Selective regulation of acid-sensing ion channel 1 by serine proteases

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Running title: protease regulation of ASIC1

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Abbreviations: ASIC, acid-sensing ion channel; ENaC, epithelial Na⁺ channel; GTP-γS, guanosine S'-o-(3-thiotriphosphate), IpH6, peak current amplitude induced by acidification to pH 6; pH0.5, pH for half-maximal activation; pHln0.5, pH for half-maximal inactivation; TLCK, tosyl-L-lysyl-chlormethan; t-PA, tissue plasminogen activator.
Summary

Acid-sensing ion channels (ASICs) are neuronal $\text{Na}^+$ channels which belong to the epithelial $\text{Na}^+$ channel/degenerin family. ASICs are transiently activated by a rapid drop in extracellular pH. Conditions of low extracellular pH, such as ischemia and inflammation in which ASICs are thought to be active, are accompanied by increased protease activity. We show here that serine proteases modulate the function of ASIC1a and ASIC1b but not of ASIC2a and ASIC3. We show that protease exposure shifts the pH dependence of ASIC1a activation and steady-state inactivation to more acidic pH. As a consequence, protease exposure leads to a decrease in current response if ASIC1a is activated by a pH drop from pH 7.4. If however acidification occurs from a basal pH of ~7, protease-exposed ASIC1a shows higher activity than untreated ASIC1a. We provide evidence that this bi-directional regulation of ASIC1a function also occurs in neurons. Thus, we have identified a mechanism that modulates ASIC function and may allow ASIC1a to adapt its gating to situations of persistent extracellular acidification.

Keywords: ASIC, serine protease, trypsin, regulation
Introduction

Acid-sensing ion channels (ASICs) are non-voltage-gated Na\(^+\) channels that are transiently activated by a rapid drop in extracellular pH (1-3). They are members of the epithelial Na\(^+\) channel (ENaC)/degenerin family of channel proteins. Functional ASICs are formed by homo- or heterotetrameric assembly of ASIC subunits 1a, 1b, 2a, 2b and 3. Each subunit has two transmembrane domains that are separated by a large extracellular loop. ASIC1a, 2a and 2b are expressed in the peripheral and the central nervous system, whereas expression of ASIC1b and 3 is restricted to the peripheral nervous system. ASICs in sensory neurons play a role in the sensation of pain due to inflammation or ischemia (4-6). ASICs in central neurons might contribute to the neuronal death associated with brain ischemia or epilepsy, which are accompanied by extracellular acidification (7,8). In addition, fluctuations in extracellular pH also occur during normal brain function. Consistent with a role in physiological functions, ASIC1a knockout mice showed a defect in spatial learning and fear conditioning (9,10).

To date, the only known activators of ASICs are protons. However, the ASIC response to pH depends on the extracellular Ca\(^{2+}\) concentration and can be modulated by extracellular Zn\(^{2+}\) and by FMRFamide and related peptides (11-15). It is known that ENaC, which belongs to the same family as ASICs, is the target of serine proteases, which either inactivate (16,17) or activate this channel (18,19). Proteases are released or activated in many situations in which ASICs are thought to be active. Inflammation, which likely activates ASICs in the periphery, also leads to release and activation of proteases (20). In the central nervous system several proteases are present and are among other functions involved in the regulation of neuroplasticity (21). For these reasons we considered that modulation of ASIC
function by proteases might be possible and physiologically relevant. We have examined such a potential modulatory role of serine proteases and show that serine proteases affect pH-dependent gating of ASIC1a and ASIC1b, but not of ASIC2 and ASIC3. Extracellular exposure to the protease shifts the pH dependence of channel activation and – in the case of ASIC1a - of steady-state inactivation to more acidic pH, thus leads to a shift of the dynamic range of ASIC function. As a consequence, protease exposure has a dual effect. ASIC currents elicited by acidification from a basal physiological pH (≥ 7.3) are diminished after protease exposure due to the shift in the activation curve. During pathological situations such as ischemia or inflammation the extracellular pH decreases. At decreased basal extracellular pH (< pH 7.0) recombinant ASIC1a and ASICs of hippocampal neurons are inactivated and cannot be activated by further acidification. However, after protease exposure, the same channels can be substantially activated under these conditions due to the shift in the steady-state inactivation curve, indicating a potential mechanism for adaptation of ASIC gating to situations of decreased basal extracellular pH.
Experimental Procedures

**Recombinant expression of ASICs**
For the expression of heteromeric ASICs we performed transient transfections in COS cells by using the PerFectin reagent (Gene Therapy Systems, San Diego, CA, USA), as described previously (22). Cells were studied on days two and three after transfection.

Cell lines that stably expressed ASICs were established for the homomultimeric ASIC1a, 1b, 2a and 3. The original cDNA constructs were kindly provided by D. Corey (human ASIC1a, 2a), M. Lazdunski (rat ASIC3) and S. Grunder (rat ASIC1b). The cDNAs were subcloned into the pEAK8 expression vector (Edge Biosystems, Gaithersburg, MD, USA). A unique EcoRV restriction site within the N-terminal part of the coding region of ASIC1b was used for the sub-cloning, in order to obtain a construct that corresponds to ASIC1b-M3, as described by Bassler et al. (23). Stable cell lines were established in CHO cells as described previously (22).

*Xenopus laevis* oocyte experiments were carried out as described previously (24). Complementary RNAs were synthesized in vitro. Healthy stage V and VI *Xenopus* oocytes were pressure injected with 100 nl of a solution containing the cRNA at 100 ng/µl and oocytes were kept in modified Barth solution during the expression phase.

**Isolation and culture of mouse hippocampal neurons**
Mouse embryonic hippocampal neurons were obtained from timed pregnant mice at 17 days gestation as described previously (22). Hippocampi were dissociated mechanically and isolated cells were plated in Neurobasal (Gibco), supplemented by 2% B27 (Gibco) and glutamine and glutamate at a final concentration of 500 and 25
µM, on poly-lysine coated cover slips at a density of usually 10000 cells/cover slip of 11 mm diameter and kept in 95% air 5% CO₂ at 37°C. The neurons were used after 6-14 days in culture.

**Biochemistry**

The HA (hemagglutinin) tag sequence YPYDVPDYA was introduced by PCR immediately upstream of the stop codon of hASIC1a and verified by sequencing. Trypsin exposure of ASIC1a-HA expressing oocytes was done 24 hours after oocyte injection at room temperature in 110 mM NaCl, 1 mM CaCl₂, 10 mM Hepes-NaOH pH 7.5, trypsin 200 µg/ml for 5 minutes. After trypsin exposure, oocytes were washed twice in recording solution and protein extraction was performed in 25 mM Tris-HCl, 5 mM EDTA, 100 mM NaOH, 0.5 % SDS, 40 µl per oocyte. Oocytes were vortexed, left 5-10 minutes at room temperature and centrifuged 15 minutes at 4°C at 15,000 rpm. Proteins were then recovered from the intermediate layer (between the pellet and the lipid layer) and separated by 10% SDS-PAGE. After a transfer onto a nitrocellulose membrane (Schleicher & Schuell), polypeptides were exposed to mouse anti-HA primary antibody (Eurogentec, Geneva, Switzerland) and goat anti-mouse horse radish peroxidase (Amersham) as secondary antibody and visualized using Super Signal® West Dura Extended Duration Substrate (Pierce).

**Venom, chemicals and enzymes**

The Psalmopoeus cambridgei venom was obtained from Spider Pharm (Yarnell AZ, USA) and was used in all experiments at a 1:20'000-fold dilution. The P. cambridgei venom inhibits ASIC1a currents due to a toxin contained in the venom, Psalmotoxin 1 (25). Amiloride, guanosine 5’-O-(3-thiotriphosphate) (GTP-γS), proteinase K, soybean
trypsin inhibitor and trypsin (DPCC-treated trypsin from bovine pancreas, a form of trypsin with low level of chymotrypsin contamination) were obtained from Sigma (Buchs, Switzerland). Chymotrypsin (TLCK-treated α-chymotrypsin, a form of chymotrypsin with a low level of trypsin contamination) was purchased from Fluka (Buchs, Switzerland). All other chemicals were obtained from Sigma or Fluka.

**Electrophysiological measurements and analysis**

We used an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany) and Pulse and PulseFit software for data acquisition and analysis. The sampling interval was 50-100 µs for current-clamp experiments and 1-5 ms for voltage-clamp experiments of ASIC currents and low-pass filtering was set to 5 kHz in all experiments. Experiments were carried out in the whole-cell configuration of the patch-clamp technique. In voltage-clamp experiments cells were clamped to –60 mV and in current-clamp experiments current injection was adjusted to obtain in the absence of stimuli a membrane potential of –60 mV. For rapid changes of extracellular solutions we used a micromanifold that brings 8 or 9 tubes into one outlet tube (MPRE8, Cell MicroControls, Norfolk, VA, USA and MSC-200 Biologic, Claix, France). The solution flow was controlled by computer-driven solenoid-valves. Extracellular solutions contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM MES, 10 mM HEPES, 10 mM Glucose and pH was adjusted to 7.5 or the values indicated, with NaOH. Proteases were applied in the extracellular solution at pH 7.5. Where not noted explicitly, the pH between acidifications was held at 7.5. This pH was chosen because ASIC1a currents have substantially less rundown at pH 7.5 compared to pH 7.4. Pipettes were pulled from Borosilicate glass (World Precision Instruments, Sarasota, FL, USA). Pipettes had a resistance of 2 – 4 MΩ, when filled with the
pipette solution. Series resistance was compensated for by 40-60%. The pipette solution contained 90 mM K Gluconate, 60 mM Hepes, 10 mM KCl, 10 mM NaCl, 5 mM MgCl₂, 10 mM EGTA, pH 7.3, and in some experiments 140 mM KCl, 10 mM Hepes, 5 mM MgCl₂, 10 mM EGTA, pH 7.3, adjusted with KOH.

The pH activation curves were fit by using the Hill equation:

\[ I = \frac{I_{max}}{1 + \left(\frac{10^{-pH_{0.5}}}{10^{pH}}\right)^{H_n}} \]

where \( I_{max} \) is the maximal current, pH0.5 is the pH at which half of the channels are opened and Hn is the Hill coefficient, using KaleidaGraph (Synergy software). Steady-state inactivation curves were fit by an analogous equation. Data are presented as mean ± SEM.
Results

*Subtype-specific modification of ASIC function by trypsin*

We have studied ASIC1a currents in stably transfected CHO cells in whole-cell voltage-clamp to $-60$ mV. These cells were exposed to the serine protease trypsin at 200 $\mu$g/ml at pH 7.5, and ASIC currents were induced every 40 s by a 5-s extracellular acidification to pH 6. The application of the acidic solution to ASIC1a-expressing CHO cells elicits a fast activating, transient inward current (Fig. 1A). Figure 1A illustrates that trypsin exposure leads to a decrease in pH6-induced peak current ($I_{pH6}$). The mean time course of $I_{pH6}$ inhibition is shown in Fig. 1B for different trypsin concentrations. It shows that the $I_{pH6}$ decreases faster at higher trypsin concentrations. This decrease in $I_{pH6}$ was not reversible upon washout of the protease (data not shown). Activity of a related channel, ENaC, is known to be increased at low trypsin concentration (2 $\mu$g/ml). At this trypsin concentration, ASIC1a $I_{pH6}$ showed a slow decrease (Fig. 1B). The decrease in $I_{pH6}$ by trypsin was prevented by the presence of soybean trypsin inhibitor or by pre-treatment of trypsin with Tosyl-L-lysyl-chlormethan (TLCK, Fig. 1C). TLCK is a small reagent that modifies irreversibly a histidin residue in the catalytic site of trypsin. Our observation therefore suggests that the proteolytic action of trypsin is required for its effect on ASIC1a currents. To test whether cleavage occurs, we have exposed intact *Xenopus* oocytes expressing HA-epitope tagged ASIC1a during 5 min to 200 $\mu$g/ml trypsin and have subsequently analyzed the protein by Western blot. The ASIC1a $I_{pH6}$ decrease upon trypsin exposure of ASIC1a-expressing oocytes was not different from that in ASIC1a-CHO cells (data not shown). The biochemical analysis revealed a strong signal at $\sim 70$ kDa in ASIC1a-injected, but not in non-injected oocytes (Fig. 1D).
Trypsin exposure of ASIC1a-injected oocytes led to the appearance of an additional, lower molecular band (~55 kDa), which corresponds to the cleaved channel protein. As the HA epitope in this construct is located at the C-terminus, our finding indicates cleavage in the N-terminal part of the extracellular loop. Because the isolated ASIC1a protein comes to a large extent from intracellular compartments which are not reached by the extracellular trypsin, the upper (70 kDa) band is not completely cleaved.

It is known that proteases can activate protease-activated receptors, a class of receptors that couple to G protein. To evaluate a potential role of G protein in the response to trypsin, we monitored the change in IpH6 during exposure to extracellular trypsin in the presence of the non-specific G protein activator GTP-γS in the pipette solution. The baseline IpH6 and its change with time was similar in the control and the GTP-γS condition and the IpH6 was similarly decreased by trypsin in both conditions (Fig. 1E). This indicates that the effect of trypsin on ASIC current is independent of G proteins.

To test whether the inhibition by trypsin is specific for ASIC1a, we have exposed CHO cells expressing ASIC1b, ASIC2a or ASIC3 to 200 µg/ml trypsin and have monitored the IpH6 (IpH5 in the case of ASIC2a) during a period of 5 min. The currents mediated by ASIC1b, 2a and 3 were not affected by the exposure to trypsin (Fig. 2A, Table 1), indicating that trypsin specifically modifies the function of ASIC1a. The trypsin modification of ASIC1a function was further confirmed by experiments with two other serine proteases, chymotrypsin and proteinase K. Interestingly, chymotrypsin and proteinase K affected in addition to ASIC1a also 1b currents, but not ASIC2a and 3 (Table 1). The time course of the ASIC1a IpH6 decrease was similar in the presence of 200 µg/ml of each of the three enzymes tested (Fig. 2B).
Papain, a member of the class of cysteine proteases, and different types of collagenase, which belongs to the class of metalloproteinases, did not significantly change ASIC1a IpH6 (data not shown).

We have then tested whether proteases can modify the function of ASIC1-containing heteromeric ASICs. This analysis showed that the IpH6 of ASIC1a2a decreased rapidly in the presence of trypsin or proteinase K, while ASIC1a3-mediated IpH6 decreased in the presence of chymotrypsin and proteinase K, but was insensitive to trypsin (Table 1). In brain, most functional ASICs appear to be either ASIC1a homomultimers or heteromers that contain ASIC1a together with ASIC2a and/or ASIC2b (26-28). All these channels can thus be regulated by proteases.

**Proteases change the functional properties of ASIC1**

Protease exposure did not affect the inhibition of the ASIC1a current by amiloride (data not shown). However, after protease exposure, the ASIC1a current was no longer inhibited by the venom of Psalmopoeus cambridgei, an ASIC1a-specific inhibitor (25). This is shown for trypsin-exposed ASIC1a in Fig. 3A.

To understand the mechanisms that underlie ASIC current inhibition by proteases and to detect related functional changes induced by protease exposure, we have compared the following functional properties of ASIC1a and ASIC1b after protease exposure with those of untreated channels: (1) the pH dependence of activation, (2) the time course of open-channel inactivation, (3) the pH dependence of steady-state inactivation, and (4) the time course of recovery from inactivation. In Fig. 3B, representative traces of ASIC1a currents induced by acidification from pH 7.5 to different pH values are shown, which were obtained from untreated cells (left panel) or from cells that had been exposed to 200 µg/ml trypsin during 315 s (Fig. 3B, right
In Fig. 3C, the ASIC peak currents, normalized to the IpH6 measured before trypsin exposure, are plotted as a function of pH. Figure 3C shows that trypsin exposure resulted in a reduction of the maximal peak current, and in a shift of the pH dependence of ASIC activation to more acidic pH values. A fit of ASIC1a currents before exposure to trypsin to the Hill equation yielded a pH for half-maximal activation (pH0.5) of 6.6 ± 0.0 and a Hill coefficient of 4.6 ± 1.5 (n = 4), while the corresponding values after trypsin exposure were 5.8 ± 0.1 and 1.7 ± 0.4 (pH0.5, Hn, n=6).

ASIC current responses to extracellular acidification are transient because ASICs inactivate (or desensitize) in the continued presence of the low pH solution. The rate of open-channel inactivation increases with the acidity of the stimulus in ASIC1a-, 1b- and 2a-mediated currents (29). This is consistent with the observation that in classical ligand-gated channels, inactivation is induced by ligand binding and becomes faster with increasing agonist concentration (30). Because exposure to proteases shifted the pH-dependence of ASIC1a activation by ~1 unit to more acidic pH, we have verified whether the pH dependence of the time course of open-channel inactivation was also shifted. Direct comparison at pH 6 showed that protease exposure did not change the time course of open-channel inactivation (inset Fig. 3D, Table 2) and the comparison of the rate of open-channel inactivation, k, over the pH range 3.5 – 6.8 indicated that trypsine exposure did not shift its pH dependence (Fig. 3D).

There is evidence that inactivation can occur at pH values that are not sufficiently acidic to activate ASICs and it is known that ASIC1a is at pH 7.5 already partially inactivated (1,31). This steady-state inactivation of ASICs is physiologically
important, because it determines for a given basal pH the fraction of ASICs at the plasma membrane available for activation. We have determined the pH dependence of the steady-state inactivation by exposing ASIC-expressing cells during 40 s to the conditioning pH in the range of 8.0 to 6.6, before switching to pH 5 for ASIC activation. Figure 4A illustrates the protocol and shows current traces from representative experiments before and after trypsin exposure. Figure 4B plots the normalized pH5-induced peak current as a function of the conditioning pH. The pH for half-maximal inactivation (pHIn0.5) before protease exposure was 7.2 ± 0.0 (n=5) for ASIC1a. The pH dependence of inactivation was steep with a Hill slope of 5.0 ± 0.2, consistent with previous studies (2,32). The main effect of trypsin exposure was a shift in the pH-dependence of steady-state inactivation to more acidic pH, leading to an increase in the number of channels available for opening in the pH range 6.8 – 7.3 relative to untreated ASIC1a (Fig. 4B). The steady-state inactivation curve contained in addition a minor component with a pHIn0.5 of 7.7, leading to a small current decrease relative to untreated ASIC1a at conditioning pH of 7.4 – 8.0 (Fig. 4B, Table 2). The acidic shift in the pH dependence of steady-state inactivation leads to the situation that at basal pH in the range of 7.3 – 6.7 protease exposure will result in an increase of channels available for activation.

Inactivated ASICs require exposure to sufficiently alkaline pH for a certain duration before they can be activated again by an acidification. It has previously been shown that the time course of the recovery from inactivation depends on the ASIC type (33,34). A fast recovery from inactivation may be physiologically important during rapid pH oscillations (7). To measure the time course of the recovery from inactivation we have separated two acidic stimulations to pH 6 by a short exposure to pH 7.5 for channel recovery from inactivation. This basic protocol was repeated
several times with intervals of increasing duration between the two stimulations, until the interval was sufficiently long to allow complete recovery of the pH6-induced current. Figure 4C illustrates the protocol and shows current traces from representative experiments before and after exposure to trypsin. In Fig. 4D the current fraction recovered in the second stimulus is plotted against the interval between the two acidic stimulations, thus showing the time course of current recovery from inactivation. Untreated ASIC1a required ~10 s exposure to pH 7.5 for complete recovery from inactivation, consistent with previous studies (33,34). An exponential fit of the data yielded a rate of recovery of $0.42 \pm 0.02 \text{ s}^{-1}$. After trypsin exposure, recovery was complete within 2-3 s, with a rate of recovery of $2.44 \pm 0.32 \text{ s}^{-1}$ (Fig. 4B, Table 2). These experiments indicate that protease-modified ASIC1a responds better to high frequency stimulation.

Most effects of trypsin, chymotrypsin and proteinase K on ASIC1a and of chymotrypsin and proteinase K on ASIC1b function were similar. They are summarized in Table 2. We observed the following differences dependent on the channel type and the protease used: The shift in the pH dependence of activation and the acceleration of recovery from inactivation were slightly larger in ASIC1a compared to ASIC1b. In ASIC1a, chymotrypsin and proteinase K induced a larger shift in the pH dependence of activation compared to trypsin. On steady-state inactivation however, protease exposure had different effects in ASIC1a and ASIC1b. Unmodified ASIC1a and ASIC1b have a different pH sensitivity to steady-state inactivation, with pHIn0.5 values of $7.2 \pm 0.0$ (n=5) for ASIC1a and $6.7 \pm 0.0$ (n= 4) for ASIC1b. After protease exposure, the pH dependence of steady-state inactivation was in most cases complex and was best fitted by two components and was intermediate between that of untreated ASIC1a and ASIC1b. In ASIC1a, the main
effect of protease exposure was a shift of the inactivation curve to more acidic pH, thus increasing the number of channels available for opening, while for ASIC1b the protease exposure led to a shift of the inactivation curve to more alkaline pH (Table 2).

Different time course of the shift in the activation curve and the decrease in maximal ASIC current
The decrease observed in IpH6 of ASIC1a and 1b during exposure to proteases (Figs. 1 and 2) is in part due to the shift in the activation curve that leads to a reduction of the fraction of channels activated by pH 6. In addition, exposure to the proteases tested led to a substantial decrease in the maximal peak current of both, ASIC1a and ASIC1b, measured as response to acidification by pH 4 (IpH4). This is illustrated in Fig. 3C for trypsin modification of ASIC1a. For ASIC1b, the shift in the steady-state inactivation curve due to protease action reduces the number of channels that can be opened by acidification and thus also contributes to the decrease in IpH6 and IpH4 observed. However, for both ASIC1a and 1b, additional mechanisms must exist that lead to the decrease observed for IpH4 and IpH6. It is possible that several cleavage events can occur sequentially, which first modify channel gating and eventually may disrupt channel activity. To compare in trypsin-exposed ASIC1a the time course of loss of channel activity to that of the shift in the activation curve, we have used a time-course protocol in which we alternated stimulation by pH 4 and pH 6. The IpH4 is independent of the trypsin-induced changes in pH-dependence of ASIC1a activation and reflects the maximal peak current, while both, the maximal current amplitude and the pH dependence of ASIC1a gating determine the IpH6. The time course of the change in IpH4 and IpH6
is shown in Fig. 5. If the same cleavage affected both, the pH dependence of activation and the maximal current, then the rate of decrease of IpH4 and of IpH6 determined by the exponential fit should be the same, only the amplitude should be different. The dashed line in Fig. 5 represents the IpH6 time course scaled to that of IpH4. This illustrates that the time course of IpH6 and IpH4 has different rates. The exponential fits to IpH4 and IpH6 are shown as solid lines. The IpH4 was well fitted by a single exponential, while the time course of IpH6 was best fitted by the sum of two exponentials, a major, fast component, and a slower component that had the same time constant as that of IpH4. This observation is consistent with sequential cleavage events induced by trypsin exposure, which first lead to the shift in the activation curve that is responsible for the rapid decrease in IpH6 during the first 3-4 min of trypsin application, and then gradually induce the loss of channel activity, which is reflected by the slow, but consistent decrease of IpH4 and by the late phase of the IpH6 decrease.

Trypsin regulates acid-induced depolarization in cultured hippocampal neurons

To examine the effect of proteases on neuronal ASICs, we have exposed cultured hippocampal neurons to trypsin at 200 µg/ml for 4 min. As shown in Fig. 6A, the pH dependence of ASIC activation is shifted to more acidic pH after exposure to trypsin. ASIC currents in hippocampal neurons are mediated by homomultimeric ASIC1a and heteromeric assemblies of ASIC1a with ASIC2a and/or 2b (22,26,35). The shift in the pH dependence of ASIC activation in hippocampal neurons is consistent with our findings that such a shift occurs in ASIC1a and ASIC1a2a. We have then studied the effect of trypsin exposure on acid-induced membrane depolarization and action
potential generation in cultured hippocampal neurons. Traces from representative experiments of acid-induced action potential generation before and after exposure to trypsin are shown in Fig. 6B and C. The extracellular solution was acidified from a basal pH of 7.5 in panel B, and from a basal pH of 7.0 in panel C, to mimic a persistent decrease in basal extracellular pH that may occur under ischemic conditions (8). As expected, the acidification-induced depolarization $\Delta V_m$ from the basal pH 7.5 was decreased after exposure to trypsin (Fig. 6B, D). At a basal pH of 7.0, untreated ASIC1a is mostly inactivated, while a substantial fraction of ASIC1a that has been exposed to proteases remains activatable (Fig. 4B). Acid-induced depolarization from a basal pH of 7.0 was smaller in amplitude than from pH 7.5. However, relative to untreated neurons, trypsin-exposed neurons showed an increased acid-induced depolarization $\Delta V_m$ when activated from pH 7.0 (Fig. 6C, E). This observation is consistent with the shift in inactivation of cloned ASICs and reveals a positive regulation of ASICs by proteases in conditions of consistent extracellular acidification, that may be physiologically relevant.
Discussion

We show in this study that several serine proteases modify the function of ASIC1 and ASIC1a-containing heteromeric ASICs, but not of ASIC2 and ASIC3. Exposure to extracellular proteases leads to a shift of the pH dependence of ASIC activation and in the case of ASIC1a also of inactivation to more acidic pH and it accelerates recovery from inactivation.

Regulation of other ion channels by proteases

Proteases have previously been shown to regulate the activity of the nicotinic α9 acetylcholine receptor, of NMDA receptors, and of ENaC, which belongs to the same family as ASICs (18,36,37). Early reports that studied protease effects on ENaC in toad urinary bladder found an inhibitory effect of trypsin and other proteases at high concentrations (16,17). More recent studies with cloned ENaC found substantial channel activation with low concentrations (2 µg/ml) of trypsin or chymotrypsin and could not reproduce the inhibitory effect at higher concentrations. The protease-dependent activation of ENaC is not mediated by G protein and is likely due to an increase of the open probability of channels already present at the cell surface, through a proteolytic action of the enzyme (18). Membrane-associated proteases have been identified that are co-localized with ENaC and may regulate its activity (19,38,39). ENaC regulation by proteolysis is thought to occur at two developmental states, first during maturation and later after having been inserted into the plasma membrane (18,19,40,41). A recent, extensive study followed maturation of recombinant ENaC subunits and provided evidence for cleavage at two sites in the α and γ ENaC extracellular loop (40,41). Cleavage occurs at consensus sites for furin
cleavage, which are located in the N-terminal part of the large extracellular loop of α and γ ENaC. This study further showed that cleavage is part of the channel maturation and that the fragments remain stably associated with the channel. Functional studies showed that channels containing mutated furin cleavage sites had smaller basal currents than wild type channels, but that the currents could be increased by exposure to extracellular trypsin (41). This observation indicates that trypsin cleaves ENaC subunits at sites different from furin consensus sites.

*Direct effect of serine proteases on ASIC1*

We show for trypsin, that the modification of ASIC function involves proteolytic activity on the ASIC protein, since inhibition of trypsin by soybean trypsin inhibitor or modification of its catalytic site by TLCK prevented the modification of ASIC1a function, and cleavage of the channel protein is evident from western blot analysis. Most likely, the other serine proteases also act directly on ASICs, because (1) they modify ASIC function in very different cellular systems, (2) functional modification by chymotrypsin and proteinase K is faster for ASIC1a than ASIC1b, and (3) the protease effects are irreversible on the time scale of the duration of our experiments. All ASIC subunits contain 50 or more predicted cleavage sites for trypsin, chymotrypsin and proteinase K in their extracellular loop. Our observation that functional changes after exposure to these proteases only occur in ASIC1a and 1b suggests that only few of the predicted sites are indeed cleaved by the proteases. Likely, the extracellular portion of the ASIC subunits is folded in a way that protects most of the potential cleavage sites from the extracellular solution. Because trypsin affects the function of ASIC1a but not ASIC1b, it is likely that the ~100 most N-terminal amino acids of the extracellular loop, which are different between ASIC1a
and 1b, are involved in ASIC regulation by serine proteases. Our observation that trypsin exposure modifies the pH dependence of ASIC activation and steady-state inactivation and leads to an acceleration of the recovery from inactivation suggests that activation and steady-state inactivation depend on the same part of the channel protein. However, the time course of open-channel inactivation is not changed by the protease exposure. In this context it is interesting to note that a recent study which compared rat and fish ASIC1a showed that open-channel inactivation was 28 times faster in fish than in rat ASIC1a, while the pH dependence of steady-state inactivation was not different (42). This suggests that steady-state inactivation and open state inactivation are achieved by different conformational changes. The three proteases tested disrupted P. cambridgei venom inhibition of ASIC1a. Psalmopoeus cambridgei venom, which selectively blocks ASIC1a (25), requires the unique N-terminal portion of ASIC1a for its action. Protease exposure of ASIC1a either disrupts the toxin binding site on ASIC1a or uncouples this part of the channel protein from the channel gate.

The functional effects of all three tested proteases on pH-dependent gating were qualitatively the same, consistent with cleavage by the different proteases of the same functional domain. The small differences in the parameters of channel gating after exposure to different proteases might be due to cleavage by the different proteases of the channel protein at different sites within the exposed domain. In addition, our observation that the shift in pH dependence of ASIC1a activation and the decrease in the maximal channel current occur at different time scales (Fig. 5) suggest that several cleavage events may occur upon protease exposure, which eventually lead to complete channel inactivation.
Possible physiological importance of ASIC regulation by proteases

It is known that ASICs can induce action potentials in neurons (22,26,43). We have recently shown in hippocampal neurons that ASIC activity, when occurring simultaneously with other excitatory stimuli, can either facilitate action potential generation or inhibit action potential bursts, depending on the amplitude and the time course of the induced depolarization (22). Here we show that protease exposure strongly affects the ASIC-mediated depolarization, which suggests that by this pathway proteases can affect neuronal excitability.

Several well-characterized serine proteases, as e.g. t-PA (tissue plasminogen activator), urokinase-type plasminogen activator, thrombin and chymotrypsin B are present under physiological conditions in the central nervous system (21,44). Besides, many brain-specific proteases exist, as for example neuropsin and neurotrypsin (45,46). The activity of brain serine proteases is tightly regulated and depends on their equilibrium with their serine protease inhibitors (serpins) (reviewed in (21)). Proteases in the brain are involved in the catabolism of the extracellular matrix, and there is strong evidence for an involvement of several proteases in the regulation of neuroplasticity in both the developing and mature brain (21,47), thus in a function that is also modulated by ASICs (9,10). ASICs in central neurons may contribute to the neuronal death associated with brain ischemia or epilepsy, which are accompanied by extracellular acidification (7,48). Various studies have shown that the expression of serine proteases is modulated by brain injury, and there is also evidence that the integrity of the blood brain barrier is compromised in head trauma, stroke, status epilepticus and other pathological conditions, allowing proteases from the blood to gain access to the extracellular spaces that surround neurons and glia (21,49). Over-expression of the protease inhibitor neuroserpin is neuroprotective in
focal ischemic stroke (50). Thus, there is evidence for various roles of proteases in physiological and pathological processes in the central nervous system, in which ASIC are also likely involved. It is possible that in these situations the proteases modulate ASIC function.

ASICs are also expressed in the peripheral nervous system and there is evidence that ASICs are involved in pain sensation (4-6). Regarding functional roles of proteases in the periphery, it has been long known that proteases are liberated during inflammation (20). t-PA and other proteases have been shown to be involved in the development of neuropathic pain (51-53).

In this study we provide evidence for subtype-specific modification of ASIC function by three prototypes of serine proteases. Our analysis of acid-induced depolarization in hippocampal neurons shows that at physiological basal extracellular pH, ASIC activity is downregulated by protease exposure. At more acidic basal extracellular pH, that can occur during hypoxia or ischemia, thus processes which may be associated with increased protease activity, the proteases adapt ASIC gating and allow increased ASIC activity compared to ASICs that have not been exposed to proteases. It is highly probable that the same functional effects that we have observed with three prototypes of serine proteases can be exerted by proteases that are present in the brain or are released during inflammation.
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Figure 1. Decrease in pH 6-induced ASIC1a current during exposure to trypsin

All measurements were obtained in whole-cell voltage-clamp to –60 mV from CHO cells that stably expressed ASIC1a. The extracellular pH was kept at pH 7.5 and was interrupted every 40s by a 5-s acidification to pH 6 to activate ASICs. Trypsin was added immediately after the third control application, as indicated. A, representative current traces measured during the 5-s acidifications at the times indicated. B, C, E, current response to pH 6 (IpH6), normalized to the control applications before addition of trypsin to the extracellular solution, is plotted as a function of time. The lines represent an exponential fit to the data. B, Time course of IpH6 under control conditions (○), and in the presence of trypsin at 2 µg/ml (■), 20 µg/ml (▲), 100 µg/ml (◆) and 200 µg/ml (●), n = 5 - 9. C, time course of IpH6 under control conditions (○), in the presence of 200 µg/ml trypsin (●), in the presence of 200 mg/ml trypsin and 50 µg/ml Tosyl-L-lysyl-chlormethan (TLCK, which had been added to trypsin prior to the experiment, □) and in the presence of each 200 mg/ml trypsin and trypsin inhibitor (▲), n=3-5. Rates of current decrease (k, min⁻¹) obtained from an exponential fit to the current decrease were 0.036 ± 0.001 min⁻¹ (control), 0.031 ± 0.009 min⁻¹ (TLCK), 0.040 ± 0.003 min⁻¹ (trypsin inhibitor) and 0.344 ± 0.039 min⁻¹ (trypsin). D, Western blot immunostaining of solubilized ASIC1a. Oocytes were not injected or injected with RNA encoding ASIC1a carrying an HA epitope at its C-terminus. Where indicated, intact oocytes were exposed during 5 min to 200 µg/ml trypsin. Solubilized proteins were subjected to SDS-PAGE and ASIC1a was visualized on Western blots using a monoclonal anti-HA antibody. Molecular markers are indicated. E, Time course of IpH6 in the absence of extracellular trypsin with 0.3 mM GTP-γS in the pipette solution (□, k = 0.038 ± 0.04 min⁻¹) and in the presence of
200 µg/ml trypsin with (■, k = 0.807 ± 0.602) and without (●, k = 0.916 ± 0.049 min⁻¹) 0.3 mM GTP-γS in the pipette solution, n= 4-5.

**Figure 2. Specific modification of ASIC1 by serine proteases.**

The extracellular pH was kept at pH 7.5 and was interrupted every 40s by a 5-s acidification to pH 6 (pH 5 in the case of ASIC2a) to activate ASICs. The current response to pH 6 or pH 5, normalized to the control application before addition of the protease to the extracellular solution, is plotted as a function of time. Proteases were added immediately after the third control application, as indicated by the bars. The lines represent an exponential fit to the data; the rate constants determined are presented in Table 1. A, time course of IpH6 of ASIC1a (●), ASIC1b (■), ASIC3 (▲) and of IpH5 of ASIC2a (●) upon exposure to 200 µg/ml trypsin. B, time course of ASIC1a IpH6 upon exposure to 200 µg/ml trypsin (●), chymotrypsin (▲) and proteinase K (■). All measurements were obtained in whole-cell voltage-clamp to –60 mV, n ≥ 4.

**Figure 3. Changes in P. cambridgei venom block and ASIC1a activation after trypsin exposure**

Measurements were made from CHO cells stably expressing ASIC1a in whole-cell voltage-clamp to –60 mV, before or after a 315-s exposure to 200 µg/ml trypsin. A, representative current traces illustrating ASIC1a current inhibition by the venom of P. cambridgei and bar graph summarizing the data (n=5). Cells were exposed to P. cambridgei venom for 90 s to allow development of the inhibition. B, representative current traces obtained by short extracellular acidification to the pH values indicated, for control ASIC1a, and ASIC1a after trypsin exposure. C, pH dependence of
activation before (○) and after trypsin (●). Currents are normalized to the IpH6 before trypsin application. Solid lines represent fits to the Hill equation; the fit parameters are shown in Table 2., n = 4-6). D, rate of inactivation, k, obtained from exponential fits to the time course of current inactivation, plotted against the activating pH. (○), control; (●), after exposure to trypsin, n=4. The inset in D shows representative current traces of pH 6-induced current before and after exposure to trypsin.

Figure 4. Changes in ASIC1a pH dependence of steady-state inactivation and in the time course of recovery from inactivation.

The data presented were obtained from control ASIC1a expressing cells or after exposure to 200 µg/ml trypsin for the duration of 315 s. A, B, pH dependence of steady-state inactivation. ASIC currents were induced by 5-s acidification to pH 5 after exposure for 40 s to the conditioning pH. (A) illustrates the protocol used and shows representative current traces and (B) plots the current response, normalized to the response with the conditioning pH of 8.0 against the conditioning pH, n = 4-8. C, D, recovery from inactivation. Two 5-s extracellular solution changes to pH 6 were separated by an interval at pH 7.5. (C) illustrates the protocol and shows current traces from representative experiments. The normalized peak amplitude of the second current response is plotted in (D) against the duration of the interval at pH 7.5, n = 3-4. The solid lines represent exponential fits to the data. All measurements were obtained in whole-cell voltage-clamp to –60 mV.
Figure 5. Comparison of time course of the shift in the activation curve and the decrease in maximal ASIC current. Measurements were made from CHO cells stably expressing ASIC1a, in whole-cell voltage-clamp to –60 mV. The extracellular pH was kept at pH 7.5 and was interrupted every 40s by a 5-s acidification that alternated between pH 6 and pH 4, to activate ASICs. In half of the experiments the series of acidifications was started by pH 4, in the other half by pH 6. The current response to pH 6 and pH 4 (IpH6, ○, and IpH4, ■), normalized to the control application before addition of the protease to the extracellular solution, is plotted as a function of time. Proteases were added immediately after the third control application, as indicated by the bar. Solid lines are exponential fits to the time course, k = 0.00223 s⁻¹ (IpH4) and k1 = 0.01666 s⁻¹, k2 = 0.00215 s⁻¹ (IpH6, relative weight of first component is 0.67), the dashed line represents the fit to IpH6, scaled to the IpH4 at 640 s.

Figure 6. Bi-directional regulation of ASICs in hippocampal neurons by trypsin. A, pH dependence of ASIC activation in cultured hippocampal neurons before and after 4-min exposure to 200 µg/ml trypsin, obtained from whole-cell voltage-clamp to –60 mV. The solid lines represent a fit of the data to the Hill equation (see Experimental Procedures) and yielded a pH for half-maximal current, pH0.5 of 6.3 ± 0.1 and a Hill coefficient of 1.7 ± 0.6 before exposure to trypsin, and 5.8 ± 0.1 and 1.3 ± 0.4 after trypsin exposure (n= 10-11). B, C, representative experiments showing acidification-induced depolarization and action potential generation in whole-cell current clamp. Current injection was chosen to obtain a membrane potential of –60 mV in the absence of stimuli. Extracellular pH was changed from a basal pH of 7.5
(panel B) or 7.0 (panel C) to the pH values indicated for 15 s. The traces in each panel are from the same neuron, before or after 4-min exposure to trypsin, as indicated in the figure. Bars are 1s and 20 mV. D, E, summary of trypsin effects on ASIC-mediated depolarization $\Delta V_m$. Acidification-induced depolarization $\Delta V_m$, measured between action potentials, is shown as a function of pH and protease exposure. *, P < 0.05 (t-test, n = 7-10).
Table 1. Rate of current decrease, $k (\text{min}^{-1})$ due to protease action

The functional consequence of exposure to the protease at 200 $\mu$g/ml at pH 7.5 was monitored as the change in $I_{pH6}$ (or $I_{pH5}$ for ASIC2a). The rate $k (\text{min}^{-1})$ from an exponential fit to the current decrease is presented. The number of experiments was $\geq 3$ per condition.

|           | ASIC1a | ASIC1b | ASIC2a | ASIC3 | ASIC1a/2a | ASIC1a/3 |
|-----------|--------|--------|--------|-------|-----------|----------|
| control   | < 0.02 | < 0.02 | < 0.02 | < 0.02| < 0.02    | < 0.02   |
| trypsin   | 0.630 ± 0.020 | < 0.02 | < 0.02 | < 0.02| 0.482 ± 0.000 | < 0.02   |
| Chymotrypsin| 0.545 ± 0.176 | 0.167 ± 0.009 | 0.028 ± 0.009 | < 0.02| 0.300 ± 0.059 | 0.276 ± 0.022 |
| proteinase k | 1.248 ± 0.084 | 0.385 ± 0.016 | < 0.02 | < 0.02| 1.262 ± 0.000 | 0.204 ± 0.000 |
Table 2. Functional properties of ASIC1a and ASIC1b currents before and after protease exposure

ASIC-expressing cells were exposed to the proteases at 200 µg/ml for a time period that revealed the functional changes without leading to an extensive loss of maximal current. The duration was for ASIC1a, 315 s (trypsin), 205 s (chymotrypsin), 105 s (proteinase K) and for ASIC 1b, 280 s (chymotrypsin) and 290 s (proteinase K). For ASIC activation, the pH for half-maximal activation, pH0.5 and the Hill coefficient Hn were determined from a fit of the data to the Hill equation (see Experimental Procedures). The rate k of open-channel inactivation was determined from an exponential fit to the current decrease after ASIC activation by the pH indicated. The fit parameters of steady-state inactivation were determined as described in the legend to Fig. 4. The pH dependence was fitted with a Hill equation containing 1, or 2 components if necessary. pHIn0.5, pH for half-maximal inhibition; Hn, Hill coefficient; F1, relative weight of the first component. The rate of recovery from inactivation was determined as described in the legend to Fig. 4 from an exponential fit to the current recovery. *, activation pH. The number of experiments was 2-19 per condition.

| pH dependence of activation | ASIC1a | ASIC1b |
|----------------------------|--------|--------|
| pH0.5 (1)                  | control | trypsin | chymotrypsin | proteinase K | control | chymotrypsin | proteinase K |
|                            | 6.6 ± 0.0 | 5.8 ± 0.1 | 5.5 ± 0.0 | 5.5 ± 0.0 | 6.3 ± 0.0 | 5.6 ± 0.2 | 5.5 ± 0.1 |
| Hn (1)                     | 4.5 ± 1.4 | 1.7 ± 0.4 | 1.5 ± 0.1 | 2.6 ± 0.2 | 3.8 ± 0.8 | 0.8 ± 0.4 | 1.2 ± 0.2 |

| Rate of open-channel inactivation (k, s⁻¹) | ASIC1a | ASIC1b |
|------------------------------------------|--------|--------|
| pH 6*                                    | control | trypsin | chymotrypsin | proteinase K | control | chymotrypsin | proteinase K |
|                                          | 2.49 ± 0.11 | 3.23 ± 0.14 | 2.97 ± 0.20 | 2.48 ± 0.04 | 1.13 ± 0.08 | 1.01 ± 0.01 | 1.02 ± 0.09 |
| pH 5*                                    | 3.29 ± 0.18 | 4.35 ± 0.11 | 3.91 ± 0.26 | 4.13 ± 0.46 | 2.07 ± 0.12 | 1.90 ± 0.05 | 1.96 ± 0.18 |

| pH dependence of steady-state inactivation | ASIC1a | ASIC1b |
|-------------------------------------------|--------|--------|
| pHIn0.5 (1)                               | control | trypsin | chymotrypsin | proteinase K | control | chymotrypsin | proteinase K |
|                                          | 7.3 ± 0.0 | 7.0 ± 0.0 | 7.4 ± 0.0 | 7.0 ± 0.0 | 6.7 ± 0.0 | 7.5 ± 0.0 | 6.7 ± 0.9 |
| Hn (1)                                    | 5.0 ± 0.2 | 9.1 ± 5.2 | 2.4 ± 0.5 | 4.3 ± 1.0 | 5.7 ± 0.6 | 4.3 ± 0.2 | 1.9 ± 0.0 |
| F1                                        | 0.77 | | | | 0.54 | 0.75 | |
| pHIn0.5 (2)                               | 7.7 ± 0.1 | | | | 6.7 ± 0.0 | 7.7 ± 0.1 | |
| Hn (2)                                    | 3.3 ± 1.5 | | | | 4.0 ± 0.2 | 7.2 ± 2.6 | |

| Rate of recovery from inactivation (k, s⁻¹) | ASIC1a | ASIC1b |
|-------------------------------------------|--------|--------|
| pH 6*                                     | control | trypsin | chymotrypsin | proteinase K | control | chymotrypsin | proteinase K |
|                                          | 0.42 ± 0.02 | 2.44 ± 0.32 | 1.38 ± 0.23 | 0.61 ± 0.04 | 0.40 ± 0.01 | 0.83 ± 0.08 | 0.97 ± 0.06 |
| pH 5*                                     | 0.34 ± 0.02 | 1.66 ± 0.09 | 1.33 ± 0.28 | 1.62 ± 0.17 | 0.41 ± 0.02 | 0.76 ± 0.05 | 0.69 ± 0.10 |
Figure 1  Poirot et al.

A

![Graph showing trypsin 200 µg/ml over time](image)

B

![Graph showing normalized pH over time for different trypsin concentrations](image)

C

![Graph showing normalized pH over time for trypsin +/− inhibitor](image)

D

![Image showing trypsin and kDa bands](image)

E

![Graph showing normalized pH over time for GTPγS and trypsin+GTPγS](image)
Figure 2 Poirot et al.

A

B

ASIC1a control
ASIC1a trypsin
ASIC1b trypsin
ASIC2a trypsin
ASIC3 trypsin

control
trypsin
chymotrypsin
proteinase k

ASIC1a
Figure 3  Poirot et al.

A

| Control pH 5 | pH 5 + venom |
|--------------|-------------|
| 2 nA 1 s     | 2 nA 1 s    |

| Trypsin pH 5 | pH 5 + venom |
|--------------|-------------|
| 1 nA 1 s     | 1 nA 1 s    |

B

| pH 7.5 | pH 6.8 | 6.6 | 6.4 | 6 | 5 | 6.4 | 6 | 5.5 | 5 | 3.5 |
|--------|--------|-----|-----|---|---|-----|---|-----|---|-----|
| 2 nA 2 s | 1 nA 2 s |

C

Graph showing the rate of inactivation (k, s⁻¹) as a function of pH:
- Control: solid line
- Trypsin: dashed line

D

Graph showing the ratio of currents at pH 6 before and after trypsination:
- Control: square markers
- Trypsin: circle markers
Figure 4 Poirot et al.

A
pH
8.0 7.5 7.0
control
5 nA 2 s

trypsin
1 nA 2 s

B

I/I_{max}

conditioning pH

control trypsin

C
pH
7.5 6.0
control Δt
Δt
0.25 s

Δt
1 s

Δt
5 s

1 nA 1 s

400 pA 1 s

D
l/I_{max}

time (s)

control trypsin
Figure 5 Poirot et al.
Figure 6  Poirot et al.