The δ-subunit of epithelial Na⁺ channels (ENaC) is predominately expressed in brain, heart, and pancreas. The amiloride sensitivity, Na⁺ conductance, and critical domains for gating are characterized as a cross between proton-activated Na⁺ channels and α-ENaC. The hypothesis that external protons may activate human δ-ENaC was addressed by expressing δβγ-hENaC in Xenopus oocytes and evaluating proton-activated current with the two-electrode voltage clamp technique. Our results showed that protons transiently evoked a Na⁺ current with an EC₅₀ of pH 6 overlapped on the basal current of δβγ-hENaC. Proton-activated current was not observed in uninjected oocytes. Studies on gating kinetics revealed that activation, desensitization, and recovery times of proton-activated Na⁺ current were 3.8 ± 0.5 s, 253 ± 9.5 s, and 10 ± 3.6 s, respectively (n = 4–12). Alkali metal cation selectivity of the proton-activated current was identical to that of the basal current of δβγ-hENaC. The metabolic acids, lactate, pyruvate, and formate, modified the proton-activated current, as did hypo-osmotic stress. EDTA, hypo-osmolarity, and lactate enhanced proton activation synergistically. Our results suggest that δ-hENaC subunit is essential for proton-activated current and γ-subunit may potentially regulate the response of δ-ENaC to protons. We have concluded that δβγ-hENaC is a proton-activated cation channel whose closing gate can be regulated by a proton-induced conformational change. Proton-sensitivity of δβγ-hENaC may be an important mechanism for integrating external ischemic signals in inflamed and hypoxic tissues.

Acidosis is very common under physiological and pathological conditions. Systematic acidosis, including respiratory and metabolic acid-base disorders, increases the extracellular proton concentrations throughout the body. Local hypoxia, inflammation, and ischemia generally lead to accumulation of metabolic acids, resulting in a pH decrease of the extracellular fluid. On the other hand, a pH drop is also a normal physiological occurrence. For example, external and cellular pH changes happen by the H⁺ ion and neurotransmitter translocation.

δ-subunit of epithelial sodium channels (ENaCs) belongs to the ENaC/degenerin superfamily, which was originally identified in Caenorhabditis elegans and colonic epithelia. So far, five isoforms of the ENaC branch of this superfamily have been identified. When δ-ENaC is expressed in oocytes, channels with low amiloride sensitivity, more Na⁺ permeability over Li⁺, and a unitary Na⁺ conductance of 12 pS resulted (1, 2). δ-ENaC is originally cloned from human kidney epithelial cells and also distributes in both epithelial tissues (i.e. pancreas, lung, testis, and ovary, etc.) and non-epithelial cells (i.e. astrocytes, cardiovascular cells, neurons, and skeletal muscles). β- and γ-ENaC subunits have been found in lung (3, 4), testis (2, 5), and astrocytes (6). Tissue distribution of δ-ENaC subunit mostly matches that of the proton-activated Na⁺ channels (ASIC), another member of the ENaC/degenerin superfamily. Location of these channels in non-epithelial tissues may reflect a function and regulation of δ-ENaC as distinct and diverse as its epithelial counterpart, namely α-ENaC subunit.

There is ample evidence of regulation of native amiloride-sensitive Na⁺ conductance in epithelial tissues and cloned ENaC by external pH. Ussing (7) found in 1949 that the short circuit current of frog skin decreased when external pH was below 5. Comparable findings were reported by several groups (8–10), and these observations were verified by Roussaud and Sladen (11) and Chang and Johnson (12) in mammalian epithelia. In contrast, a stimulation of amiloride-sensitive Na⁺ conductance in frog skin was observed as external pH declined (13–15). Recently, Chalfant et al. (16) reported that external acidic pH (pH 6.4) did not have any effect on rat αβγ-ENaC (rENaC) expressed in oocytes when examined by the two-electrode voltage clamp and cell-free planar lipid bilayer assays. Zhang et al. (17) found with cell-attached patches that only the αβ-ENaC channel (and not αβγ-ENaC) was down-regulated by external acidic pH. Acidic pH elevated the apical Na⁺ conductance in A6 cells within the first hour but decreased αβγ-ENaC activity expressed in oocytes following a slight transient rise (18). However, little is known about the effects of external protons on cloned δ-ENaC (19, 20).

The similarities in cation selectivity, amiloride affinity, and tissue location of δ-ENaC and ASIC channels prompted us to test the hypothesis that δ-ENaC is activated by external protons. This idea has been addressed by co-expressing δ-hENaC with human βγ-subunits in Xenopus oocytes and measuring the effects of protons on δ-hENaC channel activity.

MATERIALS AND METHODS

cRNA Preparation—cRNA for human δ-, β-, and γ-ENaC (hENaC) and their degenerin mutants were prepared as previously described (1, 2).
Oocyte Expression and Whole-cell Assay—A detailed description of this technique has been previously published (24). In brief, defolliculated oocytes were cytosolically injected with wild type and mutant hENaCs in 50 nl of RNase free water/oocyte of cRNA and incubated in half-strength L-15 medium. Whole-cell cation currents were measured by the two-electrode voltage clamp technique. Oocytes were impaled with two electrodes having resistances of 0.5–2 MΩ when filled with 3 mM KCl. A TEV 200 voltage clamp amplifier was used to clamp oocytes with concomitant recording of currents. Two reference electrodes were connected to the bath. Sampling protocols were given by pCLAMP 8.0, and current at ~60 mV was simultaneously monitored with a chart recorder. The control superfusate was ND96 (in mM: 96 NaCl, 1 MgCl₂, 1.8 CaCl₂, 2 KCl, and 5 HEPES, pH 7.5). Superfusate with pH 6 or below was buffered with MES. pH was adjusted with NaOH and HCl. Na salt was replaced with equal molar Li⁺, K⁺, and Cs⁺ for ion selectivity determination. Current-voltage relationships were acquired by stepping the holding potential in 20-mV increments from −120 to +80 mV after the monitoring currents were stable, with and without application of amiloride. To test the effects of hypo-osmolarity, extracellular divalent cations, lactate, and their combinations on the proton-activated δβγ-hENaC current, 40 mM NaCl (ND-40) was included in the bath solution (200 mosM) and mannitol was used to prepare the corresponding iso-osmotic medium (200 mosM). 5 mM EDTA or EGTA and 15 mM lactate were added.

Data were sampled at the rate of 1 kHz and filtered at 500 kHz. Because most proton-activated channels have fast activation and desensitization times of up to few seconds, pH switching was controlled by a SF-77B perfusion fast step system (Warner Instrument Corp., Hamden, CT) to deliver solutions with neutral or acidic pH to a tiny chamber with a 0.1 ml volume.

Data Analysis—Proton-activated Na⁺ current was computed by subtracting the basal current from the peak current in the presence of protons. The activation time constant (τa) and desensitization time constant (τd) were calculated by fitting the current trace of activation and decay with the built-in first order exponential function of CLAMPFIT 9. The fitted curves are depicted in Fig. 5. Recovery time (τrec) was calculated by fitting the fraction of proton-activated Na⁺ currents as a function of time interval between proton applications.

Computation of the EC₅₀ of external pH was performed by fitting the acid-activated current with the Hill equation as shown in Equation 1:

\[
I_{Na} = I_{max} \frac{pH^n}{pH_{EC50}^n + pH_{EC50}^n}
\]

where \(I_{Na}\) is the proton-activated current, \(I_{max}\) is the maximal acid-activated current, pH stands for the extracellular pH, EC₅₀ represents the value of pH, that results in half the maximal current of the ASIC channel, and \(n\) is the Hill coefficient.

All results are presented as mean ± S.E. One-way analysis of variance computation combined with the Bonferroni test was used to analyze data with unequal variance between each group. A significance level of 0.05 was employed.

RESULTS

Activation of δβγ-hENaC by Protons—To address the question of whether or not δ-hENaC is activated by external protons, human δ-ENaC was expressed in Xenopus oocytes with various compositions and acidic pH 4 was applied by a rapid perfusion system that has been successfully used to study ASIC currents (24). Fig. 1A shows a current trace elicited in an oocyte expressing δ-subunit alone. Acidic external pH 4.0 activated δ-ENaC-associated Na⁺ current at ~60 mV. The proton-activated Na⁺ current was not observed in uninjected oocytes; instead, protons decreased the background current of oocytes contributed by endogenous anion and cation channels (Fig. 1B). Proton-activated current levels of δβ (−2.4 ± 2 nA, n = 8) and δβγ-hENaC channels (−55 ± 11 nA, n = 8) were not markedly different from that of δ-subunit alone (−50 ± 16 nA, n = 9). In contrast, the proton-activated Na⁺ current of δβ-γ-hENaC was 44-fold greater (−1886.5 ± 269 nA, n = 35) than that of δ-ENaC itself (Fig. 1B). An EC₅₀ of 6.0 ± 0.03 for proton activation on δ-hENaC alone was retrieved by fitting the dose-response curve (Fig. 1C, n = 11). A similar observation expressed in oocytes has just been reported (25). Although the proton-activated current can be elicited in oocytes expressing δ-ENaC, it is too small for systematic study of its properties. Therefore, we amplified the δ-ENaC current magnitude by co-expressing δβγ-hENaC subunits based on the fact that there is no difference between the basic features of δ- and δβγ-hENaC channels (2).

As shown in Fig. 2A, inward current of δβγ-hENaC was reversibly inhibited by amiloride. Exposure to solution with pH 4.0 resulted in an ~50% increase in basal current. Proton-activated current returned to basal levels immediately upon restoration of the pH of the bathing solution to 7.5. Replacement of external Na⁺ with N-methyl-D-glucamine abolished Na⁺ conductance associated with δβγ-hENaC (Fig. 2B). If the basal current associated with δβγ-hENaC was inhibited by
amiloride, external protons could not elicit an increase in inward current (Fig. 2B).

To characterize the proton dependence of δβγ-hENaC, solutions of different pH were superfused over δβγ-expressing oocytes. Fig. 2C shows representative current traces elicited by external pH ranging from 8.3 to 4. Alkali pH 8.3 slightly depressed basal current, but acidic pH activated basal current in a concentration-dependent manner. An EC₅₀ of pH 6.0 ± 0.5 (n = 8) was determined by computer fitting the proton-activated current versus pH data (Fig. 2D). These results are consistent with our previous observations and identical to that of δ-ENaC alone (19, 20).

The response of rat and human αβγ-ENaC expressed in oocytes to external protons was also determined with the same protocol. Superfusion of oocytes with a solution at pH 4.0 decreased human αβγ-ENaC current (Fig. 3A). Similar results were obtained in oocytes expressing the rat αβγ-ENaC channel (not shown). Interestingly, in the absence of an external Na⁺ ion, an inward current was activated at pH 4.0 as observed in oocytes expressing δβγ-hENaC (Fig. 2A). In contrast to δβγ-hENaC, the basal current of αβγ-hENaC was significantly down-regulated by external protons (Fig. 3B), suggesting that the responses to protons between αβγ- and δβγ-hENaC are different (19).

Regulation of δβγ-hENaC by Intracellular pH—Intracellular acidosis down-regulated apical Na⁺ conductance of epithelial cells and cloned ENaC expressed in oocytes within hours (26).

Proton-activated current associated with δβγ-hENaC showed a slower activation (along the lines of several seconds) compared with those of the ASIC channels (27). Thus, the question arises as to whether the effect of external protons on δβγ-hENaC is mediated by cytosolic acidification. To test this hypothesis, membrane-permeable acetate was used to lower intracellular pH (16). Similar to the effect of intracellular acidification on αβγ-rENaC (18), acetate down-regulated basal current of δβγ-hENaC progressively over 20 min (Fig. 4A). In contrast to the stimulatory effect of external protons on δβγ-hENaC, internal protons significantly decreased both basal and proton-activated currents (Fig. 4B).

Gating Properties of Proton-activated Current—To characterize gating kinetics of the proton-activated current associated with δβγ-hENaC, we examined activation, desensitization, and recovery times. The activation time of δβγ-hENaC by external protons was longer (3.8 ± 0.5 s, n = 12) than that of the ASIC channel (<1 s). To estimate the desensitization time constant, oocytes were exposed to protons for a time sufficient to permit the current to return to basal levels (Fig. 5A). The average desensitization time was 253 ± 9.5 s (n = 4). Recovery time was determined by application of acidic pH at various time intervals. Proton-activated currents were normalized to the proton-activated current recorded post the maximal interval (25 s). Fig. 5B shows that the recovery time for proton-activated current of δβγ-hENaC was 10.4 ± 3.6 s (n = 8).

Ionic Selectivity of Proton-activated Current—To answer the
question of whether the proton-activated current of δβγ-ENaC has the same selectivity as the basal current, cation selectivity for basal and proton-activated currents was determined simultaneously. Fig. 5C shows representative traces of basal and proton-activated Na⁺ currents at potentials stepped from −120 to +80 mV. Their current-voltage relationship revealed an identical reversal potential (+40 mV), indicating that basal and proton-activated currents are both predominately Na⁺-permeable (Fig. 5D).

Whole-cell basal and proton-activated currents for δβγ-ENaC-expressing oocytes bathed in solutions having an alkali-metal cation different from Na⁺ were measured and summarized in Fig. 5E. These experiments revealed that proton-activated Li⁺ current is smaller than Na⁺ current, consistent with the findings for the basal current (1, 2). Estimated from the whole-cell current, the cation selectivity ratio in the order of Na⁺/Li⁺/K⁺/Cs⁺ for the basal current (1/0.70/0.44/0.07) was similar to that of the proton-activated current (1/0.50/0.44/0.1).

Protons Modify the Closing Gate via Conformational Change—Two channel gates formed by separate pre-H1 and pre-H2 domains have been proposed in ENaC channels (28–30). Mutating a degenerin site located in the pre-H2 domain (pore region) added a basal current to ASIC channels (31). Similar to ENaC, the basal current of degenerin-mutated ASIC channels was sensitive to amiloride and impermeable to K⁺. To explore the idea that protons may stimulate the basal current of δβγ-hENaC by modifying a closing gate, three degenerin mutants, δS526F, βS520F, and γS529F, were engineered and injected into oocytes with their conjugate wild type partners.

Fig. 6A shows representative proton-activated Na⁺ current traces evoked with pH 4.0. Either δS526F or γS529F alone resulted in the loss of total proton-activated Na⁺ current; in some cases, the response was reversed (Fig. 6B). βS520F likewise diminished the proton-activated current to 34.6% of the wild type response (p <0.05). In the case of co-expressing three degenerin mutants together, there were no basal and proton-activated currents in oocytes 3 days postinjection of δS526F, βS520F, and γS529F (data not shown). These oocytes had resting membrane potential identical to those of uninjected oocytes (from −30 to −60 mV).

To test the role of the cytoskeleton, we disrupted cytoskeletons of oocytes expressing δβγ-ENaC with cytochalasin D and/or nocodazole, 20 μg/ml (32). Both cytoskeletal disruptors affected neither the basal nor the proton-activated Na⁺ currents of δβγ-hENaC in voltage-clamped oocytes (data not shown).

Metabolic Acids and Hypotonicity Facilitate Proton-activated Current—The postulated physiological relevance for ASIC channels includes transduction of acid-mediated pain, mechanical stretch, and temperature (27). Even though metabolic acids generally take several minutes to produce a significant drop of external pH, it is reasonable to hypothesize ASIC as an integrator of all the aforementioned stimuli. To test the hypothesis that acidic metabolites facilitate proton-activated Na⁺ current associated with δβγ-hENaC, proton-activated current of δβγ-ENaC was evaluated in the presence and absence of lactate, pyruvate, and formate in an acidic superfusate.

As shown in Fig. 7, 15 mM lactate nearly doubled proton-activated Na⁺ current. Pyruvate and formate facilitated activation of protons on δβγ-hENaC less, but their effects were statistically significant. As observed by Imkne and McClesky (33), even 20-mM acidic metabolites still cannot reach the saturating level of stimulated current. Continuing to increase acidic metabolite concentration leads to marked changes in osmolality and pH. This prohibited us from determining the EC₅₀ of each acidic metabolite for the proton-activated δβγ current.

Hyponcotic generally develops a few minutes following ischemia (34, 35). To examine the response of proton-activated δβγ-hENaC current to hyponcotic, oocytes were perfused with ND-40 solution, which has an osmolality of 80 mosM. Hyponcotic exposure of oocytes increased proton-activated current by 92% (Fig. 7E). A combination of hyponcotic and lactate stimulated proton-activated δβγ-hENaC synergistically (Fig. 7E).

Several publications have reported that gating of ASIC channels by extracellular protons is regulated by intracellular and extracellular Ca²⁺ and Mg²⁺ (33, 36, 37). To test the hypothesis that extracellular divalent cations regulate the proton-activated current of δβγ-ENaC, we pretreated oocytes for 30 min with the membrane-permeable Ca²⁺ chelator, BAPTA_AM (50 μM), in the absence of extracellular Ca²⁺. The proton-activated peak current associated with δβγ-ENaC was not significantly affected either by pretreatment with BAPTA_AM (control: −2016 ± 125 nA versus BAPTA_AM: −1726.3 ± 335.4 nA, n = 8, p >0.05) or by removal of bath Ca²⁺ (1.8 mM: −1573.3 ± 154 nA versus 0 mM: −1696.7 ± 173 nA, n = 8, p >0.05). However, EDTA, but not EGTA, can augment the response to hyponcotic and lactate up to 2.5-fold (Fig. 7E). In the presence of both EDTA and lactate, the increased proton-activated current was stably elicited by repeated application of acidic solution, whereas the increment of proton-activated current with either hyponcotic or lactate ran down in a few minutes. These results indicate that the extracellular Ca²⁺/Mg²⁺ ratio rather than Ca²⁺ alone affects the proton-induced current of δβγ-hENaC.

DISCUSSION

Our results indicate that δβγ-ENaC is activated by extracellular protons. Proton activation of δβγ-ENaC current is re-
moved by degenerin mutations, leading to a conformational change as well as a decrease in closing time. Ion selectivity of the proton-activated \( H^+/Na^+ \)-hENaC current was identical to that of the basal current, suggesting that an unaltered channel pore is used for both processes. Regulation of the proton-activated current of \( H^+/Na^+ \)-ENaC by hypo-osmolarity and EDTA suggested that \( H^+/Na^+ \)-ENaC may integrate ischemia-related signals. In epithelial tissue, \( H^+/Na^+ \)-ENaC may combine with \( H^+/Na^+ \)-ENaC subunits to regulate salt and water balance across the epithelium under hypoxic conditions. In non-epithelial tissues, \( H^+/Na^+ \)-ENaC may share physiological function with proton-activated channels in the transduction of acidosis, stretch, and other ischemia-induced signals.

**Mechanisms for Proton-activated Conductance**—Amiloride sensitivity and alkali metal salt permeabilities of proton-activated current in oocytes expressing \( H^+/Na^+ \)-ENaC exclude the involvement of endogenous cation channels. MES and Hepes pH buffers are membrane-impermeable, and Na⁺ substitution eliminated proton-activated currents more than 80%, so it is unlikely that the acid-activated current is carried by protons. However, external proton-induced inward currents of \( H^+/Na^+ \)- and \( H^+/Na^+ \)-hENaC channels in the absence of bath sodium indicate that ENaC channels may be slightly permeable to H⁺ ion (38). A second line of evidence comes from examination of the effect of intracellular protons on basal and proton-activated currents (Fig. 2). Intracellular acidosis, instead of enhancing proton-activated current, abolished it gradually over minutes. A similar response to external protons was not observed in uninjected oocytes and in oocytes expressing \( H^+/Na^+ \)-ENaC, further confirming that activation of \( H^+/Na^+ \)-ENaC by protons is specific.

Fast activation kinetics suggested that protons may not activate \( H^+/Na^+ \)-ENaC by recruiting new channel proteins from a subplasma membrane channel pool. Trafficking and internalizing measurements of ENaC in oocytes revealed that these processes generally occurred in minutes or even hours (39). It is well known that unitary conductance is not altered when co-expressed with \( H^+/Na^+ \)-ENaC; an increased open probability, then, may mediate pH activation of \( H^+/Na^+ \)-ENaC. As reported by Kellenberger et al. (40), only ~10% of ENaC channels located at the plasma membrane can be detected electrically at any given time. Thus, another mechanism of proton activation is that acidic pH values increase the percentage of active ENaC channels.

It is not surprising to see that the pH sensitivity of \( H^+/Na^+ \)-ENaC is identical to that of \( H^+/Na^+ \)-ENaC. Several groups have found that \( H^+/Na^+ \)-like ENaC, including \( H^+/Na^+ \), \( H^+/Na^+ \), and \( H^+/Na^+ \)-ENaC, exhibit the same biophysical properties as those of channels co-expressed with \( H^+/Na^+ \)-subunits. Co-expression of \( H^+/Na^+ \)-ENaC did not influence
ion selectivity, amiloride sensitivity, or conductance. However, mutagenesis revealed domains in \( \gamma \)-ENaC as well as in \( \alpha \)-ENaC that affect self-inhibition and amiloride sensitivity (41, 42). Our results confirmed that activation of protons is mainly mediated by the degenerin site of \( \delta \) - and \( \gamma \)-subunits. Because \( \delta \)-ENaC alone was activated by protons, and neither regulatory subunit \( \beta \)-nor \( \gamma \)-ENaC co-expression increased the proton-activated \( \mathrm{Na}^{+} \) current (Fig. 1B), \( \gamma \)-ENaC may potentially regulate the response of \( \delta \)-subunit to protons as revealed by degenerin site mutations (Fig. 6).

Although proton activation on \( \delta \beta \gamma \)-ENaC requires the presence of a degenerin site, protons may not interact with this residue directly. Serine is not a proton-sensitive amino acid, and an identical site in \( \alpha \beta \gamma \)-rENaC showed a different response to protons. On the other hand, the gating kinetics of proton-activated current associated with \( \alpha \beta \gamma \)-ENaC is reminiscent of the acid-activated current produced by a degenerin mutant, namely, G430F-ASIC1a (24). The response of G430F-ASIC1a to protons excluded the interaction of protons with the degenerin site. Instead, the \( \mathrm{pK}_a \) of the His residue (6.0) is identical to the
EC$_{50}$ of protons activating $\delta$- and $\delta\beta\gamma$-ENaC currents (Figs. 1C and 2D). Glu and Asp amino acid residues showing a pK$_a$ close to 4.0 may also contribute to the stimulatory effect of protons on $\delta\beta\gamma$-ENaC. Our results support the view that protons titrate pH-sensitive residues in the extracellular loop, leading to a conformational change of channel protein and then causing an action on the closing gate mimicking that of ASIC. Yamamura et al. (25) very recently reported an EC$_{50}$ of 5.0 for $\delta$-hENaC expressed in Chinese hamster ovary cells, which differs from our observation (6.0) in oocytes. Inconsistent expression systems and time required for recovery between every proton exposure may contribute to this diversity.

As we mentioned before, there are 26 His, 11 Asp, and 22 Glu amino acid residues located in the extracellular loop of $\delta$-ENaC that are potential proton regulatory sites. Amiloride at micromolar concentration may not be able to shield all of these 59 acid-sensitive residues in total. Instead, protons may bind to these regulatory sites and lead to a conformational change of ENaC proteins and finally stimulate channel activity. Based on our observations as shown in Figs. 1 and 2, protons may not competitively bind to the out mouth of the channel with amiloride and Na$^+$. Two amiloride binding sites in the extracellular domain of ENaC have been identified (1, 44). Amiloride binding most likely prevents the conformational change resulting from acidic external pH and/or blocks Na$^+$ entry through the channel pore.

Protons may not influence the coordination and interactions of ENaC subunits. A body of evidence demonstrated that both $\beta$- and $\gamma$-ENaC subunits amplify the current amplitude of $\delta$- and $\alpha$-ENaC (2, 22). The current density of $\alpha\beta$ and $\alpha\gamma$ channels is approximately as low as that formed by $\alpha$-subunit alone (22). Regardless of subunit composition, no studies showed that changes in subunit ratio, normally 2$c$:1$\beta$:1$\gamma$ or 3$a$:3$\beta$:3$\gamma$, regulate ENaC channel activity.

**Proton-activated ENaC as an Integrator of Ischemia-related Signals**—Predominate expression of $\delta$-ENaC in heart and brain implies that the functions of this isoform may differ from those distributing at epithelial tissues (2). pH sensitivity of $\delta$-ENaC led us to hypothesize that $\delta$-ENaC may serve as a pH sensor by itself or as an element of a proton receptor complex in excitable cells. Lactic acid, pyruvic acid, and formic acid are generally produced during cardiac and brain ischemia and result in acidosis within minutes (45–47).

Lactate, pyruvate, and formate may accumulate in cytosol via their transporters, which results in intracellular acidification and elevation of Ca$^{2+}$ (48–50). However, intracellular acidification and BAPTA treatment do not support the idea that these metabolic acids cytosolically enhance proton activation on $\delta$-ENaC; instead, they potentiate $\delta$-ENaC, possibly by chelating extracellular divalent cations. A similar mechanism has been proposed to explain potentiation of proton-gated Na$^+$ channels by lactate in ischemia-sensing neurons (33, 43, 51). Facilitation of proton-activated current by these acidic metabolites strongly indicates that $\delta\beta\gamma$-ENaC is most likely involved in ischemic signal transduction. Not unique, this phenomenon has also been reported in ischemia-sensing neurons (33).

Membrane stretch occurs after a decrease in osmolarity of extracellular fluid during brain and heart stroke (34, 35). A potentiation of the proton-activated $\delta$-ENaC current was observed following a hypotonic challenge (Fig. 7E). Generally, both hypotonic stress and removal of extracellular Ca$^{2+}$ have been proved to cause an increment in cytosolic Ca$^{2+}$. However, BAPTA pretreatment cannot affect the proton-induced current, indicating that cytosolic Ca$^{2+}$ may not play a role in hypotonicity-facilitated current. A significant rise in the proton-induced current by hypotonic medium with 1.8 mm, but not in the absence of external Ca$^{2+}$, suggests that external Ca$^{2+}$ regulates $\delta$-ENaC in a dose-dependent pattern or Ca$^{2+}$ exerts two separate effects. A dual effect of external Ca$^{2+}$ on ASIC channel has also been observed (36).

The diverse action of hypotonicity in the presence and absence of extracellular Ca$^{2+}$ implies that external Mg$^{2+}$ may also be involved in modification of proton activation. Additional supportive evidence comes from EDTA and EGTA application in the presence of external Ca$^{2+}$ and Mg$^{2+}$. EGTA is a specific chelator of Ca$^{2+}$, whereas EDTA is a chelator for both Ca$^{2+}$ and Mg$^{2+}$. The facilitating effects of EDTA and EGTA on proton-induced current are inconsistent, indicating that not only Ca$^{2+}$...
itself but also the Ca\(^{2+}\)/Mg\(^{2+}\) ratio regulate proton activation on \(\delta\)-ENaC. Taken together, enhancement of proton-activated current by hypo-osmolarity, decreased extracellular divalent cation, or a combination of acidic metabolites further suggest that \(\delta\)-ENaC may integrate ischemia-related signals as well as ASIC channels (43). 

**Potential Impact**—In brain and heart, acidic metabolites can produce a fall of more than one pH unit in few minutes post-stroke (45, 46). ASIC channels are thought to be activated to transduct ischemia-related signals. However, the process of pH fall generally requires minutes to develop. The millisecond activation of ASIC may be an additional limitation for them as effective pH sensors. Compared with ASIC channels, \(\delta\)-ENaC is distinguished by a very slow activation and desensitization process, allowing ischemic cells to have enough time to detect changes in proton concentration and osmolarity. Another striking feature of \(\delta\)-ENaC is its slow desensitization. The ASIC channel generally desensitized in less than a second (31), whereas \(\delta\)-ENaC took minutes. Thus, the gating properties of \(\delta\)-ENaC indicated that it is an ischemic sensor different from ASIC channels.

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Acid Sensing of \( \delta \)-ENaC

26947

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