Role of NHE1 in calcium signaling and cell proliferation in human CNS pericytes

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The central nervous system (CNS) pericytes play a pivotal role in brain microcirculation. Na+/H+ exchanger isoform 1 (NHE1) has been suggested to regulate the proliferation of nonvascular cells through the regulation of intracellular pH, Na+, and cell volume; however, the relationship between NHE1 and intracellular Ca2+, an essential signal of cell growth, is still not known. The aim of the present study was to elucidate the role of NHE1 in Ca2+ signaling and the proliferation of human CNS pericytes. The intracellular Ca2+ concentration was measured by fura 2 in cultured human CNS pericytes. The cells showed spontaneous Ca2+ oscillation under quasi-physiological ionic conditions. A decrease in extracellular pH or Na+ evoked a transient Ca2+ rise followed by Ca2+ oscillation, whereas an increase in pH or Na+ did not induce the Ca2+ responses. The Ca2+ oscillation was inhibited by an inhibitor of NHE in a dose-dependent manner and by knockdown of NHE1 by using RNA interference. The Ca2+ oscillation was completely abolished by thapsigargin. The proliferation of pericytes was attenuated by inhibition of NHE1. These results demonstrate that NHE1 regulates Ca2+ signaling via the modulation of Ca2+ release from the endoplasmic reticulum, thus contributing to the regulation of proliferation in CNS pericytes.

Na+/H+ exchanger; central nervous system; cerebral ischemia; microcirculation

PERICYTES ARE LOCATED AT THE abluminal surface of microvessels such as arterioles, venules, and particularly capillaries, where smooth muscle cells are lacking (1, 5). Pericytes closely communicate with endothelial cells and regulate the maturation, remodeling, and maintenance of the vascular system via the secretion of growth factors or the modulation of the extracellular matrix (2, 11, 32). Recent studies have demonstrated that, in the central nervous system (CNS), pericytes play a pivotal role in brain angiogenesis, regulation of blood flow, immune responses, and maintenance of blood-brain barrier (3, 12, 27). CNS pericytes may cause CNS diseases due to a disruption of the microcirculation.

Intracellular Ca2+ is a ubiquitous second messenger controlling a broad range of cellular functions. The concentration of Ca2+ in the cytosol is maintained at a low level, and it is finely tuned by numerous proteins, including receptors, transducers, channels, Ca2+ pumps, and exchangers (7). A large body of evidence indicates that temporal and spatial changes in intracellular Ca2+ regulate many divergent cellular processes, such as exocytosis, contraction, metabolism, transcription, and proliferation (6, 8, 24). It is assumed that the rapid and highly localized Ca2+ spikes regulate fast responses, whereas slower responses are controlled by either repetitive global Ca2+ transients or intracellular Ca2+ waves.

Similar to intracellular Ca2+, pH also plays a central role in the regulation of many aspects of cell physiology. Protons appear to function as a second messenger similarly to Ca2+. Intracellular protons are supposed to be critical for linking external stimuli to proliferation, motility, apoptosis, and differentiation (28, 29). Intracellular pH is modified by intrinsic buffering capacity and actively controlled by proteins such as Na+/H+ exchanger (NHE). NHE1 is ubiquitously expressed in the plasma membrane of virtually all tissues and is a primary regulator for intracellular pH, Na+ concentration, and cell volume (10, 22, 25, 26, 29). In the heart, intracellular acidosis causes Ca2+ overload, possibly via activation of NHE1 and the Na+/Ca2+ exchanger (NCX). In addition to acting as a housekeeping protein involved in Na+ and Ca2+ homeostasis, NHE1 has been suggested to regulate cell proliferation, differentiation, and apoptosis; the organization of the cytoskeleton; and immune responses (10, 29).

In the brain, pericytes form neurovascular units, and a malfunction of the cells causes a serious disruption of microcirculation, leading to CNS diseases. During pathological states such as ischemia or hypoxia, intra- and extracellular pH drop, which may affect the cellular function of pericytes and impair microcirculation. However, the change in intracellular signaling during acidic conditions and the interplay between intracellular Ca2+ and proton in CNS pericytes are not known. The present study focused on the cross-talk between extracellular pH and intracellular Ca2+ in CNS pericytes. The results showed NHE1 to be a regulatory protein coupling pH and Ca2+ signal, thereby affecting the cell proliferation in CNS pericytes.

MATERIALS AND METHODS

Cell culture. Human brain microvascular pericytes were purchased from Cell Systems (Kirkland, OR). Culture of human brain microvascular pericytes was initiated from normal brain cortical tissues. The cells were plated on collagen-coated dishes (Iwaki Glass, Tokyo, Japan) and were cultured in a CS-C complete medium kit (Cell Systems), which is a combination of DMEM and Ham’s F-12 medium, supplemented with 10% fetal bovine serum, 15 mmol/l HEPES,
acidic FGF, and heparin. The cells were cultured at 37°C in 5% CO₂ in a humidified incubator. Pericytes that had been subcultured four to eight times were used for the present experiments.

**Measurement of intracellular Ca²⁺ concentration.** A fluorescent Ca²⁺ indicator, fura 2-AM, was used for the measurement of the intracellular Ca²⁺ concentration ([Ca²⁺]i), according to the method described previously (14, 15). The pericytes were plated on 35-mm dishes with a glass coverslip bottom (MatTek, Ashland, MA) in 2 ml of CS-C medium and were used 2–5 days later. The cells were loaded with 5 μmol/l fura 2-AM for 30 min and then were washed out with HEPES-buffered saline (in mmol/l: 132 NaCl, 5.9 KCl, 1.2 MgCl₂, 1.5 CaCl₂, 11.5 glucose, 11.5 HEPES, 1.2 Na₂HPO₄, pH 7.35). The fura 2-loaded cells were perfused with HEPES-buffered saline and were illuminated alternately at wavelengths of 340 and 380 nm through a rotating filter wheel. The fluorescence was measured at a wavelength of 510 nm (C4742–95-ER; Hamamatsu Photonics, Hamamatsu, Japan), and the ratio of that illuminated at 340 and 380 nm (F₃₄₀/F₃₈₀) was calculated by using a dual-excitation microfluorescence system (Aquacosmos v.1.3; Hamamatsu Photonics). In Na⁺-free solution, NaCl was replaced with equimolar N-methyl-d-glucamine. CaCl₂ was omitted, and 1 mmol/l EGTA was added in Ca²⁺-free solution. All experiments were performed at room temperature.

To compare Ca²⁺ response after stimulation, percentage of cells showing Ca²⁺ oscillations in each experiment and the maximum increase in the fluorescence ratio from baseline ([Ca²⁺]i), peak) were evaluated in each cell.

**Measurement of intracellular pH.** The intracellular pH (pHi) of the pericytes was measured with a pH-sensitive dye, 2′, 7′-bis-(2-carboxyethyl)-5-(6) - carboxyfluorescein (BCECF), by using a similar procedure to that used for fura 2. The cells were loaded with 1 μmol/l BCECF-AM for 30 min and were illuminated alternately at wavelengths of 490 and 450 nm; then the ratio (F₄₉₀/F₄₅₀) was calculated. The calibration of pHᵢ was accomplished by using the high-K⁺-nigericin method (30).

Cell pHᵢ recovery was examined after acidification with the NH₄Cl prepulse technique (9). After 3-min exposure to NH₄Cl (20 mmol/l), the cells were perfused with HEPES-buffered saline or Na⁺-free solution, as described above. We calculated the rate of pHᵢ recovery (ΔpHi/Δt) for pH units/min) over 5 min from the initiation of acidification to compare the pHᵢ response.

**RT-PCR and real-time PCR.** Total RNAs from the cultured pericytes were prepared by using the TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was reverse transcribed with avian myeloblastosis virus transcriptase (Roche Diagnostics, Basel, Switzerland) in a total volume of 20 μl. With the use of 1 μl of the product as a template, PCR was performed with primers specific for human NHE1 (forward, 5′-GGGCTTTTCACTGGTGTG-3′; reverse, 5′-CCTTCTTCCTCAGGTAGT-3′; 264 base pairs) and for human β-actin (forward, 5′-CAAGAGATGGGACGGCTGCT-3′; reverse, 5′-CCTTCTTCCTCAGGTAGT-3′; 275 base pairs; Sigma, St. Louis, MO). After preincubation at 94°C for 5 min, PCR was performed with 25 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and elongation at 72°C for 30 s. The PCR products were separated by 1.5% agarose gel electrophoresis and were stained with ethidium bromide.

Quantitative real-time PCR was performed by using a LightCycler (Roche). The RT products were amplified in the reaction mixture (20 μl) containing 2 μl of LightCycler-FastStart DNA Master SYBR Green I (Roche), 0.5 μmol/l of each primer described above, and 3 mmol/l MgCl₂. The copy numbers of mRNA were standardized to those of GAPDH or β-actin.

**Western blotting.** The cultured pericytes were homogenized in RIPA lysis buffer (10 mmol/l Tris⋅HCl, pH 8.0, 150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide; Santa Cruz Biotechnology, Santa Cruz, CA) containing PMSF, sodium orthovanadate, and protease inhibitor cocktail at 4°C and were centrifuged at 10,000 g for 10 min. Protein concentration was determined by using the standard Bradford method. The lysate with an equal volume of 2× electrophoresis sample buffer was boiled for 3 min, subjected to 7.5% SDS-PAGE (15 μg/lane), and transferred to a PVDF membrane. The membrane was incubated 1 h with Block Ace (DS Pharma Biomedical, Osaka, Japan) for blocking at 37°C, probed with primary anti-NHE1 antibody (1: 500 dilution, Santa Cruz Biotechnology) overnight at 4°C, washed, and incubated in secondary anti-goat antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were developed by using an ECL-Plus immunoblotting detection reagent kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer instructions.

**Knockdown of NHE1.** RNA interference (RNAi) was performed for the specific knockdown of the NHE1 expression according to the electroporation-based gene-transfer technique. The double-strand small interfering RNAs (siRNAs) targeting NHE1 were designed as 3′-overhanged form (forward, 5′-CGAAGAGAUCCACACACGtt-3′; reverse, 5′-CUGUUGGUGAUUCGUCCG-3′; Ambion, Austin, TX). The Nucleofector system (Amamax Biosystems, Cologne, Germany) was used for the transfection of pericytes with siRNA according to the manufacturer’s protocol. Briefly, the pericytes were suspended in Nucleofector solution at a final concentration of 0.5–1 × 10⁶ cells/100 μl. The cell suspension was combined with 5 μg of siRNA duplex, transferred into a certified cuvette, and subjected to the Nucleofector. After transfection, the cells were cultured for 48 h in CS-C medium and then were used for the experiments.

**Investigation of cell proliferation.** Cell proliferation was determined by using cell count. Briefly, the culture dishes were washed with PBS (in mmol/l: 137 NaCl, 2.68 KCl, 8.10 NaHPO₄·2H₂O, and 1.47 KH₂PO₄), trypsinized, and resuspended in fresh culture medium. The cell suspension was transferred into an Eppendorf tube, and then the total number of cells was counted by a hemocytometer.

**Chemicals.** BCECF-AM, benzamil, 5-[N,N-hexamethylen] amilorida (HMA), nigericin, carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP), and EGTA were purchased from Sigma, KB-R7943 mesylate was from Tocris (Bristol, UK), and fura 2-AM,amilorida, thapsigargin, N-methyl-d-glucamine, LaCl₃, GdCl₃, and all other materials were purchased from Wako (Osaka, Japan).

**Statistical analysis.** All data are expressed as means ± SD; n is the number of experiments or the number of cells examined. Statistical analysis was done by using Student’s t-test or a one-way ANOVA. Post hoc comparisons were made by using Dunnett’s multiple-comparison tests. A P value <0.05 was considered to be significant.

**RESULTS**

**Ca²⁺ oscillation induced by extracellular Na⁺ and pH.** When human brain microvascular pericytes were perfused with normal HEPES-buffered saline containing 132 mmol/l Na⁺ (pH 7.35), a periodic spike-shaped increase in [Ca²⁺]i, (Ca²⁺ oscillation) spontaneously occurred in 21.9 ± 16.6% of the cells (n = 11, Fig. 1A).

Acidification of the extracellular pH to 6.50 increased the percentage of the cells with Ca²⁺ oscillation to 43.5 ± 23.8% (n = 4, Fig. 1, B–D). Under these conditions, 45.7 ± 31.7% of the cells showed a transient increase in [Ca²⁺]i, followed by a Ca²⁺ plateau (n = 4, Fig. 1, B and C). Therefore, extracellular acidification generated a transient Ca²⁺ increase followed by Ca²⁺ oscillation or a Ca²⁺ plateau. The initial [Ca²⁺]i peak increased after extracellular acidification (Fig. 1E). In contrast, the alkalization of the extracellular solution to pH 8.0 completely abolished Ca²⁺ oscillation (0.0 ± 0.0%, n = 4, Fig. 1, B and D), thus diminishing the peak Ca²⁺ (Fig. 1E).

A decrease in extracellular Na⁺ evoked a similar Ca²⁺ oscillation as that by low pH, and a transient increase in the [Ca²⁺]i, followed by a Ca²⁺ plateau was also observed (Fig.
2A). Figure 2B shows a representative response of $[\text{Ca}^{2+}]_i$ to different concentrations of extracellular $\text{Na}^+$. Therefore, the percentages of the cells presenting the $\text{Ca}^{2+}$ oscillation and the magnitude of transient increase in $[\text{Ca}^{2+}]_i$ were conversely correlated with the concentration of extracellular $\text{Na}^+$ (Fig. 2, C and D). On the other hand, $\text{Ca}^{2+}$ oscillation and $[\text{Ca}^{2+}]_i$ peak were suppressed when the cells were exposed to high concentrations of external $\text{Na}^+$ up to 166 mmol/l. The percentages of the cells with $\text{Ca}^{2+}$ oscillation in response to 0, 66, 132, and 166 mmol/l $\text{Na}^+$ were $65.2 \pm 27.8$ ($n = 6$), $56.9 \pm 21.0$ ($n = 3$), $21.9 \pm 16.6$ ($n = 11$), and $3.1 \pm 6.3\%$ ($n = 4$), respectively.

**Mechanisms of low-$\text{Na}^+$-induced $\text{Ca}^{2+}$ oscillation.** The removal of extracellular $\text{Ca}^{2+}$ could not inhibit the $\text{Ca}^{2+}$ oscillation. Neither nicardipine (1 $\mu$mol/l), an inhibitor of L-type voltage-dependent $\text{Ca}^{2+}$ channels; $\text{La}^{3+}$ (100 $\mu$mol/l), an inhibitor of capacitative $\text{Ca}^{2+}$ entry; nor $\text{Gd}^{3+}$ (100 $\mu$mol/l), an inhibitor of nonselective cation channels inhibited the $\text{Ca}^{2+}$ oscillation. Moreover, changes in the external osmolarity by mannitol (50 mmol/l) did not affect the low-$\text{Na}^+$-induced $\text{Ca}^{2+}$ oscillation (Fig. 3A, $n = 3$ for each experiment). Therefore, voltage-dependent $\text{Ca}^{2+}$ channels, nonselective cation channels, or cell volume may not be involved in the generation of $\text{Ca}^{2+}$ oscillation.

Amiloride (50 $\mu$mol/l), a nonselective inhibitor of $\text{Na}^+$ channels, or KB-R7943 (10 $\mu$mol/l), a selective inhibitor of NCX, had little effect on low-$\text{Na}^+$-induced $\text{Ca}^{2+}$ oscillation. On the other hand, the $\text{Ca}^{2+}$ oscillation was inhibited by benzamil (50 $\mu$mol/l), which is a weak inhibitor of NHE, to $38.7 \pm 4.9\%$ ($n = 3$). Low-$\text{Na}^+$-induced $\text{Ca}^{2+}$ oscillation was dose-dependently inhibited by HMA, a specific inhibitor of NHE (Fig. 3B). The percentages of the cells showing $\text{Ca}^{2+}$ oscillation in the presence of 5, 10, and 50 $\mu$mol/l HMA were $71.5 \pm 13.1$, $43.9 \pm 2.8$, and $0.0 \pm 0.0\%$, respectively ($n = 3$ for each experiment). Similarly, HMA dose-dependently inhibited low-pH-induced $\text{Ca}^{2+}$ oscillation (Fig. 3C). The percentages of the cells showing $\text{Ca}^{2+}$ oscillation in the presence of 5, 10, and 50 $\mu$mol/l HMA were $32.4 \pm 5.7$, $21.7 \pm 7.2$, and $0.0 \pm 0.0\%$, respectively ($n = 3$ for each experiment).

**$\text{pH}_i$ regulation by NHE.** We used RT-PCR analysis and immunoblotting to identify NHE expression in human brain.
microvascular pericytes. RT-PCR revealed that transcripts of NHE1 were expressed in human CNS pericytes (Fig. 4A). Western blot analysis showed a discrete band for NHE1 protein corresponding to the previously reported size (Fig. 4B). We also detected transcripts of NHE7 by using RT-PCR in the pericytes (data not shown).

To investigate the H\(^+\)/H\(_{11001}\) extrusion systems in human CNS pericytes, the intracellular pH recovery after cell acidification was examined by using the NH\(_4\)Cl prepulse technique. By means of a prepulse of the cells with 20 mmol/l NH\(_4\)Cl, intracellular acidification was induced by NH\(_3\) efflux (Fig. 4C). In the control cells, the rate of pH\(_i\) recovery in the first 5 min was 0.030 ± 0.037 pH U/min in HEPES-buffered saline (n = 12) and −0.007 ± 0.011 pH units/min in Na\(^+\)-free solution (n = 12, Fig. 4D). Therefore, the recovery from intracellular acidification is dependent on the extracellular Na\(^+\) in human CNS pericytes, thus suggesting that intracellular pH may be primarily regulated by NHE.

Mechanisms for Ca\(^{2+}\) oscillation and Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) store.
Thapsigargin, an inhibitor of sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA), caused a transient [Ca\(^{2+}\)]\(_i\) increase due to a Ca\(^{2+}\) leak from stored Ca\(^{2+}\) in the control cells, the rate of pH\(_i\) recovery in the first 5 min was 0.030 ± 0.037 pH U/min in HEPES-buffered saline (n = 12) and −0.007 ± 0.011 pH units/min in Na\(^+\)-free solution (n = 12, Fig. 4D). Therefore, the recovery from intracellular acidification is dependent on the extracellular Na\(^+\) in human CNS pericytes, thus suggesting that intracellular pH may be primarily regulated by NHE.

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the sarcoendoplasmic reticulum (Fig. 5A). After addition of 1 μmol/l of thapsigargin, the removal of extracellular Na\(^+\) did not cause [(Ca\(^{2+}\)]\(i\) oscillation or a transient [(Ca\(^{2+}\)]\(i\) increase (Fig. 5, A and C). On the other hand, 1 μmol/l FCCP, the mitochondrial uncoupler, did not affect the Ca\(^{2+}\) oscillation (Fig. 5, B and C). These results indicate that Ca\(^{2+}\) oscillation may be supplemented by Ca\(^{2+}\) release, not from the mitochondria, but from the sarcoendoplasmic reticulum.

**Effect of NHE1 knockdown.** To analyze the role of NHE1 in Ca\(^{2+}\) oscillation, mRNA of NHE1 was inhibited by RNAi. A significant reduction of NHE1 mRNA expression, but not of \(\beta\)-actin, was detected in pericytes treated with anti-NHE1 siRNA (Fig. 6A, 7.9 ± 2.7% of control, \(n = 3\)). Nontargeting (control) siRNA did not affect the mRNA expression of NHE1 or \(\beta\)-actin.

The intracellular pH\(_i\) regulation was evaluated by using the NH\(_4\)Cl prepulse technique in the NHE1 knockdown cells. This showed that the rate of pH\(_i\) recovery in the first 5 min was \(-0.012 \pm 0.008\) pH units/min in HEPES-buffered saline (Fig. 4, B, \(n = 7\)).

Ca\(^{2+}\) oscillations induced by Na\(^+\) removal were almost completely suppressed in the NHE1 knockdown pericytes (Fig. 6, B and C, 1.8 ± 4.1%, \(n = 5\)). In contrast, Ca\(^{2+}\) oscillations were observed in the pericytes treated with nontargeting siRNA (43.2 ± 21.1%, \(n = 5\)), thus indicating that the application of siRNA itself may not alter Ca\(^{2+}\) signaling.

Extracellular acidification-induced Ca\(^{2+}\) oscillations were also suppressed in the NHE1 knockdown pericytes (Fig. 6, D and E, 9.0 ± 6.4%, \(n = 5\)) compared with the cells with control siRNA (35.4 ± 9.4%, \(n = 5\)).

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**Fig. 4.** Expression of NHE1 and intracellular pH recovery after acidification using the NH\(_4\)Cl prepulse technique. A: expression of NHE1 and \(\beta\)-actin mRNA was examined by RT-PCR. B: expression of NHE1 protein was examined in human microvascular pericytes and positive control (K562 whole cell lysate; Santa Cruz Biotechnology). C: representative traces of changes in intracellular pH (pH\(_i\)) in human microvascular pericytes perfused with HEPES-buffered saline (black line) or Na\(^+\)-free solution (gray line) after a 3-min exposure to 20 mmol/l NH\(_4\)Cl. Gray dotted line represents cells transfected with anti-NHE1 siRNA. D: rate of pH\(_i\) recovery (dpH\(_i\)/dt, pH units/min) during first 5 min from initiation of acid recovery. *\(P < 0.05\) vs. control by ANOVA.

**Fig. 5.** Effects of depletion of intracellular Ca\(^{2+}\) stores on Ca\(^{2+}\) oscillation. Representative traces of changes in [(Ca\(^{2+}\)]\(i\), during Na\(^+\)-free external solution after depletion of Ca\(^{2+}\) store by 1 μmol/l thapsigargin (A) or 1 μmol/l carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (B). C: percentage of cells showing low-Na\(^+\)-induced Ca\(^{2+}\) oscillations after application of thapsigargin or FCCP. *\(P < 0.05\) vs. control by ANOVA.
When the pericytes were incubated in 5–50 μmol/l HMA, the proliferation of the cells was dose-dependently inhibited (Fig. 7A). Moreover, the proliferation of the cells transfected with anti-NHE1 siRNA was significantly suppressed compared with those treated with nontargeting siRNA (Fig. 7B).

**DISCUSSION**

The present study showed that Ca^{2+} oscillation spontaneously occurred in ~20% of the pericytes under quasi-physiological ionic conditions. Extracellular acidification (pH < 6.50) increased the proportion of the cells with Ca^{2+} oscillation. In contrast, extracellular alkalization did not induce the Ca^{2+} oscillation. Extracellular pH in the brain is reported to fall by 0.8–1.2 during ischemia and/or hypoxia (20, 33). Therefore, extracellular pH may be transformed into Ca^{2+} signaling in CNS pericytes. The linkage between extracellular pH and Ca^{2+} signaling potentially mediates diverse cellular functions of the cells and modulates microcirculation during cerebral ischemia.

Decreasing extracellular concentration of Na^{+} caused augmentation of the Ca^{2+} oscillation in a dose-dependent manner, although the frequency and magnitude of each Ca^{2+} spike were varied in the individual cells. On the other hand, high concentration of extracellular Na^{+} (166 mmol/l) abolished the oscillation. The osmotic change was not involved in the low-external Na^{+}-induced Ca^{2+} signaling. Pharmacological profiles with benzamil, HMA, and KB-R7943 suggest that NHE may be involved in the generation of the Ca^{2+} oscillation. NHE mediates the exchange of one proton in the intracellular or extracellular space and one Na^{+} in the opposite side across the membrane by using the energy of the sodium gradient (10, 17, 22, 25, 26, 29). Changing the ionic conditions of extracellular Na^{+} and protons may drive NHE in either direction according to the ionic gradient. Therefore, NHE may play a role in the transduction of the change in extracellular Na^{+} into Ca^{2+} signaling.

At least nine isoforms of NHE have so far been identified (22). Among them, NHE1 is expressed ubiquitously in most...
cell types. These experiments detected NHE1 in the human CNS pericytes. The recovery of intracellular pH from acidosis following the withdrawal of NH4Cl was dependent on external Na+, thus suggesting that intracellular pH may be regulated predominantly by NHE mechanism in CNS pericytes. The finding that knockdown of NHE1 by RNAi abolished recovery from intracellular acidosis in CNS pericytes supports the interpretation that NHE1 may be a principal regulator of intracellular pH in human CNS pericytes.

The knockdown of NHE1 abolished the Ca2+ oscillation induced by low Na+ as well as low pH level in the pericytes. In contrast, transfection with nontargeting siRNA did not affect the Ca2+ oscillation. These results indicate that NHE1 is essential for the generation of the Ca2+ oscillation in the pericytes. A chemical gradient of either Na+ or protons drives the NHE1 to countertransport the other, and both extracellular acidification and Na+ depletion can operate NHE1 in reverse mode, i.e., influx of proton and extrusion of Na+. Therefore, NHE1 causes the generation of the Ca2+ oscillation when it is operated in the reverse mode. NHE1 may play a pivotal role not only in maintenance of ionic homeostasis but also in the transduction of the extracellular environment of Na+ and protons into Ca2+ signaling. The link between the activity of NHE1 and Ca2+ signaling has not been previously reported. This novel function of NHE1 would regulate the signaling cascade by turning spatial and temporal changes of intracellular Ca2+. On the other hand, the knockdown of NHE1 did not abolish the transient Ca2+ increase. Therefore, the mechanism of the Ca2+ transients induced by low Na+ and low pH may be associated with factors other than NHE1 such as Ca2+ influx via NCX or acid-sensing ion channel.

Even in the absence of extracellular Ca2+, the Ca2+ oscillation was still induced by external low Na+. Moreover, the oscillation was not affected by a variety of inhibitors that block the numerous pathways for Ca2+ entry, including capacitative Ca2+ entry (La3+), nonselective cation channels (Gd3+), and L-type voltage-dependent Ca2+ channels (nicardipine). Coactivation of NCX was not involved in the Ca2+ oscillation, because the oscillation was not affected by KB-R7943. Neither stretching of the cells nor a change in cell volume was involved in the oscillation, because elevated osmotic pressure in the external solution by mannitol did not affect the oscillation. Ca2+ oscillation appears to be related to Ca2+ release from intracellular Ca2+ stores. In the present study, FCCP, a mitochondrial uncoupler, did not affect the oscillation; however, depletion of the ER by inhibiting SERCA with thapsigargin completely abolished the Ca2+ oscillation. These results suggest that low-Na+-induced Ca2+ oscillation is due to release and replenishment of Ca2+ from the ER. NHE1 comprises two domains: an NH2-terminal membrane domain that functions to transport ions and COOH-terminal cytoplasmic regulatory domain that regulates the activity and mediates cytoskeletal interactions (22, 29). NHE1 in the plasma membrane changes the concentration of Na+ and protons in the microdomain in the close vicinity of the ER. These changes may modulate the release of Ca2+ from the ER in the pericytes. On the other hand, a number of recent reports have suggested that NHE1 acts as a structural anchor regulating cytoskeletal organization (22, 29). Therefore, conformational coupling of NHE1 to enzymes or cytoskeletal elements may be involved in the regulation of the Ca2+ oscillation.

Intracellular Ca2+ is a ubiquitous second messenger regulating a wide range of cellular functions, including cell growth and proliferation. The spatial and temporal pattern of Ca2+ signaling, which is composed of the amplitude, frequency and duration of Ca2+ increase in the microdomain, is considered to be essential for controlling the cellular functions (6, 8, 24). A transient Ca2+ increase activates rapid cellular responses, such as secretion and muscle contraction. On the other hand, when signals are transmitted over longer time periods, repetitive spikes of Ca2+ oscillations mediate longer cellular processes, such as cell proliferation, cell migration, and fertilization (6, 19). Recent studies indicate that Ca2+ oscillation modifies the transcription of genes via activation of Ca2+-dependent transcriptional factors, such as cAMP response element binding protein and nuclear factor of activated T cells (4, 19, 31), thus leading to the modification of the cell cycle and the cell proliferation. Future investigation is needed to confirm the NHE1-Ca2+ oscillation-cell proliferation pathway.

It has been reported that NHE1 plays an important role not only in the regulation of ion homeostasis but also in the regulation of a wide variety of cellular functions (22, 29). However, the precise mechanisms of NHE in the modulation of physiological functions such as proliferation, migration, and morphology are still poorly understood. It is possible that NHE1-mediated change in pH, and/or intracellular Na+ concentration is an important signal for the regulation of diverse growth-related cellular functions. Another possibility is that direct protein-protein interaction triggers the intracellular signaling cascade of the growth of CNS pericytes. The present...
study suggested that NHE1 may play an important role in regulating periodic Ca\(^{2+}\) release from the endoplasmic reticulum as well as the proliferation of CNS pericytes. This novel role of NHE1 in Ca\(^{2+}\) signaling and cell proliferation in CNS pericytes may have pathophysiological relevance to CNS diseases. It has been reported that an inhibitor of NHE is protective against ischemic brain injury in vitro and in vivo; however, the precise mechanism remains unclear (13, 16, 21, 23). Disruption of retinal pericytes is acknowledged to be the initial step for diabetic retinopathy (5), and lack of CNS pericytes has been reported to cause numerous microaneurysms and an increase in vascular permeability in the brain (18). Therefore, NHE1 might modulate Ca\(^{2+}\) signaling and cell proliferation in response to external pH and regulate blood-brain barrier, capillary flow, and angiogenesis in the brain.

In conclusion, NHE1 may modulate Ca\(^{2+}\) signaling via the release of Ca\(^{2+}\) from the endoplasmic reticulum and thereby contribute to the regulation of proliferation of CNS pericytes. This novel role of NHE1 in the pericytes may therefore have pathophysiological relevance to angiogenesis in cerebral ischemia.

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