Effects of pH on Contraction and Ca\textsuperscript{2+} Mobilization in Vascular Smooth Muscles of the Rabbit Basilar Artery

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Abstract: The present study was undertaken to investigate the effects of extracellular pH (pH\textsubscript{e}) and intracellular pH (pHi) on 5-hydroxytryptamine (5-HT)-induced contraction and Ca\textsuperscript{2+} mobilization in vascular smooth muscles. Strip preparations of the rabbit basilar artery without endothelium were loaded with 40 \textmu M fura-2-AM and 2 \textmu M BCECF-AM and mounted in an organ bath. The isometric tension was recorded by using a force displacement transducer. Administration of 5-HT caused dose-dependent contraction in the rabbit basilar arteries. Acidification of pH\textsubscript{e} from 7.40 to 6.90 reduced the 5-HT-induced contraction and [Ca\textsuperscript{2+}]\textsubscript{transient}. Alkalization of pH\textsubscript{e} from 7.40 to 7.90, on the other hand, enhanced the contraction and elevation of [Ca\textsuperscript{2+}]. In the other series of experiments, pH\textsubscript{i} (7.12 in normal PSS) was selectively altered by adding either butyric acid or trimethylamine. Intracellular acidification (pHi=6.89) and alkalization (pHi=7.35) without changes in pH\textsubscript{e} produced qualitatively similar effects to those caused by extracellular acidification and alkalization, respectively. Ca-sensitivity, which is defined as \(\Delta\text{tension}/\Delta[\text{Ca}\textsuperscript{2+}]\), was not affected by the alteration of pH\textsubscript{e} nor pH\textsubscript{i}. In the Ca\textsuperscript{2+}-free solution, the addition of 5-HT produced transient increases in [Ca\textsuperscript{2+}], and isometric tension that were much smaller than those in the normal physiological salt solution. The 5-HT-induced responses of [Ca\textsuperscript{2+}] and tension in the Ca\textsuperscript{2+}-free solution were not affected by acidification nor alkalization. These results suggest that a 5-HT-induced contraction is significantly modulated by pH through changing the [Ca\textsuperscript{2+}]\textsubscript{transient}, and that the change of pH\textsubscript{i} plays, at least in part, a role in the alteration of 5-HT-induced contraction resulting from acidosis or alkalosis in the rabbit basilar artery. [Japanese Journal of Physiology, 49, 55–62, 1999]

Key words: basilar artery, vascular smooth muscle, 5-hydroxytryptamine, calcium, pH.

There is little doubt that cytosolic calcium ions play a major role in the contractile process of vascular smooth muscles. A number of hormones, neurotransmitters, and autacoids increase the concentration of cytosolic calcium ions ([Ca\textsuperscript{2+}]) and cause a contraction of vascular smooth muscles. Recent studies revealed that a secondary mechanism which can modify, independently of [Ca\textsuperscript{2+}], phosphorylation and dephosphorylation of the myosin light chain is involved in the agonist-induced contraction [1]. Such agonists can increase force in permeabilized muscles in which [Ca\textsuperscript{2+}], is maintained at a constant level, indicating an increase in Ca-sensitivity defined as the force/[Ca\textsuperscript{2+}] ratio. In cerebral arteries, 5-hydroxytryptamine (5-HT) has been known to show a potent constrictor effect [2]. The contractile response of cerebral arteries to 5-HT is mediated through the activation of 5-HT\textsubscript{1}-like and/or 5-HT\textsubscript{2} receptors [3, 4]. Activation of these receptors may increase both [Ca\textsuperscript{2+}], and Ca-sensitivity.

It is also known that alteration of the pH significantly affects the vascular smooth muscle tone [5].

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general, acidosis leads to vasodilatation and alkalois causes vasoconstriction. In cerebral arteries, extracellular acidosis plays a significant role in relaxation to hypercapnia [6-8]. There are few reports, however, in which the effects of pH on agonist-induced contraction and \([\text{Ca}^{2+}]\), transients in cerebral arteries were simultaneously examined. Deckert and coworkers [9] demonstrated that extracellular acidification significantly attenuated the 5-HT-induced contraction of the rabbit basilar artery, but [\text{Ca}^{2+}]), was not measured in this study. In addition, there are still conflicting discussions whether the changes in vascular tone are mediated through alteration of extracellular pH (pH_e) or intracellular pH (pH_i). The importance of pH_i has been shown in rat cerebral arteries [10], as well as in cat pial arteries [6, 7], dog cerebral arteries [8], and rat pial arterioles [11], whereas the dominant role of pH_e has been demonstrated in the cat cerebral arterioles [12], rat cerebral arteries [13], rat mesenteric arteries [14], the rabbit cortex [15], and guinea-pig portal veins [16].

Thus, in the present study, the effects of pH on 5-HT-induced contraction and Ca^{2+} mobilization in rabbit cerebral arteries were investigated by measuring isometric tension, [Ca^{2+}], and pH, simultaneously. The main questions are: 1) which is responsible for the pH-dependent alteration in the 5-HT-induced contraction (i.e., the change in [Ca^{2+}], or Ca-sensitivity), and 2) is the effect of pH mediated through the change in pH_e or pH_i?

**MATERIALS AND METHODS**

All procedures were reviewed and approved in accordance with the Guideline for Animal Experimentation at the Faculty of Medicine, Tottori University, Yonago, Japan, and conformed to “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences” of the Physiological Society of Japan.

**Animal preparation.** Twenty-five Japanese white rabbits of both sexes weighing 2.5-3.5 kg were sacrificed by intravenous injection of 25% urethane (6.7 ml/kg). The basilar artery (outer diameter 0.4-0.6 mm) was rapidly dissected from each animal. The arterial segment was cut into circular strips of 1.5 mm long and 2.0 mm wide. Intracellular acidification of vascular endothelial cells releases Ca^{2+} into the cytosol [17], and Ca^{2+} mobilization may cause synthesis and the release of endothelium-derived vasodilatory substances which, in turn, affect the vascular tone. Thus, in the present study, the endothelium was removed by gently rubbing the intimal surface with a cotton swab to examine the direct effects of pH on vascular smooth muscle cells.

**Measurement of isometric tension.** After each strip was loaded with fura-2-acetoxymethyl ester (fura-2-AM) and 2',7'-bis(carboxymethyl)-4 or 5-carboxyfluorescin diacetoxymethyl ester (BCECF-AM), it was mounted horizontally between hooks in an organ bath filled with 5 ml physiological salt solution (PSS) on the stage of an inverted fluorescence microscope (TMD-300, Nikon, Tokyo, Japan). The PSS was maintained at 37±0.5°C and bubbled with 100% O_2. A hook holding one end of the strip was fixed to the wall of the bath, and the other hook was connected to the lever of a force displacement transducer (UL-10GR, Minebea, Nagano, Japan). The isometric tension detected by the transducer was amplified and recorded in a data analyzer (MacLab Mk III, AD Instrument, Castle Hill, Australia). The resting tension was maintained at 4.9 mN which was optimal for obtaining a contractile response to a 80 mM potassium solution. After each experiment, the strip was removed from the organ bath, and the wet weight of the preparation was measured. The active tension produced by 80 mM KCl or 5-HT was normalized as newtons (N) of force per gram (g) of wet weight. To confirm the denudation of endothelium, 100 \mu M acetylcholine was applied after the strip had been contracted by 100 \mu M 5-HT.

**Measurement of [Ca^{2+}] and pH.** The arterial strip was loaded for 4 h at 37°C with fluorescence dyes 40 \mu M fura-2-AM and 2 \mu M BCECF-AM. Chromophor EL (0.02%) was added to improve the dye loading. After loading, the tissue was washed out 2-3 times with the PSS. The measurement of fluorescence was performed using an inverted fluorescence microscope equipped with a fluorometric system (Quanti Cell 700, Applied Imaging, Newcastle, UK). Fura-2 was excited with the light of a 100 W xenon lamp at wavelengths of 340 and 380 nm. The fluorescence emission was recorded at 510 nm and monitored with an intensified CCD camera. After the noise signal and autofluorescence had been subtracted, [Ca^{2+}], was estimated from the ratio (R) of the fluorescence obtained by excitation at 340 and 380 nm. Two wavelengths (440 and 490 nm) were used to excite BCECF, and the emission was recorded at 535 nm. The pH_i was estimated from the 490/440 ratio (R_4) of the fluorescence.

**Calibration of the fura-2 fluorescence ratio signals.** The [Ca^{2+}], was calculated according to the following formula:

\[
[\text{Ca}^{2+}] = K_d \frac{S_{380}/S_{340}}{(R - R_{\text{min}})/(R_{\text{max}} - R)},
\]

where \(K_d\) is the dissociation constant (224 nM; Gryniewicz et al. [18]), \(R_{\text{min}}\) and \(R_{\text{max}}\) are the mini-
mmum and maximum values of $R_\text{f}$ and $S_\text{f}$ and $S_\text{r}$ are the fluorescence values for the free and bound forms of the dye at 380 nm, respectively. In order to calibrate the signals, we determined $R_\text{min}$ and $R_\text{max}$ values of the fluorescence ratio. The strip was incubated in a high K⁺ HEPES buffered, Ca²⁺-free solution (130 mM KCl, 20 mM NaCl, 10 mM HEPES) containing 2 µM ionomycin, a Ca²⁺ ionophore. Also, 2 mM NaCN, 0.5 mM monoiodoacetic acid, and 2 µM carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) were added to inactivate the function of mitochondria [19]. After 10 min incubation, the $R_\text{min}$ signal was obtained in a solution containing 5 mM glycodeither-diamine tetraacetic acid (EGTA), 0.2 µM thapsigargin, a Ca²⁺ pump inhibitor, and 100 µM N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), a calmodulin antagonist [20]. The $R_\text{max}$ signal was measured in a solution containing 10 mM CaCl₂. The values for $S_\text{f}/S_\text{r}$, $R_\text{min}$, and $R_\text{max}$ were estimated to be 3.103, 0.401, and 2.122, respectively. The value for the $K_\text{d}$ of fura-2 is pH dependent and markedly increases as pH falls to less than 6.5 [21–23]. In this study, we used a constant value for the $K_\text{f}$ as pH in our studies was always well above 6.5. Under these conditions, $K_\text{d}$ corrections have only a little impact on the final estimation of [Ca²⁺] [22, 23].

Calibration of the BCECF fluorescence ratio signals. The pH was calculated according to the following formula:

$$\text{pH} = pK_\text{B} + \log\{ (R_\text{B} - R_{\text{Bmin}}) / (R_{\text{Bmax}} - R_\text{B}) \},$$

where $pK_\text{B}$ is the apparent $pK$ for BCECF, and $R_{\text{Bmin}}$ and $R_{\text{Bmax}}$ are minimum and maximum values of $R_\text{B}$, respectively. After loading with the two dyes, the strip was suspended in a depolarizing high K⁺ buffer solution (130 mM KCl, 20 mM NaCl, 10 mM HEPES) which contained 10 µg/ml nigericin, a K⁺/H⁺ exchanger [19], and pH was adjusted to 6.80, 7.40, or 8.00 by adding 1.0 N NaOH for 30 min. The values for $pK_\text{B}$, $R_{\text{Bmin}}$, and $R_{\text{Bmax}}$ were estimated to be 7.032, 0.381, and 1.356, respectively.

Experimental protocol. Arterial strips were allowed to equilibrate for 90 min at a resting tension of 4.9 mN. After the equilibration period, the strips were contracted by 80 mM KC1 solution, and then concentration-response curves for 5-HT were obtained. The magnitude of contraction was expressed as a percentage of the KCl-induced contraction. In these experiments, [Ca²⁺] and pH were not measured.

In the next series of experiments, isometric tension, [Ca²⁺] and pH were measured simultaneously, and the effects of pH alteration on 5-HT-induced responses were examined. The pH of the PSS was adjusted to 6.90, 7.40, or 7.90 by changing the amount of 1.0 N NaOH. Responses to 80 mM KCl and 100 µM 5-HT were obtained 60 min after the change in pH. The order of these three pH levels was randomized. Because the change in pH is associated with the alteration of pH, it is interesting to examine whether the pH-dependent alteration of 5-HT-induced responses is attributable to the change in pH itself or secondary to the change in pH. Thus, the pH was selectively altered by adding either butyric acid (BA) or trimethylamine (TMA) into the PSS. In these experiments, the pH was kept constant at 7.40 by adding an adequate amount of NaOH or HCl. Responses to 100 µM 5-HT were measured 10 min later. The order of the three pH levels was again randomized.

To investigate the mechanism of pH-dependent alteration in response to 5-HT, the isometric tension, [Ca²⁺], and pH, were recorded during the application of 100 µM 5-HT in a Ca-free solution. The responses to 100 µM 5-HT were measured 1 min after changing the solution.

Solutions. The composition of the standard PSS was as follows (mM): 130.0 NaCl, 5.4 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 5.5 glucose, and 10.0 HEPES. A high K⁺ solution (80 mM) was made by substituting KCl for equimolar NaCl in the standard PSS. To make the Ca²⁺-free solution, CaCl₂ was replaced by 2 mM EGTA in the standard PSS.

Drugs and chemicals. The following compounds were used: fura-2 AM and BCECF-AM (Dojin, Kumamoto, Japan), EGTA (Nacalai Tesque, Kyoto, Japan), NaCN (Wako Pure Chem Ind., Osaka, Japan), monoiodoacetic acid (Aldrich, Deisenhofen, Germany), 5-HT, thapsigargin, CCCP, and cremophor EL (Sigma, St. Louis, USA). Stock solutions of 5-HT were made in distilled water and diluted in the PSS. The concentrations of 5-HT and EGTA were expressed as the final organ bath concentration. Fura-2 and BCECF were dissolved in dimethyl sulfoxide (DMSO).

Statistics. In the present study, the changes in [Ca²⁺], (Δ[Ca²⁺]), tension (Δtension), and Ca sensitivity (Δtension/Δ[Ca²⁺]) were assessed. Data are given as mean values ± SEM (n), where n refers to the number of animals. Results were statistically analyzed by ANOVA with repeated measures followed by a post hoc Scheffe’s test. The difference in means was considered significant at $p<0.05$.

RESULTS

Figure 1 shows the concentration-response curve of 5-HT-induced contraction in rabbit basilar arteries. The addition of 5-HT ranging from $10^{-8}$ M (10 nM) to
Fig. 1. Dose-response curve to 5-HT in isolated rabbit basilar arteries. The maximum contraction induced by 5-HT was 83.1±7.0% of the KCl-induced contraction (n=5). The data are expressed as means±SEM.

Fig. 2. Time course for tension and intracellular calcium ([Ca^{2+}]) induced by 80 mM KCl (A) and 100 μM 5-HT (B) at three pH levels. Acidification reduced 5-HT-induced contraction and the [Ca^{2+}] transients and alkalization enhanced them. The responses to KCl were not affected by the alteration of pH.

10^{-3}M (1 mM) caused concentration-related contractions. The concentration required to develop a detectable response was 10^{-8}M. The maximum contraction induced by 5-HT was 83.1±7.0% of the KCl-induced contraction.

Figure 2 demonstrates typical responses of isometric tension and [Ca^{2+}], to 80 mM KCl solution (Fig. 2A) and 100 μM 5-HT (Fig. 2B) at three different pH levels. Acidification of pH_{i} to 6.90 decreased the pH_{i} by 0.21±0.01 units, and alkalization of pH_{i} to 7.90 increased the pH_{i} by 0.16±0.01 units. Both acidification and alkalization did not significantly affect the resting tension or [Ca^{2+}]. Acidification reduced the Δ[Ca^{2+}], and Δtension induced by 5-HT, but did not change the Ca sensitivity (Fig. 3). Alkalization, on the other hand, enhanced the Δ[Ca^{2+}]; and Δtension caused by 5-HT. Ca sensitivity was again not altered. Neither acidification nor alkalization markedly changed the Δ[Ca^{2+}]; and Δtension induced by 80 mM KCl (Fig. 3).

The addition of 30 mM BA into the PSS decreased
the pH by 0.22 ± 0.02 units from 7.12 ± 0.04 in the standard PSS, and 20 mM TMA increased the pH by 0.24 ± 0.04 units. Intracellular acidification attenuated the Δ[Ca²⁺] and Δtension produced by 100 μM 5-HT, and intracellular alkalization enhanced them (Fig. 4).

Changing the perfusate from normal PSS to Ca²⁺-free solution decreased the [Ca²⁺] from 56.0 ± 7.1 to 33.3 ± 4.4 nM and the resting tension from 5.5 ± 0.3 to 5.0 ± 0.1 mN (n = 15). In the Ca²⁺-free solution, the addition of 100 μM 5-HT produced a transient increase in [Ca²⁺]; and isometric tension which were much smaller than those in the normal PSS (Fig. 5). The 5-HT–induced responses of [Ca²⁺] and tension in the Ca²⁺-free solution were not affected by acidification nor alkalization.

**DISCUSSION**

The present results demonstrated that: 1) alterations in pH of the bathing medium significantly affected the maximum contraction of the rabbit basilar artery induced by 100 μM 5-HT but not the one induced by the 80 mM KCl solution; 2) acidification and alkalization of the bathing medium caused a decrease and an increase in the 5-HT–induced [Ca²⁺] transients, respectively, without any change in Ca sensitivity; 3) selective changes in pH produced similar effects in 5-HT–induced responses of contraction and [Ca²⁺] transients to those elicited by altering the pH of the bathing medium, and 4) the contraction and [Ca²⁺] transients induced by 5-HT in the Ca²⁺-free solution were not affected by the changes in pH of the bathing medium.

It has been reported that the contractile responses of rat cerebral arteries to a high potassium solution are not influenced by pH changes in the range from 6.9 to 7.8 [24], which is consistent with the present results. Acute acidification produced marked relaxation in the rat aorta precontracted with noradrenaline but not when the aorta was contracted by 80 mM KCl solution [25]. These results suggest that an alteration in pH of the bathing medium does not significantly affect Ca²⁺ influx through a voltage-gated Ca²⁺ channel, although there has been one report that suggests the effects of pH on KCl-induced contraction in the canine coronary artery [26]. It is not likely that the contraction produced by the 80 mM KCl solution is too strong and masks the effect of pH [24] because 5-HT caused a contraction similar to that produced by 80 mM KCl (Fig. 1). The 5-HT–induced contraction of the rabbit basilar artery, on the other hand, was reduced by acidification and enhanced by alkalization. Similar results were observed in the noradrenaline-induced contraction of the rat aorta [25] and 5-HT–induced contraction of the rabbit cerebral artery [9]. Little atten-
tion was paid, however, to [Ca$^{2+}$]i in these studies.

It is now widely accepted that secondary mechanism(s) can regulate, independently of [Ca$^{2+}$], myosin light-chain phosphorylation in the agonist-induced contraction of smooth muscle cells. Thus, it is interesting to examine which factor is more influenced by pH (i.e., [Ca$^{2+}$]), transients or the secondary mechanism (Ca-sensitivity)). The present results clearly show that the effect of pH is solely mediated through the change in [Ca$^{2+}$]i transients, and the change in Ca sensitivity (i.e., ∆Tension/∆[Ca$^{2+}$]) does not play a role in the phenomenon (Fig. 3). The [Ca$^{2+}$]i transients induced by 5-HT were reduced by acidification and enhanced by alkalization in the rabbit basilar artery. Alkalization of the bathing medium by adding NH$_4$Cl produced a transient reduction followed by a sustained elevation of [Ca$^{2+}$]i in isolated rat mesenteric arterial segments which had been stimulated with NA [27]. The transient reduction may represent the acute effect of pH which is often opposite to what might be expected [5]. In the present study, responses to the agonists were observed 60 min after the pH had been changed. Therefore, the acute effect of pH could not be detected. These findings suggest that the elevation of pH in bathing medium enhances the agonist-induced [Ca$^{2+}$]i transients in steady-state vascular smooth muscles and vice versa. The effect of pH on the Ca sensitivity of smooth muscles is still controversial. A reduction of the Ca sensitivity due to acidification was observed in isolated, perfused rat tail artery [28], whereas an increase of sensitivity was found in skinned smooth muscles of the rat tail artery exposed to acidification [29]. The difference in the method used may contribute to the discrepancy.

Since the alteration in the pH of bathing medium leads to a change in pHi, it is interesting to examine the relative roles of pHi and pH in response to extracellular acidosis or alkalosis. In the present study, pHi decreased by 0.21 units 60 min after changing the pH of the bathing medium from 7.40 to 6.90. Alkalization of the pH in bathing medium to 7.90 increased the pHi by 0.16 units. These values are comparable to those obtained in the rat portal vein [30], rat uterus [31], and guinea-pig vas deferens [32]. Recently, procedures to produce selective changes in pHi have been developed. Intracellular pH can be altered at a constant pHi by using a weak acid or base [14, 30, 33]. The addition of 30 mM BA decreased the pHi by 0.22 units and 20 mM TMA increased it by 0.24 units in our study. Although the changes in pHi are rapid, they are usually followed by a continuous change in pHi towards the baseline level [30]. Thus, in the present experiments, effects of 5-HT were obtained 10 min after the change in pHi. Intracellular acidification and alkalization caused similar effects on the 5-HT–induced contraction and [Ca$^{2+}$]i transients as those produced by extracellular acidification and alkalization, respectively (Fig. 4). These findings suggest that pH plays a certain role in the regulation of tension in the rabbit basilar artery. A number of studies suggested the importance of pHi in regulating vascular smooth muscle tone. Most of them, however, demonstrated that acidification of pHi caused a transient increase in tension [33, 34], which is opposite to our results. In those studies, pHi was changed in isolated vessels which had been already contracted with an agonist. Therefore, the observed response is presenting the acute effect of the changing pHi, which is opposite, as mentioned before, of what might be expected in a steady state.

In a calcium-free solution, 100 μM 5-HT elicited a transient contraction which was smaller to that obtained in the normal PSS. This is consistent with previous results observed in the guinea-pig basilar artery [35]. Therefore, the 5-HT–induced contraction of the rabbit basilar artery seems to be mediated by both calcium release from the intracellular stores and calcium influx through the ligand-gated calcium channel. Since the 5-HT–induced contraction and [Ca$^{2+}$]i transients in the calcium-free solution were not affected by pH, it is likely that the alteration of pH modifies mainly the calcium influx through the ligand-gated calcium channel.

It is known that a large amount of 5-HT is released from platelets during the period of aura in patients with migraine [36]. The release of 5-HT may cause a potent constriction of cerebral arteries which produces regional ischemia. The ischemia in turn leads to local acidosis, and it may attenuate 5-HT–induced vasoconstriction as shown in the present study. Thus, the acidosis may play a role in migraine headaches through the dilatation of cerebral arteries. To understand the mechanism of pH-dependent alteration in vascular contractility will provide us clinically important information as well as advances in physiological knowledge.

In conclusion, agonist-induced contraction of the rabbit cerebral artery is modified by altering the pH of the bathing medium. The effect of pH is mediated through a change in the agonist-induced [Ca$^{2+}$]i transients but not Ca sensitivity. The change in pHi is responsible, at least in part, for the effects of changing pH on vascular contractility.

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