996; Aalkjær and Peng, 1997; Smith et al., 1998; Austin and Wray, 2000; Wray and Smith, 2004). Because it is somewhat difficult to manipulate pH_o and pH_i independently, apparent pH_o-dependent vascular responses may be due to concomitant changes in pH_i (Roos and Boron, 1981; Smith et al., 1998; Wray and Smith, 2004). However, pH_o may be more physiologically relevant when considering the effects of acid–base status on vascular resistance, because most (if not all) acid–base disturbances arise from non-vascular tissue.

pH-induced vasoactivity is most often correlated with changes in intracellular calcium ($[Ca^{2+}_i]$), which is then followed by a corresponding change in force (Aalkjær and Poston, 1996; Aalkjær and Peng, 1997; Smith et al., 1998; Austin and Wray, 2000; Wray and Smith, 2004). Much of this Ca^{2+} may be from an extracellular source as L-type Ca^{2+} channels have been shown to be closed by acidosis and opened by alkalosis. Other pH-sensitive targets that have been identified include potassium channels, intracellular Ca^{2+} stores

(sarcoplasmic reticulum; SR), and Ca^{2+}-ATPases involved in translocating Ca^{2+} into the SR (SERCA pumps). Upon these generalizations there is a continuum of variables that include differences between vessels, differences between resting or pre-contracted vessels, the nature of the stimulant used for precontraction, and the mechanism with which the change in pH is achieved.

These vagaries notwithstanding, acidotic vasodilation and alkalotic vasoconstriction are assumed to be physiologically significant. In systemic vessels, where blood flow is coupled to metabolism, it is easy to envision how acidosis attendant with hypoxia would contribute to the well-known hypoxic vasodilation. Conversely, hypoxia contracts pulmonary arteries (Madden et al., 1992), and although both acidosis and alkalosis have been reported to produce vasoconstriction (Krampetz and Rhoades, 1991), Madden et al. (Madden et al., 2001) clearly showed in canine pulmonary arteries that the effects of hypoxia on vessel tension and pH_i depend on the size of the vessel; hypoxia relaxes large vessels and decreases pH_i, whereas it contracts small arteries and increases pH_i. Thus, although the responses of systemic and large pulmonary arteries to hypoxia are different from those of small pulmonary arteries, all responses appear to be consistent with a corresponding change in pH_i. It should be noted, however, that a mechanistic link between hypoxia and pH_i has not been clearly demonstrated (Taggart and Wray, 1998) and it is unclear if this connection is coincidental.

Acid–base status in mammals is largely regulated from within by the interplay between metabolism, ventilation and renal function. Other than diet, there is little, if any, environmental load of either acid or base equivalents. Conversely, acid–base status in fish is strongly linked to the environment. Ambient water is a large sink for respiratory CO_2. This keeps blood P_{CO_2} low (~1–2 mmHg) (Janssen and Randall, 1975), but increases sensitivity of blood pH to ambient P_{CO_2}. Many aquatic environments experience rapid and large variations in ambient P_{O_2}, P_{CO_2} and pH, singularly and in various combinations, and these have a substantial impact on blood pH (Dejours, 1972; Janssen and Randall, 1975; Thomas and Le Ruz, 1982). Although the effect of acid–base disturbances on the fish heart has been examined in some detail (Farrell et al., 1983), there is scant information regarding the effects of pH on fish blood vessels (Canty and Farrell, 1985).

The purpose of the present experiments was to examine the effect of pH_o and pH_i on fish VSM in vitro. Afferent and efferent branchial (ABA and EBA) and celiaco-mesenteric (CMA) arteries, ventral aorta (VA) and anterior cardinal veins (ACV) were mounted in myograph chambers and the vasoactive effects of manipulating pH_o, and in some instances (EBA) pH_i, were examined in unstimulated vessels and in vessels pre-contracted with ligand- and voltage-mediated agonists. We also examined the effects of pH_o on vascular resistance of the isolated perfused gill whose sensitivity to hypoxia (Smith et al., 2001), multifunctionality (Olson, 2002) and close apposition to the environment make it especially relevant.

Materials and methods

Experimental animals

Steelhead trout (*Oncorhynchus mykiss* Walbaum, Skamania strain; 3–7 kg) were obtained from the Indiana Department of Natural Resources. Studies on steelhead vessels were conducted from January to March. Rainbow trout (*Onchorhynchus mykiss* Walbaum, Kamloops strain; 0.5–1 kg) were obtained from a commercial hatchery (Homestead Trout Farm, Grand Haven MI, USA). Rainbow trout were housed in 2000 liter fiberglass tanks with circulating well water (12–15°C), maintained on a 12 h:12 h, L:D schedule, and fed Purina trout chow. Studies on rainbow trout vessels and gills were conducted throughout the year.

Isolated vessels

Steelhead were anesthetized in (25 mg l^{-1}) tricaine methanesulfonate (MS-222) and efferent branchial arteries (EBA) from the third and fourth gill arches, afferent branchial arteries (ABA) from the first arch, ventral aorta (VA), anterior cardinal vein (ACV), and coeliaco-mesenteric arteries (CMA) were removed, gently cleaned of extra-adventitial tissue, and placed in 4°C Hepes-buffered saline. Vessels were cut transaxially into 2- to 3-mm-long segments, individually mounted on 280 μm diameter stainless steel hooks and suspended in 5 or 20 ml water-jacketed (12–14°C) smooth muscle chambers. The baths were aerated with room air (Hepes buffer) or 21% O_2 1% CO_2 78% N_2 (Cortland buffer). Tension was measured by a Grass FT-03 force-displacement transducer and recorded on a Grass model 7 polygraph calibrated to detect tension changes as low as 5 mg. Analog data was digitally converted by computer interface and written directly to disk by Labtech Notebook (Laboratory Technologies, Andover, MA, USA) and displayed with Sigmaplot software (Jandel Scientific, San Rafael, CA, USA). Resting tension (equilibrium tension) of 500–1000 mg was applied to all arteries and 300 mg was applied to the ACV. The rings were then allowed to stabilize for at least 30 min, contracted with 80 mmol l^{-1} KCl, washed with buffer, and allowed to equilibrate an additional 30 min before experimentation. Resting tension was continuously adjusted to the desired level during this period. The response of EBAs to contractile agonists and pH was generally more pronounced than those of other vessels and these vessels were selected for additional study.

pH_o was adjusted by replacing the entire bath with pre-titrated 14°C buffers. The ammonium-pulse technique was used to change pH_i. With this technique, 40 mmol l^{-1} NH_4Cl is added to the bath and the dissociate NH_3 rapidly diffuses into the cell and produces a transient intracellular alkalosis as the NH_3 buffers intracellular H^+ (Roos and Boron, 1981). After the cells have presumably re-established pH_i (~30 min), the bath is replaced with ammonium-free buffer creating an outward diffusion of NH_3 and a transient intracellular acidosis. Other agonists/antagonists present during the pH_o perturbation were added to the new buffers prior to addition to the cells.

The L-type calcium channel blocker methoxyverapamil (D600, 0.1 mmol^{-1}) was added 30 min prior to experimentation.

Perfused gills

Rainbow trout were killed by a blow to the head and vessels removed as above, or the gill arches isolated and cannulated as described elsewhere (Olson et al., 1986). Briefly, after the fish was stunned, the heart was exposed through a midventral incision and 1 ml of heparinized (15 mg ml^{-1}) saline (0.9 g% NaCl) was injected into the ventricle and allowed to circulate for ~2 min. The head was then severed and the second pair of gill arches were isolated. The afferent branchial artery was cannulated with an 18-gauge beveled (45°) needle connected to polyethylene tubing (PE-90). A 'T' was inserted 8–10 cm proximal to the cannula through which perfusion pressure could be monitored *via* a Gould-Statham (Detroit, MI, USA) pressure transducer and Grass polygraph (Astromed, Providence, RI, USA). The gill was suspended in aerated tapwater (<1 mOsm) and continuously perfused with filtered (0.2 μm) phosphate-buffered saline (PBS) at 12°C *via* a peristaltic pump. Pump speed was adjusted (nominally 0.6 ml min^{-1}) to produce stable input pressures of ~40 mmHg (1 mmHg=133 Pa). This was estimated to be equivalent to ~35 mmHg at the gill arch. A four-way stopcock on the aspiration end of the pump was used to switch between perfusates without interrupting flow. All treatments were perfused for a sufficient time (10 min or more) to achieve steady-state input pressures.

Chemicals

The composition of Hepes (*N*-2-hydroxyethyliperazine-*N*′-2-ethanesulfonic acid) buffer was (in mmol l^{-1}): NaCl, 145; KCl, 3; $MgSO_4 \cdot 7H_2O$, 0.57; $CaCl_2 \cdot 2H_2O$, 2; Hepes acid, 3; Hepes Na^+ salt, 7; glucose, 5. The composition of Cortland saline was (in mmol l^{-1}): NaCl, 124; KCl, 3; $CaCl_2 \cdot 2H_2O$, 2; $MgSO_4 \cdot 7H_2O$, 1.1; NaH_2PO_4, 0.09; Na_2HPO_4, 1.8; $NaHCO_3$; glucose, 5.5. The composition of PBS was as follows (in mmol l^{-1}): NaCl, 126; KCl, 4.16; $CaCl_2 \cdot 2H_2O$, 0.68; $MgSO_4 \cdot 7H_2O$, 0.57; KH_2PO_4, 3.38; Na_2HPO_4, 14.23; glucose, 5. Buffers were set to pH 7.8 (control) or adjusted to the desired pH with 1 mol l^{-1} NaOH or 1 mol l^{-1} HCl. Changes in bath or perfusate pH were made by complete exchange of appropriate buffer at constant temperature. If pH was changed during an agonist contraction, the agonist was added to the new buffer prior to exchange.

Choice of contractile agonist and dose (50–80% of maximal contraction; EC_{50} to EC_{80}) was based on previous experience with these vessels. Stock solutions were prepared as follows: arginine vasotocin (AVT; 1 μmol l^{-1}), acetylcholine (ACh; 10 mmol l^{-1}), the thromboxane A_2 mimetics U-44069 or U-46619; 10 mmol l^{-1}) and epinephrine (EPI; 10 mmol l^{-1}). Propanolol (final concentration 0.1 mmol l^{-1}) was added to the baths 15 min prior to EPI to block β-receptor-mediated relaxation in CMAs (Olson and Meisheri, 1989). All compounds except U-46619 were purchased from Sigma (Chemical Co., St Louis, MO, USA) and dissolved in distilled H_2O. U-44069 was a generous gift from Dr K. Meisheri of the Upjohn Company (Kalamazoo MI, USA). Both U-44069 and U-46619 were dissolved in 95% ethanol. Ethanol was not vasoactive at the concentrations used in these studies.

Data analysis

Values are expressed as mean ± s.e.m., unless indicated otherwise. Vessel tension is presented in mg. Gill resistance (R_{GILL}) was calculated from input pressure (in mmHg) divided by pump flow (ml min^{-1}) and normalized for gill wet mass after blotting. Venous pressure was assumed to be zero because the efferent branchial artery was not cannulated.

Comparisons were made by Students' *t*-test or paired *t*-test where appropriate. One-way ANOVA followed with Student-Newman-Keul's test was used for multiple comparisons of means. Significance was assumed when $P<0.05$.

Results

Effects of pH_o on isolated vessels

Increasing pH_o above 7.8 contracted and decreasing it below 7.8 relaxed otherwise un-stimulated EBA and ACV (Fig. 1). This sigmoidal, dose-dependent response was quantitatively similar in both vessels with an EC_{50} around pH_o 8.0. Similar responses, albeit somewhat reduced in magnitude, were observed in CMA and VA (not shown).

The effect of initial pH_o on an arginine vasotocin (AVT), potassium chloride (KCl) or potassium acetate (KAc) contraction of EBA is shown in Fig. 2. AVT contractions were significantly stronger at the lowest pH_o examined (7.4), whereas KCl and KAc contractions were significantly stronger at the highest pH_o (8.2 and 8.6). In contrast, U-44069 or KCl contractions in ACV were not significantly affected by initial

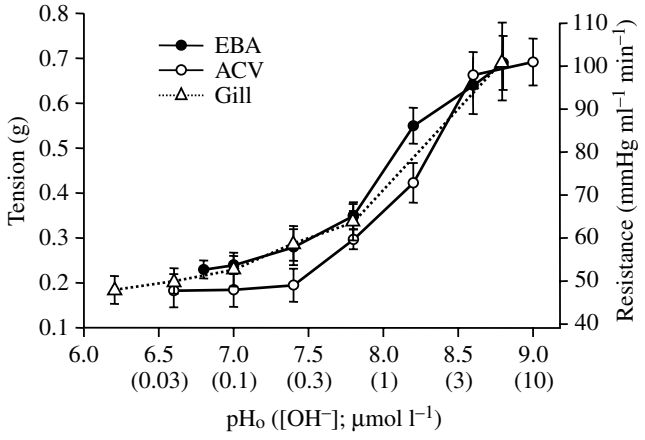

Fig. 1. Effects of extracellular pH (pH_o) on tension of unstimulated efferent branchial arteries (EBA; *N*=11) and anterior cardinal veins (ACV; *N*=11) in Hepes buffer, and on vascular resistance of rainbow trout gills (11 gills from four trout) perfused with phosphate-buffered saline. Values are means ± s.e.m. EBA and ACV values are significantly different from their respective control (pH 7.8) except at pH 7.4; all vascular resistances except at pH 7.4 and 7.0 are significantly different from that at pH 7.8.

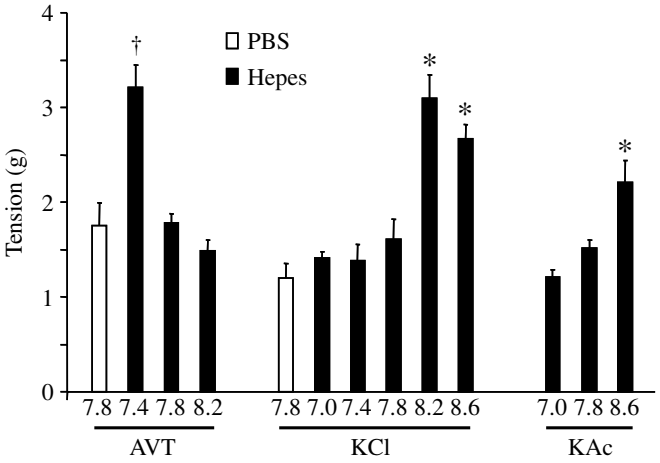

Fig. 2. Effects of extracellular pH on efferent branchial artery contractions produced by arginine vasotocin (AVT; 1 nmol l^{-1}), potassium chloride (KCl; 50 mmol l^{-1}) or potassium acetate (KAc; 50 mmol l^{-1}) in phosphate (PBS) or Hepes buffer. Values are means ± s.e.m. (*N*=4). *Different from same agonist (KCl or KAc) at all pH values lower than 8.2 or 8.6; †different from AVT at higher pH.

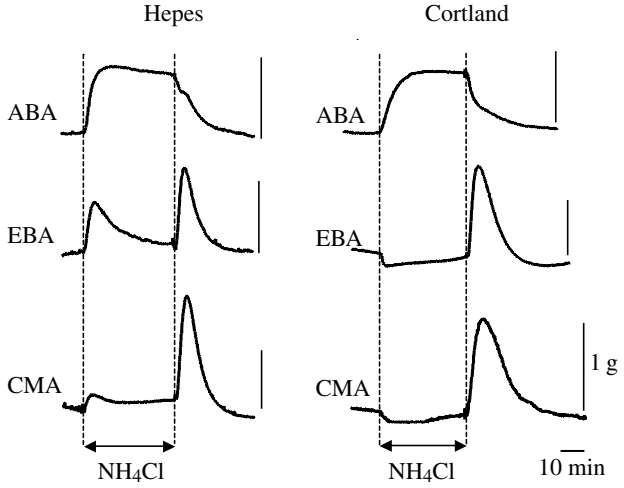

Fig. 4. Representative traces showing the effects of changing intracellular pH on resting tension of afferent (ABA) and efferent (EBA) branchial and celiacomesenteric (CMA) arteries in Hepes and Cortland buffer. Application of 40 mmol l^{-1} NH_4Cl produces intracellular alkalosis and its removal produces acidosis. Vertical scale bar = 1 g tension in all traces.

pH_o between 7.4 or 8.2 (not shown). Lowering pH_o during an AVT or KCl contraction reduced tension (Fig. 3A,B). However, tension did not significantly increase in pre-contracted vessels when pH_o increased (Fig. 3A), even though noticeable contractions were evident in a number of individual vessels (Fig. 3B).

Effects of pH_i on isolated vessels

Application of NH_4Cl increases pH_i in cells and its removal from the extracellular compartment decreases pH_i (Roos and Boron, 1981). The increased pH_i following addition of NH_4Cl, contracted ABA in either Hepes or Cortland buffer and tension was sustained until pH_i was lowered by removing NH_4Cl (Fig. 4). 41 out of 48 EBA and all (eight) CMA in Hepes buffer transiently contracted when pH_i was increased and then returned toward near baseline within 10 min (Fig. 4). The ensuing decrease in pH_i during NH_4Cl washout produced an even stronger, but also transient, contraction. The response of the other seven EBA in Hepes was similar to the ABA and these vessels were not examined further. Increasing pH_i in Cortland buffer produced a slight, but prolonged relaxation of EBA and CMA, whereas the fall in pH_i during NH_4Cl washout again produced a strong, transient, contraction similar to that in Hepes buffer (Fig. 4). EBA responses to increased or decreased pH_i in Hepes buffer were not affected by addition of 10 mmol l^{-1} $NaHCO_3$ (Fig. 5), 1 mmol l^{-1} NaH_2PO_4 (*N*=8; not shown), or 10 mmol l^{-1} $NaHCO_3$ plus 1 mmol l^{-1} NaH_2PO_4 (*N*=8; not shown). In Cortland buffer, both the alkalotic relaxation and enhanced acidotic contraction were significantly different from the respective responses in Hepes and in Hepes buffer with bicarbonate (Fig. 5), Hepes buffer with phosphate, and Hepes buffer with bicarbonate plus phosphate.

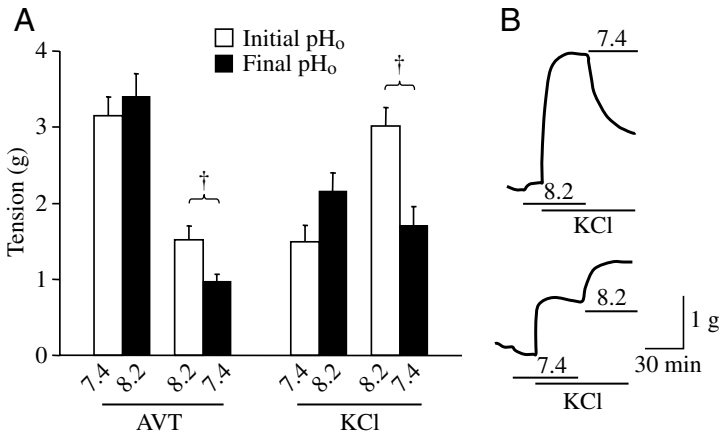

Fig. 3. Effects of changing extracellular pH on tension produced during continuous exposure of efferent branchial arteries to arginine vasotocin (AVT; 1 nmol l^{-1}) or KCl (50 mmol l^{-1}) in Hepes buffer. (A) Raising pH from 7.4 to 8.2 did not significantly affect contraction, whereas AVT and KCl contractions were significantly (†) decreased when pH was lowered from 8.2 to 7.4. (B) Representative tracings of the KCl responses. Values are means ± s.e.m. (*N*=4).

The effect of increasing pH_i on pre-contracted EBA in Hepes buffer was dependent upon the nature of the pre-contraction stimulus; KCl-contracted vessels contracted further following NH_4Cl application, while vessels contracted with the thromboxane A_2 agonist, U-46619, relaxed (Figs 6–8). Both responses lasted 20 min or longer (Fig. 6). NH_4Cl washout transiently contracted both KCl and U-46619 pre-contracted vessels (Figs 6–8). Responses of pre-contracted vessels in Cortland buffer were qualitatively similar to those in Hepes although the acidotic contraction of KCl-contracted vessels in Cortland buffer was significantly weaker than the corresponding

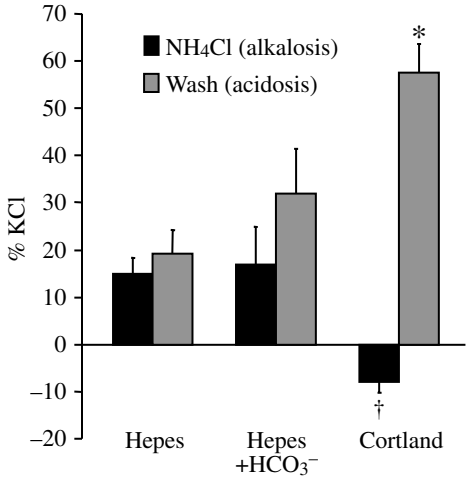

Fig. 5. Effects of buffer composition on the response of efferent branchial arteries to addition of 40 mmol l^{-1} NH_4Cl (intracellular alkalosis) and NH_4Cl washout (Wash; intracellular acidosis) 30 min later. Addition of 10 mmol l^{-1} $NaHCO_3$ to Hepes (Hepes +HCO_3^-) did not affect the contraction accompanying NH_4Cl addition or washout, whereas in Cortland buffer, addition of NH_4Cl produced a significant relaxation (†) and washout produced a contraction that was significantly greater than the corresponding contraction in either Hepes or Hepes + HCO_3^- (*). Values are means ± s.e.m.; Hepes (*N*=14), Hepes+HCO^-_3 (*N*=4), Cortland (*N*=8).

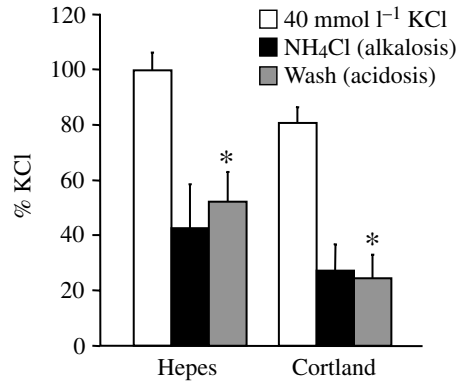

Fig. 7. Effects of buffer composition on the response of KCl (40 mmol l^{-1}) pre-contracted efferent branchial arteries to addition of 40 mmol l^{-1} NH_4Cl and NH_4Cl washout (Wash) 30 min later. Buffer composition did not affect the KCl contraction nor the contraction accompanying NH_4Cl addition. *The contraction accompanying NH_4Cl washout was significantly weaker in Cortland than in Hepes buffer. Values are means ± s.e.m.; Hepes (*N*=4), Cortland (*N*=4).

contraction in Hepes buffer (Fig. 7) and the alkalotic relaxation in U-46619-contracted vessels in Cortland buffer was significantly greater than the corresponding relaxation in Hepes buffer (Fig. 8).

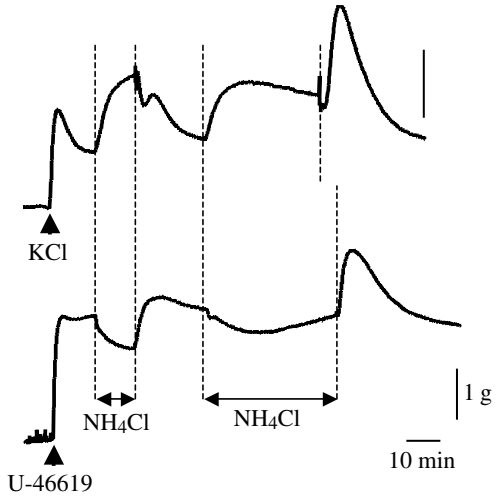

Fig. 6. Representative traces showing the effects of intracellular alkalosis (addition of 40 mmol l^{-1} NH_4Cl) and acidosis (removal of NH_4Cl) on tension of KCl (50 mmol l^{-1})- and U-46619-contracted efferent branchial arteries in Hepes buffer. Two NH_4Cl exposures, 10 and 30+ min, are shown. Intracellular alkalosis contracts, and acidosis relaxes, KCl-stimulated vessels; these responses are reversed when the vessels are pre-contracted with U-46619. Vertical scale bars = 1 g.

Relationship between pH_i and $[Ca^{2+}]_o$ in isolated vessels

The contribution of extracellular calcium ($[Ca^{2+}]_o$) to contractions accompanying intracellular alkalosis and acidosis in otherwise un-stimulated EBA in Hepes buffer is shown in Fig. 9. The magnitude of contraction during the initial increase in pH_i following NH_4Cl application was unaffected by either D600 or zero extracellular calcium ($0[Ca^{2+}]_o$). The second contraction, associated with the decrease in pH_i, was significantly lower in $0[Ca^{2+}]_o$ and appeared to be reduced by D600, although this was not statistically significant.

KCl contractions were reduced by 70% in D600 and abolished in $0[Ca^{2+}]_o$ (Fig. 10). The initial contraction

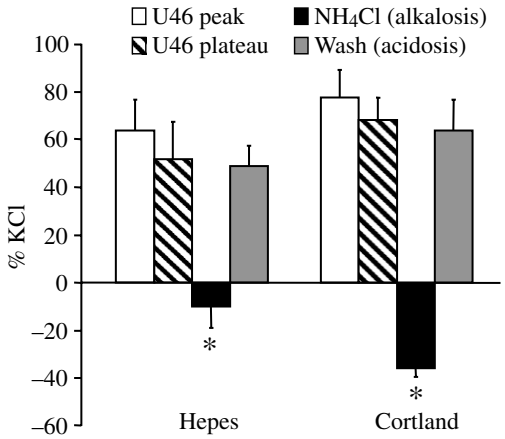

Fig. 8. Effects of buffer composition on the response of U-46619 (1 μmol l^{-1}) pre-contracted efferent branchial arteries to addition of 40 mmol l^{-1} NH_4Cl and NH_4Cl washout (Wash) 30 min later. Only alkalosis was affected by buffer composition. Values are means ± s.e.m.; Hepes (*N*=6), Cortland (*N*=4). *Significantly different from each other.

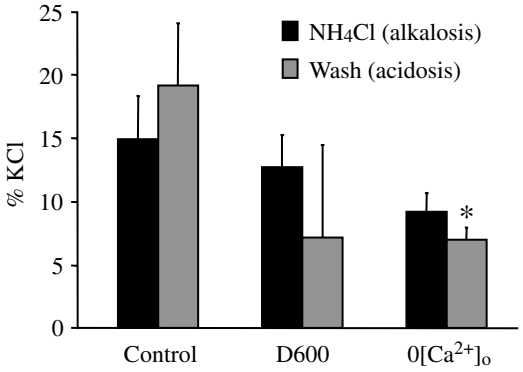

Fig. 9. Effect of L-type calcium channel inhibition (D600= methoxyverapamil; 0.1 mmol l^{-1}) and zero extracellular calcium ($0[Ca^{2+}]_o$) on response of efferent branchial arteries to intracellular alkalosis (NH_4Cl addition) and acidosis (NH_4Cl washout) in Hepes buffer. Values expressed as percentage of an 80 mmol l^{-1} KCl contraction. *Significantly different from control. Values are means ± s.e.m.; Control (*N*=14), D600 (*N*=10), $0[Ca^{2+}]_o$ (*N*=8).

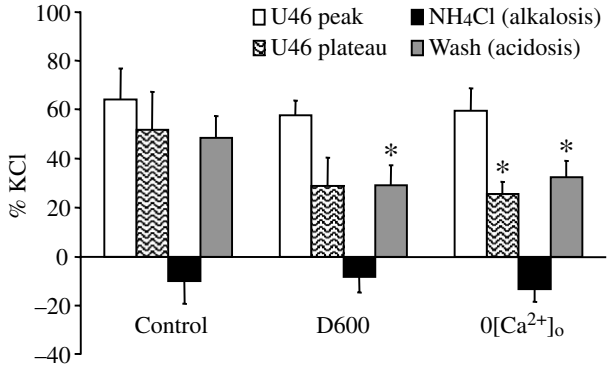

Fig. 11. Role of L-type calcium channels and extracellular calcium on U-46619 contractions (U-46 peak and U-46 plateau) and on intracellular alkalosis and acidosis (NH_4Cl and wash, respectively) in U-46619 pre-contracted efferent branchial arteries in Hepes buffer. *Significantly different from respective control. All values (means ± s.e.m.) expressed as a percentage of an 80 mmol l^{-1} KCl contraction. Control (*N*=6), D600 (*N*=4), $0[Ca^{2+}]_o$ (*N*=4).

accompanying NH_4Cl application to KCl-contracted EBA was not affected by either D600 or $0[Ca^{2+}]_o$ whereas the contraction following washout of NH_4Cl appeared to be slightly inhibited by D600 and was reversed to a slight relaxation in $0[Ca^{2+}]_o$ (Fig. 10).

In many EBA, U-46619 produced an initial peak contraction that was followed by a sustained plateau at lower tension. This peak contraction was not affected by either D600 or $0[Ca^{2+}]_o$, whereas the plateau was partially inhibited in $0[Ca^{2+}]_o$ and appeared to be inhibited by D600, although this was not significant (Fig. 11). The relaxation accompanying NH_4Cl addition to U-46619-contracted vessels was unaffected by D600 or $0[Ca^{2+}]_o$, whereas the contraction accompanying NH_4Cl washout was partially inhibited by both treatments (Fig. 11).

Effects of pH_o on vascular resistance of the perfused gill

Vascular resistance of the perfused gill significantly increased when perfusate pH was increased from 7.8 to 8.8 and decreased when perfusate pH was lowered from 7.8 to 6.6 or below (Fig. 1). Even the lowest pH was well tolerated by the gill as the acidotic vasodilation and ensuing recovery was reproducible when perfusate pH was cycled between 7.8 and 6.2 (Fig. 12).

Discussion

The present experiments show that isometric tension in trout blood vessels is sensitive to both pH_o and pH_i and that these effects can be produced in both pre-contracted and otherwise-unstimulated vessels. The responses of pre-contracted EBA to altered pH_o and pH_i depend on the type of pre-stimulus with

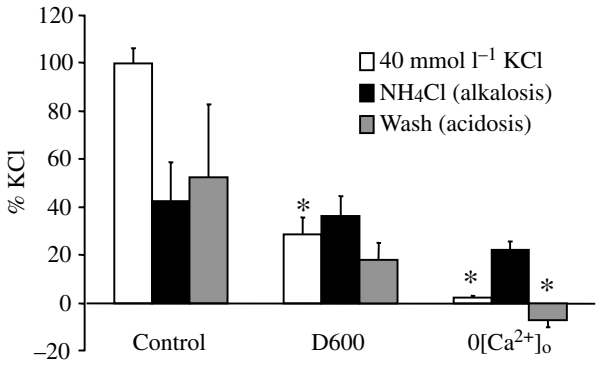

Fig. 10. Role of L-type calcium channels and extracellular calcium on 40 mmol l^{-1} KCl contractions and on intracellular alkalosis and acidosis (NH_4Cl and wash, respectively) in KCl pre-contracted efferent branchial arteries in Hepes buffer. *Significantly different from respective control. All values (means ± s.e.m.) expressed as a percentage of an 80 mmol l^{-1} KCl contraction.

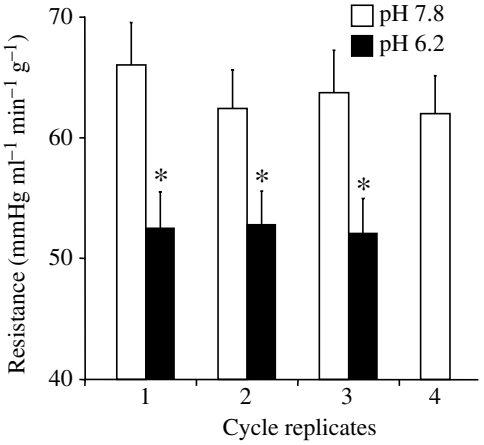

Fig. 12. Effectiveness and reproducibility of acidity-induced reductions in gill resistance in isolated perfused gills of rainbow trout. Values are means ± s.e.m.; *N*=9 gills from five trout. *Significantly different from initial resistance at pH 7.8.

notable differences between ligand-mediated (AVT or U-46619) and voltage-mediated (KCl) agonists. These differences may be related to agonist-specific mechanisms of calcium mobilization. Vascular responses to transient changes in pH_i produced by the ammonium pulse technique could not always be predicted from the vascular responses to tonic changes in pH_o and even differences between vessels were observed, suggesting a variety of transcellular mechanisms for regulating and/or responding to pH_i. The long-term and reproducible effects of pH_o on vascular resistance of perfused gills were consistent with the effects of pH_o on isolated vessels, suggesting that blood pH may be a tonic regulator, or modulator, of vascular resistance. This may be problematic in fish where environmental pH can directly impact upon blood pH. Finally, these studies question whether blood vessels are actually responding to changes in pH [H^+], as is the general perception in the literature, or if pOH ([OH^-]), acting through its anionic properties, is actually driving the vascular responses.

pH_o and pH_i

Essentially all mammalian vessels dilate when pH_o falls, and constrict when pH_o increases (Aalkjær and Poston, 1996; Aalkjær and Peng, 1997; Smith et al., 1998; Austin and Wray, 2000; Wray and Smith, 2004). Much, but not all, of this response in both systemic and pulmonary vessels appears to be due to concomitant and parallel changes in pH_i when pH_o is manipulated (Madden et al., 2001; Wray and Smith, 2004). As these responses are also observed in trout arteries, veins, perfused gills (Fig. 1), and the perfused trunk of the ocean pout (Canty and Farrell, 1985) it is likely that they are indicative of fundamental properties of vertebrate vascular smooth muscle. The ammonium pulse technique, which increases pH_i when ammonium is applied due to rapid non-ionic diffusive entry of NH_3 and subsequent absorption of intracellular H^+, and which lowers pH_i when the process is reversed, has been used extensively in mammalian vessels to alter pH_i independent of pH_o (Roos and Boron, 1981). In our experiments, the mechanical response of trout vessels to alterations in pH_i did not always correlate with those produced by a change in pH_o.

Afferent branchial arteries (ABA) contract when ammonium is added to the bath and they remain contracted for the duration of the ammonium exposure, independent of the incubation buffer (Fig. 4). This implies that intracellular alkalosis produces contraction and it suggests that these vessels are unable to restore pH_i, with or without extracellular bicarbonate. ABA are pre-gill vessels and their unique response to pH may be related to their association with systemic venous blood.

Efferent branchial (EBA) and celiacomesenteric (CMA) arteries are post-gill, systemic, vessels and their response to ammonium was similar, but unlike that of the ABA. Both EBA and CMA in Hepes buffer contract after ammonium addition (Fig. 4), but the contraction wanes within 10–20 min. This suggests that these vessels are able to restore pH_i. Conversely, in Cortland buffer, both EBA and CMA slightly relax after ammonium addition and recovery is slow (Fig. 4). Hepes buffers have been shown to inhibit contraction in mammalian vessels (see below), however, this is clearly different from our findings in that trout vessels contract in the presence of Hepes, Hepes plus bicarbonate, Hepes plus phosphate, and Hepes plus bicarbonate and phosphate, yet they relax in Cortland. This suggests that the effect is due to Hepes, but the mechanism is different from that in mammalian vessels.

A number of studies have shown that Hepes buffers inhibit mammalian vascular smooth muscle (Altura et al., 1980a; Altura et al., 1980b; Kane et al., 1997) whereas others show little effect (Sigurdsson, 1983; Douglas et al., 1993). In many of the earlier studies, Hepes buffer was substituted for bicarbonate-based buffers and the authors did not examine whether the effect was in fact due to the presence of Hepes or the absence of bicarbonate. In studies where Hepes solutions contained bicarbonate, the inhibition has been variously attributed to Hepes [i.e. canine basilar arteries (Kane et al., 1997)], or bicarbonate [i.e. rat aortas (Lamb and Barna, 1998)]. How Hepes acts is unclear. In canine basilar arteries, Hepes inhibits KCl, serotonin and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) contractions by an unknown mechanism that does not include chloride channels, generation of H_2O_2, or release of vasodilators from the endothelium (Kane et al., 1997). Regardless, the inhibitory effects of Hepes or bicarbonate are not observed in trout EBA as these vessels contract during ammonium alkalinization in the presence of Hepes, with or without bicarbonate, but relax in a bicarbonate buffer. Thus in trout EBA, Hepes appears to promote alkaline-initiated contraction and its absence prevents it. The nature of the vascular response becomes even more problematic in pre-stimulated EBA where the type of pre-stimulation (voltage or ligand) determines whether ammonium alkalinization contracts or relaxes vessels in Hepes buffer. Furthermore, Hepes effects appear to be vessel specific as they were observed in EBA and CMA, but not in ABA. Yamamoto and Suzuki proposed (Yamamoto and Suzuki, 1987) that Hepes enters *Drosophila* neurons and blocks Cl^- channels on the cytoplasmic side of the membrane. If Hepes affects transmembrane Cl^- flux in EBA it would either have to decrease Cl^- entry or increase Cl^- efflux, neither of which have been demonstrated.

Calcium signaling and alkalosis

Whereas both extracellular and intracellular alkalosis contract EBA, the magnitude of the response in pre-contracted vessels varies with the pre-stimulus, i.e. alkalosis enhances voltage-mediated (KCl) contractions and decreases ligand-mediated (AVT or U-46619) contractions. As described below, the interaction between alkalosis and voltage-mediated (KCl) contractions appears relatively straightforward, how alkalosis affects ligand-mediated contractions is less obvious.

EBA responses to alkalosis and KCl appear to be independent and additive. The contraction produced by an increase in pH_i in otherwise un-stimulated EBA in Hepes buffer does not utilize extracellular calcium $[Ca^{2+}]_o$: it is not significantly affected by the L-type calcium channel inhibitor, D600, or by removal of $[Ca^{2+}]_o$ (Fig. 9). This is different from the responses of mammalian vessels where L-type channels are generally involved in alkalotic contractions (Wray and Smith,

2004). Alkalotic contraction of trout must therefore depend either on release of intracellular calcium (Ca^{2+}_i) or Ca^{2+} sensitization of the contractile apparatus; this was not addressed in our experiments. Lack of involvement of Ca^{2+}_o in an alkaline contraction of EBA is even more evident in KCl-contracted vessels where the KCl response was greatly decreased by D600 and completely inhibited in zero $[Ca^{2+}]_o$, whereas the response to ammonium application was not significantly affected by either treatment (Fig. 10). As is evident from our study (Fig. 10), and others on mammalian vessels (Nobe and Paul, 2001), KCl-mediated contraction of vascular smooth muscle is essentially due to influx of Ca^{2+}_o. Our study shows that this is probably the result of a potassium-mediated cellular depolarization, because identical results were observed when acetate was substituted for chloride (Fig. 2). Thus the total contraction in KCl-alkalinized (either by increased pH_i or pH_o) vessels probably represents the sum of two independent events; (1) KCl-mediated depolarization and resultant influx of Ca^{2+}_o, and (2) alkalinity-mediated release of Ca^{2+} from an intracellular store. This also explains why increasing either pH_i or pH_o augments KCl contractions.

It is not clear how alkalosis relaxes ligand (AVT and U-46619)-contracted vessels (Figs 2, 3, 6). Ligand-mediated contraction of mammalian vessels is usually a two-step process, involving an initial increase in intracellular Ca^{2+} (Ca^{2+}_i) firstly due to Ca^{2+} release from intracellular stores, and secondly due to entry of Ca^{2+}_o. Entry of Ca^{2+}_o is brought about by the initial increase in $[Ca^{2+}_i]$ which opens calcium-activated chloride channels (ClC) thereby increasing Cl^- efflux. This depolarizes the cell membrane and opens the L-type (voltage-gated) calcium channels (Lamb and Barna, 1998). U-46619 appears to contract EBAs through a similar two-step process in that removal of Ca^{2+}_o does not affect the initial contraction but reduces the plateau (Fig. 11). However, an alkalosis-mediated inhibition of either ClC or L-type Ca^{2+} channels does not appear to be involved in the alkalotic relaxation of U-46619-contracted vessels because the relaxation is independent of Ca^{2+}_o (Fig. 11). Thus the increase in pH_i either decreases total Ca^{2+} release from intracellular stores, or desensitizes the contractile proteins to Ca^{2+}.

Calcium signaling and acidosis

All trout vessels relax when pH_o is decreased (Fig. 1), whereas, with the exception of ABA, they transiently contract when pH_i is increased, irrespective of pre-stimulation, the nature of the pre-stimulus or the presence or absence of bicarbonate in the buffer (Figs 4–8). It is generally accepted that the relaxation accompanying extracellular acidosis in mammalian vessels is due to decreased $[Ca^{2+}]_i$ (Austin and Wray, 2000). A number of mechanisms have been shown to contribute to this effect in mammalian vessels, including inhibition of L-type channels, inhibition of receptor-operated channels, opening of ATP-dependent (K_{ATP}), voltage-dependent (K_V) and Ca^{2+}-activated (K_{Ca}) potassium channels, inhibition of passive and capacitative Ca^{2+} entry, and possibly systems that remove Ca^{2+}_i or affect myofilament Ca^{2+} sensitivity (Austin and Wray, 2000). These mechanisms may be operative in trout as well, although trout do not appear to have K_{ATP} channels (Smith and Olson, unpublished observation).

Brief, but substantial contractions are also frequently observed in mammalian vessels when pH_i is transiently decreased (Aalkjær and Poston, 1996). This is similar to our findings in EBA, CMA and ACV (Fig. 4). In mammalian vessels, this has been attributed to a rise in $[Ca^{2+}]_i$ from both extracellular and intracellular sources (Aalkjær and Poston, 1996). It is unclear how trout vessels regulate Ca^{2+}_o during ammonium washout. In both un-stimulated and U-46619 pre-contracted vessels, $0[Ca^{2+}]_o$ and D600 only partially inhibited the acidotic contraction (Figs 9, 11) suggesting that both Ca^{2+}_o and Ca^{2+}_i are involved. However, in KCl-contracted vessels the acidotic contraction was completely inhibited by $0[Ca^{2+}]_o$ (Fig. 10). In all vessels D600 was less effective than $0[Ca^{2+}]_o$ in inhibiting the transient acidotic contraction. This could be due to the presence of non-voltage gated Ca^{2+} channels as in mammalian vessels (Austin and Wray, 2000), or poor specificity of D600 for trout Ca^{2+} channels. The latter seems more likely as D600 only inhibited around 70% of the KCl response (Fig. 10).

pH_o effects in the gill

The effect of pH_o on vascular resistance of the perfused gill was consistent with its effect on isolated conductance arteries (EBA) and veins (ACV), even to the degree of pH sensitivity (Fig. 1). Tissue hypoxia resulting from a decrease in the ratio of O_2 delivery (perfusion) to O_2 consumption (metabolism), such as that encountered by fish during exercise, typically results in tissue and circulatory acidosis (Milligan, 1996). In mammalian vessels, both acidosis (Aalkjær and Poston, 1996; Wray and Smith, 2004) and hypoxia (Thorne et al., 2004) dilate systemic vessels thereby producing a concerted increase in blood flow. Even in large mammalian pulmonary arteries a hypoxic vasodilation is accompanied by a fall in pH_i (Madden et al., 2001). However, the response of small mammalian pulmonary arteries is different as hypoxia produces vasoconstriction and an increase in pH_i (Madden et al., 2001). We did not measure pH_i in our study, but it is probable that resistance vessels in the gill are similar to small pulmonary arteries; they are relaxed by acidosis (Fig. 1) and constricted by hypoxia (Smith et al., 2001). Conversely, hypoxic vasoconstriction in conductance arteries, such as EBA, is uncommon (Smith et al., 2001).

Acidotic dilation (Figs 1, 12) and hypoxic vasoconstriction (Smith et al., 2001) of gill resistance vessels is undoubtedly of homeostatic benefit. Acidotic dilation may enhance gas exchange and it certainly would decrease cardiac afterload at a time, such as that accompanying exhaustive exercise in trout (Milligan, 1996), when myocardial contractility would be most vulnerable (Farrell et al., 1986). Hypoxic vasoconstriction in fish would have similar beneficial effects as it does in the mammalian lung by preventing over perfusion of under ventilated lamellae thereby maintaining O_2 saturation of systemic arterial blood. In fact, it is likely that this unique response originally developed in the gill microcirculation and was retained during evolution to become an integral component of the mammalian pulmonary circulation.

Homeostatic and environmental implications

Pollution aside, fish often encounter rapid and long-term variations in ambient pH, P_{O_2} and P_{CO_2} that directly affect blood acid–base status (Dejours, 1972; Janssen and Randall, 1975; Thomas and Le Ruz, 1982; Moyle and Cech, Jr, 1996). The consequences of these perturbations on homeostatic mechanisms regulating systemic and branchial perfusion, as well as blood pressure are unknown, but clearly deserve further investigation.

pH or pOH?

Although the difficulty in separating pH from pOH effects is well known (Roos and Boron, 1981), virtually all recent reviews and primary articles on acid–base effects in mammalian vascular smooth muscle discuss the relative contribution of pH_o and pH_i (i.e. $[H^+]$) to vascular smooth muscle tension, but do not consider OH^- (Aalkjær and Poston, 1996; Aalkjær and Peng, 1997; Smith et al., 1998; Austin and Wray, 2000; Wray and Smith, 2004). We feel that a case can be made for OH^- as the vasoactive moiety. First, an increase in tension is directly correlated with an increase in $[OH^-]$, but inversely related to $[H^+]$. It seems intuitively easier to envision how a contraction would dose-dependently increase with increasing agonist concentrations, rather than decreasing concentrations. This is especially evident in otherwise un-stimulated vessels (Fig. 1) where there does not appear to be large resting tone. When these (resting) vessels are acidified from physiological pH (7.8) to 6.8 there is little further change in tonus, even though $[H^+]$ has now increased 10-fold from 0.016 to 0.16 μmol l^{-1}. However, increasing $[OH^-]$ from physiological pH of 7.8 to 8.8 increases $[OH^-]$ from 0.63 μmol l^{-1} to 6.3 μmol l^{-1} and more than doubles the tension. This response is also obvious in pre-contracted vessels. Second, at the upper range of pH effects (e.g. pH 9; Fig. 1), the concentration of H^+ is 1 nmol l^{-1}, whereas the OH^- concentration is 10 μmol l^{-1}. Not only is this a 10 000-fold difference, but it seems more realistic that a 10 μmol l^{-1} increase in OH^- (pH 8 to 9) would produce a half-maximal contraction, than would a 10 nmol l^{-1} decrease in H^+. Granted, H^+ can have substantial effects on amphoteric molecules and buffers, however, when one considers the alkalinity at which most vasoactivity is observed we feel that it is more likely an OH^- effect. Perhaps this is through the variety of anion channels and transporters present in smooth muscle.

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