Effects of Serotonin on Intracellular pH and Contraction in Vascular Smooth Muscle


Serotonin (5-HT) and other contractile agonists stimulate Na⁺-H⁺ exchange in vascular smooth muscle. Since intracellular alkalization, per se, stimulates contraction, we tested whether 5-HT-induced contraction was associated with an increased pH. In HCO₃⁻-free buffer (pH 7.4), 5-HT (10⁻⁵ M) increased pH, as measured by 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein fluorescence, from 7.10±0.03 to 7.34±0.03 (p<0.01) in primary cultures of canine femoral artery vascular smooth muscle cells grown to confluence in the presence of 10% fetal calf serum. In HCO₃⁻ buffer (24 mM, pH 7.4), resting pH was 7.26±0.04 (p<0.01 versus HCO₃⁻-free buffer) but was not altered by 5-HT. In both types of buffer, 5-HT stimulated 5-(N-ethyl-N-isopropyl)amiloride-sensitive ²²Na⁺ uptake (Na⁺-H⁺ exchange). In HCO₃⁻ buffer and in Na⁺ and HCO₃⁻-free buffer, 5-HT increased 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid-sensitive ³⁶Cl⁻ uptake, suggesting that 5-HT stimulated Na⁺-independent Cl⁻-HCO₃⁻ and Cl⁻-Cl⁻ exchange activities, respectively. Individual vascular smooth muscle cells were then cultured on rat tail tendon collagen gels in the presence of 0.5% fetal calf serum, and cell length and pH were measured by video and epifluorescence microscopy. 5-HT contracted cells in a dose-dependent, reversible, and ketanserin-inhibitable manner. These cells, like cells grown in 10% fetal calf serum, exhibited Na⁺-H⁺ and Na⁺-independent Cl⁻-HCO₃⁻ exchange activity. In HCO₃⁻ buffer, 5-HT contracted cells without an associated change in pH. We concluded the following: 1) 5-HT stimulated both Na⁺-H⁺ and Na⁺-independent Cl⁻-HCO₃⁻ exchange activities in cultured vascular smooth muscle cells in parallel. 2) As a result of enhanced H⁺ and HCO₃⁻ efflux, pH was not altered. 3) In the presence of HCO₃⁻, 5-HT-induced contraction was not associated with a change in pH. (Circulation Research 1992;71:1294-1304)

Key Words: Na⁺ transport • Cl⁻ transport • HCO₃⁻ transport • 5-(N-ethyl-N-isopropyl)amiloride • 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid

The signal transduction systems responsible for excitation–contraction coupling in vascular smooth muscle are not completely understood. Several investigators have reported that in HCO₃⁻–free media contractile agonists, such as angiotensin II, vasopressin, endothelin, and thrombin, stimulate Na⁺-H⁺ exchange activity in the sarcolemma with resultant intracellular alkalization. Although these agonists stimulate contraction via mechanisms unrelated to changes in pH, it has been suggested that agonist-stimulated contraction may be related to an increase in pH. This hypothesis is in keeping with other studies showing that artificially raising pH increases vascular smooth muscle tone.

Whether Ca²⁺-mobilizing agonists are capable of inducing intracellular alkalization in a more physiological HCO₃⁻–containing buffer is controversial. In some cells, certain of these agonists increase pH, whereas in others, the same agonists cause either no change in pH or a decrease in pH. Although these agents may stimulate Na⁺-H⁺ exchange activity, recent studies have reported that the same agents also stimulate Na⁺-independent Cl⁻-HCO₃⁻ exchange activity. Since the latter transport system mediates HCO₃⁻ efflux, the combined effect of stimulating the activity of both transport systems may be that pH does not increase and in fact may fall. Indeed, several recent studies performed with cultured vascular smooth muscle cells and mesangial cells have shown that selected contractile agonists increase pH in the nominal absence of HCO₃⁻ but fail to do so in media that contain HCO₃⁻.

The majority of previous studies that have examined pH regulation in vascular smooth muscle and the vascular smooth muscle–like mesangial cell were performed with passaged cultured cells, which usually have different phenotypic characteristics from cells in intact tissue. In fact, vascular smooth muscle cells under such culture conditions have usually lost their ability to contract. The present study was designed to test the hypothesis that serotonin (5-HT)–induced contraction in vascular smooth muscle is associated with intracellular alkalization. We have previously shown that 5-HT induced contraction of primary cultured canine saphenous vein vascular smooth muscle cells and stimulated dimeth-
yalimloride-sensitive Na⁺ influx in primary cultured canine femoral artery vascular smooth muscle cells. In the present study, we measured the effects of 5-HT on contraction and pH in individual primary cultured canine femoral artery vascular smooth muscle cells and found that 5-HT contracted cells without changing pH. Similarly, we also demonstrated that 5-HT did not affect pH in confluent monolayers of these cells. The data indicated that 5-HT simultaneously stimulated Na⁺-H⁺ and Na⁺-independent Cl⁻-HCO₃⁻ exchange activities.

Materials and Methods

Cell Culture

Adult mongrel dogs of either sex were killed with intravenous pentobarbital sodium, and the femoral arteries were dissected free. Primary cultures of vascular smooth muscle cells were prepared as previously described. In brief, the media of the arteries were minced and incubated at 37°C in a solution containing elastase (type V, Sigma Chemical Co., St. Louis, Mo.) and collagenase (type I, Worthington Biochemical Corp., Freehold, N.J.). After 2 hours, the enzyme solution was discarded and replaced with fresh solution, and the tissue was incubated for an additional 2 hours. The dispersed cells were pelleted and suspended in Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories, Grand Island, N.Y.) that contained 10% fetal calf serum (FCS, Cyclone), 1% glutamine, and 1% PS (10,000 units/ml penicillin, 10 mg/ml streptomycin, Sigma). To grow cells on coverslips, the cell suspension was adjusted to 1.7x10⁵ cells/ml, and 0.3 ml was placed on the surface of 1.5x22-mm plastic coverslips (Thermanox, Miles). To grow cells on the bottom of culture dishes, the suspension of dispersed cells was adjusted to 2x10⁵ cells/ml, and 1 ml was placed in 35-mm plastic dishes (Falcon). The coverslips and dishes were placed in a humidified tissue culture incubator maintained at 37°C and equilibrated with 5% CO₂-95% air. After 48 hours and every 48 hours thereafter, the media were replaced with 1 ml fresh media. The cells reached confluence between the 10th and 15th day. The identity of the cultured cells as smooth muscle cells was confirmed, as previously described, by the "hill and valley" pattern of cell growth and by an actin-to-myosin heavy chain ratio characteristic of intact vascular smooth muscle.

Contraction Assay

Dispersed vascular smooth muscle cells from canine femoral artery were obtained by the enzyme digestion method described above. The cells were washed three times in Hanks' balanced salt solution (GIBCO) and suspended to a density of 2x10⁵ cells/ml in DMEM that contained 0.5% FCS, 1% glutamine, and 1% PS. One milliliter of this suspension was placed in 35-mm culture dishes on top of rat tail tendon collagen gels, which were prepared as follows. Sprague-Dawley rat tail tendons were sterilized in 70% ethanol for 4 hours, minced, and extracted with 0.1% acetic acid for 48 hours at 4°C. The protein concentration of the supernatant was adjusted to 0.15 mg/ml and titrated to pH 8.0 with NaOH at 4°C. One milliliter was placed in 35-mm culture dishes at room temperature. The gels formed within 20 minutes. The gels were incubated with DMEM overnight before seeding with dispersed vascular smooth muscle cells.

After seeding with cells, the dishes were incubated in the tissue culture incubator described above. After 48 hours and every 48 hours thereafter, the media were replaced with 1 ml of the same fresh media. After 5–8 days, the dishes were placed on the heated (37°C) stage of a Nikon Diaphot inverted-phase contrast microscope. The culture medium was replaced with a physiological salt solution (PSS) that contained (mM) NaCl 140, KCl 4, CaCl₂ 2, MgCl₂ 1, glucose 5, and HEPES-Tris 10, pH 7.4 (HEPES PSS). After 20 minutes, a field of at least 10 cells was photographed at x200 to obtain baseline images. The medium was replaced with the desired experimental solution, and at the indicated times, another photograph was taken of the same field. The lengths of the longest axes of 10 arbitrarily chosen cells were measured in the first photograph, and lengths of the same cells were measured in subsequent photographs. For each cell, the percent contraction from the baseline length was calculated, and these values were averaged for all 10 cells, as previously described. This average was taken as the response for that particular culture dish.

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Effect of serotonin (5-HT) on pH of confluent monolayers of vascular smooth muscle cells from canine femoral artery in HCO₃⁻-free buffer. Confluent monolayers were grown on coverslips and mounted in a cuvette inside a fluorescent spectrophotometer that was perfused with physiological salt solution containing HEPES (HEPES PSS). pH was continuously measured by monitoring the fluorescence emission of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein. Left panel: A representative pHₐ-time course before and after adding 10⁻⁵ M 5-HT to the perfusate. Right panel: Bar graph showing steady-state pH, of confluent monolayers under baseline conditions in HEPES PSS (n=8), after 20 minutes of continued perfusion with HEPES PSS (n=4), and after 20 minutes of perfusion with 10⁻⁵ M 5-HT in HEPES PSS (n=4). *p<0.05 versus 20-minute control.
Na\(^+\) and Cl\(^-\) Uptake

The uptakes of Na\(^+\) or Cl\(^-\) in the presence and absence of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) or 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), respectively, were assayed as previously described. \(^{30,31}\) Confluent cells grown in the presence of 10% FCS in plastic dishes were preincubated at 37°C for 45 minutes in 1 ml HEPES PSS (Figure 2A) or HCO\(_3^-\) PSS (Figures 2B and 3A) that contained (mM) NaCl 116, NaHCO\(_3\) 24, KCl 4, CaCl\(_2\) 2, MgCl\(_2\) 1, and glucose 5, at 5% CO\(_2\)-95% O\(_2\). The cells were then incubated in 0.7 ml of solutions of the same composition containing 1.0 \(\mu\text{Ci}\ {}^{22}\text{Na}^+\) plus 1 mM ouabain (Figure 2), or 0.5 \(\mu\text{Ci}\ {}^{36}\text{Cl}^-\) (Figure 3A). In one experiment (Figure 3B), \(^{36}\text{Cl}^-\) uptake was measured in the absence of HCO\(_3^-\) and Na\(^+\). The cells were depleted of HCO\(_3^-\) and Na\(^+\), and pH\(_i\) was preset to 7.26 by preincubating them for 2 hours in HCO\(_3^-\)- and Na\(^+\)-free media containing 140 mM K\(^+\), pH 7.26, plus 7 \(\mu\text{M}\) nigericin, a K\(^+\)−H\(^+\) exchanger. Some of the cells in the experiment shown in Figure 3B were Cl\(^-\)-depleted by substituting gluconate \(^-\) for Cl\(^-\) in the preincubation media. \(^{22}\text{Na}^+\) and \(^{36}\text{Cl}^-\) uptakes were terminated after 2 minutes, and the cells were washed by rapidly rinsing the dishes six times with 5.5 ml ice-cold 100 mM MgCl\(_2\). The cells were dissolved in 1 ml of 1 M NaOH. \(^{22}\text{Na}^+\) or \(^{36}\text{Cl}^-\) content was measured by liquid scintillation spectroscopy, and protein was measured by the method of Lowry. In each experiment, the isotopic uptake solution was applied to cells and then removed 1–2 seconds later, and the cells were washed, dissolved in NaOH, and counted to estimate binding and/or trapping of isotope by the cells and plastic dishes. This value was subtracted from all uptake values. Each isotope uptake experiment was performed in triplicate at least four times.

pH\(_i\), Measurements of Confluent Cells Grown on Coverslips

pH\(_i\) was measured as previously described. \(^{30,31}\) A coverslip with confluent cells was placed in a polystyrene cuvette inside a fluorescent spectrophotometer (model LS-3B, Perkin-Elmer Corp., Norwalk, Conn.) so that the coverslip was anchored at its bottom and top and sat at a 45° angle to the excitation beam. The cuvette, which was held in a thermostatic holder, was perfused with solution at 37°C containing HEPES PSS or HCO\(_3^-\) PSS. A peristaltic perfusion pump delivered solution at 3 ml/min into the bottom of the cuvette via
polyethylene tube that ran on the opposite side of the coverslip from the excitation light and pumped solution at 3 ml/min from the top of the cuvette. The storage flasks of solutions were kept at 37°C, and in the case of HCO₃⁻ PSS, the fluid was vigorously bubbled with 5% CO₂-95% O₂. Gas-impermeable tubing was used for flow to and from the peristaltic pump. The pump itself was enclosed in a plastic bag that was inflated with 5% CO₂-95% O₂ to avoid diffusion of CO₂ out of the silicone rubber tubing of the pump. The pH values of the perfused solutions in the cuvette were the same as in the storage flasks. Before the cells were exposed to pH-sensitive dye, they were excited at 450 nm, the light at 530 nm was monitored, and the intensity was blanked to zero units. The excitation beam was changed to 506 nm, and the emission signal at 530 nm was arbitrarily set to 150 units. This value was subtracted from all subsequent values obtained after cells had been loaded with dye to obtain fluorescence data from the dye itself without the contribution of autofluorescence from the cells or other components in the cuvette.

To load the cells with the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), the perfusion was stopped, and 10 μM of the permeable acetoxymethyl derivative, BCECF AM (Molecular Probes Inc., Eugene, Ore.) was added to the cuvette from a stock of 2 mM in dimethyl sulfoxide. The cells were preincubated for 30 minutes at 37°C without perfusion, and in the case of HCO₃⁻ PSS, the cuvette was gently bubbled with 5% CO₂-95% O₂. Perfusion was restarted at 3 ml/min, and fluorescence was continuously monitored by activating at 506 nm and recording the emission at 530 nm with a strip-chart recorder. The excitation wavelength was changed to 450 nm every minute for 5 seconds, and the emission at 530 nm was recorded. pH was determined by the fluorescence ratio

FIGURE 3. Bar graphs showing the effect of serotonin (5-HT) and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) on ³⁶Cl⁻ uptake by confluent monolayers of vascular smooth muscle cells. Panel A: Confluent monolayers of cells were preincubated for 45 minutes with physiological salt solution containing HCO₃⁻ (HCO₃⁻ PSS) or for 40 minutes with HCO₃⁻ PSS and then 5 minutes with HCO₃⁻ PSS plus 10⁻³ M 5-HT. The preincubation solutions were removed, and 0.7-ml solutions of the same composition plus ³⁶Cl⁻ (0.5 μCi/ml) with or without 0.2 mM DIDS were added to the dishes for an additional 2 minutes. The experiment was performed in an atmosphere of humidified 5% CO₂-95% O₂. The graph shows ³⁶Cl⁻ uptake with or without 5-HT in the absence (left bars) and presence (middle bars) of DIDS. The DIDS-sensitive component of ³⁶Cl⁻ uptake with and without 5-HT is also shown (right bars). Values are expressed as a percentage of ³⁶Cl⁻ uptake in the absence of DIDS and 5-HT, which averaged 73.0 nmol/mg protein for 2 minutes. Data are from four separate experiments. Panel B: Confluent monolayers of cells were preincubated for 2 hours with (mM) KCl 140, CaCl₂ 2, and MgCl₂ 1 (left and middle bars) or potassium gluconate 140, calcium gluconate 10, and magnesium gluconate 5 (right bars) plus glucose 5, and HEPES-Tris 10, pH 7.26, along with 7 μM nigericin. The cells were preincubated with or without 10⁻³ M 5-HT for the last 5 minutes. The preincubation solutions were removed, and the 2-minute uptake of 11.5 mM ³⁶Cl⁻ was assayed by incubating the cells in the presence of (mM) K⁺ 140, Ca²⁺ 10, Mg²⁺ 5, N-methylglucamine⁺ 1.5, gluconate⁻ 160, glucose 5, and HEPES-Tris 10, pH 7.4, along with 10 mg/ml bovine serum albumin, in the absence and presence of 10⁻³ M 5-HT and in the absence (left and right bars) or presence (middle bars) of 0.2 mM DIDS. Values are expressed as a percentage of ³⁶Cl⁻ uptake by Cl⁻-replete cells in the absence of 5-HT and DIDS, which averaged 21.1 nmol/mg protein for 2 minutes. Data are from four separate experiments. *p<0.05 vs. control.
method (530-nm emission intensity at 506-nm excitation divided by that at 450-nm excitation). A fluorescence ratio-pH, calibration curve was obtained at the end of each experiment by determining the fluorescence ratios at pH values of 7.5, 7.25, 7.0, and 6.5. pH was set by incubating the coverslip at 37°C in (mM) KCl 140, CaCl2 2.0, MgCl2 1, glucose 5, and HEPES 16 (buffered to the desired pH with Tris), along with 7 μM nigericin. Unknown pH, values were determined from a linear regression line obtained by the least-squares fit of the calibration data.

Simultaneous pH, and Length Measurements of Individual Cells

pH, and length of individual cells were simultaneously measured using a custom-built epifluorescence microscope coupled to a photomultiplier tube and a video camera. This instrument has been described in detail elsewhere. In brief, cells on the stage of a Nikon Diaphot inverted microscope were illuminated with an excitation beam reflected to the cells by a 510-nm high-pass dichroic mirror. The beam originated from a mercury arc lamp and passed through a filter wheel and shutter assembly that alternated 100-msec pulses of light at 500 and 448 nm. The cells were also illuminated with red light from the bright field lamp to obtain a visual image. The fluorescence emission and red image of the cell were separated by a short-pass dichroic mirror that reflected wavelengths of 598 nm and above to a Newvicon-type video camera and transmitted the shorter wavelength BCECF emission through a 530-nm filter to a photomultiplier tube. A computer controlled the filter wheel and shutter, collected the output from the photomultiplier, and analyzed these data.

Cells grown on a collagen gel in the presence of 0.5% FCS were preincubated with HEPES PSS for 20 minutes at 37°C on the stage of the microscope. A single cell was identified on the video monitor and centered in the field of view, and a variable aperture proximal to the photomultiplier tube was adjusted so that emissions from all other cells were excluded from the photomultiplier tube. Background intensity of emitted light (530 nm) on exposure to the two excitation wavelengths (500 and 448 nm) was stored in the computer. Cells were preincubated with 10 μM BCECF AM in HEPES PSS for 30 minutes at 37°C. The culture dish was then perfused with HCO3- PSS at 3 ml/min. The fluorescence measurements were serially obtained and stored by the computer, the background fluorescence was subtracted, and the 500/448 intensity ratio was calculated by the computer. The image of the cell was continuously recorded on videotape. The perfusion solution was changed to HCO3- PSS plus 10-5 M 5-HT once a stable fluorescence signal was obtained. As was the case for pH, measurements of cells grown on coverslips, pH, of the single cell was determined by the fluorescence ratio method. A fluorescence ratio-pH, calibration curve was obtained for the cell at the end of each experiment by use of the high potassium–nigericin technique as described above. The length of the longest axis of the cell was measured on the video monitor at selected times during the course of the experiment.

5NaCl (1,598 mCi/mg) and 36Cl (15.6 mCi/g) were obtained from New England Nuclear, Boston. EIPA was synthesized as previously described. Ketanserin and DIDS were obtained from Sigma. Statistical analysis was performed on paired and unpaired data by use of Student’s t test.

Results

Effect of 5-HT on Acid-Base Transport and pH, in Confluent Monolayers

The effect of the contractile agonist 5-HT on pH, of confluent monolayers in HEPES PSS is shown in Figure 1. An effect was observed within 4 minutes (left panel). Baseline pH, averaged 7.10±0.03 and increased to 7.34±0.03 (p < 0.01) after a 20-minute exposure to 10-5 M 5-HT (right panel). pH, did not rise over the same time period in the absence of the agonist (right panel) or in the presence of both 10-3 M 5-HT plus 50 μM EIPA (data not shown).

To test directly whether 5-HT increased Na+-H+ exchange activity in these cells, the uptake of 140 mM 22Na+ by confluent monolayers was assayed in HEPES PSS in the presence and absence of 10-5 M 5-HT. This experiment was performed with and without 50 μM EIPA in the uptake media. 5-HT stimulated Na+ uptake in the absence of EIPA (Figure 2A, left bars) but not in its presence (Figure 2A, middle bars). As shown in the right bars of Figure 2A, the EIPA-sensitive component of Na+ uptake, which represents Na+-H+ exchange activity, was stimulated sixfold by 5-HT in HEPES PSS.

To determine whether Na+-H+ exchange activity in cells incubated in HCO3- PSS was also stimulated by 5-HT, we measured the effect of the agonist on 22Na+ uptake by confluent monolayers in the presence and absence of 50 μM EIPA. As shown in the left and middle bars of Figure 2B, 10-3 M 5-HT caused a small stimulation of 22Na+ uptake in the absence of EIPA but not in its presence. The EIPA-sensitive uptake of 22Na+, which represents Na+-H+ exchange activity, was stimulated threefold by 5-HT (right bars). The 5-HT–stimulated component of Na+-H+ exchange activity was smaller under these conditions than when cells were incubated with HEPES PSS (9.0±2.9 versus 30.8±4.3 nmol/mg protein for 2 minutes, p < 0.05, n = 4).

Na+-independent Cl--HCO3- exchange activity in HCO3- PSS was estimated by measuring the DIDS-sensitive uptake of 36Cl-. As shown in the left and middle bars of Figure 3A, 36Cl- uptake in confluent monolayers bathed in HCO3- PSS was stimulated by 10-3 M 5-HT in the absence of 0.2 mM DIDS but not in its presence. As shown in the right bar, the DIDS-sensitive uptake of 36Cl- was stimulated threefold by 5-HT. These data suggest that in HCO3- PSS, Na+-independent Cl--HCO3- exchange activity was stimulated by 5-HT.

We have previously shown that in the nominal absence of Na+ and HCO3-, the Na+-independent Cl--HCO3- exchanger in these cells mediates DIDS-sensitive Cl--Cl- exchange. To support the possibility that 5-HT stimulated the Na+-independent Cl--HCO3- exchanger, we tested whether 5-HT would stimulate Cl--Cl- exchange activity. The 2-minute uptake of 36Cl- was measured in cells that had been preincubated for 2 hours in Na+- and HCO3-–free solution. pH, was preset to 7.26 by the high K+-nigericin technique during the preincubation period. As shown in the left bars of Figure 3B, 10-3 M 5-HT stimulated 36Cl- uptake. In the
 presence of 0.2 mM DIDS (middle bars) or in Cl⁻-depleted cells (right bars), ³⁶Cl⁻ uptake was not stimulated by 10⁻⁵ M 5-HT. The data in Figure 3B demonstrate that 5-HT stimulated DIDS-sensitive Cl⁻-Cl⁻ exchange activity and support the notion that, in HCO₃⁻ PSS, HCO₃⁻ efflux via the Na⁺-independent Cl⁻-HCO₃⁻ exchanger was stimulated by 5-HT.

To determine whether the putative 5-HT-stimulated efflux of intracellular HCO₃⁻ from cells in confluent monolayers due to stimulated Na⁺-independent Cl⁻-HCO₃⁻ exchange activity would offset the efflux of protons consequent to 5-HT-stimulated Na⁺-H⁺ exchange activity, the effect of 5-HT on pHᵢ was measured in these cells bathed in HCO₃⁻ PSS. As shown in Figure 4, basal pHᵢ of confluent cells in HCO₃⁻ PSS was 7.26±0.02, which was greater than the pHᵢ of these cells bathed in HEPES PSS (7.10±0.02, p<0.01). When these cells were incubated with 10⁻⁵ M 5-HT, pHᵢ did not rise over a 20-minute time interval and was not significantly different from basal pHᵢ or from time-controlled pHᵢ values obtained in the absence of 5-HT. Thus, as opposed to the results with confluent monolayers incubated in HEPES PSS, basal pHᵢ of cells in HCO₃⁻ PSS was higher and did not rise after exposure to 5-HT.

Effect of 5-HT on Contraction and pHᵢ in Individual Cells

To determine whether 5-HT contracted individual cells, the following experiments were performed. Canine femoral artery vascular smooth muscle cells were cultured for 5–8 days on rat tail tendon collagen gels in the presence of 0.5% FCS. Cells do not increase in number throughout this period when grown under these conditions, and their contractile and pHᵢ regulatory responses to 5-HT were not affected by withdrawing FCS entirely for 24 hours before the experiments. It was assumed that these cells maintain the quiescent state during the culture period. Figure 5 shows a photomicrograph of a field of cells before (left panel) and 20 minutes after (right panel) exposing them to 10⁻⁵ M 5-HT. The majority of cells that had spread out on the collagen gel contracted after exposure to 5-HT. Figure 6A shows a time course of the effect of 10⁻⁵ M 5-HT on the contraction of these cells. Maximal contraction was achieved within 10 minutes, in a dose-dependent manner (Figure 6B), with maximal contraction occurring at 10⁻⁵ M and half-maximal contraction occurring at approximately 5×10⁻⁸ M. The data in Figure 7 (left panel) show that 5-HT-induced contraction was inhibited by 10⁻⁴ M ketanserin, which will inhibit 5-HT₁-like and 5-HT₂ receptor occupancy. In addition, cells contracted by 5-HT relaxed when the agonist was washed off (right panel).

Figure 4. Bar graph showing the effect of serotonin (5-HT) on pHᵢ of confluent monolayers of vascular smooth muscle cells in HCO₃⁻-containing buffer. Confluent monolayers were grown on coverslips and mounted in a cassette inside a fluorescent spectrophotometer that was perfused with physiological salt solution containing HCO₃⁻ (HCO₃⁻ PSS). pHᵢ was measured by monitoring the fluorescence emission of 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein. pHᵢ was measured at baseline (n=8) and after perfusion with HCO₃⁻ PSS with (n=4) or without (n=4) 10⁻⁵ M 5-HT for an additional 20 minutes.

Figure 5. Photomicrographs showing serotonin-induced contraction of vascular smooth muscle cells. Vascular smooth muscle cells were cultured on rat tail tendon collagen gels. Left panel: Cells were incubated in physiological salt solution containing HEPES for 20 minutes on the stage of a Nikon Diaphot inverted microscope and photographed. Right panel: Serotonin (10⁻⁵ M) was added to the incubation medium, and the same field of cells was photographed after 10 minutes. Magnification, ×154.
The primary confluent cultured vascular smooth muscle cells (grown in 10% FCS), which we used in the transport experiments shown in Figures 1–4, come from the same source as the individual cells (maintained in 0.5% FCS), which we used in the contraction experiments shown in Figures 5–7. Nevertheless, the two preparations could have important phenotypic differences. To determine whether the individual cells that were used in the contraction studies have acid-base transport characteristics similar to the primary confluent cultured cells, the following experiments were performed.

pH of individual cells grown on rat tail tendon collagen gels in the presence of 0.5% FCS was monitored by measuring the fluorescence emission of BCECF. Figure 8 shows the pH of a cell incubated in HEPES PSS. When 20 mM NH₄⁺ was added to the perfusion solution, cell pH immediately rose and then slowly fell toward the resting value. When the NH₄⁺ perfusion solution was replaced by an NH₄⁺- and Na⁺-free solution, pH fell to and remained at an acidic value. When the perfusion solution was changed to one containing Na⁺, pH rose back to the resting value. When 50 μM EIPA was present in the latter solution, pH remained at the acidic value (data not shown). The data in Figure 8 indicate that the cells used in the contraction assays mediate Na⁺-H⁺ exchange. pH responded to the above manipulations in the same manner as primary cultured vascular smooth muscle cells from the same source grown to confluence in 10% FCS (compare Figure 8 in this study with Figure 1 in Reference 30).

Figure 9 shows the pH of a cell grown under the same conditions as the cells used in the contraction assays and incubated in HCO₃⁻ PSS. When the perfusion solution was changed to one containing 20 mM acetate, pH fell because of the nonionic diffusion of acetic acid into the cell. When acetate was removed from the perfusion solution, pH acutely increased because of nonionic diffusion of acetic acid out of the cell but spontaneously decreased to the resting value. In three experiments, a second acetate prepulse was followed by an increase and then an immediate decrease in pH, which equaled 94±14% and 103±12%, respectively, of the pH changes obtained after the first acetate prepulse. In the experiment shown in Figure 9, the second acetate prepulse was followed by perfusion with a Cl⁻ free solution. The acute alkalization following the second acetate prepulse was sustained in the absence of external Cl⁻. On perfusing the cell with control HCO₃⁻ PSS, pH fell to the resting value. In three experiments identical to the one shown in Figure 9, pH fell by 0.20±0.03 after the solution was switched from Cl⁻ free to control HCO₃⁻ PSS (p<0.05 versus no change in pH). The data in Figure 9 show that external Cl⁻ mediates base efflux from acutely alkalinized cells and indicate that the cells mediate Na⁺-independent Cl⁻–HCO₃⁻ exchange activity. Similar results were obtained with primary cultured vascular smooth muscle cells from the same source grown to confluence in 10% FCS (compare Figure 9 in
Vascular smooth muscle cells were cultured on rat tail tendon collagen gels. Left panel: Cells were preincubated with physiological salt solution containing HEPES (HEPES PSS) for 20 minutes, photographed at baseline, and photographed again after 10 minutes incubation with HEPES PSS plus 10^{-3} M 5-HT with or without 10^{-4} M ketanserin. Data represent the mean±SEM of the mean percent contraction from baseline of 10 cells per dish. Results were obtained from four dishes with 5-HT and four dishes with 5-HT plus ketanserin. *p<0.05 vs. 5-HT. Right panel: Cells were preincubated with HEPES PSS for 20 minutes, photographed at baseline, incubated with HEPES PSS plus 10^{-3} M 5-HT for 5 minutes, photographed, washed three times with 1-mI alignments of HEPES PSS, and photographed after 5 minutes. Data represent the mean±SEM of the mean percent contraction from baseline of 10 cells per dish. Results were obtained from five dishes. *p<0.05 versus 5-HT.

This study with Figure 5 in Reference 31). Taken together, the data in Figures 8 and 9 indicate that individual cells grown on collagen gels in the presence of 0.5% FCS have pH\(_i\) regulatory mechanisms similar to those found in primary cultured confluent cells grown in 10% FCS.30,31

The effects of 20-minute exposure to 10^{-3} M 5-HT on pH\(_i\) and cell length of vascular smooth muscle cells grown in the presence of 0.5% FCS on collagen gels and incubated in HCO\(_3^-\) PSS were then measured. As shown in Figure 10, contraction of individual cells and the pH\(_i\) response of the same cells after a 20-minute exposure to 5-HT were not related (r=−0.03, p=0.79). Contraction averaged 13.7±3.5%. The change in pH\(_i\) averaged −0.05±0.04 (not different from zero).

The time courses for contraction and pH\(_i\) change of three individual cells exposed to 10^{-3} M 5-HT are shown in Figure 11. Alkalization of these cells did not occur despite a progressive contraction in two of them over a 20-minute period.

To evaluate the possibility that a lower concentration of 5-HT might alkalize cells in HCO\(_3^-\) PSS, we determined the 5-HT dose–response effect for pH\(_i\) change in individual cells grown on collagen gels. As shown in Figure 12, pH\(_i\) was not significantly affected by 5-HT over a dose range of 10^{-9}–10^{-3} M.

**Discussion**

These studies were designed to test the hypothesis that agonist-induced contraction of vascular smooth muscle cells is associated with intracellular alkalization. This hypothesis was posed since various contractile agonists stimulate sarcolemmal Na\(^+-\)H\(^+\) exchange activity and elevate pH\(_i\) under certain experimental condi-
In addition, other studies demonstrated that intracellular alkalinity, achieved artificially in the absence of conventional agonists, increased vascular smooth muscle tone. In the present study, we have shown that 5-HT contracted individual vascular smooth muscle cells in HCO$_3^-$-PSS without affecting pH$_i$. The effect of 5-HT on contraction occurred in a dose-dependent, inhabitable, and reversible manner. Using confluent monolayers of these cells, we showed that 5-HT increased Na$^+$/H$^+$ exchange activity and pH$_i$ in the nominal absence of HCO$_3^-$-PSS. However, in the presence of media containing HCO$_3^-$, the data suggest that 5-HT-stimulated both Na$^+$/H$^+$ and Na$^+$/independent Cl$^-$/HCO$_3^-$ exchange activities without affecting pH$_i$. Taken together, the data in the present study demonstrate that in physiological media containing HCO$_3^-$, 5-HT induces contraction without an associated change in pH$_i$. The data also indicate that in HCO$_3^-$-containing media, 5-HT stimulates Na$^+$/H$^+$ exchange activity, but the concomitant stimulation of Na$^+$/independent Cl$^-$/HCO$_3^-$ exchange activity prevents pH$_i$ from rising.

It needs to be emphasized that the vascular smooth muscle cells in the confluent monolayers, which were grown in the presence of 10% FCS and which we used in these studies to quantitate the basal and 5-HT-stimulated activities of Na$^+$/H$^+$ and Na$^+$/independent Cl$^-$/HCO$_3^-$ exchange activities, did not contract when exposed to 5-HT (authors' unpublished observations). We have previously reported that these cells have additional phenotypic differences from freshly dispersed vascular smooth muscle cells. In the present study, we used these confluent cells as a model to understand the acid-base transport mechanisms operative in the contracting cells grown on collagen gels in the presence of 0.5% FCS.

It is noteworthy that 5-HT-stimulated Na$^+$/H$^+$ exchange activity was approximately three times greater in HEPES PSS than in HCO$_3^-$-PSS in confluent cells. This may be related to the facts that resting pH$_i$ is higher in HCO$_3^-$ PSS than in HEPES PSS (7.26 versus 7.10) and that the intracellular pK for Na$^+$/H$^+$ exchange activity in these cells is substantially lower (approximately 6.5). This modest response may have contributed to the lack of intracellular alkalization of cells exposed to 5-HT in HCO$_3^-$ PSS.

In the present studies, we estimated the activity of the Na$^+$/independent Cl$^-$/HCO$_3^-$ exchanger by measuring the DIDS-sensitive component of 36Cl$^-$ uptake in HCO$_3^-$ PSS. It should be pointed out that although this transport system is inhibited by DIDS, the Na$^+$/dependent Cl$^-$/HCO$_3^-$ exchanger in these cells is also inhibited by this agent. Thus, it is possible that a portion of DIDS-sensitive 36Cl$^-$ uptake in HCO$_3^-$ PSS is mediated by the latter transport system. Nevertheless, on the basis of results of the present study, we concluded that 5-HT stimulated the activity of the Na$^+$/independent Cl$^-$/HCO$_3^-$ exchanger. In support of this conclusion, we have also shown in the present study that DIDS-sensitive Cl$^-$/Cl$^-$ exchange activity, which was measured in the nominal absence of HCO$_3^-$ and Na$^+$ and is mediated by the Na$^+$/independent Cl$^-$/HCO$_3^-$ exchanger, was stimulated by 5-HT.

The shortening of the vascular smooth muscle cells grown on collagen gels in response to 5-HT reported in the present study may have been caused by interaction of actin-myosin filaments, but this was not directly tested. The observation that 5-HT–shortened cells

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**Figure 10.** Effect of serotonin on pH$_i$ and contraction in individual vascular smooth muscle cells in HCO$_3^-$-buffered media. Vascular smooth muscle cells were cultured on rat tail tendon collagen gels. A dish of cells was perfused with physiological salt solution containing HCO$_3^-$ on the stage of an epifluorescence microscope coupled to a photomultiplier tube and a video camera. The pH$_i$ of one cell per dish was measured by monitoring the fluorescence emission of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein, and the length of that cell was measured on a video monitor before and after adding $10^{-5}$ M serotonin to the perfusion media. The change in pH$_i$ and corresponding percent contraction of cells after 20 minutes of exposure to serotonin is plotted (n=8).

**Figure 11.** Time course of the effect of $10^{-5}$ M serotonin on contraction (top panel) and pH$_i$ (bottom panel) in individual vascular smooth muscle cells. Data are from three of the cells from Figure 10 at 5, 10, 15, and 20 minutes after adding $10^{-5}$ M serotonin.
elongated when 5-HT was washed off (Figure 7, right panel) suggest that the cells were under tension during the shortening period. We think it is unlikely that the shortening process was due to loss of cell volume since 5-HT–stimulated increases in Na⁺-H⁺ and Na⁺-independent Cl⁻-HCO₃⁻ exchange activities should cause net Na⁺ and Cl⁻ influx. Cell swelling could theoretically simulate cell contraction by causing retraction of extended myoplasmic processes as the central part of the cell body fills with fluid. This scenario, however, could not explain the cell shortening observed in the present study since the photomicrographs shown in Figure 5 do not show ballooning of the cell bodies after exposure to 5-HT. For these reasons, the cell shortening observed after exposure to 5-HT in the present study probably represents contraction under tension caused by actin-myosin filament interaction.

Vascular smooth muscle is known to contain two of the three classes of 5-HT receptors, the 5-HT₁-like and 5-HT₂ subtype. It has been reported that both 5-HT₁ and 5-HT₂ receptor occupancy can cause contraction of canine vascular smooth muscle. We have recently shown that cultured vascular smooth muscle cells from canine femoral artery contain 5-HT₁ (EC₅₀ of 0.032 μM) and 5-HT₂ (EC₂₀ of 1.8 μM) receptors, which were involved in stimulating the Na⁺-K⁺ pump. Occupancy of 5-HT₁ receptors stimulated Na⁺ influx via an intracellular Ca²⁺-dependent mechanism, whereas occupancy of 5-HT₂ receptors stimulated phosphoinositide metabolism and protein kinase C. Thus, it is possible that 5-HT–induced contraction of cultured vascular smooth muscle cells from canine femoral artery in the present study is mediated by 5-HT₁ and 5-HT₂ receptor occupancy. This conclusion is based on data from cultured cells and may not pertain to native tissue from this or other vessels. It should be pointed out that the contraction dose–response curve of intact canine femoral arteries to 5-HT spans two orders of magnitude, has an ED₅₀ of approximately 5 × 10⁻⁸ M, and is principally mediated by 5-HT₂ receptor occupancy. In the present study, the ED₅₀ for 5-HT–induced contraction of isolated cultured cells was also approximately 5 × 10⁻⁸ M, but the dose–response curve spanned four orders of magnitude. The reasons for this difference between intact canine femoral arteries and the cells used in the present study are not known. In the cultured cells, both 5-HT₁ and 5-HT₂ receptor occupancy may mediate contraction, and the affinities of one or both of these receptors for 5-HT may be different from the affinities in intact vessels.

Kikkeri et al² recently examined pH regulation in A10 cells, which are derived from embryonal rat thoracic aorta. They reported findings similar to those in the present study. Arginine vasopressin increased pH in A10 cells in HEPES buffer because of a concomitant stimulation of the Na⁺-independent Cl⁻-HCO₃⁻ exchanger. The effects of agonists on cell contraction were not reported in these studies with vascular smooth muscle or mesangial cells.

Serially passaged cultured vascular smooth muscle cells usually lose the ability to contract and may have other phenotypic characteristics that differ from the native cell. In the present study, we used primary cultured vascular smooth muscle cells and found that the contractile agonist 5-HT contracted cells in HCO₃⁻ PSS without changing pH. The explanation for the effects of agonist on pH in the present study was the
same as for the passed cells described above. Aalkjaer and colleagues \(^{16, 39}\) reported that noradrenaline and angiotensin II contracted rat resistance vessels bathed in HCO\(_3^-\) buffer but did not affect pH. Thus, the present study confirms and extends the findings obtained with passed cultured vascular smooth muscle and mesangial cells and intact resistance vessels.

In conclusion, we have found that 5-HT–induced contraction of isolated canine femoral artery vascular smooth muscle cells is not associated with a change in pH. Thus, excitation–contraction coupling under these conditions is not partially mediated by an increase in pH. Whether 5-HT or other agonist-induced contractions in other canine arteries or in arteries from different species is caused, in part, by increased pH, has not yet been determined.

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