pH_o, pH_i, and P_{CO_2} in Stimulation of IP_3 and $[Ca^{2+}]_c$ in Piglet Cerebrovascular Smooth Muscle (44336)

MARIA LUIZA C. ALBUQUERQUE*,† AND CHARLES W. LEFFLER*,[1]

The Laboratory for Research in Neonatal Physiology, Departments of Physiology and Biophysics and Pediatrics, University of Tennessee, Memphis, Tennessee, 38163; and Division of Critical Care Medicine,† St. Jude Children's Research Hospital, Memphis, Tennessee 38101*

Abstract. **Hypocapnia produces cerebral vasoconstriction. The mechanisms involved in hypocapnia-induced elevation of vascular smooth muscle tone remain unclear. We addressed the hypothesis that, in cerebrovascular smooth muscle, increases in extracellular pH (pH_o) cause increases in Ins(1,4,5)P_3 and cytosolic calcium ($[Ca^{2+}]_c$). Superfused primary cultures of piglet cerebral microvascular smooth muscle cells were exposed to artificial CSF (aCSF) of control (pH_o 7.4, PCO_2 36 mm Hg), metabolic alkalosis (pH_o 7.7, PCO_2 36 mm Hg), or respiratory alkalosis (pH_o 7.7, PCO_2 19 mm Hg). Intracellular pH (pH_i) and $[Ca^{2+}]_c$ were measured, using BCECF and fura-2, respectively, with dual wavelength spectroscopy. Ins(1,4,5)P_3 was determined by a protein binding assay. Both metabolic and respiratory acidosis treatments increased pH_i from the control value of about 7.2 to 7.35. Metabolic and respiratory alkalosis increased Ins(1,4,5)P_3, as we showed previously. Metabolic and respiratory alkalosis increased $[Ca^{2+}]_c$ about 80% and 110%, respectively. Neither Ins(1,4,5)P_3 nor $[Ca^{2+}]_c$ increased in cells treated with aCSF that produced control pH_o with increased pH_i (7.3). In contrast, when pH_o increased (7.7), but pH_i was maintained at control (7.2), Ins(1,4,5)P_3 increased from 123 pmol/well to 307 pmol/well and $[Ca^{2+}]_c$ increased 46%. However, the increase of $[Ca^{2+}]_c$ was less than with either respiratory or metabolic alkalosis. Thus, hypocapnia-induced cerebral vasoconstriction could involve production of Ins(1,4,5)P_3 with resultant elevation in $[Ca^{2+}]_c$. While the Ins(1,4,5)P_3 signal appears to be dependent on an increase in extracellular pH, a role for intracellular pH cannot be completely excluded.** [P.S.E.B.M. 1998, Vol 219]

Carbon dioxide tension plays a major role in the regulation of the fetal, newborn, and adult cerebral circulations. While cerebral vasodilation to hypercapnia and acidosis involves prostanoid-dependent mechanisms, the opposite response, cerebral vasoconstriction in response to hypocapnia, occurs *via* mechanisms that are prostanoid independent (1–6). The signals by which a decrease in CO_2 tension or an increase in pH augment cerebral vascular tone have only recently begun to be investigated. Previously, we showed that metabolic alkalosis and respiratory alkalosis increased Ins(1,4,5)P_3 in piglet cerebral microvascular smooth muscle cells (7). We found that increasing pH *via* a lowering of PCO_2 (respiratory alkalosis) increased Ins(1,4,5)P_3 more than increasing pH with fixed base (metabolic alkalosis). Thus, the signal transduction cascade involving Ins(1,4,5)P_3 with stimulation of intracellular calcium release, may participate in altering vascular tone (8).

More than 20 years ago, it was proposed that an increase in pH_o, irrespective of the CO_2 tension, produces an increase in cerebrovascular tone (9). Depending on the species, tissue bed, and experimental conditions, either pH_o, pH_i, or both pH_o and pH_i have been implicated in alterations of $[Ca^{2+}]_c$ associated with changes in vasomotor tone (10–12). An increase in pH (more specifically, pH_o) or decrease in PCO_2, may affect a membrane protein that is coupled to

The research was supported in part by the National Institutes of Health and in part by Cancer Center Support CORE Grant P30 CA-21765 and the American Lebanese Associated Charities.

[1] To whom requests for reprints should be addressed at Department of Physiology, The University of Tennessee, 894 Union Avenue, Nash Building, Room 426, Memphis, TN 38163. E-mail: cleffler@physiol.utmem.edu

Received January 28, 1998. [P.S.E.B.M. 1998, Vol 219]
Accepted August 3, 1998.

0037-9727/98/2193-0226$10.50/0
Copyright © 1998 by the Society for Experimental Biology and Medicine

a phosphoinositide-specific phospholipase C. This complex may then produce the calcium mobilizing second messenger Ins(1,4,5)P_3 and result in cerebral vasoconstriction. As an extension of our previous work, the present studies were designed to test the hypothesis that there is a positive correlation among pH_i, Ins(1,4,5)P_3, and $[Ca^{2+}]_c$. In addition, it was our goal to determine more precisely the contributions of changes in pH_o and pH_i to Ins(1,4,5)P_3 production and changes in $[Ca^{2+}]_c$ in cerebral microvascular smooth muscle cells.

Materials and Methods

Procedures for collection of cells to culture were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee, Memphis. All procedures were done using sterile techniques.

Chemicals and Supplies. 2′,7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM), fura 2-AM, Pluronic F-127, and Ca^{2+} calibration buffer kits were purchased from Molecular Probes (Eugene, OR). Leighton tissue culture tubes and glass coverslips were from Bellco Biotechnology (Vineland, NJ). Fetal bovine serum was purchased from Hyclone (Logan, UT). Vitrogen 100 was from Collagen (Palo Alto, CA). Matrigel was from Collaborative Biomedical Products (Bedford, MA). D-*myo*-Inositol 1,4,5-trisphosphate (IP_3) ^{3}[H] protein-binding assay system kits were obtained from Amersham International (Amersham, UK). All other chemicals or biochemicals were purchased from Sigma Chemical (St. Louis, MO). Disposable culture plates were from Costar (Cambridge, MA). Nylon Mesh screens were obtained from Spectrum (Houston, TX).

Isolation and Culture of Microvascular Smooth Muscle Cells from Newborn Pig Brain. Brains were removed from newborn 1 to 3-day-old pigs. Cerebral microvascular smooth muscle cells were prepared as described previously (7). Briefly, newborn pig brain cortex was removed under ketamine hydrochloride (33 mg/kg im) and acepromazine (3.3 mg/kg im) anesthesia, placed into a beaker with 40 ml of cold isolation solution containing medium 199 (M199), 0.015 *M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 1 U/ml sodium heparin, and antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B). The meninges and attached vessels were removed from the tissue, and the tissue was washed three times with M199 isolation solution. The tissue was minced into tiny pieces using two scalpels in 20 ml of M199 isolation solution and then transferred to a 40-ml Dounce homogenizer and homogenized with 10 strokes of a loose-fitting pestle. The homogenate was passed through a 300-μm nylon mesh screen. The passage was refiltered over a 60-μm nylon mesh screen. The screen was removed and placed in a 50-ml centrifuge tube containing 50 ml of M199 isolation solution. The microvessels (60–300 μm) were washed off by agitation and scraping and then centrifuged at 500*g* for 5 min. The pellet was resuspended using preincubated culture medium consisting of Dulbecco's Modification of Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 2 mg/ml sodium bicarbonate, 1 U/ml sodium heparin, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. Isolated microvessels were seeded directly onto a 9-mm × 35-mm Matrigel-coated coverslip within a Leighton tissue culture tube (16 mm × 93 mm), or into collagen-coated 12-well (5 ml per well) Costar plates. Microvessels that grew out from the ends of the vessels were grown to confluence in DMEM(SM). These cells stain positively for α-smooth muscle actin, form hills and valleys characteristic of vascular smooth muscle (7), and demonstrate phenotypic characteristics of vascular smooth muscle ultrastructurally (Fig. 1).

Measurement of pH_i. The pH_i of adherent smooth muscle cells was measured using the pH-sensitive fluorescent dye BCECF. Cells attached to the coverslip were incubated for 5 min at 37°C in 1 ml artificial cerebrospinal fluid (KCl 3.0 m*M*, $MgCl_2$ 0.6 m*M*, $CaCl_2$ 2.0 m*M*, glucose 3.7 m*M*, urea 6.7 m*M*, NaCl 127 m*M*, $NaHCO_3$ 27.4 m*M*; pH 7.4, PCO_2 36 mm Hg) containing 4.3 μ*M* BCECF-AM. Stock solutions of 1.44 m*M* BCECF-AM were made in dimethyl sulfoxide (DMSO) and stored at −20°C for 1 month. For experimentation, BCECF stock was thawed and added to loading media. After 5 min of loading, the cells were washed once in fresh control aCSF for 15 min. Next, the coverslip containing the cells was placed into a 1-ml cuvette, contained in an LS-50B spectrofluorophotometer (Perkin-Elmer) at a 45° angle to the excitation beam. Cells were then superfused at a rate of 3 ml/min with artificial cerebrospinal fluid for 10 min prior to excitation. The temperature of the experimental chamber was kept at 37°C for all of the experiments. Measurement of the fluorescence intensity of BCECF was performed with an excitation wavelength pair of 505/439 nm (slit, 15 nm) and an emission wavelength of 530 nm (slit, 10 nm). After a relatively constant fluorescence ratio was observed, cells were superfused for an additional 5 min with aCSF to obtain basal values for pH_i. The medium was then changed to an experimental medium for an additional 5 min.

The ratio of fluorescence at 530 nm was corrected for autofluorescence (measured for each coverslip before loading BCECF). At the end of each experiment, calibration of the fluorescence ratio to pH_i was performed on each coverslip by using the monovalent cation ionophore nigericin to equilibrate pH_i with extracellular pH (pH_o) when intracellular $[K^+]$ equalled extracellular $[K^+]$ (13, 14). The calibration solution contained KCl 145 m*M*, HEPES 22 m*M*, nigericin 5 μ*M*, and all other components of aCSF except NaCl and $NaHCO_3$, and was titrated to values of pH_o between 7.0 and 7.8 by using 5 *N* KOH. The ratio of the fluorescence intensities 505/439 (minus background) was linearly related to the pH_i. Perkin Elmer FL Data Manager Software Products (Beaconsfield, Bucks, England) were used to convert the fluorescence intensity ratios to absolute

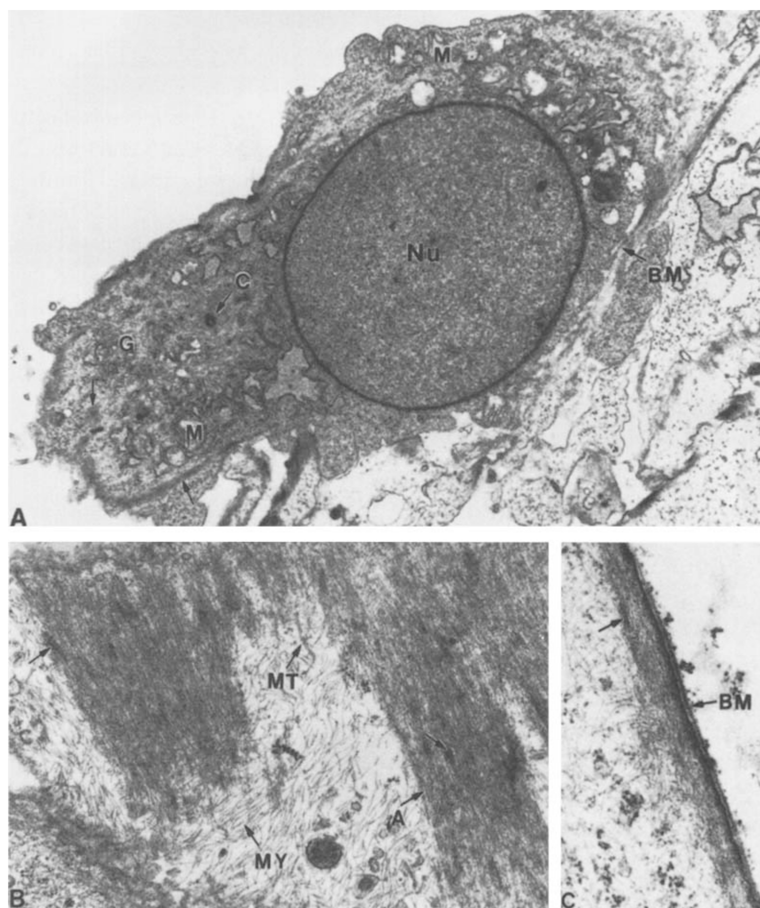

Figure 1. Electron micrograph of a piglet cerebral microvascular smooth muscle cell. Panel A. Nu = nucleus, M = mitochondria, BM = basement membrane, C = centriole, G = golgi. Panel B detail of microtubules (MT), myosin filaments (MY), and actin filaments (A). Panel C, detail of basement membrane (BM) and classical alignment of actin-myosin complexes. In all panels single arrows denote dense bodies, a smooth muscle contractile unit. One can see abundent actin and myosin filaments that form dense bodies important in the generation of constriction.

pH_i *via* linear regression methodology. High frequency noise reduction was accomplished using the binomial smoothing filter component of the Data Manager software that is a quadratic function of the slit width.

Measurement of $[Ca^{2+}]_c$. The $[Ca^{2+}]_c$ of piglet cerebral microvascular smooth muscle cells was measured using the fluorescent Ca^{2+} indicator fura 2-AM (3, 15). Smooth muscle cells attached to a Matrigel-coated coverslip were incubated for 2 hr in 1 ml of aCSF containing 5 μM fura 2-AM and 0.08% Pluronic F-127. Incubations were performed in the dark at room temperature. The coverslip containing the cells was then placed in the 1-ml cuvette of an LS-50B spectrofluorophotometer as described above. Cells were then superfused at 3 ml/min with aCSF. Measurement of the fluorescence intensity of fura 2 was performed with an excitation wavelength pair of 340/380 nm (slit, 15 nm) and an emission wavelength of 510 nm (slit, 10 nm). After a relatively constant fluorescence was observed, cells were superfused for an additional 5 min with aCSF to obtain basal values for $[Ca^{2+}]_c$. The medium was then changed to an experimental medium for another 5 min (Table I).

Calibration of the fura 2-AM signal was carried out on each coverslip after the experiment by measuring the maximal fluorescence in the presence of 4 mM $CaCl_2$ and 5 μM ionomycin and the minimal fluorescence in the presence of 4 mM ethylene glycol-bis(b-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 5 μM ionomycin. The ratio of fluorescence at 510 nm was corrected for autofluorescence (measured for each coverslip before loading fura 2-AM). $[Ca^{2+}]_c$ was calculated with the ICBC software package provided for the LS-50B spectrofluorophotometer (Perkin Elmer) using the formula $[Ca^{2+}]_c = K_d \times [(R-R_{min})/(R_{max}-R)] \times (S_{f2}/S_{b2})$, where K_d is the dissociation constant of the Ca^{2+}/fura 2 complex, R is the 340/380 nm ratio, R_{max} and R_{min} are the maximum and minimum ratios, respectively, and S_{f2}/S_{b2} is the Ca^{2+}-free/Ca^{2+} replete ratio of emissions at 380 nm excitation.

K_d varies with pH, ionic strength, and temperature (3, 16, 17). Previously we showed that the effect of pH from 6.40 to 7.20 on a K_d of Ca^{2+} binding to fura 2 was described by the equation $K_d = 713 - 89pH$ (3). We also found that pHs from 7.20 to 7.36 intracellular pH also affected our K_d values. We obtained Ca^{2+} calibration buffer kits with magnesium II C-3722 from Molecular probes to measure the K_d under each experimental situation.

While pH_i may influence other intracellular proteins and even the intracellular dye fura 2, we have found previously that essentially all fura 2 in our endothelial cells is in the cytoplasm. We have previously treated endothelial cells

Table I. Composition of Control and Test Solutions

Control or test solutions	Composition
Control	KCl 3.0 m*M*, $MgCl_2$ 0.6 m*M*, $CaCl_2$ 2.0 m*M*, glucose 3.7 m*M*, urea 6.7 m*M*, NaCl 127 m*M*, $NaHCO_3$ 27.4 m*M*; pH_o 7.40, PCO_2 36
Metabolic alkalosis	pH_o was increased to 7.74 by using the Henderson-Hasselbach equation to manipulate the $NaHCO_3$ concentration while maintaining a PCO_2 of 36 mm Hg with 5% CO_2. $NaHCO_3$ was increased to 60 m*M*, and NaCl was appropriately decreased to 97 m*M* for osmolarity balance.
Respiratory alkalosis	pH_o was adjusted to 7.7, PCO_2 to 19 mm Hg by equilibrating control aCSF with 2.5% CO_2.
Control pH_o/increased pH_i	pH_o was held constant relative to an increased pH_i by decreasing $NaHCO_3$ to 10.7 m*M*, increasing NaCl to 146.3 m*M*, and equilibrating the solution with 2.5% CO_2.
Increased pH_o/control pH_i	pH_o was increased relative to pH_i by increasing $NaHCO_3$ to 65.9 m*M*, decreasing NaCl to 90.9 m*M*, adding propionate 8 m*M*, and equilibrating the solution with 7% CO_2.

with Digotonin (disrupts plasma membrane but leaves organelle membranes largely intact) and found that almost all the cellular fura 2 was released. In addition, Triton-X 100, which would disrupt all the membranes, did not cause any further release of the dye. Therefore, we believe that fura 2 remained free in the cytoplasm, not bound to intracellular constituents of large molecular weights. Lack of such dye-intracellular protein binding would tend to diminish overestimation of increases in K_d (3, 18).

Determination of $Ins(1,4,5)P_3$ by Protein-Binding Assay. The protein content of the cells was determined by the Bradford method and found to be similar among the wells. Each well of cells was stimulated with 900 μl of control or test artificial cerebrospinal fluid for 30 sec (maximal response in preliminary experiments) in the appropriate CO_2 atmosphere. The reactions were terminated by addition of 100 μl of 1.0 *N* HCL. Cells were sonicated for 20 sec and then centrifuged at 15,000*g* for 20 min at 4°C. The supernatant was used for quantitative determinations of $Ins(1,4,5)P_3$ with a D-*myo*-Inositol 1,4,5-trisphosphate ^{3}[H] protein-binding assay system assembled by Amersham International. Since an acid extraction method was used, each sample was neutralized with 1.0 *N* tris base to achieve a pH between 7.5 and 8.5 before performing the protein-binding assay.

General Experimental Design. Confluent cerebral microvascular smooth muscle cells (9 days in culture) were incubated in 2% FBS DMEM for 24 hr prior to measurement of pH_i, $[Ca^{2+}]_c$, or $Ins(1,4,5)P_3$. Controls or treatments were performed with specific artificial cerebrospinal fluid solutions. The solutions were pre-equilibrated with the desired partial pressure of CO_2, 21% O_2, and the balance with N_2 at 37°C for 1 hr. The pH, PCO_2, and PO_2 were measured before addition to the cells. For pH_i and $[Ca^{2+}]_c$ measurement, after 5 min of stable control baseline, the superfusion solution was switched to a specific aCSF as delineated in Table I.

Statistical Analysis. Data are presented as means ± SEM. Statistical analysis was performed using analysis of variance and a *post hoc* Tukey's protected *t* test for planned comparisons. Values of $P \leq 0.05$ were regarded as significant.

Results

Effect of Manipulation of pH_o or pH_i on $Ins(1,4,5)P_3$ Production. Previously we showed that pure respiratory alkalosis (increase in pH resulting from low CO_2) increased $Ins(1,4,5)P_3$ more than metabolic alkalosis (increase in pH resulting from fixed base) in cerebral microvascular smooth muscle cells (7). In the current study, aCSF of increased pH_o (pH_o 7.7/pH_i 7.18) significantly increased $Ins(1,4,5)P_3$ from 123 ± 13 pmol/well to 307 ± 18 pmol/well as compared to the control (pH_o, 7.4/pH_i 7.18), or increased pH_i (pH_o 7.40/pH_i 7.29) aCSF (*n* = 8) (Fig. 2). Thus, elevation of pH_o increased $Ins(1,4,5)P_3$ but elevation of pH_i alone did not.

These measurements of $Ins(1,4,5)P_3$ were made at 30 sec of stimulation. In preliminary experiments, we deter-

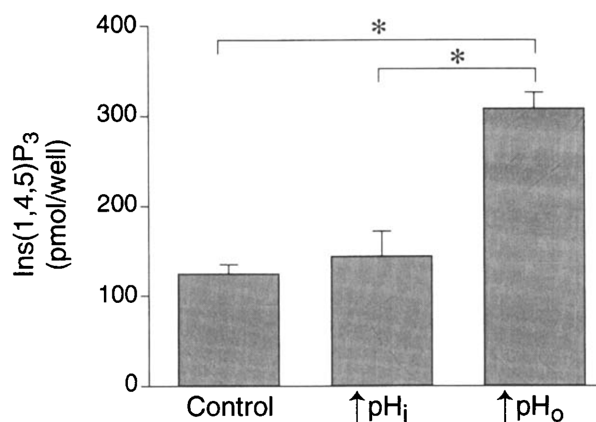

Figure 2. Effect of control, increased pH_i, or increased pH_o artificial cerebrospinal fluid on $Ins(1,4,5)P_3$ production in piglet cerebral microvascular smooth muscle cells as determined by protein binding assay. Stimulation time: 30 sec. Values are mean ± SEM. *$P \leq 0.0001$ as compared to control aCSF (*n* = 8).

mined that Ins(1,4,5)P_3 responses were rapid and transient. Thus, at both P_{CO_2} = 10 mm Hg and P_{CO_2} = 25 mm Hg, Ins(1,4,5)P_3 at 30 sec stimulation was double that at 1 and 5 min. At all time points, Ins(1,4,5)P_3 at P_{CO_2} = 10 mm Hg was about three times that after the same stimulation time at 25 mm Hg.

Effect of Control, Metabolic Alkalosis, or Respiratory Alkalosis on pH_i and $[Ca^{2+}]_c$. The mean basal pH_i of cells exposed to a CSF control (pH_o 7.4, PCO_2 36 mm Hg), was 7.18 ± 0.02 (n = 6). Figure 3A is a representative pH_i tracing of piglet cerebral microvascular smooth muscle cells perfused in control (0–250 sec) followed by exposure to metabolic alkalosis (250–500 sec). Metabolic alkalinization caused pH_i to rise to 7.36 over approximately 200 sec. Subsequently, the cells were reexposed to control conditions at 500 sec (open arrow) to show the reversibility of the metabolic alkalosis affect (n = 3). Figure 3B is a representative pH_i tracing of cerebral microvascular smooth muscle cells perfused with control aCSF (0–425 sec) followed by exposure to respiratory alkalosis (425–700 sec). There was a more rapid cytosolic alkalinization to 7.35 in response to respiratory alkalosis (70 sec to peak) as compared to metabolic alkalosis. Immediately upon reexposure to control aCSF (open arrow, 700 sec), pH_i returned to basal (n = 7). These data show that pH_i under control conditions is approximately 0.2 units lower than pH_o, and that changes in pH_o in the alkalotic direction directly influence pH_i as well. Given the lipid solubility of CO_2, the increase in pH_i to 7.35 from respiratory alkalosis was more acute and greater in magnitude than from metabolic alkalosis.

Figures 4A and 4B show $[Ca^{2+}]_c$ measurements during metabolic and respiratory alkalosis. In response to metabolic alkalosis, $[Ca^{2+}]_c$ increased from 28 ± 7 nM to 49 ± 10 nM (Figure 4A). Respiratory alkalosis caused an increase in $[Ca^{2+}]_c$ from 36 ± 8 nM to 75 ± 13 nM (Figure 4B). These data suggest that both pH_o and pH_i may play a role in stimulating an increase in $[Ca^{2+}]_c$ in response to alkalosis.

Effect of Manipulations of pH_o Relative to pH_i on $[Ca^{2+}]_c$. To determine whether pH_i or pH_o has a greater stimulatory effect on $[Ca^{2+}]_c$, stimulation of cerebral microvascular smooth muscle cells was performed with pH_i increased relative to pH_o. Figure 5A demonstrates that we were able to achieve a stable pH_o at a control value of 7.4 while increasing pH_i from 7.2 to 7.3 (n = 5). Figure 5B shows that pH_o was able to be increased while pH_i was maintained at a control value (n = 3). To maintain pH_i in the 7.18 ± 0.02 range, propionate (8 mM) was added to the solution. The weak acid, propionic acid, acidifies the poorly buffered intracellular environment while being effectively neutralized extracellularly. Propionate caused a decrease in pH_i to 7.14 which recovered to 7.20 within 100 sec. These data demonstrate that we were able to manipulate pH_o with respect to pH_i. Conditions producing control pH_o with increased pH_i did not increase $[Ca^{2+}]_c$ (data not shown). In contrast, increased pH_o, (7.66) with normal pH_i (7.18), increased $[Ca^{2+}]_c$ from 78 ± 29 nM to 114 ± 41 nM (Fig. 6). These data suggest that pH_o is more important in stimulation of elevated $[Ca^{2+}]_c$ in response to alkalosis than is pH_i.

Figure 7 shows the comparison of changes in $[Ca^{2+}]_c$ in response to respiratory alkalosis, metabolic alkalosis, control pH_o/increased pH_i, or increased pH_o/control pH_i. Increases in both pH_o and pH_i increase $[Ca^{2+}]_c$ more than when only pH_o is increased.

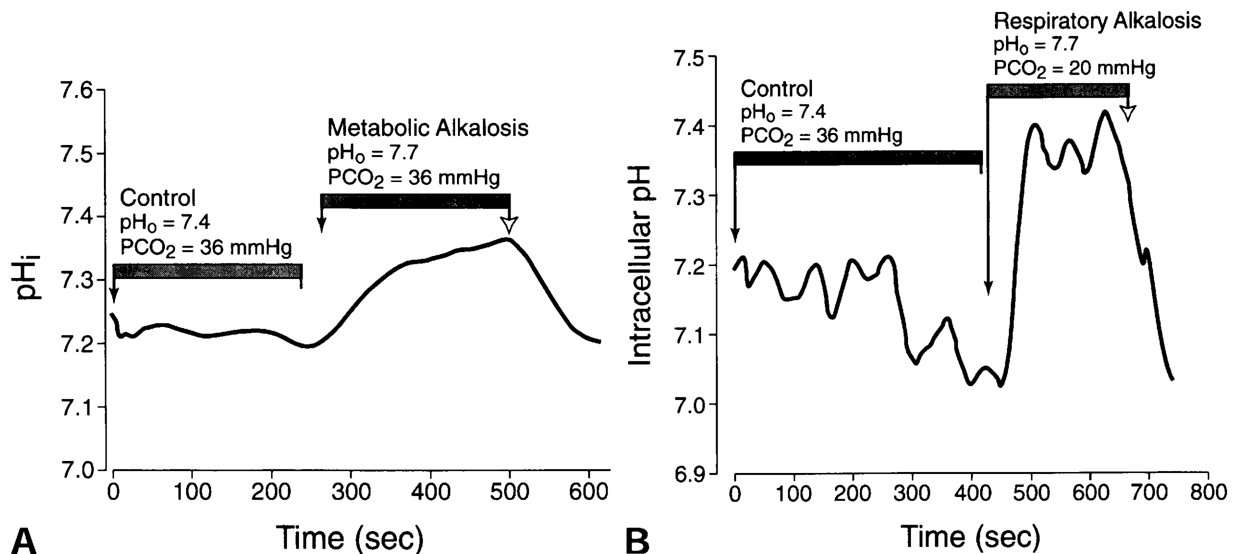

Figure 3A. Effect of metabolic alkalosis on pH_i in piglet cerebral microvascular smooth muscle cells. At 250 sec, the perfusate was changed to metabolic alkalosis (second filled-in arrow). At 500 sec, the perfusate was changed back to control (open arrow). Identical experiments were performed on three independent cultures in duplicate.

Figure 3B. Effect of respiratory alkalosis on pH_i in piglet cerebral microvascular smooth muscle cells. At 425 sec, the perfusate was changed to respiratory alkalosis (second filled-in arrow). At 700 sec, the perfusate was changed back to control (open arrow). Identical experiments were performed on seven independent cultures in duplicate.

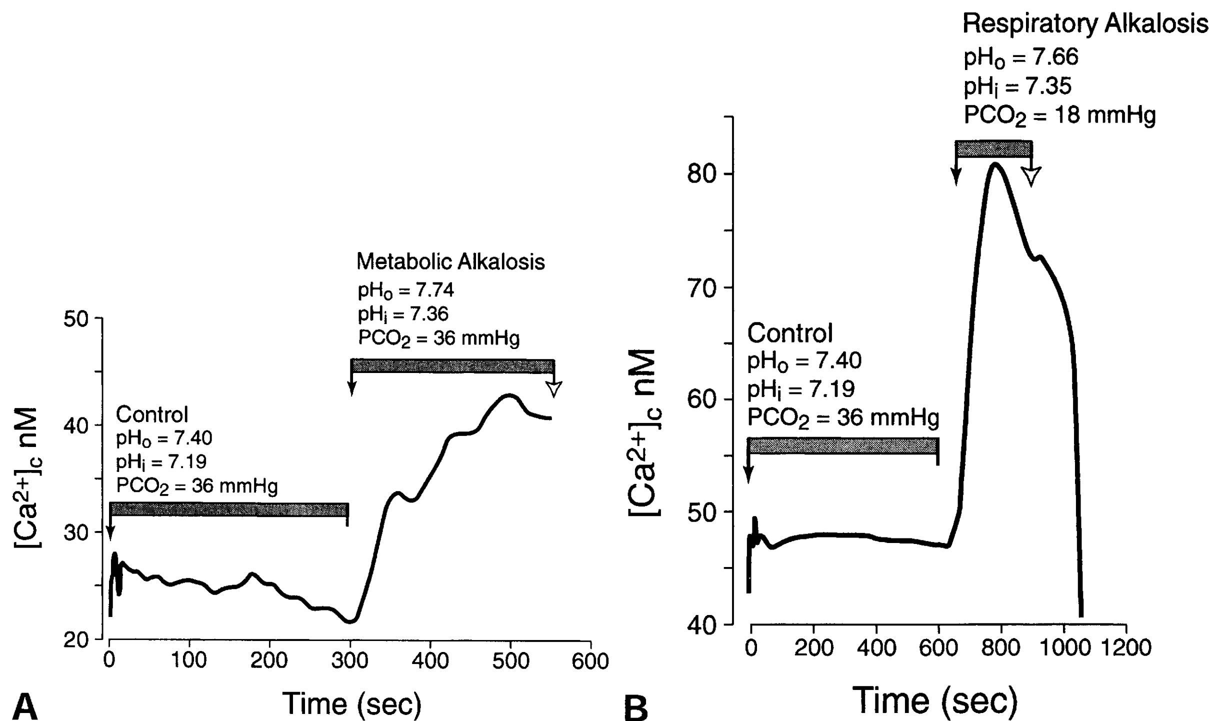

Figure 4A. Effect of metabolic alkalosis on $[Ca^{2+}]_c$ in piglet cerebral microvascular smooth muscle cells. At 300 sec, the perfusate was changed to metabolic alkalosis (second filled-in arrow). Identical experiments were performed on six independent cultures in duplicate, $^*P \leq 0.001$ versus control.

Figure 4B. Effect of respiratory alkalosis on $[Ca^{2+}]_c$ in piglet cerebral microvascular smooth muscle cells. At 600 sec, the perfusate was changed to respiratory alkalosis (second filled-in arrow). At 900 sec, the perfusate was changed back to control (open arrow). Identical experiments were performed on 12 independent cultures in duplicate, $^*P \leq 0.001$ versus control.

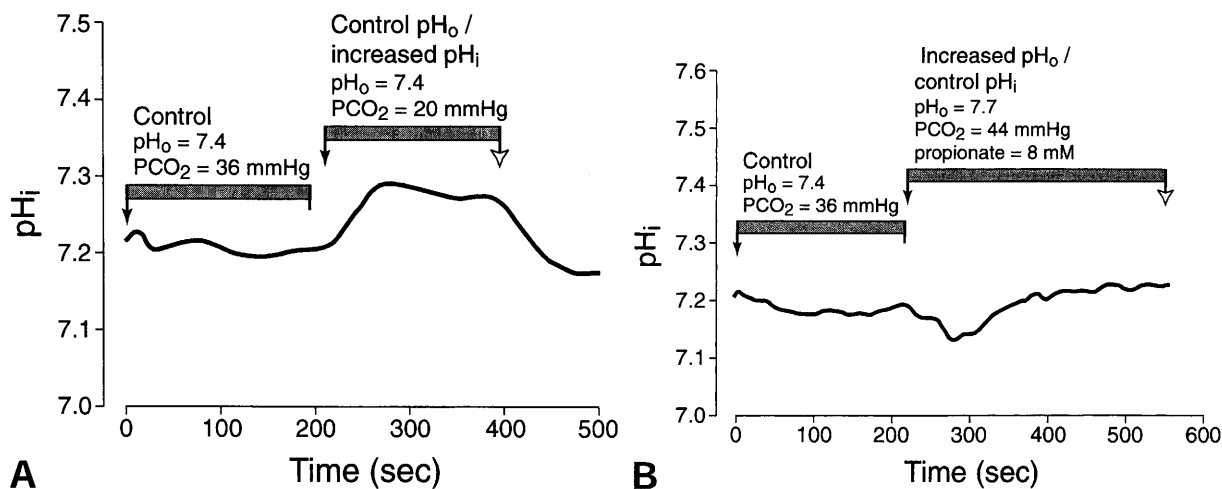

Figure 5A. Effect of control pH_o/increased pH_i on pH_i in piglet cerebral microvascular smooth muscle cells. At 200 sec, the perfusate was changed to control pH_o/increased pH_i (second filled-in arrow). At 400 sec, the perfusate was changed back to control (open arrow). Identical experiments were performed on five independent cultures in duplicate.

Figure 5B. Effect of increased pH_o/control pH_i on pH_i in piglet cerebral microvascular smooth muscle cells. At 200 sec, the perfusate was changed to increased pH_o/control pH_i (second filled-in arrow). Identical experiments were performed on seven independent cultures in duplicate.

Discussion

The new findings in this study are that respiratory alkalosis and metabolic alkalosis increase pH_i, $Ins(1,4,5)P_3$, and $[Ca^{2+}]_c$ in primary cultures of piglet cerebral microvascular smooth muscle cells assayed in a HCO_3^--containing medium. Additional new findings are that, when pH_o is kept constant and pH_i is increased, $Ins(1,4,5)P_3$ and $[Ca^{2+}]_c$ are not increased. When pH_o is increased and pH_i is kept constant, $Ins(1,4,5)P_3$ and $[Ca^{2+}]_c$ are increased; however,

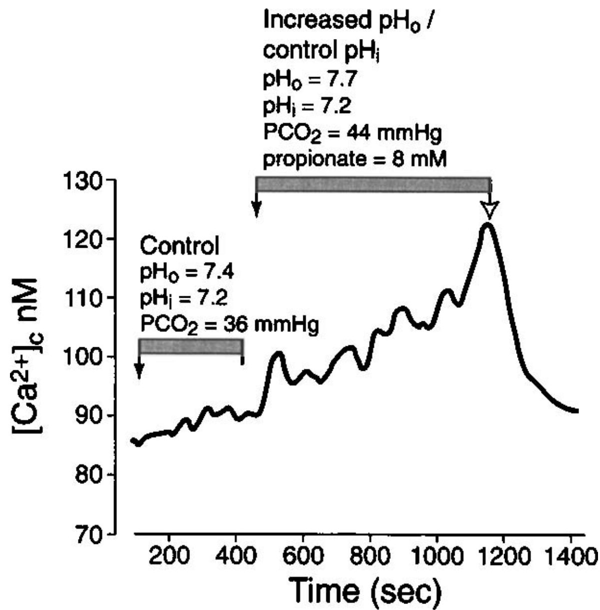

Figure 6. Effect of increased pH_o/control pH_i on $[Ca^{2+}]_c$ in piglet cerebral microvascular smooth muscle cells. At 400 sec, the perfusate was changed to increased pH_o/control pH_i (second filled-in arrow). At 1200 sec, the perfusate was changed back to control (open arrow). Identical experiments were performed on 14 independent cultures in duplicate $^*P \leq 0.0018$ versus control.

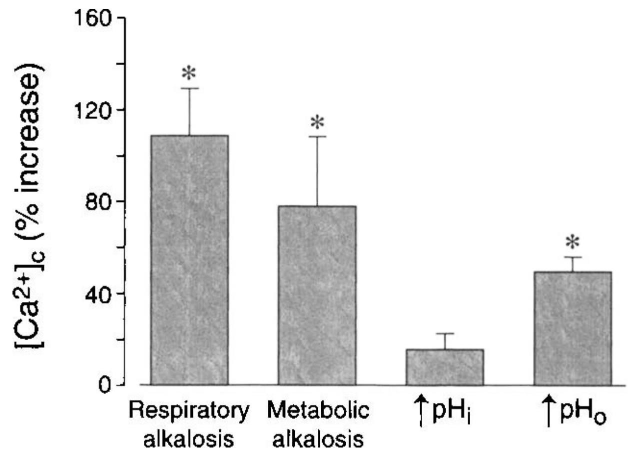

Figure 7. Comparison of the effect of metabolic alkalosis (n = 6), respiratory alkalosis (n = 12), control pH_o/increased pH_i (n = 6), and increased pH_o/control pH_i (n = 14) on $[Ca^{2+}]_c$ in piglet cerebral microvascular smooth muscle cells. $^*P \leq 0.05$.

$[Ca^{2+}]_c$ is not increased to the same extent as in respiratory alkalosis or metabolic alkalosis. These studies provide new findings on the role of pH_o versus pH_i on the calcium mobilizing second messenger, Ins(1,4,5)P_3, in the immature piglet cerebral circulation. Specifically, these data suggest that, at least in part, hypocapnia-induced cerebral vasoconstriction may involve stimulation of inositol phosphate turnover, and Ins(1,4,5)P_3-initiated $[Ca^{2+}]_c$ rises in piglet cerebral microvascular smooth muscle cells. However, whereas stimulation of this second messenger appears to be dependent on an increase in pH_o, a role for pH_i cannot be excluded completely.

The time course of changes in pH_i, IP_3, and $[Ca^{2+}]_c$ are consistent with pH_i-induced-IP_3-transient initiation of $[Ca^{2+}]_c$ elevation. With "respiratory alkalosis," pH_i increased abruptly whereas with "metabolic alkalosis," pH_i began increasing immediately with progressive elevation continuing over at least 4 min of exposure (Fig. 3A). While technical difficulties made precise time course measurements within the first 30 sec of stimulations ambiguous, upon exposure to "respiratory" acidosis, maximal elevation of Ins(1,4,5)P_3 occurred on or before 30 sec of stimulation. In contrast, the $[Ca^{2+}]_c$ rise appeared to trail the IP_3 with both stimuli but trailed pH_i with "respiratory alkalosis" and led pH_i with "metabolic alkalosis." Thus, with "respiratory alkalosis" $[Ca^{2+}]_c$ began to increase at about 30 sec and reached a peak at about 150 sec (Fig. 4B). With "metabolic alkalosis," $[Ca^{2+}]_c$ increased rapidly initially, as with "respiratory acidosis," and then rose more slowly as pH_i continued to rise (Figs. 3A and 4A). One could speculate that pH_i is having direct effects on Ca^{2+} channels that are more easily seen with metabolic alkalosis, because the IP_3 signal has passed.

We find it particularly intriguing that alkalosis increases inositol phosphate turnover and $[Ca^{2+}]_c$ in cerebral microvascular growth muscle cells, since it has been reported in coronary endothelial cells, neuroblastoma, and umbilical artery smooth muscle that *decreasing* pH_o stimulates phosphoinositide turnover and releases Ca^{2+} from intracellular stores (19). In endothelial cells, one can conceptualize the function of Ins(1,4,5)P_3-induced $[Ca^{2+}]_c$ elevation to enhance production of endothelial-derived relaxing factors that could induce relaxation of adjacent smooth muscle. However, the data from umbilical artery smooth muscle are puzzling since the $[Ca^{2+}]_c$ signal detected in response to declining pH would be expected to produce constriction. In rat aortic rings precontracted with norepinephrine, decreasing pH_o from 7.4 to 7.0 did result in decreased $[Ca^{2+}]_c$ as a result of intracellular Ca^{2+} sequestration (20).

Therapies for several critical illnesses employ hyperventilation to effect a change in vascular tone. In the brain, hypocapnia can cause intense vasoconstriction. For more than two decades, the control mechanisms mediating changes in cerebral resistance vessels have been thought to be linked to changes in pH and alteration of smooth muscle intracellular calcium concentrations (9, 12, 16, 21, 22). However, the relationships between pH (pH_i versus pH_o), CO_2 tension, and contractile force in vascular tissues are complex and dependent on the experimental methodology and tissue bed studied. For example, Yu *et al.* demonstrated that, in dog basilar artery rings, decreasing pH_i, at constant pH_o, did not change tension, but increasing pH_i, at constant pH_o, did increase tension (23). In contrast, Apkon *et al.* reported that contraction of cultured rat cerebral vascular smooth muscle cells in response to elevated pH is due solely to changes in pH_o and that pH_i changes actually produce changes in the opposite direction (24). Furthermore, the signal transduction mechanism(s) involved in the generation

of vasomotor tone in response to hypocapnia are poorly understood.

Arterial reactivity and contractility change dramatically during maturation (25, 26). In addition, it has been demonstrated that the ratio of calcium uptake to force production varies widely with age, artery size, and method of contraction in cerebral arteries. In our study, only primary cultures of cerebral microvascular smooth muscle cells were used, and the studies were performed on cells that had been in culture for the same number of days. This was done to minimize issues of differentiation that may occur with number of days in culture and to limit interexperiment variability.

Methodological differences exist for inducing alkalinization in vascular smooth muscle cells. One method for inducing cell alkalinization (and acidification) involves addition and washout of NH_4 to the perfusate. This technique produces a transient, but significant, intracellular alkalinization followed by a more prolonged intracellular acidification. However, the transients in pH_i from this technique and possible resultant effects on cytosolic calcium, made use of a CO_2/HCO_3^- more attractive and more physiologic. In this study, and in our previous investigations, we performed all experiments in CO_2/HCO_3^- buffered solutions. Use of CO_2/HCO_3^- solutions more accurately reflects the physiological milieu to which pial arteriolar resistance vessels are exposed (17). Regulation of pH_i is generally accomplished *via* the plasma membrane transporters, Na^+/H^+ exchange, Na^+-dependent Cl^-/HCO_3^- exchange, and Na^+-independent Cl^-/HCO_3^- exchange. These membrane transporters operate physiologically in a CO_2/HCO_3^- buffer system, and may also be affected by changes in intracellular second messenger activation. For example, increased cytosolic calcium from $Ins(1,4,5)P_3$ generation may cause activation of calcium-dependent protein kinase C species capable of altering plasma membrane Na^+/H^+ exchange (27). Therefore, we felt it important to perform our experiments in CO_2/HCO_3^- containing solution (16, 17, 28). Another methodologically important point is that we have determined the fluorescent dye, fura 2, to be both pH and temperature sensitive (3). In contrast to some studies that report no need for K_d correction when pH_i is above 7.0, we found pH_i greatly affects $[Ca^{2+}]_c$ readings well above pH_i of 7.0 (3).

This study and previous investigations from our laboratory demonstrated that changing pH_o concomitantly changes pH_i unless external measures are taken to control pH_i relative to pH_o (29). We found that an increase in pH_o increases pH_i during normocapnia, and that this response is amplified in the presence of hypocapnia. These effects are demonstrated in Figures 3A and 3B, metabolic alkalosis and respiratory alkalosis, respectively. Data regarding the cell membrane's capability of partitioning extracellular and intracellular pH, and the significance of such a separation, are conflicting (12, 29–31). Studies differ on whether pH_o or pH_i is more important in causing a given cellular response. The data in this study show that the $[Ca^{2+}]_c$ levels are further accentuated by an increase in pH_i compared to pH_o alone. Functionally, this finding suggests that both the extracellular and intracellular milieu could be involved in changes in $[Ca^{2+}]_c$, even though pH_o appears to be most important.

The temporal nature of a change in pH_i relative to pH_o may be critical to how and when second messenger systems may influence $[Ca^{2+}]_c$. Daugirdas *et al.* showed that, in rat aortic vascular smooth muscle cells, pH_i changes follow alterations in pH_o in a delayed fashion (32). In our cells, both pH_i and $[Ca^{2+}]_c$ steadily increased upon changing the extracellular buffer to an alkalotic mixture. Generally, the maximum increase was achieved in less than 2 min. We also found that pH_i followed changes in pH_o in a delayed fashion. This may in part account for the steady, but delayed, rise in our $[Ca^{2+}]_c$ levels. A delay in pH_i may then be causally related to changes in calcium flux even if pH_i activates second messengers such as $Ins(1,4,5)P_3$ in less than 1 min (8).

In the increased pH_o/control pH_i experiments, the significant increase in $[Ca^{2+}]_c$ contrasts with the insignificant responses to control pH_o increased pH_i (Figs. 5A and 5B). The greater increase in $[Ca^{2+}]_c$ in response to respiratory alkalosis and metabolic alkalosis, contrasts with the delayed, but steady rise in $[Ca^{2+}]_c$ from increased pH_o/control pH_i (Figs. 4A, 4B and 6). Such a delayed effect may in part be explained by the addition of propionic acid in our system producing a transient decrease in pH_i. That is, after the nadir in pH_i, $[Ca^{2+}]_c$ increased. Significant increases in $Ins(1,4,5)P_3$ levels occurred only when pH_o was increased, regardless of pH_i. While it would be tempting to suggest that pH_o may be the sole determinant of $[Ca^{2+}]_c$ release as shown in Figure 6, the percentage increase in $[Ca^{2+}]_c$ was most pronounced in the respiratory alkalosis group (Fig. 7). This finding prevents dismissing a role for pH_i in the hypocapnia-induced $[Ca^{2+}]_c$ signal.

To further characterize the mechanisms by which hypocapnia may effect a change in cerebrovascular tone, several additional studies may be entertained. Such studies include: 1) investigation of the effect of changes in CO_2 tension and manipulation of pH_o and pH_i on calcium efflux and influx; 2) examination of intracellular versus extracellular calcium sources and potential voltage-dependent and receptor-operated calcium channels involved in the $[Ca^{2+}]_c$ signal to hypocapnia; and 3) measurement of $[Ca^{2+}]_c$ signal following inhibition of phospholipase C and $Ins(1,4,5)P_3$ in the presence of decreasing tensions of CO_2.

The authors thank Alex Fedinec for technical assistance, Linda Rawlison, Danny Morse and Laura Malinick for illustration assistance, and Margaret Brown for electron micrographs of cerebral microvascular smooth muscle cells.

1. Eidson TH, Edrington JL, Albuquerque MLC, Zuckerman SL, Leffler CW. Light/dye microvascular injury eliminates pial arteriolar dilation in hypotensive piglets. Pediatr Res **37:**10–14, 1995.

2. Hsu P, Shibata M, Leffler CW. Prostanoid synthesis in response to high CO_2 in newborn pig brain microvascular endothelial cells. Am J Physiol **264**(Heart Circ Physiol 33):H1485–H1492, 1993.
3. Hsu P, Albuquerque MLC, Leffler CW. Mechanisms of hypercapnia stimulated PG production in piglet cerebral microvascular endothelial cells. Am J Physiol **268**(Heart Circ Physiol 37):H591–H603, 1995.
4. Leffler CW, Mirro R, Armstead WM, Shibata M. Topical arachidonic acid restores pial arteriolar dilation to hypercapnia of postischemic newborn brain. Am J Physiol **263**(Heart Circ Physiol 32):H746–H751, 1992.
5. Leffler CW, Mirro R, Shibata M, Parfenova H, Armstead WM, Zuckerman SL. Effects of indomethacin on cerebral vasodilator responses to arachidonic acid and hypercapnia in newborn pigs. Pediatr Res **33**:609–614, 1993.
6. Mirro R, Pharris LJ, Armstead WM, Shibata M. Effects of indomethacin on newborn pig pial arteriolar responses to PCO_2. J Appl Physiol **75**(3):1300–1305, 1993.
7. Albuquerque MLC, Lowery-Smith L, Hsu P, Parfenova H, Leffler CW. Low CO_2 stimulates inositol phosphate turnover and increased inositol 1,4,5-trisphosphate levels in piglet cerebral microvascular smooth muscle cells. Proc Soc Exp Biol Med **209**:14–19, 1995.
8. Somlyo AP. Signal transduction and regulation in smooth muscle. Nature **372**:231–236, 1994.
9. Kontos HA, Raper AJ, Patterson JL. Analysis of vasoactivity of local pH, PCO_2, and bicarbonate on pial vessels. Stroke **8**(3):358–360, 1977.
10. Austin C, Wray S. Extracellular pH signals affect rat vascular tone by rapid transduction into intracellular pH changes. J Physiol **466**:1–8, 1993.
11. Harder DR, Madden JA. Cellular mechanisms of force development in cat middle cerebral artery by reduced PCO_2. Pflugers Arch **403**:402–404, 1985.
12. West GA, Leppla DC, Simard JM. Effects of external pH on ionic currents in smooth muscle cells from the basilar artery of the guinea pig. Circ Res **71**:201–209, 1992.
13. Krampetz IK, Rhoades RA. Intracellular pH: Effect of pulmonary arterial smooth muscle. Am J Physiol **260**(Lung Cell Mol Physiol 4):L516–L521, 1991.
14. Thomas JA, Buchsbaum RA, Zimniak A, Racker E. Intracellular pH measurement in Ehrlich ascites tumor cell utilizing spectroscopic probes generated *in situ*. Biochemistry **18**:2210–2218, 1979.
15. Wang W, Hayama N, Robinson TV, Kramer RE, Schneider EG. Effect of osmolality on cytosolic free calcium and aldosterone secretion. Am J Physiol **262**(Endocrinol Metab 25):E68–E75, 1992.
16. Batlle DC, Peces R, LaPointe MS, Ye M, Daugirdas JT. Cytosolic free calcium regulation in response to acute changes in intracellular pH in vascular smooth muscle. Am J Physiol **264**(Cell Physiol 33):C932–C943, 1993.
17. Daugirdas JT, Batlle DC. Interactions of pH_i and Ca_i^{2+} in vascular smooth muscle tissue. J Lab Clin Med **119**:667–675, 1992.
18. Hollingworth S, Zhao M, Baylor SM. The amplitude and time course of the myogenic free $[Ca^{2+}]$ transient in fast-twitch fibers of mouse muscle. J Gen Physiol **108**:455–469, 1996.
19. Smith JB, Dwyer SD, Smith L. Lowering extracellular pH evokes inositol phosphate generation and calcium mobilization. J Biol Chem **264**:8723–8728, 1989.
20. Loutzenhiser R, Matsumoto V, Okawq W, Epstin IM. H^+-induced vasodilation of rat aorta is mediated by alterations in intracellular calcium sequestration. Circ Res **67**:426–439, 1990.
21. Betz E, Enzenross HG, Vlahov V. Interaction of H^+ and Ca^{2+} in the regulation of local pial vascular resistance. Pflugers Arch **343**:79–88, 1973.
22. Tian R, Vogel P, Lassen NA, Andreasen MF, Alkjaer C. Role of extracellular and intracellular acidosis of hypercapnea-induced inhibition of tension of isolated rat cerebral arteries. Circ Res **76**:269–275, 1995.
23. Yu J, Ong Y, Zheng J, Cragoe EJ, Bose D, Bose R. Effects of anion exchange on intracellular pH and tension in basilar artery of dogs. Am J Physiol **271**(Heart Circ Physiol 40):H1912–H1917, 1997.
24. Apkon M, Weed RA, Boron WF. Motor responses of cultured rat vascular smooth muscle cells to intra- and extracellular pH changes. Am J Physiol **273**(Heart Circ Physiol 42):H434–H445, 1997.
25. Akopov SE, Zhang L, Pearce WJ. Intracranial-extracranial differences in the Ca^{2+} sensitivity of rabbit arteries. Proc Soc Exp Biol Med **214**:76–82, 1997.
26. Pearce WJ, Elliott SR. Maturation enhances the sensitivity of ovine cerebral arteries to the ATP-sensitive potassium channel activator lemakalim. Pediatr Res **35**:729–732, 1994.
27. Ganz MB, Boyarsky G, Sterzel RB, Boron WF. Arginine vasopressin enhances pH_i regulation in the presence of HCO_3^- by stimulating three acid:base transport systems. Nature **337**:648–651, 1989.
28. Kikeri D, Zeidel ML, Ballerman BJ, Brenner BM, Herbert SC. pH regulation and response to AVP in A10 cells differ markedly in the presence versus absence of CO_2-HCO_3. Am J Physiol **259**(Cell Physiol 28):C471–C483, 1993.
29. Hsu P, Haffner J, Albuquerque MLC, Leffler CW. pH_i in piglet cerebral microvascular endothelial cells: Recovery from an acid load. Proc Soc Exp Biol Med **212**:256–262, 1996.
30. Matthews JG, Graves JE, Poston L. Relationships between pH_i and tension in isolated rat mesenteric resistance arteries. J Vasc Res **29**:330–340, 1992.
31. Nagesetty R, Paul RJ. Effects of pH_i on isometric force and $[Ca^{2+}]_i$ in porcine coronary artery smooth muscle. Cir Res **75**:990–998, 1994.
32. Daugirdas JT, Arrieta J, Ye M, Flores G, Batlle DC. Intracellular acidification associated with changes in free cytosolic calcium: Evidence for Ca^{2+}/H^+ exchange *via* a plasma membrane Ca^{2+} ATPase in vascular smooth muscle cells. J Clin Invest **95**:1480–1489, 1995.