Acid-sensing ion channels (ASICs) are neuronal Na⁺ channels that are members of the Epithelial Na⁺ channel/degenerin family and are transiently activated by extracellular acidification. ASICs in the central nervous system have a modulatory role in synaptic transmission and are involved in cell injury induced by acidosis. We have recently demonstrated that ASIC function is regulated by serine proteases. We provide here evidence that this regulation of ASIC function is tightly linked to channel cleavage. Trypsin cleaves ASIC1a with a similar time course as it changes ASIC1a function, while ASIC1b, whose function is not modified by trypsin, is not cleaved. Trypsin cleaves ASIC1a at Arg145, in the N-terminal part of the extracellular loop, between a highly conserved sequence and a sequence that is critical for ASIC1a inhibition by the venom of the tarantula Psalmopoeus cambridgei. This channel domain controls the inactivation kinetics and co-determines the pH dependence of ASIC gating. It undergoes a conformational change during inactivation, which renders the cleavage site inaccessible to trypsin in inactivated channels.

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 separated by a large extracellular loop.

Several putative physiological roles of ASICs have been proposed. Expression in nociceptors and activation by protons suggest that the ASICs may act as pain receptors (4), and a role for ASIC3 has been shown in pseudo-chronic hyperalgesia induced by repeated acid injections in muscle (5). In the central nervous system ASIC1a is the most abundant and most ubiquitously expressed of all ASICs. Evidence exists for a role of ASIC1a in modulating synaptic transmission, memory and fear conditioning (6,7). Recently it was shown that ASIC1a mediates cell injury induced by conditions associated with acidosis in the mammalian nervous system (8). On the cellular level, we and others have shown that ASIC activity modulates action potential signalling (9,10). The ASICs themselves are targets of various regulatory mechanisms, one of them being serine proteases such as trypsin. Extracellular trypsin can cleave ASIC1a in the extracellular loop and thereby shift the pH dependence of channel activation and inactivation to more acidic pH (11). This regulation may adapt the ASIC1a pH dependence to situations in which the extracellular pH is constitutively lowered, as e.g. ischemia. In addition, the trypsin-modified ASIC1a channel shows a reduced permeability towards divalent cations (12).

Identification of a protease cleavage site on ASIC1a would be a starting point for the search for endogenous proteases related to trypsin that may regulate ASIC function in vivo. With regard to ASIC function, the mechanism by which protons control ASIC gating is currently unknown. Knowledge of channel parts involved would make it possible to test hypotheses about gating mechanisms. The observation that trypsin cleaves ASIC1a in the extracellular loop and induces significant functional changes suggests that cleavage occurs at a functionally important site of the channel protein. The goal of this study was therefore first to verify that the cleavage event and the functional changes induced by trypsin are correlated, and second to identify the cleavage site(s). The data show a correlation...
between cleavage and functional effect in ASIC1. The mutagenesis approach showed that trypsin cleaves ASIC1a in the N-terminal part of the extracellular loop, between a highly conserved domain and a channel domain that is critical for ASIC1a inhibition by the venom of the spider Psalmopoeus cambridgei. This channel portion is important for channel inactivation properties and undergoes conformational changes during channel inactivation.

**EXPERIMENTAL PROCEDURES**

**Construction of chimeras, site-directed mutagenesis and expression in Xenopus oocytes**

The ASIC1a clone used in this study corresponds to EST clone IMAGE 6849487 and was entirely sequenced. Its sequence is identical to the published mouse ASIC1a sequence BC067025. On the amino acid level its sequence is identical to the rat ASIC1a sequence and downstream of V186 to that of rat ASIC1b, except for a S476N substitution in the intracellular C-terminus. Other cDNA constructs were kindly provided by M. Lazdunski (rat ASIC3) and S. Gründer (rat ASIC1b).

Chimeras were made with ASIC1a and either ASIC1b or ASIC3. To make the constructs that contained mainly ASIC1a (“ab” chimera) we introduced a BstEII restriction site at position F103 and an AgeI site at position T214. We used in addition a ScaI site at position Q66 after removal of a second ScaI site in the vector sequence, and an Eco47III site at position F174. Except for the BstEII site which introduced the mutation S104T, these insertions did not change the protein sequence. The corresponding ASIC1b or ASIC3 sequence to be inserted between restriction sites was amplified by PCR using primers that contained the appropriate restriction sites. For chimera ab4 and ab5, the ASIC1a/b sequence between positions 102 and 213 was amplified by PCR and then introduced in the BstEII and AgeI sites. For the construction of the ba1 chimera, the DNA encoding the N-terminal portion of the chimera was prepared by PCR and then cloned in the ASIC1bM3 sequence (13) using a BamHI site in the multiple cloning site and the BstBI site at F239 in ASIC1bM3. Point mutations and combined point mutations were introduced either by a PCR strategy or by Quikchange (Stratagene). The portions of the chimera and mutants that had been made by PCR and the entire coding sequence of mutants obtained by Quikchange were verified by sequencing (Synergene Biotech, Zurich, Switzerland).

Expression in *Xenopus laevis* oocytes was carried out as described previously (14). Complementary RNAs were synthesized in vitro. Oocytes were surgically removed from the ovarian tissue of female *Xenopus laevis* which had been anaesthetized by immersion in MS-222 (2 g l⁻¹; Sandoz, Basel, Switzerland). The oocytes were defolliculated and healthy stage V and VI *Xenopus* oocytes were isolated and pressure-injected with 50-100 nl of cRNA solution and oocytes were kept in modified Barth solution during the expression phase.

**Trypsin incubations**

Oocytes were used for biochemical analysis 20 - 36 h after cRNA injection. Trypsin incubations were done in the following way. 20-30 oocytes per condition were incubated in standard bath solution (110 mM NaCl, 2.0 mM CaCl₂, 10 mM Hepes-NaOH, pH 7.5), containing 40 or 200 µg/ml trypsin for 5 min at room temperature. Then, the oocytes were washed twice in standard bath solution that contained 10 µg/ml aprotinin in order to inhibit residual trypsin activity. For the time course protocol, the oocytes were incubated in trypsin solution for the times indicated. For incubations at different pH, trypsin activity was first measured (as described below) at different pH values. The trypsin concentration at pH 7.0 and 6.8 was increased accordingly to obtain the same proteolytic activity as at pH 7.5. In these experiments, oocytes were incubated for 1 min in the standard bath solution of the test pH. Oocytes were then incubated at room temperature for 2 min in standard bath solution at the test pH, containing 100 µg/ml trypsin in the case of pH 7.5 and 109 and 126 µg/ml trypsin for pH 7.0 and 6.8, respectively. The oocytes were then washed in the test pH solution containing 10 µg/ml aprotinin, followed by a wash in pH 7.5 solution containing 10 µg/ml aprotinin. The biotinylation protocol was performed directly after the trypsin incubation and washes.

**Cell surface protein biotinylation and western blot analysis**

20-30 oocytes per condition were used and the complete protocol was performed on ice. Oocytes were washed three times with modified
Barth solution and then incubated in biotinylation buffer (10 mM triethanolamine, 150 mM NaCl, 2 mM CaCl2, pH adjusted to 9.5) containing 1 µg/ml EZ-link sulfo-NHS-SS-Biotin (Pierce) for 15 min. Then, oocytes were washed twice and incubated for 5 min in quench buffer (0.192 M glycine, 0.025 M Tris-HCl, pH7.5 in modified Barth solution) to stop the biotinylation reaction. Oocytes were then lysed in 20 µl of lysis buffer per oocyte in lysis buffer (1% triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl) and centrifuged at 12 000 rpm for 10 minutes at 4°C. During the centrifugation step, 50 µl of immunopure immobilized streptavidin beads (Pierce) per condition were washed with the lysis buffer and the supernatant from centrifugation was added to the washed beads. The samples were incubated overnight at 4°C. The samples were then centrifuged for 1 min at 14 000 rpm at 4°C and 10 µl of supernatant (representing the intracellular protein fraction) per condition were mixed with protein sample buffer, while the beads (containing biotinylated membrane protein fraction) were washed three times with the lysis buffer and then mixed with protein sample buffer. After boiling at 95°C for 5 min, the samples were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell). The polypeptides were exposed to rabbit anti-ASIC1 primary antibody (1:200, Alomone, Israel) and goat anti-rabbit horse radish peroxidase (1:20 000, Amersham Biosciences) as secondary antibody and visualized using Super Signal® West Dura Extended Duration Substrate (Pierce).

Electrophysiological analysis
Electrophysiological measurements were taken at 18 - 36 h after cRNA injection. Macroscopic currents were recorded using the two-electrode voltage-clamp technique at a holding potential of −60 mV. Currents were recorded with a Dagan TEV-200 amplifier (Minneapolis, MN, USA) equipped with two bath electrodes. The standard bath solution contained 110 mM NaCl, 2.0 mM CaCl2, 10 mM Hepes-NaOH, (or MES-NaOH for pH < 7.0), and pH was adjusted by NaOH to the values indicated. Oocytes were placed in a recording chamber (500 µl) and perfused by gravity at a rate of 5-15 ml/min. Oocytes were exposed to trypsin directly in the recording chamber at pH 7.5, except for the experiments in which the pH dependence of the channel modification by trypsin was determined. In these experiments, incubations were carried out as described for the biochemistry experiments, before recording the pH activation curve. The pH activation curves were fit by using the Hill equation:

\[ I = I_{max}/\left[1+(10^{pH_0.5}/10^{pH_H})^n\right] \]

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. Differences between ASIC1 wt and chimera or mutants in absolute pH0.5 values, the changes in pH0.5 induced by trypsin exposure and of the rates of IpH6.5 decrease during trypsin exposure were analyzed by using ANOVA and Bonferroni post test (GraphPad Prism 4.0).

Chemicals and determination of trypsin activity
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 (15). Trypsin (DPCC-treated trypsin from bovine pancreas, a form of trypsin with low level of chymotrypsin contamination) was obtained from Sigma (Buchs, Switzerland). All other chemicals were obtained from Sigma or Fluka. For inactivation of trypsin by tosyl-L-lysyl-chlormethan (TLCK), 2 mg/ml trypsin and 0.5 mg/ml TLCK in standard bath solution at pH 7.5 were incubated for 10 min at RT and then further diluted to the working concentrations. The activity of trypsin in the extracellular solution at different pH was determined as the rate of hydrolysis of Nα-Benzoyl-DL-arginine 4-nitroanilide (BAPNA). Ten microliters of concentrated protease solution was added to 1 ml of standard bath solution containing 0.5 mM of the substrate and with pH adjusted to pH values between 8.0 and 6.8, and the increase in absorbance at 410 nM was measured every 20 s over a period of 2 min and used to calculate the rate of absorbance increase.

RESULTS
Selective cleavage of ASIC1a by trypsin
In a previous study we showed that exposure of cells expressing ASIC1a channels to extracellular
proteases causes a shift in the pH dependence of ASIC activation to more acidic pH and that the trypsin exposure cleaves the ASIC1a protein (11). The aim of the present study was to test the hypothesis that the functional changes are correlated to the cleavage event, and to identify the cleavage site. The present study was carried out in Xenopus oocytes expressing murine ASIC1a (chosen for compatibility in the chimera approach), while in the previous study we had investigated human ASIC1a in mammalian cells. In a first experiment we confirmed that a 5-min exposure to 200 µg/ml trypsin induces a shift in the activation curve of ASIC1a to more acidic pH, and that ASIC1b pH dependence is not modified (Fig. 1A). In addition to the shift in the pH dependence of ASIC1a we observed a decrease of the maximal peak current as measured at pH 4, of 19 ± 4 % (n=12) in ASIC1a and of 7 ± 4 % (n=10) in ASIC1b after the 5-min trypsin exposure (Fig. 1A). A 5-min exposure to trypsin-free control solution did not result in a significant I\textsubscript{pH4} change (1 ± 4 % (n=3) decrease in ASIC1a, 2 ± 6% (n=3) increase in ASIC1b), indicating that the peak current decrease observed during trypsin exposure is not due to channel rundown but is a consequence of trypsin exposure. One part of the current decrease (that is also seen in ASIC1b) may be a non-specific effect of trypsin, while the more important part of the maximal current decrease in ASIC1a can be attributed to the interaction of trypsin with the channel protein that also induces the shift in pH dependence, as shown previously (11).

For a biochemical characterization of the effect of trypsin we used the same incubation conditions as in the electrophysiology experiments (5 min trypsin 200 µg/ml), and purified the cell surface proteins by biotinylation followed by isolation of biotylated proteins with streptavidin beads. ASIC1 proteins were detected on Western blots with an antibody directed against the C-terminus, which is identical in ASIC1a and ASIC1b. Figure 1B shows that under control conditions, both, ASIC1a and ASIC1b have an apparent molecular mass of ~64 kDa. The ASIC1a cDNA encodes a protein of 526 amino acids with a predicted molecular mass of 60 kDa. After enzymatic deglycosylation (Endoglycosidase H, N-glycosidase F) the ASIC1a protein migrated with a predicted mass of ~64 kDa (not shown), suggesting that ASIC1a has 1-2 natural N-linked glycosylation sites. Exposure to trypsin led to almost complete disappearance of the 64 kDa band and the appearance of a lower molecular weight band of ~49 kDa in ASIC1a. In contrast, ASIC1b was not cleaved (Fig. 1B). The molecular mass of the cleaved ASIC1a suggests cleavage in the N-terminal part of the extracellular domain, which is different between ASIC1a and 1b.

To test whether the cleavage correlates with the change in ASIC1a function, we have incubated ASIC1a-expressing oocytes for different time periods in 40 µg/ml trypsin, followed by biochemical analysis. The Western blot of a typical experiment in panel C shows that with increasing duration of the incubation the intensity of the upper (non-cleaved) ASIC1a band decreases and that of the lower (cleaved) ASIC1a band increases. We have quantified the intensity of these bands and plot the fraction of uncleaved/total signal in Fig. 1D (■, n = 4 independent experiments). To study the time course of the change in ASIC1a function during trypsin exposure, we have measured every 40 s the pH6.5-induced ASIC current (I\textsubscript{pH6.5}) during the trypsin incubation at 40µg/ml, as shown in Fig. 1D (●). This analysis shows that cleavage and the change in function have a similar time course. Together with the observation that ASIC1b, which is not functionally modified by trypsin, is not cleaved (Fig. 1B), this indicates that the functional change is tightly linked to protein cleavage.

**Chimera experiments**

ASIC1a and 1b result from alternative usage of the first exon and are different in the first part of the sequence (residues 1-186 in ASIC1a), but identical in the rest of the sequence (13,16). Because ASIC1a but not ASIC1b is cleaved and functionally modified by trypsin, we constructed chimeras between ASIC1a and 1b to identify the region which is responsible for the modification of ASIC1a function by trypsin and where likely cleavage occurs. The extracellular part of the channel region different between ASIC1a and 1b contains a sequence that is highly conserved in all ENaC/degenerin family members, corresponding to F86-N95 in ASIC1a, which is followed by a sequence having low homology even within the ASIC family (3). The constructs made are illustrated in Fig. 2A. They are designated “ab” or “ba” and numbered as indicated in Fig. 2A, with “ab” chimera containing a majority of ASIC1a sequence and
“ba” chimera containing a majority of ASIC1b sequence. Of the chimera constructed, all except for the chimera ab5 gave rise to acid-induced ion currents. The ab5 chimera appeared at the cell surface as verified by biotinylation (data not shown) but did not mediate any ionic currents. Oocytes expressing the functional chimera were exposed for 5 min to 200 µg/ml trypsin and their pH dependence of activation before and after trypsin exposure was compared, as illustrated in Fig. 2B and 2C. If trypsin exposure induced a shift in the activation curve, it was interpreted as intact channel modification. In the first experiments we exchanged the complete extracellular part that is different between ASIC1a and 1b (residues Q66-V186 in ASIC1a). As expected, replacement of this part in ASIC1a by the corresponding sequence of ASIC1b (chimera ab1) resulted in a channel whose pH dependence of activation was not shifted by trypsin, while the inverse chimera (ba1) was sensitive to trypsin (Fig. 2B, upper panels). The pH dependence of activation of chimera ab2, but not of ab3 was shifted by trypsin and finally, the chimera ab4, whose pH dependence of activation was not shifted by trypsin, allowed narrowing down the trypsin target region to the domain spanning residues 103-152 in ASIC1a. We have in addition determined for selected chimera whether trypsin cleaves the chimeric protein. This biochemical analysis indicates that in correlation with the functional analysis, the chimera ab1 and ab4 are not cleaved by trypsin, while chimera ba1 is cleaved (Fig. 2D).

**Mutagenesis experiments**

The trypsin target region identified by the ab4 chimera, V103-F152, contains seven lys or arg residues, which can potentially form cleavage sites for trypsin (17) (Fig. 3A). For the mutagenesis analysis we have also included R99, which is close to the chimera border. To identify the residues involved we mutated either the first four (R99, K105, R121, K133; = 4RK1), the last four (K141, R145, K148, K150; = 4RK2) or all 8 sites (8RK) to ala. Functional analysis of the mutant channels showed that a 5-min 200 µg/ml trypsin exposure induced marked shifts in the 4RK1 and 4RK2 activation curve and still a small shift in the 8RK activation curve (Fig. 3B, summarized in Fig. 3F). To detect more subtle effects of the mutations, we have followed the time course of the IpH6.5 during exposure to trypsin at either 40 µg/ml or 200 µg/ml (Fig. 3C; filled symbols for 200 µg/ml, open symbols for 40 µg/ml). The decrease in IpH6.5 is used to monitor the rightward shift of the activation curve but is in itself not an absolute measure of channel modification. Surprisingly, the IpH6.5 decrease in the 4RK1 mutant was much faster than that of wt (compare open rectangles (4RK1) with open circles (wt) in Fig. 3C). The rate of IpH6.5 decrease during trypsin exposure, determined from such experiments, was ~10-fold increased in the 4RK1 mutant in comparison to the ASIC1a wt (Table 1). In contrast, the IpH6.5 of 8RK decreased by ~30 % at 200 µg/ml and not at all at 40 µg/ml trypsin and the IpH6.5 decrease was markedly slowed in the 4RK2 mutant. The higher rate of IpH6.5 decrease upon trypsin exposure in the 4RK1 mutant suggests that the four lys or arg to ala mutations change the conformation of the extracellular loop, leading to increased accessibility or reactivity of the ASIC1a protein towards extracellular trypsin. To verify that the functional modification of 4RK1 depends on the intact active site cleft of trypsin where substrate recognition and cleavage occur (18), we pre-treated trypsin with TLCK (tosyl-L-lysyl-chlormethane, see **Experimental Procedures**), which cross-links the Ser and His residue of the catalytic triad in the active site cleft of trypsin (19). Pre-treatment of trypsin with TLCK prevented its modification of 4RK1 function, as evidenced by the reduction of the rate of IpH6.5 decrease during the exposure to 10 µg/ml trypsin from 105 ± 8 min⁻¹ mg⁻¹ ml (trypsin) to 2 ± 1 min⁻¹ µg⁻¹ ml (TLCK-treated trypsin, n=4 each). TLCK pre-treatment of trypsin also prevented the IpH6.5 decrease of ASIC1a wt (k(40 µg/ml trypsin) = 10.8 ± 0.9 min⁻¹ mg⁻¹ ml and k(40 µg/ml TLCK-treated trypsin) = 1.1 ± 0.2 min⁻¹ mg⁻¹ ml, n=4 each). This indicates that modification of 4RK1 and ASIC1a wt function by trypsin requires a specific interaction of a part of the ASIC polypeptide with the active site cleft of trypsin. Biochemical analysis showed that the 8RK and the 4RK1 mutant are not cleaved by a 5-min 200 µg/ml trypsin incubation (Fig. 3D, n=3-4 independent experiments). For the 4RK1 mutant, this contradicts the functional analysis. A possible explanation for this apparent contradiction may be that trypsin modifies 4RK1 function by binding only, without cleavage, thus by a non-proteolytic interaction. Interestingly it has been shown that the serine protease CAP1 can modify ENaC function, even if the catalytic site of CAP1 has been inactivated by...
mutagenesis (20). In our experiments, a simple reversible binding interaction between trypsin and the 4RK1 mutant is unlikely, since the effect of trypsin on 4RK1 function is clearly not reversible upon washout or addition of aprotinin (data not shown). A possibility would be that in the 4RK1 mutant trypsin induces an irreversible conformational change in the channel protein.

The slowed IpH6.5 decrease in the 4RK2 mutant (Fig. 3C) suggested that trypsin targets one or several of the residues K141, R145, K148 and K150. To identify the cleavage site(s) in the 141-150 sequence, we have re-introduced in the 4RK2 mutant each of the four original residues, one at a time. The functional analysis showed that introduction of either residue R145 (▶ in Fig. 3E) or K150 (▼) on this mutant background allowed a rapid IpH6.5 decrease by 40 µg/ml trypsin, suggesting these two residues as trypsin cleavage sites. Further mutants were generated, on the background of the wt channel, to determine the contribution of additional arg or lys residues within these eight residues and to determine the individual contributions of R145 and K150 to the regulation of ASIC1a function by trypsin. The results are summarized in Table 1 and in Fig. 3F, which shows the pH for half-maximal current activation (pH0.5) before (open bars) and after a 5-min exposure to 200 µg/ml trypsin (filled bars). The analysis is somewhat complicated by the fact that mutation to ala of residues K105 and R121 tends to increase the channel reactivity towards trypsin, in a similar way as previously observed for the 4RK1 mutant. Nevertheless, this analysis clearly identifies the major trypsin cleavage site on ASIC1a. As expected, 200 µg/ml trypsin induced only a small pH0.5 shift in the ASIC1aR145A,K150 double mutant (Fig. 3F). The pH0.5 shift was further decreased by the introduction of the mutation K133A on the R145A,K150 background (significant difference in the direct comparison, p<0.05) but not by the K105A or R121A mutation. Of R145 and K150, mutation of K150 on the wt background (ASIC1aK150A) did not prevent the trypsin-induced shift of pH0.5, while mutation of R145A alone almost completely suppressed the trypsin-induced shift of pH0.5, similarly to the double mutant ASIC1aR145A,K150. The biochemical analysis of the three mutants ASIC1aR145A, ASIC1aR145A,K150A and ASIC1aK133A,R145A,K150A showed no cleavage after 5-min exposure to 40 µg/ml and a faint cleavage after 5-min exposure to 200 µg/ml trypsin (Fig. 3G). These analyses identify R145 as the most important target for trypsin on ASIC1a, as its mutation reduces the shift in the activation curve upon trypsin by 85% and largely suppresses the cleavage of the protein by trypsin. The residues K133 and K150 may be additional targets under the changed conditions resulting from neutralization of charges by mutagenesis.

State-dependent cleavage of ASIC1a

In the trypsin experiments described so far, extracellular trypsin was applied to ASICs that were most of the time (functional experiments) or during the complete exposure period (biochemical experiments) in the closed conformation, indicating that in this conformation the channel protein is efficiently cleaved. Control functional experiments in which the interval between acidic pH applications during the trypsin incubation was varied did not show any importance of channel opening for channel modification (data not shown). In addition to closed and open, ASICs can exist in a third conformational state, as inactivated channels, in which they enter after prolonged exposure to acidic pH (1,21). We have determined the pH dependence of entering this state of “steady-state” inactivation (SSIN) by exposing ASIC-expressing oocytes during 40 s to the conditioning pH in the range of 8.0 to 6.8, before switching to pH 6 for ASIC activation. For the SSIN curve, the normalized pH6-induced peak current is plotted as a function of the conditioning pH in Fig. 4A (grey circles). To test whether inactivation of ASIC1a may affect its reactivity towards trypsin, we have incubated ASIC1a-expressing oocytes during 2 min in 100 µg/ml trypsin in solutions of different pH, ranging from pH 6.8 to 8.0 (for protocol see Experimental Procedures). Subsequently, the pH dependence of activation was determined and from the fit to the activation curve, the fraction of modified channels was calculated and is plotted for each conditioning pH in Fig. 4A (●). Figure 4A shows that modification is maximal in the pH range 8 – 7.4. However, at more acidic pH the fraction of modified channels sharply decreases, following the pH dependence of SSIN. In control experiments we have determined the pH dependence of the trypsin activity against a synthetic substrate in our experimental solutions (open circles in Fig. 4A). The reactivity of trypsin towards the synthetic substrate showed
only a slight decrease at pH 7.0 and 6.8 compared to pH 7.5. Thus, the decreased ASIC modification at pH < 7.4 is not due to an intrinsic pH dependence of trypsin activity. Our experiments rather indicate that the reactivity of ASIC1a towards trypsin is substantially decreased in the inactivated channel, likely by a conformational change in the channel protein that renders residue R145 less accessible. To test whether inactivation prevents cleavage, we have repeated the same protocol at pH 7.5, 7.0 and 6.8 and have subsequently processed the oocytes for biochemical analysis. As shown in Fig. 4B, protein cleavage by trypsin is indeed drastically reduced when channels are inactivated.

Function of the post-M1 region
The venom of the spider Psalmopoeus cambridgei and the toxin Psalmotoxin 1 contained in it block homomeric ASIC1a, but not ASIC1b channels, indicating that most likely the region between residues Q66 and V186 of ASIC1a is necessary for the inhibitory effect of the venom. We have used the chimeric ASIC1a/1b constructs to test whether the region important for venom block and the trypsin cleavage site overlap (Fig. 5B,C). A recent study had shown that Psalmotoxin 1 inhibits ASIC1a by shifting its SSIN curve towards more alkaline pH, thus leading to completely inactivated channels at physiological conditioning pH (22). It is therefore important to choose the conditioning pH for the application of the venom as the pH just at the edge of the falling phase of the SSIN curve. In this setting, the shift in the SSIN curve by the venom will result in efficient current inhibition. Therefore we have first determined SSIN curves for the wt and chimeric constructs (Fig. 5A) and have then added the venom at the optimal pH (see legend Fig. 5C). ASICs were stimulated by pH 5.5 once per minute for 10 s. Figure 5B shows the last IpH5.5 control response before the addition of the venom (left trace) and the IpH5.5 response after the third 50-s venom incubation (right trace) for ASIC1a wt (upper traces) and the ab3 chimera (lower traces). The P. cambridgei venom thus inhibits ASIC1a wt by ~85%, while it does not inhibit currents mediated by the ab3 chimera. As a summary of such experiments Fig. 5C plots for each construct the ratio of the IpH5.5 after venom and the control IpH5.5. These experiments first confirm that ASIC1b, even if measured at the conditioning pH of 7.2, is not inhibited by the venom. The chimera ab1 and ab3 are not inhibited and their IpH5.5 increases, in contrast to ab2 and ab4, that are clearly blocked by the venom. The observation that the venom does inhibit ab3 but not ab4 currents indicates that the critical region for inhibition by the venom is between amino acid residues 152 and 186 of ASIC1a, thus not overlapping with the trypsin cleavage site. We had previously shown that trypsin-exposed ASIC1a is no longer inhibited by the venom at the conditioning pH 7.4 (11). However, trypsin shifts the SSIN curve to more acidic values (Fig. 5A), and therefore, a shift of this curve by the venom may not result in current block when the conditioning pH is pH 7.4. Indeed, the experiments show that when measured at a conditioning pH of 7.2, the venom does inhibit trypsin-exposed ASIC1a (Fig. 5C), thus it is still able to bind to the modified channel.

Our functional analysis of ASIC1a/1b chimera had shown that residues Q66-V186 are responsible for the difference in pH0.5 between ASIC1a and 1b (open symbols and bars in Figs. 2B and C). Previously, the sequence SQL83-85 in ASIC1a had been identified as critical for the rate of open channel inactivation (23), suggesting together with our present data a role of this region for pH sensing and inactivation. ASIC3 is known to display after an initial, rapidly inactivating current a second, sustained current component, that increases in amplitude at more acidic pH (24). A chimera, in which we had replaced residues Q66-V186 of ASIC1a by the corresponding sequence of ASIC3, reproduced exactly these inactivation kinetics (Fig. 5D), indicating that the sequence 66-185 contains all the structures necessary for determining the open channel inactivation kinetics.

DISCUSSION
In this study we have addressed at the molecular level the regulation of ASIC1a by trypsin. We show first that the appearance of the functional changes induced by the exposure to trypsin follows the same time course as channel cleavage and that cleavage is observed in ASIC1a that is functionally modified by trypsin, but not in ASIC1b, whose function is not modified by trypsin. These observations suggest a link between channel cleavage by trypsin and its modification of channel function. The cleavage...
site is located at R145 in the N-terminal portion of the extracellular loop, between a highly conserved domain, and a channel domain that is critical for ASIC1a inhibition by the venom of the spider Psalmopoeus cambridgei. This channel portion is important for inactivation properties and changes its conformation during inactivation thereby rendering the cleavage site inaccessible to extracellular trypsin.

Possible physiological importance of ASIC regulation by serine proteases
Currently no direct evidence exists for a regulation of ASICs by proteases in vivo. However, there is evidence for various roles of proteases in physiological and pathological processes in the central nervous system, in which ASICs are also involved. In these situations the proteases may modulate ASIC function. Several well-characterized serine proteases, as e.g. tissue plasminogen activator, urokinase-type plasminogen activator, thrombin and chymotrypsin B are present under physiological conditions in the central nervous system (25,26).

Trypsin activity in the central nervous system has been documented and might thus be a physiological modifier of ASICs (27). Besides, many brain-specific proteases exist, as for example neuropsin and neutrotispsin (28,29). Proteases in the brain are highly regulated and involved in functions such as neuroplasticity, to which ASICs also contribute (6,7). The expression of many proteases is modulated by brain injury and there is 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 (25,30). ASICs in central neurons likely contribute to the neuronal death associated with brain ischemia or epilepsy, which are accompanied by extracellular acidification (8,31,32) and might in such situations be regulated by proteases.

Link between cleavage of ASIC1a and modification of its function by trypsin
Many observations of this study suggest that channel cleavage is tightly linked to the effect of trypsin on ASIC1 function. First, the ASIC1a protein is cleaved by trypsin and its function is modified, while ASIC1b, whose function is not modified by trypsin, is not cleaved. Second, upon trypsin exposure, the appearance of functionally modified ASIC1a has a similar time course as the appearance of the cleaved channel protein. Third, except for the 4RK1 mutant, all chimeras and ASIC1a mutants in which channel cleavage by trypsin was reduced, showed also a reduced modification of channel function. The 4RK1 mutant, in which R99, K105, R121 and K133 are mutated to ala, displays an increased sensitivity to functional modification by trypsin but is not cleaved by trypsin. The neutralization of four positive charges in this mutant leads thus to an increased sensitivity of the channel for functional modification by trypsin and at the same time prevents cleavage at R145. Our observation that inhibition of trypsin by TLCK prevents the functional modification of ASIC1a wt and of the 4RK1 mutant, indicates that the intact active site cleft of trypsin, which mediates substrate recognition and cleavage (18), is required. The modification of ASIC1a function by trypsin is except for 4RK1 always associated with channel cleavage; our study does however not allow the conclusion that cleavage is absolutely required for the functional modification of ASIC1a wt. The observation that ASIC1a modification is not reversible upon washout of trypsin clearly indicates that functional modification is not due to a binding interaction alone. Rather, the changed function of ASIC1a that has interacted with trypsin is due to an irreversible conformational change in the ASIC1a extracellular loop and/or to channel cleavage. Of these two possible mechanisms, channel cleavage as a cause of the functional modification of wt ASIC1a appears to be better compatible with the known properties of trypsin.

Interestingly, an additional observation also suggests a role of the charge environment on modulation of ASIC function by trypsin. The residue K150 conferred reactivity towards trypsin to ASIC1a only in the channel that had the adjacent positively charged residues mutated to ala (Fig. 3E), but not in the wt ASIC1a or the ASIC1a R145A background (Fig. 3F). Thus it appears that charge-charge interactions in this channel region determine its conformation and its accessibility to extracellular trypsin.

Conformational changes of the extracellular loop during ASIC gating
When trypsin incubations with ASIC1a wt were carried out at sub-maximal concentrations for different times, we generally found a cleavage
product of one defined apparent molecular mass (Fig. 1C), suggesting that trypsin cleaves at one particular site or at sites that are in close proximity to each other. The mutagenesis analysis shows that the most important cleavage site is at R145, and that cleavage can occur to a minor extent also at adjacent positively charged residues. ASIC1a, similar to other ASIC subunits, contains 37 putative trypsin cleavage sites on its extracellular loop (17). The observation that ASIC1a is only cleaved in a very limited region, and ASIC1b is not cleaved at all by high concentrations of extracellular trypsin, suggest that the majority of the extracellular loop is likely not accessible to large molecules from the extracellular solution.

We show here that a conformational change occurs during inactivation, that renders the cleavage site either largely inaccessible to trypsin or decreases its reactivity towards trypsin. Previously it has been shown in the ASIC2a channel and in ENaC, that the degenerin site, which is located at the C-terminal end of the extracellular loop close to the amiloride binding site, undergoes conformational changes during gating (33-35). When the degenerin residue G430 in ASIC2a was mutated to cys, modification of this cys residue by charged methanethiosulfonate reagents induced a sustained current. However, modification occurred only when the channel was activated by lowering of the extracellular pH (33). These experiments did not allow distinguishing whether it was the channel opening or the subsequent inactivation that rendered the engineered cys residue accessible to the methanethiosulfonate reagents. These observations indicate that different parts of the extracellular loop undergo conformational changes during channel gating. Changing the mobility of these parts would likely affect channel gating, making them interesting potential drug targets.

**Determinants of ASIC gating**

Analysis of the functional properties of ASIC chimera yield some interesting information on the functional role of the region encompassing residues Q66-V186. First, this domain determines the inactivation kinetics of the channel, as most clearly shown by the transplantation of this sequence of ASIC3 into the ASIC1a channel. Second and as expected, this domain is responsible for the difference in pH dependence of activation and inactivation between ASIC1a and ASIC1b. However, it was not possible to clearly attribute the pH dependence to a defined region within residues Q66-V186, suggesting that different sites co-determine the pH dependence of ASIC gating. A previous study that compared ASIC1a and 1b function had identified residues K105 and N106 as most important for the difference in pH dependence between ASIC1a and 1b (36). In accordance with this previous work, the ab2 chimera (containing Q66-Q102 of ASIC1b) showed a similar pH dependence of SSIN as ASIC1b. However, the pH dependence of activation of the ab2 chimera was close to that of ASIC1a. Rather, our chimera analysis suggests residues V103 – V186 as important for the pH dependence of activation, because the pH0.5 of the ab3 chimera (containing V103 – V186 of ASIC1b) is close to the pH0.5 of ASIC1b. The pH0.5 of ab4 (containing V103-F152 of ASIC1b) was intermediate between that of ab3 and ASIC1a wt (Fig. 2C), suggesting that determinants of the pH dependence of activation are present in different parts of the V102-V186 sequence. Of the charge mutations tested, the 4RK1 and the 8RK mutant shifted the pH0.5 of activation to less acidic pH (Fig. 3F), indicating that some of these arg and lys residues co-define the pH sensitivity of ASIC1a activation, likely by co-determining the pKa of the residues that are protonated. Third, the P. cambridgei venom inhibits the ab2 and ab4 chimera, but not the ab3 chimera, suggesting that the region between residues F152 and V186 is important for its inhibition of ASIC current. Other residues beyond V186, common to ASIC1a and 1b might also be required for the binding and/or the inhibitory action of the venom.

Previous studies have already analyzed some of the contributions of different ASIC sequences. The SQL83-85 sequence was identified as being highly important for open channel inactivation, as its mutation to the corresponding fish sequence accelerated desensitization by a factor of 25 (23). In a very recent study the Canessa laboratory constructed chimeras between mammalian ASICs and ASICs of early vertebrates that are proton-insensitive (37). They showed that the minimal mammalian sequence requirement for proton-sensitive ASIC1a was the stretch D78 – E136, together with the residues D351 and Q358. However, this minimal construct displayed pH dependence and kinetics different from mammalian ASIC1a, indicating
that other channel regions co-determine these properties. Protease modification is a potentially important mechanism of adaptation of ASIC gating to changed extracellular pH conditions. Our identification of the cleavage site on ASIC1a highlights a potential drug target and helps in the understanding of the ASIC gating mechanisms. The cleavage site of trypsin on ASIC1a is located between two conserved cysteine residues, C93 and C172, that, in αENaC likely form a disulfide bond (38). We hypothesize therefore that a disulfide bond between C93 and C172 stabilizes the loop between them. Cleavage at R145 or nearby residues is expected to change the conformation of this loop and the interactions within the loop and with other channel parts, thereby changing the pH dependence of gating of the modified channel.

REFERENCES


FOOTNOTES

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Abbreviations
ASIC, acid-sensing ion channel; BAPNA, Nα-Benzoyl-DL-arginine 4-nitroanilide; ENaC, epithelial Na+ channel; IpH6.5, peak current amplitude induced by acidification to pH 6.5; pH0.5, pH for half-maximal activation; pHIn0.5, pH for half-maximal inactivation; SSIN, steady-state inactivation; TLCK, tosyl-L-lysyl-chlormethan; wt, wild type.

FIGURE LEGENDS

**Figure 1.** Modification of ASIC1a function by trypsin correlates with channel cleavage
Functional measurements were obtained by 2-electrode voltage-clamp at a holding potential of -60 mV. *A*, pH-dependence of ASIC activation before (open symbols) and after a 5-min application of 200 µg/ml trypsin (filled symbols), normalized to the pH4-induced current before trypsin exposure and corrected for rundown during the first activation curve (before trypsin exposure), n=10-15. The pH of half-maximal activation (pH0.5) obtained by the fit is shown in Table 1. *B,C*, cell surface proteins were isolated by biotinylation as described in Experimental Procedures and ASIC1 was visualized by a specific antibody that recognizes the C-terminus, which is common to ASIC1a and 1b. *B*, oocytes were incubated during 5 min in extracellular solution containing 200 µg/ml trypsin (lanes marked “+”) or were not exposed to trypsin (“−”). n.i. = non-injected oocytes. *C*, oocytes were exposed to 40 µg/ml for the time periods indicated on top of the lanes. Shown is a representative of 4 similar experiments. *D*, time course of pH6.5-induced current (IpH6.5, ●) of ASIC1a and fraction of uncleaved channel protein (■) during external perfusion by trypsin at 40 µg/ml. The fraction of uncleaved channel protein was
determined from experiments as that shown in panel C, as the ratio of the signal of the upper band over the total signal.

Figure 2. Chimera between ASIC1a and ASIC1b identify the target region of trypsin on ASIC1a
ASIC1a and 1b differ in the first 186 amino acids. A, illustration of the chimera that were constructed, black parts in the bars indicate ASIC1b sequence, grey parts ASIC1a sequence. B, pH dependence of ASIC activation before (○) and after a 5-min application of 200 µg/ml trypsin (●) determined as described in the legend to Fig. 1 (n=6-12). C, summary of pH0.5 values of ASIC1a and ASIC1b wt and chimera as indicated, obtained before and after a 5-min exposure to 200 µg/ml trypsin. *, pH0.5 is different from pH0.5 of ASIC1a (p<0.05); #, the change in pH0.5 upon trypsin exposure is different from the pH0.5 change of ASIC1a (p<0.05). The ab5 chimera did not produce H+-activated ionic currents and could therefore not be included in the functional analysis. D, oocytes were exposed during 5 min to 200 µg/ml trypsin (lanes marked “+”) or were not exposed to trypsin (“-”), cell-surface resident proteins were isolated by biotinylation and visualized on Western blot by a specific antibody recognizing the C-terminus (as described in Experimental Procedures).

Figure 3. Identification of target residues for trypsin on ASIC1a
A, alignment of ASIC1a and ASIC1b of the sequence corresponding to the ASIC1b insert in chimera ab4. B, pH dependence of ASIC activation of different ASIC1a mutant constructs before and after 5-min exposure to 200 µg/ml trypsin, 8RK (R99A,K105A,R121A,K133A,K141A,R145A,K148A,K150A), 4RK1 (R99A,K105A,R121A,K133A) and 4RK2 (K141A,R145A,K148A,K150A), determined as described in the legend to Fig. 1. The pH0.5 values obtained from the fit are shown in table 1. C, time course of IpH6.5 decrease (IpH6.3 for 8RK) during exposure to 40 µg/ml (open symbols) or 200 µg/ml trypsin (filled symbols), n=3-8. IpH6.5 decay rates from the fits to the data are shown in Table 1. D, Western blot of 8RK and 4RK1 mutants exposed during 5 min to 200 µg/ml trypsin (“+”) or not (“-”), as described in the legend to Fig. 1 and Experimental Procedures. E, IpH6.5 decrease upon exposure to 40 µg/ml trypsin of ASIC1a wt, the 4RK2 mutant or 4RK2 in which each of the four 4RK2 mutations had been reversed to the original residue, one at a time, as indicated in the legend. Lines are fits of the data to a mono-exponential decay equation. F, summary of pH0.5 values of ASIC1a and ASIC1b wt and ASIC1a mutants as indicated, obtained before and after a 5-min exposure to 200 µg/ml trypsin. *, pH0.5 is different from pH0.5 of ASIC1a wt (p<0.05); #, the change in pH0.5 upon trypsin exposure is different from the pH0.5 change of ASIC1a wt (p<0.05). G, western blots of three ASIC1a mutants not exposed to trypsin (“0”) or exposed during 5 min to 40 µg/ml (“40”) or 200 µg/ml trypsin (“200”); isolation and visualization as described in Experimental Procedures.

Figure 4. Inactivation prevents ASIC modification by trypsin
A, Oocytes expressing wt ASIC1a were exposed during 2 min to 100 µg/ml trypsin at different pH values ranging from pH8 to 6.8 (see Experimental Procedures), followed by the analysis of the pH-dependence of ASIC activation. The pH dependence was fitted to a Hill equation containing two components: I = f /[1+(10^pH0.5(m))/10^pH(m)Hn(m)] + (1-f)/[1+(10^pH0.5(c))/10^pH(c)Hn(c)] (see Experimental Procedures), with fixed parameters for pH0.5 and Hn, one component corresponding to non-modified channels (c, obtained from activation curves of oocytes that had not been exposed to trypsin) and the second component corresponding to modified channels (m, obtained from the fit to the pH condition, in which modification was maximal), with “f”, the fraction of modified channels, as only free value in the fit, shown in the graph as filled circles. The grey circles represent the pH dependence of steady state inactivation (SSIN) of ASIC1a. The open circles represent the trypsin activity towards a synthetic substrate, BAPNA, plotted as the rate of absorption increase (see Experimental Procedures). B, oocytes expressing ASIC1a wt were exposed to trypsin under the same conditions as for the functional analysis and subsequently isolated and western blotted. In these experiments, the trypsin concentration was increased at pH 7.0 and 6.8 to compensate for the slightly decreased proteolytic activity of trypsin at these pH values compared to pH 7.5.
Figure 5. Function of the N-terminal part of the extracellular loop

A, pH dependence of steady-state inactivation (SSIN) of wt and different chimeras, that have not been exposed to trypsin except for “ASIC1a try”, ASIC1a wt that has been exposed during 5 min to 200 µg/ml trypsin. ASIC currents were induced by 5-s acidification to pH 5.5 after exposure for 40 s to the conditioning pH. The current response, normalized to the response with the conditioning pH of 7.6, is plotted against the conditioning pH, n = 3-6. pH values of half-maximal inhibition, obtained from the fits to the data, were 7.31 ± 0.00 (ASIC1a), 7.09 ± 0.01 (ASIC1a, try), 7.10 ± 0.00 (ASIC1b), 7.03 ± 0.01 (ab1), 7.11 ± 0.01 (ab2), 7.18 ± 0.02 (ab3) and 7.28 ± 0.01 (ab4).

B, C, inhibition of ASIC1 currents by the venom of P. cambridgei was determined as described in Experimental Procedures. Conditioning pH was 7.4 (ASIC1a, ab3, ab4), 7.3 (ab2) or 7.2 (ASIC1a trypsin, ASIC1b, ab1).

B, representative current traces before venom perfusion (left trace) and after 150 s of extracellular venom perfusion (right trace) for wt ASIC1a (top) and the ab3 chimera (lower panel).

C, summary, plotting the ratio of IpH5.5 after 150 s venom perfusion relative to control.

D, Representative traces of ASIC1a wt and a chimera in which the sequence 66-186 of ASIC1a was replaced by the corresponding sequence of ASIC3.
Table 1. Functional parameters of selected ASIC channels

<table>
<thead>
<tr>
<th>Construct</th>
<th>pH0.5 control</th>
<th>pH0.5 trypsin (5 min / 200µg/ml)</th>
<th>rate k of I_pH6.5 trypsin (min⁻¹ mg⁻¹ ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASIC1a wt</td>
<td>6.55 ± 0.06</td>
<td>6.08 ± 0.05</td>
<td>10.75 ± 0.72 §</td>
</tr>
<tr>
<td>ASIC1b wt</td>
<td>5.94 ± 0.00 †</td>
<td>5.98 ± 0.05 †</td>
<td>0.12 ± 0.04 ‡ &amp;</td>
</tr>
<tr>
<td>ASIC1a 4RK1</td>
<td>6.38 ± 0.05 †</td>
<td>5.93 ± 0.04</td>
<td>109.94 ± 14.36 °&amp;</td>
</tr>
<tr>
<td>ASIC1a 4RK2</td>
<td>6.57 ± 0.02</td>
<td>6.25 ± 0.04</td>
<td>1.74 ± 0.20 † &amp;</td>
</tr>
<tr>
<td>ASIC1a 8RK</td>
<td>6.18 ± 0.03 †</td>
<td>5.97 ± 0.10 †</td>
<td>0.40 ± 0.11 ‡ &amp;</td>
</tr>
<tr>
<td>ASIC1a K133A,R145A,K150A</td>
<td>6.62 ± 0.03</td>
<td>6.55 ± 0.04 †</td>
<td>0.15 ± 0.03 ‡ &amp;</td>
</tr>
<tr>
<td>ASIC1a R145A,K150A</td>
<td>6.63 ± 0.02</td>
<td>6.52 ± 0.05 †</td>
<td>0.39 ± 0.06 ‡ &amp;</td>
</tr>
<tr>
<td>ASIC1a K133A</td>
<td>6.63 ± 0.04</td>
<td>6.19 ± 0.02</td>
<td>10.01 ± 1.46 † &amp;</td>
</tr>
<tr>
<td>ASIC1a R145A</td>
<td>6.62 ± 0.03</td>
<td>6.58 ± 0.04 †</td>
<td>0.25 ± 0.05 ‡ &amp;</td>
</tr>
<tr>
<td>ASIC1a K150A</td>
<td>6.63 ± 0.03</td>
<td>6.18 ± 0.01</td>
<td>10.13 ± 1.63 † &amp;</td>
</tr>
</tbody>
</table>

The pH for half-maximal activation, pH0.5 was determined as described in the legend to Fig. 1 and in Experimental Procedures, either in oocytes not exposed to trypsin, or oocytes that had been exposed during 5 min to 200µg/ml trypsin, n=4-15. †, pH0.5 is different from pH0.5 of ASIC1a wt (p<0.05); ‡, the change in pH0.5 upon trypsin exposure is different from the pH0.5 change of ASIC1a wt (p<0.05). The rate k of I_pH6.5 decrease during trypsin exposure was determined from an exponential fit to the current decay and normalized for the trypsin concentration used. Trypsin concentration was 5 µg/ml (°), 40 µg/ml (‡) or 200 µg/ml (†). *, for this mutant, the current induced by pH 6.3 was followed. &*, different from ASIC1a wt (p<0.05).
Figure 1 Vukicevic et al.
Figure 2 Vukicevic et al.
Figure 3 Vukicevic et al.
Figure 4 Vukicevic et al.
Figure 5 Vukicevic et al.
Trypsin cleaves acid-sensing ion channel 1A in a domain that is critical for channel gating
Marija Vukicevic, Gilles Weder, Aurélien Boillat, Anne Boesch and Stephan Kellenberger

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