# Effect of hypoxia on force, intracellular pH and $Ca^{2+}$ concentration in rat cerebral and mesenteric small arteries

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1. The effect of severe hypoxia on force, intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and $pH_i$ was studied in isolated small arteries from rat brain and rat mesenterium. The arteries were mounted for isometric force recording while $[Ca^{2+}]_i$ was measured with fura-2 or $pH_i$ was measured with bis-carboxyethylcarboxyfluorescein (BCECF).

2. Hypoxia reduced the force development in response to arginine vasopressin (AVP) while $[Ca^{2+}]_i$ was unchanged or only slightly reduced. Inhibition of acid extrusion by omission of sodium caused no force development in mesenteric arteries, but the fall in $pH_i$ was enhanced during hypoxia. In cerebral arteries, hypoxia reduced the force development associated with omission of sodium, and the fall in $pH_i$ was less than during normoxic conditions. When acid extrusion was intact, $pH_i$ was not affected by hypoxia and the changes in $pH_i$ during activation with AVP were similar during hypoxia and in the control situation.

3. Although a decrease in smooth muscle $[Ca^{2+}]_i$ may be partly responsible for the reduced force development during hypoxia, $[Ca^{2+}]_i$-independent mechanism(s) may play an even more important role. Furthermore, although hypoxia and force development are associated with enhanced acid production, acid extrusion maintains $pH_i$ near the control level and it is unlikely that a decrease in smooth muscle $pH_i$ plays any role in the reduced force development during hypoxia.

Hypoxia induces a reduction of tone in some vascular beds, although in other beds a vasoconstriction is seen (see e.g. Wadsworth, 1994). The mechanisms responsible for the reduction of blood vessel tone induced by hypoxia are not completely known (Carrier, Walker & Guyton, 1964; Hellstrand, Johansson & Norberg, 1977; Coburn, Grubb & Aronson, 1979; Lövgren & Hellstrand, 1985; Lombard, Smeda, Madden & Harder, 1986; Rodman, Hasunuma, Peach & McMurtry, 1990; von Beckerath, Cyrys, Dischner & Daut, 1991). Lövgren & Hellstrand (1987, 1988) concluded that the inhibition of active tone in the isolated portal vein by a reduction in the partial pressure of oxygen ($P_{O_2}$) is unlikely to result from an increase in the lactate concentration or a fall in phosphocreatinine levels in the vascular smooth muscle cells. Another possibility is that a reduction of ATP levels in the smooth muscle cells plays a role in mediating vascular relaxation in response to reduced $P_{O_2}$, possibly by limiting the ability of the myofilaments to develop contractile force. Although moderate hypoxia may not result in a measurable ATP depletion despite a reduction of force (Lövgren & Hellstrand, 1985; Scott & Coburn, 1985), severe hypoxia is associated with a moderate reduction of ATP (Namm & Zucker, 1973; Hellstrand *et al.* 1977).

One consequence of a reduction of ATP levels in the vascular smooth muscle cells may be hyperpolarization mediated through the opening of ATP-sensitive potassium channels (Daut, Maier-Rudolph, von Beckerath, Mehrke, Günther & Goedel-Meinen, 1990). Hypoxia is associated with hyperpolarization of cat cerebral arteries (Lombard *et al.* 1986) and rat portal vein (Ekmehag, 1989). Evidence has also been presented to suggest that hyperpolarization of coronary arteries during hypoxia is mediated through the opening of ATP-sensitive potassium channels (Daut *et al.* 1990; von Beckerath *et al.* 1991). However, there is evidence that this mechanism is unimportant in the rabbit aorta (Rodman *et al.* 1990) and other studies (Gebremedhin *et al.* 1994) indicate that hyperpolarization of isolated smooth muscle cells of cat middle cerebral artery in response to hypoxia is mediated by the opening of $Ca^{2+}$-sensitive maxi $K^+$ channels.

Another potential consequence of a reduced ATP concentration is inhibition of phosphoinositide metabolism. Coburn, Baron & Papadopoulos (1988) have presented evidence for a reduction in the phosphoinositide turnover during hypoxia, which could also contribute to the reduction of tone. A final common pathway for either activation of

ATP-sensitive potassium channels or a reduction in phosphoinositide turnover might be a fall in intracellular calcium concentration ($[Ca^{2+}]_i$). However, no measurements of $[Ca^{2+}]_i$ are available to assess this possibility.

Another potential mediator of the vasoactive effect of hypoxia is reduced pH. It is well known that prolonged changes in pH produce vasoactive effects (Wray, 1988; Aalkjær, 1990), and that acidification is associated with vasodilatation. If hypoxia is associated with acid production and a consequent fall in $pH_i$, this might have significance for the vasodilatory effect of reduced $P_{O_2}$. Using NMR, Spurway & Wray (1987) demonstrated that severe metabolic inhibition (with cyanide and fluoride) was associated with a reduction in $pH_i$ in resting rabbit arteries. However, no information is available regarding the effect of hypoxia on acid production and $pH_i$ in vascular tissue.

We have therefore addressed the following three questions in the present study. (a) Can the reduction in tone seen during hypoxia be explained by a reduction in $[Ca^{2+}]_i$ in rat cerebral and mesenteric small arteries? (b) Is hypoxia associated with acid production in these arteries? (c) Can part of the vasodilatory effect of hypoxia be mediated through a reduction in $pH_i$ in the smooth muscle? Cerebral and mesenteric small arteries were chosen for these experiments because both of these vascular beds show vasodilatation in response to hypoxia, and we have extensive experience with measurements of $[Ca^{2+}]_i$ and $pH_i$ in these vessels.

## METHODS

### Preparation

Male Wistar rats (12–16 weeks old) were killed with $CO_2$ and the brain or intestinal mesenterium was removed. A branch of the middle cerebral artery or a third- or fourth-order branch of the superior mesenteric artery was isolated and prepared as described below.

### Set-up

The arteries (approximate inner diameter of 200 $\mu$m) were mounted as ring preparations in a myograph for isometric force development by threading them onto two stainless-steel wires (Mulvany & Halpern, 1977). The internal diameter was set to an internal circumference based on the passive tension–length curve which was equal to $0{\cdot}9L_{100}$, where $L_{100}$ is the circumference the vessel would have if exposed to a transmural pressure of 100 mmHg *in vivo*.

$[Ca^{2+}]_i$ and $pH_i$ were measured with fura-2 and biscarboxyethylcarboxyfluorescein (BCECF), respectively, as described earlier (Aalkjær & Cragoe, 1988; Jensen, Mulvany & Aalkjær, 1992; Jensen, Mulvany, Aalkjær, Nilsson & Yamaguchi, 1993). Vessels mounted on the myograph were loaded at 37 °C with the acetoxymethyl ester form of BCECF (BCECF AM, 5 $\mu$M) or 10 $\mu$M fura-2 for 60 min. The BCECF-loaded vessels were excited alternately with light at 450 and 495 nm provided by a 75 W xenon lamp which fed into a monochromator. The emission from the preparation passed through a bandpass filter (515–560 nm) and a < 720 nm cut-off filter, and was fed into the computer via the photomultiplier. The ratio of emission at the two different excitation wavelengths was calculated after subtraction of background fluorescence. A ratio measurement was obtained every 20 s in these experiments. The ratio was calibrated in terms of $pH_i$ with nigericin (Thomas, Buchsbaum, Zimniak & Racker, 1979; Aalkjær & Cragoe, 1988). The fura-2-loaded vessels were excited alternately with light at 347 and 380 nm provided by a 75 W xenon lamp which passed light into two bandpass filters and the emission from the preparation passed through a bandpass filter (500–530 nm) and a < 720 nm cut-off filter. The ratio of emission at the two excitation wavelengths was calculated after subtraction of the fluorescence from the preparation obtained after quenching with manganese at the end of the experiment. A ratio measurement was obtained every 10 s in these experiments. The ratio was calibrated as described previously (Jensen, Hughes, Boonen & Aalkjær, 1993) using the $K_d$ for intracellularly trapped fura-2 ($K_d$ = 342 nM) which we have previously determined in rat mesenteric small arteries (Jensen *et al.* 1993).

### Protocol

The vessels were activated for 10 min with 2 U $l^{-1}$ arginine vasopressin (AVP), which is a near-maximal concentration in both preparations. The activation with AVP was made both in bicarbonate-containing and bicarbonate-free solution. The effect of sodium-free conditions (in the absence of bicarbonate) was also assessed in relaxed vessels and in vessels activated with AVP. The activations were made in solutions gassed with 21 or 0% $O_2$ after an equilibration period of at least 30 min. The sequence of stimulations was changed between different experiments.

In some experiments the proton buffering capacity was determined in the mesenteric arteries in bicarbonate-free solution from the change in $pH_i$ induced by addition of 10 mM $NH_4Cl$ as described by Roos & Boron (1981).

**Table 1. $pH_i$ and $pCa_i$ in unstimulated rat cerebral and mesenteric small arteries**

| | | Cerebral | Mesenteric | |
|---|---|---|---|---|
| | | $pH_i$ | $pH_i$ | $pCa_i$ |
| + Bicarbonate | Control | 7·08 ± 0·07 (4) | 7·17 ± 0·02 (8) | 7·01 ± 0·06 (8) |
| | Hypoxia | 7·08 ± 0·04 | 7·20 ± 0·02 | 6·98 ± 0·04 |
| − Bicarbonate | Control | 7·15 ± 0·03 (9) | 7·19 ± 0·01 (15) | 6·93 ± 0·05 (8) |
| | Hypoxia | 7·11 ± 0·04 | 7·19 ± 0·02 | 7·00 ± 0·05 |

Values are means ± S.E.M.; the numbers in parentheses indicate the number of vessels for which the $pH_i$ was compared at 21 and 0% $O_2$. $pCa_i$ is $-\log\{[Ca^{2+}]_i, (M)\}$.

### Solutions and chemicals

The physiological salt solution (PSS) used in these experiments had the following composition (mM): 119 NaCl, 4·7 KCl, 1·18 $KH_2PO_4$, 1·17 $MgSO_4$, 25 $NaHCO_3$, 2·5 $CaCl_2$, 0·026 ethylene diaminetetraacetic acid (EDTA), 5·5 glucose and 5 Hepes. The pH of this solution was adjusted to 7·45–7·50 when gassed with 5% $CO_2$–21% $O_2$–74% $N_2$. For the experiments in which the solution was gassed without $CO_2$, $NaHCO_3$ was replaced by NaCl on an equimolar basis. $N_2$ was used as a substitute for $O_2$ and $CO_2$. For the experiments in which sodium was omitted, NaCl was replaced by *N*-methyl-D-glucamine on an equimolar basis and the pH was adjusted with HCl to give a final chloride concentration of about 130 mM. The $O_2$ tension in the tissue chamber was measured with a micro-oxygen electrode (Microelectrodes, Inc., Londonderry, NH, USA). In four experiments the $O_2$ tension was found to be 146 ± 2·5 and 4·5 ± 0·9 mmHg, after 10 min gassing with 21 and 0% $O_2$, respectively.

Other chemicals used were fura-2 AM, BCECF AM and Pluronic F-127 (Molecular Probes, Junction City, OR, USA). Ionomycin, Cremophor EL, acetylcholine and noradrenaline-HCl were obtained from Sigma Chemical Co. (Poole, UK). Arginine vasopressin was purchased from Sandoz (Basel, Switzerland).

### Statistics

In the text values are means ± S.E.M. followed by the number of arteries, one artery per rat, in parentheses. Mean values were compared using Student's two-tailed *t* test. $P < 0·05$ was considered significant. In all figures, force is expressed as tension, which is force divided by two times the artery segment length.

## RESULTS

### Effect of hypoxia in unstimulated arteries

Figure 1 shows the effect of hypoxia on force and $[Ca^{2+}]_i$ in cerebral arteries at rest and following activation with AVP or sodium-free solution. Cerebral arteries occasionally developed a small amount of tone in the absence of a stimulus. There were, however, substantial variations of $[Ca^{2+}]_i$ in unstimulated cerebral arteries. In particular, it was notable that after wash-out of an activating solution, $[Ca^{2+}]_i$ often fell transiently to a low level (about 100 nM) which was close to the resting level in the mesenteric arteries (see below), but then rose again to a level which was occasionally associated with a small amount of force

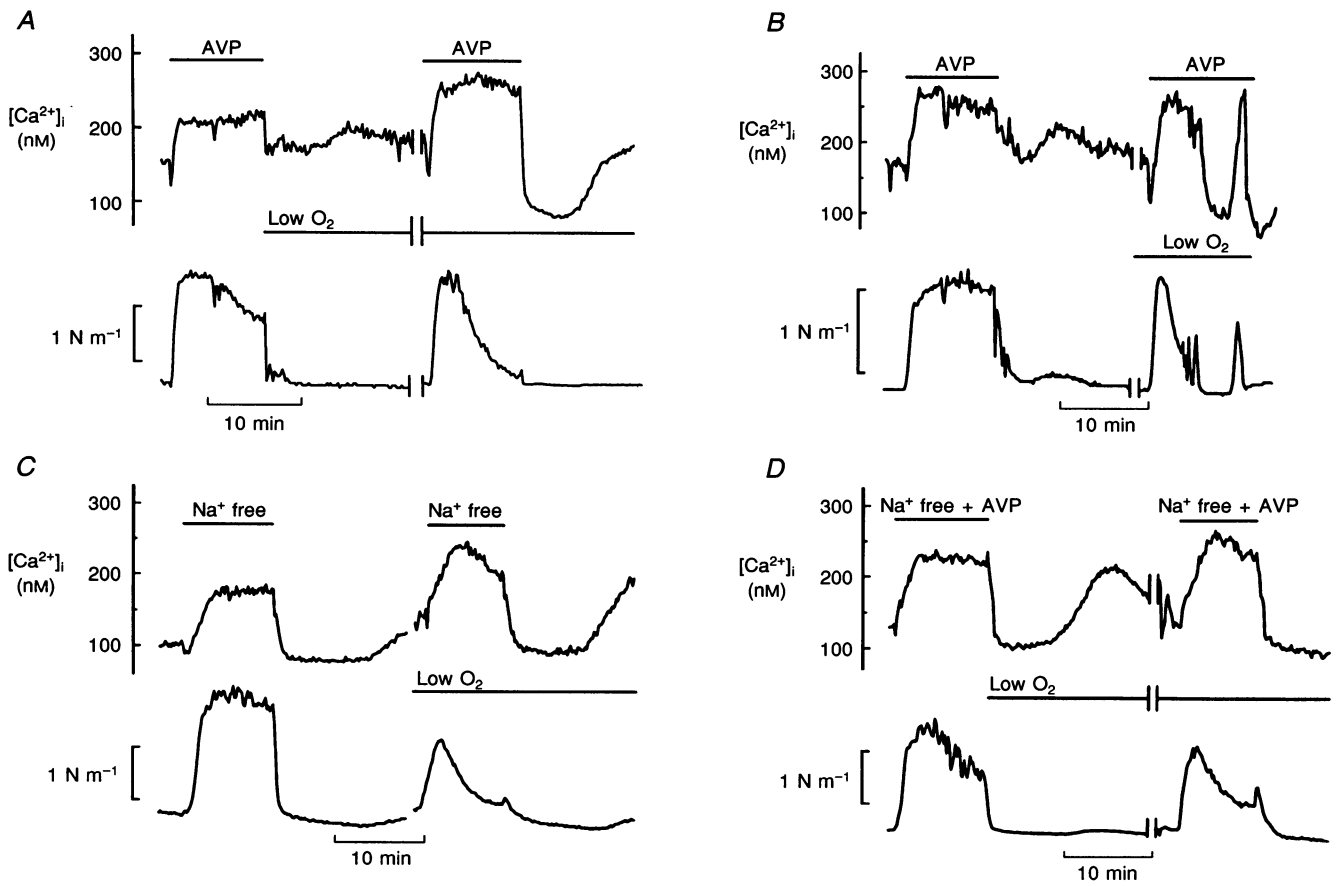


**Figure 1. Traces showing $[Ca^{2+}]_i$ and force in cerebral small arteries during hypoxia**

$[Ca^{2+}]_i$ (upper) and force (lower traces) in cerebral small arteries activated with 2 U $l^{-1}$ arginine vasopressin (AVP) or exposed to sodium-free solution (sodium replaced by *N*-methyl-D-glucamine). At the point indicated (Low $O_2$), the solution was gassed with 0% $O_2$. *A*, bicarbonate-containing solution (PSS); *B*, *C* and *D*, bicarbonate-free solution.

**Table 2. $[Ca^{2+}]_i$ and force after 5 and 10 min stimulation of eight cerebral and eight mesenteric small arteries**

| | 5 min | | | | 10 min | | | |
|---|---|---|---|---|---|---|---|---|
| | AVP, PSS | | AVP, Hepes | | AVP, PSS | | AVP, Hepes | |
| | $pCa_i$ | Force ($N\ m^{-1}$) | $pCa_i$ | Force ($N\ m^{-1}$) | $pCa_i$ | Force ($N\ m^{-1}$) | $pCa_i$ | Force ($N\ m^{-1}$) |
| Cerebral | | | | | | | | |
| Control | 6·07 ± 0·03 | 1·45 ± 0·09 | 6·06 ± 0·05 | 1·93 ± 0·35 | 6·04 ± 0·04 | 1·38 ± 0·13 | 6·05 ± 0·03 | 2·20 ± 0·50 |
| Hypoxia | 6·13 ± 0·02 | 0·90 ± 0·26 | 6·05 ± 0·04 | 1·01 ± 0·18 | 6·28 ± 0·07 | 0·75 ± 0·22 | 6·10 ± 0·05 | 0·82 ± 0·16 |
| *P* | n.s. | n.s. | n.s. | < 0·05 | < 0·01 | < 0·05 | n.s. | < 0·05 |
| Mesenteric | | | | | | | | |
| Control | 6·28 ± 0·05 | 2·96 ± 0·20 | 6·16 ± 0·05 | 2·80 ± 0·14 | 6·47 ± 0·07 | 2·04 ± 0·28 | 6·30 ± 0·05 | 2·02 ± 0·30 |
| Hypoxia | 6·24 ± 0·06 | 1·47 ± 0·28 | 6·18 ± 0·09 | 1·60 ± 0·14 | 6·59 ± 0·09 | 0·73 ± 0·28 | 6·47 ± 0·10 | 0·72 ± 0·22 |
| *P* | n.s. | < 0·01 | n.s. | < 0·01 | n.s. | < 0·01 | n.s. | < 0·01 |

Values are means ± S.E.M. $pCa_i$ is $-\log\{[Ca^{2+}]_i, (M)\}$.

development (Fig. 1*B*, *C* and *D*). In some experiments, $[Ca^{2+}]_i$ in unstimulated arteries even rose to a level which was close to the level seen in vessels activated with AVP, but with little force development. There was no apparent difference in the extent of spontaneous changes in $[Ca^{2+}]_i$ whether bicarbonate was present or not, and removal of the endothelium (see below) did not affect the spontaneous changes in $[Ca^{2+}]_i$ and tension in the cerebral arteries. Due to the substantial variations in $[Ca^{2+}]_i$, it was not meaningful to define a 'resting' value for $[Ca^{2+}]_i$ in these arteries. To define a resting value for the tension, we used the lowest value obtained during the experiment. The $pH_i$ in resting vessels was stable and not significantly different in the presence or absence of bicarbonate (Table 1), as reported previously for mesenteric small arteries (Aalkjær & Cragoe, 1988).

In the mesenteric arteries no spontaneous changes in tone, $[Ca^{2+}]_i$ (Fig. 2) or $pH_i$ (not shown) were seen. $[Ca^{2+}]_i$ and $pH_i$ were stable and of similar values in the presence and absence of bicarbonate (Table 1). Values of $pH_i$ in the mesenteric arteries were not significantly different from those obtained in the cerebral arteries.

Hypoxia did not significantly affect $pH_i$ in either the cerebral or the mesenteric arteries (Table 1), whether or not bicarbonate was present.

The proton buffering capacity determined from addition of 10 mM $NH_4Cl$ was 14·8 ± 1·2 mM (pH unit)$^{-1}$ ($n = 6$) during hypoxia and 17·1 ± 1·1 mM (pH unit)$^{-1}$ ($n = 6$) during control conditions. These values were not significantly different.

### Effect of hypoxia on force and $[Ca^{2+}]_i$ in activated vessels

Activation of cerebral and mesenteric arteries with AVP during normoxic conditions was associated with an increase of force which slowly diminished over the 10 min of activation in both vessel types, both in bicarbonate-containing and in bicarbonate-free solutions (Table 2).

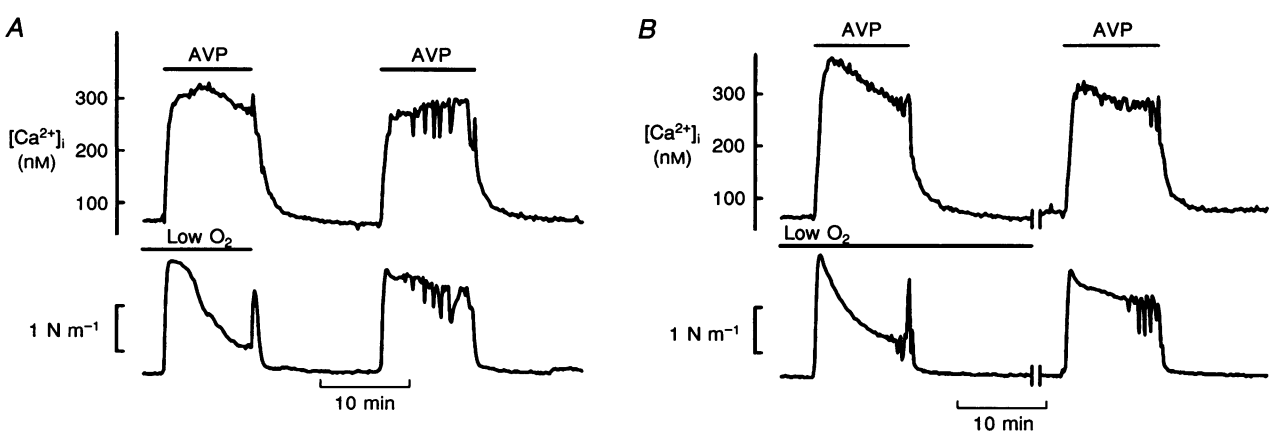


**Figure 2. Traces showing $[Ca^{2+}]_i$ and force in mesenteric small arteries during hypoxia**

$[Ca^{2+}]_i$ (upper) and force (lower traces) in mesenteric small arteries activated with 2 U $l^{-1}$ arginine vasopressin (AVP). Where indicated (Low $O_2$) the solution was gassed with 0% $O_2$. *A*, bicarbonate-containing solution (PSS). *B*, bicarbonate-free solution.

**Table 3. Change in $pH_i$ and force after 10 min stimulation of cerebral and mesenteric small arteries**

| | AVP, PSS | | AVP, Hepes | | $Na^+$ free | | AVP + $Na^+$ free | |
|---|---|---|---|---|---|---|---|---|
| | $\Delta pH_i$ | Force (N $m^{-1}$) | $\Delta pH_i$ | Force (N $m^{-1}$) | $\Delta pH_i$ | Force (N $m^{-1}$) | $\Delta pH_i$ | Force (N $m^{-1}$) |
| Cerebral | | | | | | | | |
| Control | −0·01 ± 0·02 | 1·18 ± 0·08* | −0·07 ± 0·02 | 1·32 ± 0·12* | −0·23 ± 0·03* | 1·47 ± 0·08* | −0·26 ± 0·04 | 1·67 ± 0·09* |
| Hypoxia | −0·03 ± 0·03 | 0·40 ± 0·11 | −0·07 ± 0·01 | 0·56 ± 0·09 | −0·14 ± 0·03 | 0·68 ± 0·09 | −0·18 ± 0·02 | 0·97 ± 0·14 |
| | (4) | | (9) | | (9) | | (9) | |
| Mesenteric | | | | | | | | |
| Control | −0·04 ± 0·01 | 2·67 ± 0·15* | −0·22 ± 0·04 | 2·32 ± 0·25* | −0·14 ± 0·01* | 0·02 ± 0·02 | −0·24 ± 0·02* | 1·87 ± 0·25 |
| Hypoxia | −0·09 ± 0·03 | 1·78 ± 0·33 | −0·15 ± 0·04 | 1·30 ± 0·15 | −0·23 ± 0·03 | 0·01 ± 0·03 | −0·41 ± 0·02 | 1·23 ± 0·20 |
| | (4) | | (4) | | (5) | | (5) | |

Values are means ± S.E.M.; the numbers in parentheses indicate the number of rats (vessels) for which $pH_i$ and force were compared at 21 and 0% $O_2$. * indicates that there was a significant difference in $\Delta pH_i$ or force between the hypoxic and the control situation ($P < 0·05$, using Student's $t$ test).

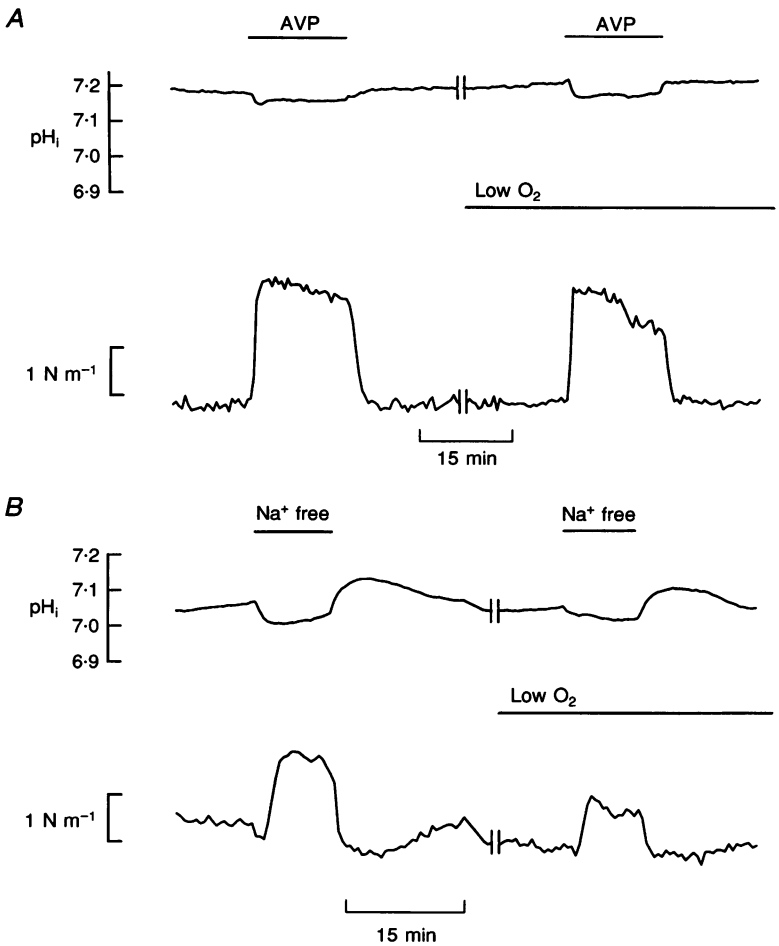


**Figure 3. Traces showing $pH_i$ and force in mesenteric and cerebral small arteries during hypoxia**

Traces showing $pH_i$ (upper) and force (lower) in mesenteric small artery (*A*) and cerebral small artery (*B*) exposed to 2 U $l^{-1}$ arginine vasopressin (AVP) in bicarbonate-containing solution or to sodium- and bicarbonate-free solution (sodium replaced by *N*-methyl-D-glucamine and bicarbonate by chloride) as indicated. Low $O_2$ indicates that the solution was gassed with 0% $O_2$.

Activation was also associated with an increase in $[Ca^{2+}]_i$, which also diminished slowly with time in the mesenteric vessels (Fig. 2 and Table 2). However, in cerebral arteries, no consistent fall of $[Ca^{2+}]_i$ was seen during the 10 min stimulation (Fig. 1 and Table 2). In cerebral vessels, replacement of sodium by NMDG caused an immediate force development and a maintained elevation in $[Ca^{2+}]_i$, irrespective of whether AVP was present (Fig. 1*D*) or not (Fig. 1*C*). In mesenteric arteries, omission of sodium caused no force development (Fig. 5*C*). Although $[Ca^{2+}]_i$ was not measured in these experiments, we have previously shown (Mulvany, Aalkjær & Jensen, 1991) that omission of sodium does not lead to a rise in $[Ca^{2+}]_i$ in these arteries.

During hypoxia, contractile force induced by AVP was transient in both preparations, as was force generation by omission of sodium in the cerebral arteries (Figs 1–5; Table 2). Although the initial peak force development was of similar magnitude to that seen in the control situation, the transience was faster during hypoxia compared to the control situation (Figs 1 and 2; Table 2), and after 10 min stimulation the force was significantly lower in the hypoxic vessels than in the control vessels (Tables 2 and 3). The transient force development during hypoxia was associated with an increase in $[Ca^{2+}]_i$. Often $[Ca^{2+}]_i$ remained elevated during the 10 min stimulation (Figs 1*A*, *C* and *D*, 2*A* and *B*), despite the fall in force. However, on some occasions, $[Ca^{2+}]_i$ suddenly fell during the 10 min stimulation, but the fall in tension always preceded the decrease in $[Ca^{2+}]_i$ (e.g. Fig. 1*B*). Furthermore, during activation of hypoxic vessels, force often fell when $[Ca^{2+}]_i$ was still increasing (e.g. Fig. 1*C* and *D*).

To assess the effect of the endothelium on the mechanical effect of hypoxia, the endothelium was removed from the arteries by rubbing the inside of the vessel with a 40 $\mu$m

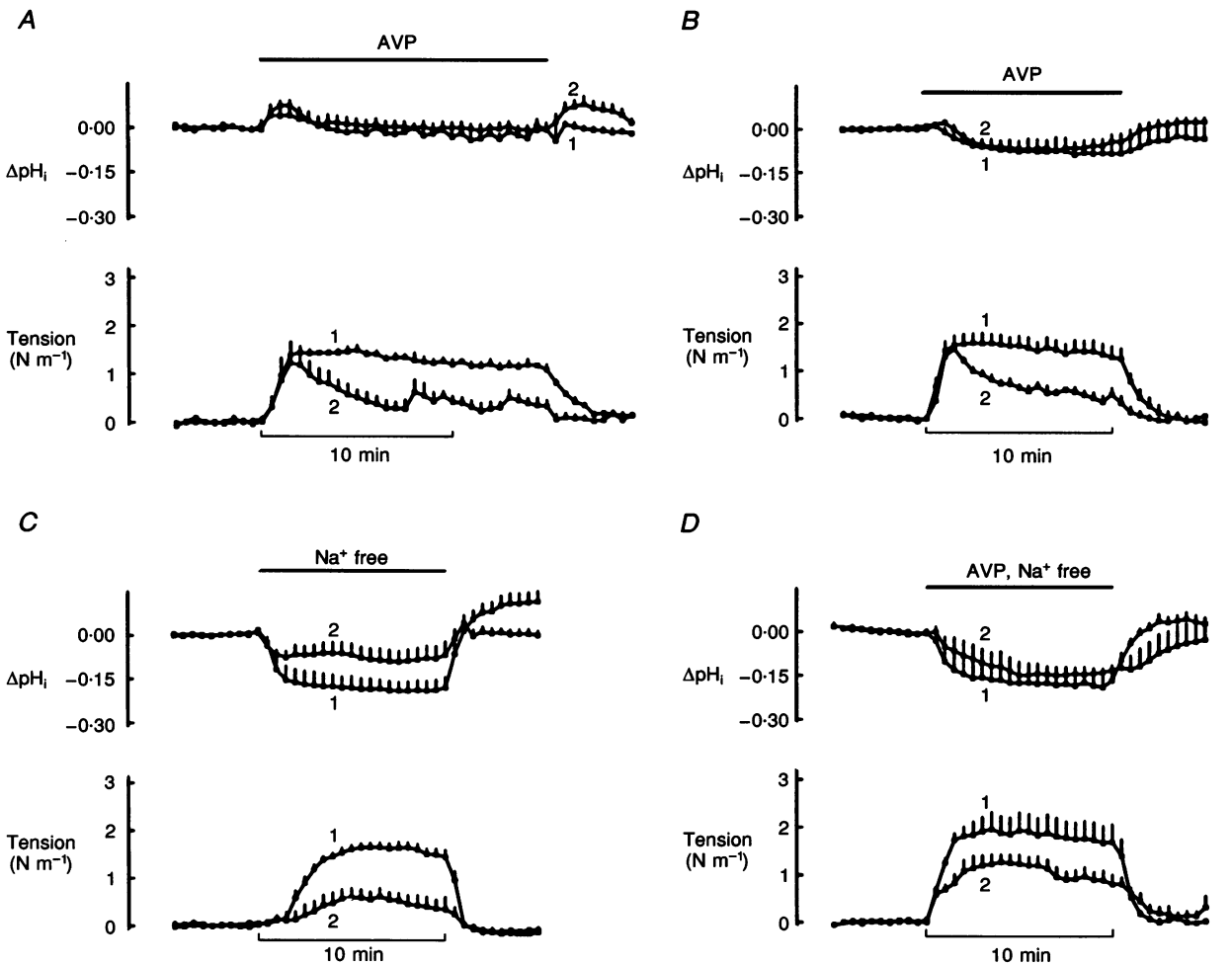


**Figure 4. Effect of hypoxia on $pH_i$ and force in cerebral small arteries**

Change in $pH_i$ ($\Delta pH_i$) and force in cerebral small arteries activated with 2 U $l^{-1}$ arginine vasopressin (AVP) or exposed to sodium-free solution (sodium replaced by *N*-methyl-D-glucamine). 1, vessels activated in solution gassed with 21% $O_2$; 2, vessels activated in solution gassed with 0% $O_2$. Each point is the mean of the number of experiments indicated below and the bars represent S.E.M. *A*, four experiments in bicarbonate-containing solution (PSS); *B*, *C* and *D*, nine experiments each in bicarbonate-free solution.

steel wire. The efficiency of this procedure was judged from the disappearance of the relaxing effect of 10 $\mu$M acetylcholine (mesenteric vessels) or 3 $\mu$M bradykinin (cerebral vessels) on AVP-induced tone. In these experiments, the response to AVP was still depressed during hypoxia, i.e. tension after 10 min stimulation in mesenteric vessels from which the endothelium was removed was $2{\cdot}27 \pm 0{\cdot}31$ N m$^{-1}$ during control conditions ($n = 3$) and $1{\cdot}06 \pm 0{\cdot}32$ N m$^{-1}$ during hypoxia ($n = 3$), ($P < 0{\cdot}05$); tension after 10 min stimulation in cerebral vessels from which the endothelium was removed was $2{\cdot}16 \pm 0{\cdot}06$ N m$^{-1}$ during control conditions ($n = 6$) and $0{\cdot}69 \pm 0{\cdot}26$ N m$^{-1}$ during hypoxia ($n = 6$; $P < 0{\cdot}05$).

### Effect of hypoxia on force and $pH_i$ in activated vessels

Figure 3 shows examples of typical traces from the experiments investigating the effect of hypoxia on $pH_i$ in activated vessels, and Figs 4 and 5 summarize the changes in force and $pH_i$ in cerebral and mesenteric arteries during exposure of the vessels to hypoxia. In the presence of bicarbonate, no significant decrease in $pH_i$ was seen during activation of cerebral arteries with AVP (Fig. 4*A*), while in the mesenteric arteries a small fall in $pH_i$ was observed (Fig. 5*A*). However, $pH_i$ was not affected differently during hypoxia when compared with control conditions, despite the reduced force development during exposure to hypoxia in both preparations (Figs 4*A* and 5*A*; Table 3). In the absence of bicarbonate, a fall in $pH_i$ was seen during activation with AVP in both vessels (Figs 4*B* and 5*B*). However, as in the response in bicarbonate-containing solution, the fall in $pH_i$ was not enhanced in the hypoxic situation (Figs 4*B* and 5*B*; Table 3).

In the cerebral arteries, the force development induced by omission of sodium was associated with a substantial

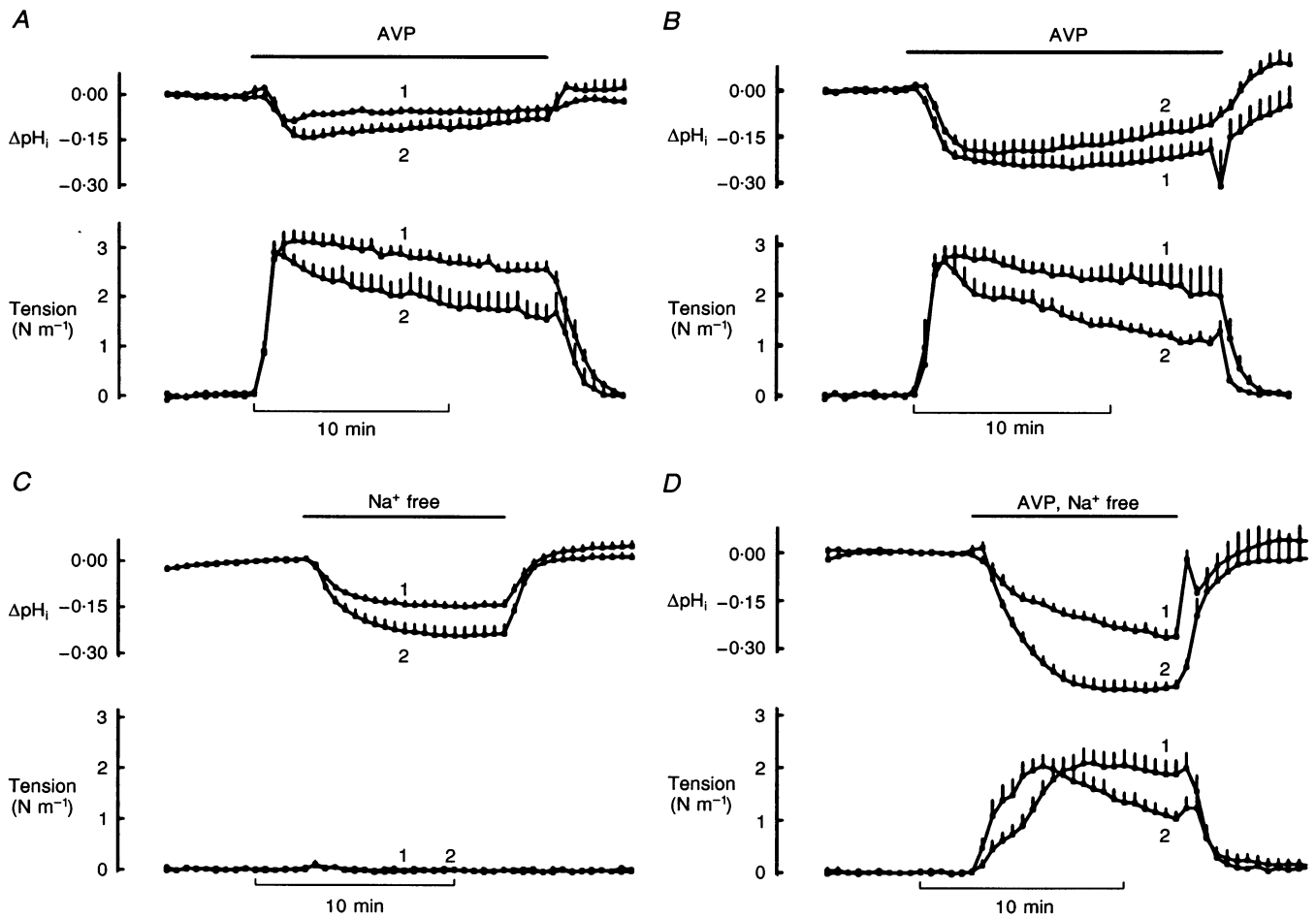


**Figure 5. Effect of hypoxia on $pH_i$ and force in mesenteric small arteries**

Change in $pH_i$ ($\Delta pH_i$) and force in mesenteric small arteries activated with arginine vasopressin (AVP) or exposed to sodium-free solution. In *A*, *B* and *C*, 2 U l$^{-1}$ AVP was used; in *D*, 1 U l$^{-1}$ AVP was used in the control situation and 2 U l$^{-1}$ in the hypoxic situation. 1, vessels activated in solution gassed with 21% $O_2$; 2, vessels activated in solution gassed with 0% $O_2$. Each point is the mean of the number of experiments indicated below and the bars represent S.E.M. *A*, four experiments in bicarbonate-containing solution (PSS); *B*, *C* and *D*: four, five and five experiments, respectively, in bicarbonate-free solution.

decrease in $pH_i$ both in the absence (Fig. 4*C*) and presence (Fig. 4*D*) of AVP. Again this fall in $pH_i$ was not enhanced during hypoxia (Fig. 4*C* and *D*; Table 3). In fact, omission of sodium alone was associated with a significantly smaller fall in $pH_i$ during hypoxia than during the control conditions (Fig. 4*C*; Table 3).

In the mesenteric arteries, omission of sodium did not induce any force development (Fig. 5*C*). However, $pH_i$ decreased (Fig. 5*C*; Table 3) consequent to inhibition of acid extrusion via $Na^+$–$HCO_3^-$ cotransport and $Na^+$–$H^+$ exchange (Aalkjær & Cragoe, 1988). The fall in $pH_i$ after replacement of sodium by NMDG was significantly greater during hypoxia than in the control situation, and there was still no force development (Fig. 5*C*; Table 3). Simultaneous addition of AVP and omission of sodium caused a force development (Fig. 5*D*). To obtain the same force development (and therefore presumably the same metabolic load under control and hypoxic conditions), only 1 U $l^{-1}$ AVP was added in the control situation while 2 U $l^{-1}$ AVP was used in the hypoxic situation. This protocol gave a more rapid but partially transient force development during hypoxia when compared with the control situation (Fig. 5*D*), and after 10 min activation, force was not significantly smaller during hypoxia. With this protocol, $pH_i$ decreased much faster than it did during omission of sodium without addition of AVP (compare Fig. 5*C* and *D*), and the fall in $pH_i$ was enhanced during hypoxia compared to the control situation.

## DISCUSSION

In this study we have investigated whether $[Ca^{2+}]_i$ and $pH_i$ play a role in the inhibition of force development associated with severe hypoxia in isolated cerebral and mesenteric small arteries. We have compared the control situation (21 % $O_2$) with a situation of severe hypoxia which resulted in bath $P_{O_2}$ of less than 5 mmHg, and examined the responses after at least 30 min of hypoxia. This was done to increase the likelihood of observing even small changes in $[Ca^{2+}]_i$ and $pH_i$ associated with hypoxia. Although the majority of the experiments were made in the presence of the endothelium, the inhibition of force development with hypoxia in these arteries was also seen in the absence of a functional endothelium, indicating that severe hypoxia has a direct effect on the smooth muscle cells.

In the normoxic situation, there were some interesting differences in the control of $[Ca^{2+}]_i$ between the mesenteric and the cerebral small arteries. As expected, stimulation of both cerebral and mesenteric small arteries with AVP was associated with an increase in $[Ca^{2+}]_i$. The control of resting calcium was, however, different in the two vessels. While $[Ca^{2+}]_i$ in unstimulated mesenteric arteries was stable (Fig. 2), $[Ca^{2+}]_i$ in unstimulated cerebral arteries was unstable, suggesting a less efficient control of $[Ca^{2+}]_i$ in these vessels. In six experiments, removal of the endothelium from the cerebral arteries did not increase the stability of the $[Ca^{2+}]_i$ or tension.

The absolute value for $[Ca^{2+}]_i$ in the two preparations depends on the calibration of the fluorescent ratio signal. In the present study, we have used a $K_d$ of fura-2 for calibration (342 nM), which we have previously determined in rat mesenteric small arteries (Jensen *et al.* 1993). If this $K_d$ is also valid for cerebral arteries, the absolute $[Ca^{2+}]_i$ levels in the two preparations can be compared. Such a comparison would suggest that while the cerebral arteries may be able to obtain a level of $[Ca^{2+}]_i$ similar to the resting level of mesenteric arteries (compare Figs 1 and 2), this low $[Ca^{2+}]_i$ level cannot always be maintained in these arteries. The reason for this relatively poor control of $[Ca^{2+}]_i$ in the cerebral arteries is unknown. Another interesting difference between the two preparations was the observation that omission of sodium has no effect on force and $[Ca^{2+}]_i$ in the mesenteric small arteries (present study and Mulvany *et al.* 1991), but leads to substantial force development and an associated rise in $[Ca^{2+}]_i$ in the cerebral small arteries. Whether the marked effect of sodium omission in the cerebral arteries reflects a more important role of the $Na^+$–$Ca^{2+}$ exchange in these arteries or an effect of the sodium-free solution on the membrane potential of the vessels has, to our knowledge, not been investigated.

### Hypoxia and $[Ca^{2+}]_i$

One important aim of this study was to evaluate the role of changes in $[Ca^{2+}]_i$ in mediating vasodilatation during exposure to severe hypoxia. If the hypoxic vasodilatation is mainly determined by reduced $Ca^{2+}$ influx mediated by changes in the membrane potential as suggested for coronary arteries (Daut *et al.* 1990), by inhibition of phosphoinositol turnover (Coburn *et al.* 1988), or by other mechanisms, e.g. direct inhibition of membrane $Ca^{2+}$ channels (Pearce, Ashwal, Long & Cuevas, 1992), relaxation of the vessels should be associated with a fall in $[Ca^{2+}]_i$ from the stimulated levels.

$[Ca^{2+}]_i$ of resting arteries was not affected by severe hypoxia in the present experiments. After 10 min stimulation in hypoxia, $[Ca^{2+}]_i$ was lower than control during some (but not all) types of activation. However, it was not lower than control values after 5 min stimulation in hypoxic conditions. This suggests that hyperpolarization, inhibition of phosphoinositol turnover, or direct inhibition of $Ca^{2+}$ influx at the level of the membrane $Ca^{2+}$ channel may play some role in intracellular calcium homeostasis during hypoxia, but also indicates that $[Ca^{2+}]_i$ may be less affected by severe hypoxia than might be expected. This is consistent with the possibility that the energy-dependent extrusion of calcium from the cells may

also suffer during hypoxia. The latter mechanism would not be apparent in studies of unidirectional $Ca^{2+}$ influx (Pearce *et al.* 1992).

It was a general finding in our study that $[Ca^{2+}]_i$ was to some extent dissociated from force during hypoxia, e.g. although force 5 min after activation was reduced during hypoxia compared to the control situation, $[Ca^{2+}]_i$ was not. Moreover, in the experiments where force fell abruptly during activation, it was a consistent finding that a decrease in force always preceded a fall in $[Ca^{2+}]_i$. These findings suggest that steps in the excitation–contraction coupling process, which are unrelated to the regulation of $[Ca^{2+}]_i$, play an important role in mediating vascular relaxation during exposure to severe hypoxia. The latter hypothesis is consistent with the conclusions of previous studies (Pearce *et al.* 1992) which reported a dissociation between hypoxic inhibition of contractile force and inhibition of $Ca^{2+}$ uptake. Based on the present experiments it is not possible to predict which mechanism(s) is involved. However, our results would be consistent with a role for decreased actomyosin ATPase activity and/or reduced phosphorylation of the myosin light chain consequent to ATP depletion in mediating the reduction of contractile force in these vessels during exposure to hypoxia.

### Hypoxia and proton homeostasis

A second aim of this study was to assess the effect of hypoxia on acid production and $pH_i$ of small arteries. In both cerebral and mesenteric small arteries, hypoxia had little effect on $pH_i$ under relaxed conditions. This contrasts with the effect of metabolic inhibition using high concentrations of cyanide and fluoride, which caused a decrease in $pH_i$ in large arteries from the rabbit (Spurway & Wray, 1987) and in the rat uterus (Wray, 1990). These differences may arise because of tissue-specific variations in the three major factors determining $pH_i$: (1) buffering capacity, (2) net acid production and (3) transmembrane transport of acid equivalents.

To assess the possibility that a change of buffering capacity affects intracellular pH during hypoxia, buffering capacity (calculated from the change in $pH_i$ associated with addition of 10 mM $NH_4Cl$; Roos & Boron, 1981) was determined in small mesenteric arteries. No difference in buffering capacity was found in these experiments, and although we cannot exclude a minor reduction of buffering capacity with hypoxia, it seems reasonable to assume that the differences in $pH_i$ under hypoxic and control conditions in these vessels reflect primarily changes in the net production or transmembrane transport of acid equivalents.

In the absence of a significant influence of altered buffering capacity in contributing to the changes in $pH_i$ in the vessels during our experiments, net acid production may be assessed after inhibition of transmembrane transport of acid equivalents. In the rat mesenteric small arteries, we have previously shown that the three main pathways for transmembrane transport of acid equivalents are $Na^+$–$HCO_3^-$ cotransport, $Na^+$–$H^+$ exchange and $Cl^-$–$HCO_3^-$ exchange (Aalkjær & Cragoe, 1988; Aalkjær & Hughes, 1991) and that all of these pathways are inhibited by omission of sodium and bicarbonate. We cannot completely rule out the possibility that sodium- and $HCO_3^-$-independent pathways may contribute to the change in $pH_i$ under these conditions. However, since the recovery from an acid load is completely inhibited by omitting sodium and bicarbonate (Aalkjær & Cragoe, 1988), the contribution of transport and diffusion of acid equivalents to changes in $pH_i$ under these conditions is probably minimal. Thus, the fall in $pH_i$ when sodium and $HCO_3^-$ are omitted presumably reflects net metabolic production of acid. The enhanced fall in $pH_i$ during hypoxia when sodium and $HCO_3^-$ are omitted (Fig. 5*C* and *D*) therefore suggests that hypoxia induces an increased net production of acid. In addition, comparison of Fig. 5*C* and *D* (see Table 3 also) indicates that force development, independent of the oxygen tension, was associated with acid production, since $pH_i$ in mesenteric arteries fell more in sodium-free conditions when AVP was added to induce force than it did during resting conditions. In the cerebral arteries, the interpretation was more difficult since omission of sodium was associated with force development in these vessels. This force development was inhibited by hypoxia, and the smaller fall in $pH_i$ during hypoxia suggests that reduced acid production as a consequence of reduced force development overrides the direct effect of hypoxia on acid production.

The observation that $pH_i$ was well maintained during hypoxia in the face of an enhanced net production of acid suggests that $pH_i$ homeostasis is very efficient in the small arteries, even under hypoxic conditions. Force development induced by vasopressin in the presence of bicarbonate caused little change in $pH_i$ in either preparation. However, in the absence of bicarbonate, $pH_i$ fell in both preparations. This confirms our previous report that bicarbonate influx is important for extrusion of acid during force development in rat mesenteric small arteries (Aalkjær & Mulvany, 1991) and extends this observation to rat cerebral small arteries. The absence of an increase in $pH_i$ in small arteries after exposure to agonists both in the absence and presence of bicarbonate has been reported by several groups (Aalkjær & Cragoe, 1988; Izzard, MacIver, Cragoe & Heagerty, 1991; Carr, Graves & Poston, 1993) and is in variance with observations obtained in isolated vascular smooth muscle cells or vascular smooth muscle cells in culture (Berk, Aronow, Brock, Cragoe, Gimbrone & Alexander, 1987; Kahn *et al.* 1992). We suggest that this difference may reflect more

metabolically produced acid in the contractile vascular smooth muscles of the intact preparations (also see above).

### Hypoxia, $pH_i$ and force development

A third aim of this study was to assess whether a decrease in $pH_i$ could partly explain the vasodilatation induced by hypoxia. There was no evidence for an important role of smooth muscle $pH_i$ for hypoxic vasodilatation in either cerebral or mesenteric small arteries. Firstly, when bicarbonate was present, $pH_i$ did not change in either vessel type during activation with AVP under hypoxic or control conditions, even though the force was reduced during hypoxia. Secondly, in bicarbonate-free conditions, the small fall in $pH_i$ seen during activation was similar during hypoxia and in the control situation, despite smaller force development during hypoxia. The conclusion that intracellular acidification does not limit contractile force during hypoxic vasodilatation is emphasized by the observation that in cerebral small arteries the decrease in $pH_i$ was most pronounced during normoxic conditions when sodium was omitted, even though this resulted in the most pronounced force development. The unimportance of intracellular acidification for relaxation is also compatible with recent observations (Ighoroje & Spurway, 1984; Aalkjær & Mulvany, 1988; Matthews, Graves & Poston, 1992; Jensen *et al.* 1993) which suggest that acute intracellular acidification may lead to force development rather than relaxation, and with more recent experiments (T. Rong, C. Aalkjaer, N. A. Lassen, P. Vogel & M. J. Mulvany, unpublished results) in which we found that a reduction in steady-state $pH_i$ (without reduction in $pH_o$) does not inhibit active tone in cerebral small arteries of rats.


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