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Interaction of Acid-sensing Ion Channel (ASIC) 1 with the Tarantula Toxin Psalmotoxin 1 is State Dependent

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Acid-sensing ion channels (ASICs) are Na^+ channels gated by extracellular H^+ . Six ASIC subunits that are expressed in neurons have been characterized. The tarantula toxin psalmotoxin 1 has been reported to potently and specifically inhibit homomeric ASIC1a and has been useful to characterize ASICs in neurons. Recently we have shown that psalmotoxin 1 inhibits ASIC1a by increasing its apparent affinity for H^+ . However, the mechanism by which PcTx1 increases the apparent H^+ affinity remained unclear. Here we show that PcTx1 also interacts with ASIC1b, a splice variant of ASIC1a. However, PcTx1 does not inhibit ASIC1b but promotes its opening; under slightly acidic conditions, PcTx1 behaves like an agonist for ASIC1b. Our results are most easily explained by binding of PcTx1 with different affinities to different states (closed, open, and desensitized) of the channel. For ASIC1b, PcTx1 binds most tightly to the open state, promoting opening, whereas for ASIC1a, it binds most tightly to the open and the desensitized state, promoting desensitization.

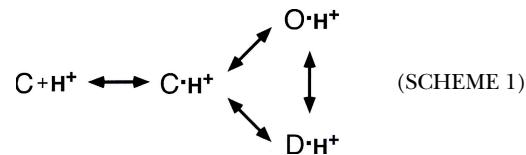
INTRODUCTION

Acid sensing ion channels (ASICs) are Na^+ -selective ion channels that are activated by extracellular H^+ (Waldmann and Lazdunski, 1998; Krishtal, 2003). They are abundantly expressed in the central and the peripheral nervous system and participate in higher brain functions, such as learning and memory (Wemmie et al., 2002), and in perception of pain (Sutherland et al., 2001; Voilley et al., 2001; Chen et al., 2002; Mamet et al., 2002), taste (Ugawa et al., 2003), and mechanical stimuli (Price et al., 2000). ASIC subunits have a simple topology with two transmembrane domains, short intracellular termini, and the bulk of the protein in the extracellular space (Saugstad et al., 2004). In the genome of mammals there are four *asic* genes. ASIC1a and ASIC1b are splice variants of the *asic1* gene, which differ in the first third of their amino acid sequence, including the first transmembrane domain TM1, whereas the remaining two thirds of the proteins are identical (Chen et al., 1998; Bässler et al., 2001). ASIC1a is highly expressed in the small neurons of the dorsal root ganglia and many regions, mostly those with excitatory input, in the brain (Waldmann et al., 1997; Wemmie et al., 2003). In contrast, ASIC1b is specifically expressed in sensory neurons (Chen et al., 1998). Native ASICs are homo- and heteromeric assemblies of probably four subunits (Sutherland et al., 2001; Baron et al., 2002; Benson et al., 2002; Xie et al., 2002).

Like the related epithelial Na channel, ENaC, ASICs are blocked by the diuretic amiloride, with an EC_{50} of

$\sim 20 \mu\text{M}$ (Waldmann et al., 1997; Paukert et al., 2004). The first potent and specific blocker of ASICs to be identified was the tarantula toxin psalmotoxin 1, PcTx1 (Escoubas et al., 2000). It was reported that PcTx1 specifically inhibits ASIC1a with an EC_{50} of $\sim 1 \text{ nM}$ (Escoubas et al., 2000). No other ASIC and also no heteromeric ASICs, even those containing the ASIC1a subunit, were inhibited (Escoubas et al., 2000). Recently, we reported that PcTx1 inhibits ASIC1a by increasing its apparent H^+ affinity (Chen et al., 2005). This increase in apparent affinity for their ligand, H^+ , is sufficient to shift ASIC1a channels into the desensitized state at a resting pH of 7.4. In addition, PcTx1 promotes the opening of ASIC1a (Chen et al., 2005).

Apparent H^+ affinity, however, is an unspecific description that does not provide much insight into the underlying mechanism (Colquhoun, 1998). According to the basic kinetic scheme



ASICs bind H^+ in the closed state C, and from this H^+ -bound closed state they either reach the open state O or the desensitized state D. H^+ -bound states would be cyclically connected so that channels could reach the desensitized state D also from the open state O. As we

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Abbreviations used in this paper: ASIC, acid-sensing ion channel; DHP, dihydropyridine; PcTx1 , psalmotoxin 1.

previously proposed (Chen et al., 2005), the increase in apparent H^+ affinity by *PcTx1* could be explained in two different ways. First, *PcTx1* could increase the true affinity of H^+ to *ASIC1a*, modifying the energetics of the binding step. Second, it could modify the energetics of the gating step, shifting the equilibrium between the closed state with H^+ bound and the open and desensitized state. Such a shift of the equilibrium between different states would be expected if *PcTx1* would have a higher affinity to the open and/or the desensitized state than to the closed state. Since *PcTx1* promoted steady-state desensitization as well as opening of *ASIC1a*, we were unable to decide if *PcTx1* directly affects the H^+ affinity of *ASICs* or if it indirectly modulates gating by state-dependent binding.

In this study, we further addressed the mechanism of inhibition of *ASIC1* by *PcTx1*. We found that *PcTx1* also interacts with *ASIC1b*. However, in contrast to *ASIC1a*, *ASIC1b* was almost not inhibited by *PcTx1* but its opening was greatly facilitated and its desensitization slowed down by *PcTx1*. These results show that facilitated binding of H^+ cannot explain the effects of *PcTx1*. They rather support a model in which *PcTx1* binds with different affinity to different states of the channel, allosterically modifying gating.

MATERIALS AND METHODS

Electrophysiology

cDNAs for rat *ASIC1a*, *ASIC1b*, and chimeras C43, C61, C92, and C98 had been previously described (Bässler et al., 2001; Babini et al., 2002). Chimera C166 was constructed as described before (Babini et al., 2002). Capped cRNA was synthesized by SP6 RNA polymerase from linearized cDNA, using the mMessage mMachine kit (Ambion).

We injected 0.01–10 ng cRNA into defolliculated stage V–VI oocytes of *Xenopus laevis* and kept oocytes in OR-2 medium for 2–4 d before measurements. OR-2 (oocyte ringer-2) solution contained (in mM) NaCl 82.5, KCl 2.5, Na_2HPO_4 1.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 5.0, PVP 0.5 g/liter, 1,000 U/liter penicillin, and 10 mg/liter streptomycin; pH was adjusted to 7.3 with NaOH. Whole cell currents were recorded with a TurboTec 03X amplifier (npi electronic) using an automated, pump-driven solution exchange system together with the oocyte testing carousel controlled by the interface OTC-20 (Madeja et al., 1995). Data acquisition and solution exchange were managed using the software CellWorks 5.1.1 (npi electronic). Data were filtered at 20 Hz and acquired at 0.1–1 kHz. Bath solution for whole oocyte current measurements contained (in mM) NaCl 140, HEPES 10, $CaCl_2$ 1.8, $MgCl_2$ 1.0; pH was adjusted with NaOH. For acidic test solutions, HEPES was replaced by MES. Glass electrodes filled with 3 M KCl were used; they had a resistance of 0.3–1.5 M Ω . The membrane potential was clamped at –70 mV.

PcTx1 synthesis and refolding was previously reported (Chen et al., 2005). Solutions containing *PcTx1* were supplemented with 0.05% BSA (Sigma-Aldrich) in order to avoid absorption by the tubing. 0.05% BSA itself did not significantly affect *ASICs* currents. In addition, we obtained results that were qualitatively similar to the results with *PcTx1*, using solutions containing the *Psalmopoeus cambridgei* venom (SpiderPharm) (not depicted). These solutions were not supplemented with BSA.

Data Analysis

Data were analyzed with the software IgorPro (Wave metrics, Lake Oswego, OR USA). pH response curves were fit with a Hill function:

$$I = a + (I_{max} - a) / \left(1 + (EC_{50}/[H])^n \right), \quad (1)$$

where I_{max} is the maximal current, a is the residual current, $[H]$ is the concentration of the H^+ , EC_{50} is the concentration at which half-maximal response occurs, and n is the Hill coefficient. The pH of half-maximal activation, pH_{50} , was calculated as $-\log(EC_{50})$. The activation and desensitization curves of *ASICs* currents were fit with a mono-exponential function. Before fitting, currents from each measurement were normalized to the maximal value measured. Results are reported, in the text, as means \pm SD or, on the figures, as means \pm SEM. They represent the mean of n individual measurements on different oocytes. Statistical analysis was done with the paired or unpaired *t* test, as appropriate.

RESULTS

PcTx1 Only Weakly Affects Steady-state Desensitization of *ASIC1b* but Strongly Promotes H^+ Activation

It has been reported that *PcTx1* does not inhibit *ASIC1b* at pH 7.4 (Escoubas et al., 2000). However, since *ASIC1b* has a lower H^+ affinity than *ASIC1a* and its steady-state desensitization curve is shifted by 0.25 pH units compared with *ASIC1a* (Babini et al., 2002), we reevaluated inhibition at a conditioning pH of 7.1. Fig. 1 shows that also at pH 7.1 *ASIC1b* currents were not inhibited, even by a high concentration (500 nM) of synthetic *PcTx1* ($n = 5$). At a more acidic conditioning pH of 6.9, 100 nM *PcTx1* slightly inhibited *ASIC1b* currents ($n = 5$). To more systematically address the interaction of *PcTx1* with *ASIC1b*, we determined the steady-state desensitization curve of *ASIC1b* in the absence and in the presence of 100 nM *PcTx1*. As is shown in Fig. 1, the toxin slightly ($P = 0.04$) shifted the pH_{50} of steady-state desensitization from pH 6.95 ± 0.03 ($n = 6$) without *PcTx1* to pH 6.99 ± 0.03 ($n = 6$) with *PcTx1*. This shift is tiny compared with *ASIC1a*, for which steady-state desensitization is shifted by 0.27 pH units by 30 nM *PcTx1* (Chen et al., 2005), consistent with the finding that *PcTx1* does not inhibit *ASIC1b* as clearly as it inhibits *ASIC1a*.

Next we examined the apparent H^+ affinity for activation of *ASIC1b* in the absence and presence of 100 nM *PcTx1*. As shown in Fig. 2 B, and similar to a previous reports (Babini et al., 2002), *ASIC1b* currents were half-maximally activated at a pH of 5.70 ± 0.04 ($n = 7$) in the absence of *PcTx1*. In the presence of 100 nM *PcTx1*, the pH_{50} of activation was significantly ($P < 0.01$) leftward shifted by 0.38 pH units to pH 6.08 ± 0.06 ($n = 6$). Hence *PcTx1* robustly increased the apparent H^+ affinity of *ASIC1b* for activation. The increased apparent H^+ affinity of *ASIC1b* is also illustrated by a potentiation of *ASIC1b* currents by *PcTx1* as shown in

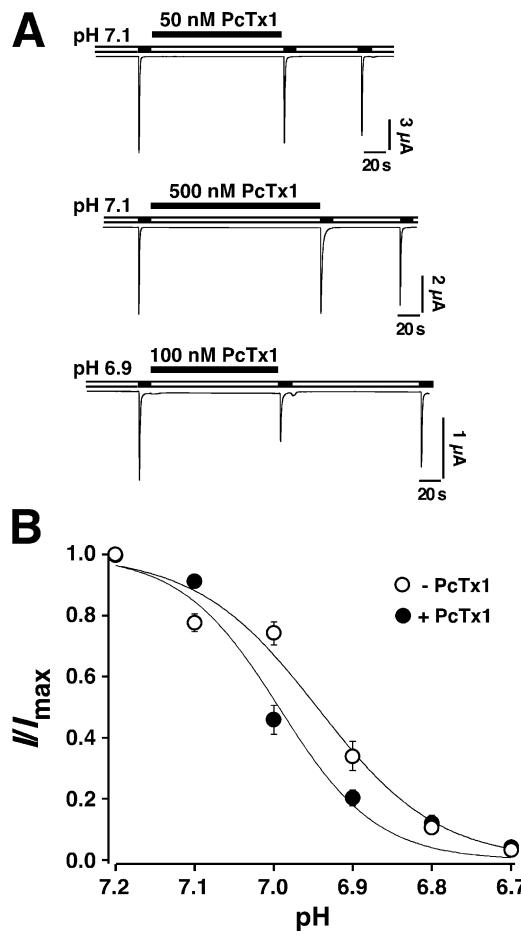


Figure 1. PcTx1 did not strongly inhibit ASIC1b. (A) ASIC1b currents were repeatedly activated by pH 5.0 for 10 s. Even 500 nM PcTx1 did not inhibit the current, when it was applied at pH 7.1 ($n = 5$). When applied at pH 6.9, 100 nM PcTx1 slightly inhibited the current ($n = 6$). (B) H^+ dependence of steady-state desensitization of ASIC1b currents in the absence ($n = 6$, open circles) or presence ($n = 6$, filled circles) of 100 nM PcTx1. PcTx1 was applied for 120 s in the conditioning period; available channels were assessed with pH 5.0. Solid lines are fits of the mean values of each data point to a Hill function (Eq. 1). PcTx1 slightly but significantly ($P = 0.04$) shifted the steady-state desensitization curve of ASIC1b leftwards.

Fig. 3 A. Application of 100 nM PcTx1 in the conditioning period increased ASIC1b currents elicited by pH 6.0 by 1.9-fold (1.9 ± 0.8 , $n = 9$). This result clearly shows that PcTx1 can bind to and interact with ASIC1b. It promotes the opening of ASIC1b but does not induce strong desensitization at steady state.

PcTx1 Binds with Different Affinities to Different States of the Channel

PcTx1 directly opens ASIC1a when either the H^+ concentration is simultaneously increased or the Ca^{2+} concentration is simultaneously lowered (Chen et al., 2005). We, therefore, examined whether PcTx1 opens ASIC1b in a similar way. pH 6.6 by itself does not open ASIC1b

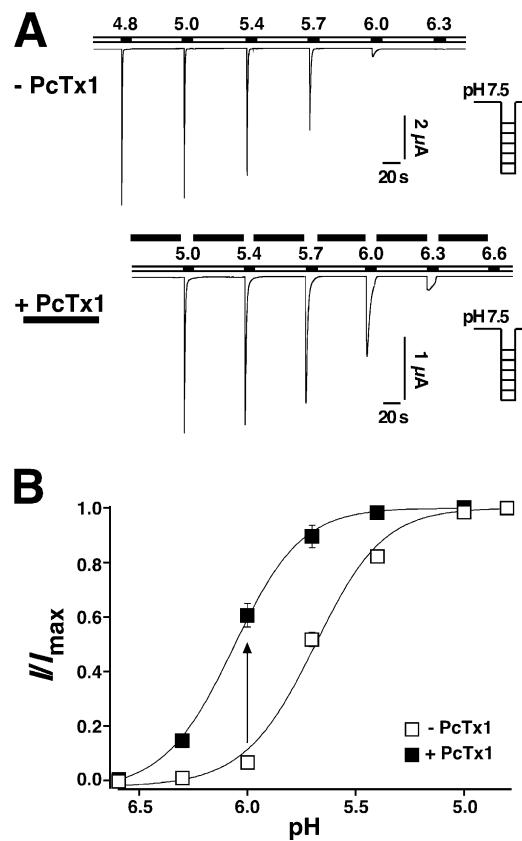


Figure 2. PcTx1 facilitated opening of ASIC1b. (A) Representative current traces elicited by pH ranging from 4.8 to 6.6 with conditioning pH 7.5 (applied for 60 s). Top, without PcTx1; bottom, with 100 nM PcTx1. (B) H^+ dependence of ASIC1b activation in the absence ($n = 7$, open squares) or in the presence ($n = 6$, filled squares) of 100 nM PcTx1. Solid lines are fits of the mean values of each data point to a Hill function (Eq. 1). PcTx1 significantly ($P < 0.01$) shifted the pH activation curve to lower H^+ concentrations. The arrow illustrates how PcTx1 potentiates currents at pH 6.0, as shown in Fig. 3 A.

(Fig. 3 B). However, as illustrated in Fig. 3 B, coapplication of 200 nM PcTx1 and pH 6.6 indeed opened ASIC1b. We estimated the apparent affinity of ASIC1b for PcTx1 by coapplying different concentrations of the toxin with pH 6.6. This analysis yielded an EC_{50} of ~ 100 nM (Fig. 3 B). From a similar analysis with ASIC1a we estimated a lower limit for the EC_{50} of 150 nM when PcTx1 was coapplied with pH 7.1 (Fig. 3 C). Due to desensitization of the channel, these EC_{50} values were not measured at steady state and, therefore, provide only a rough estimate for the apparent affinity of the open state of the channels. They suggest that apparent affinities of PcTx1 for open ASIC1a and open ASIC1b channels are on the same order of magnitude.

We previously estimated the apparent IC_{50} of PcTx1 for ASIC1a to be ~ 3 nM at pH 7.4 (Chen et al., 2005). This value was obtained by measuring the inhibition of the ASIC1a current and thus provides an estimate of the apparent affinity of PcTx1 for the desensitized state of

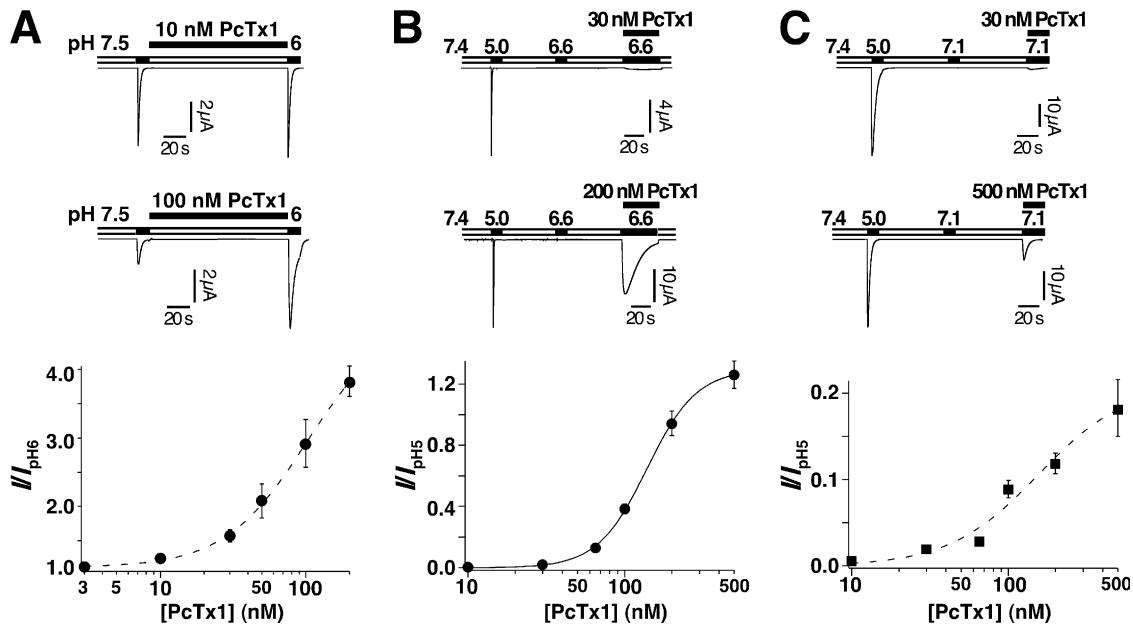


Figure 3. (A) PcTx1 potentiated ASIC1b currents elicited by pH 6.0. Top, representative current traces. Conditioning pH was 7.5. PcTx1 was applied in the conditioning period for 120 s. Bottom, concentration–response relationship ($n = 5$ –9). Fit to the Hill equation revealed half-maximal potentiation at 101 nM (dashed line). Since a maximal response was not reached, this EC_{50} value provides a lower limit for the apparent affinity. (B) PcTx1 directly and robustly opened ASIC1b. Top, different concentrations of PcTx1 were coapplied with pH 6.6. pH 6.6 alone did not open ASIC1b. Bottom, concentration–response relationship. Solid line represents a fit to the Hill equation and revealed half-maximal potentiation at 139 nM ($n = 4$ –8). (C) PcTx1 directly opened ASIC1a. Top, different concentrations of PcTx1 were coapplied with pH 7.1 ($n = 4$ –10). pH 7.1 alone did not open ASIC1a. Bottom, concentration–response relationship. Fit to the Hill equation revealed half-maximal potentiation at 156 nM (dashed line), providing a lower limit for the apparent affinity.

ASIC1a. As shown above, the apparent IC_{50} of PcTx1 for ASIC1b was much larger than for ASIC1a (>500 nM at pH 7.1). It is not possible to directly compare the estimates of the IC_{50} with the estimates of the EC_{50} , because IC_{50} values have been measured at steady state, whereas, as already mentioned, EC_{50} values cannot be measured at steady state.

PcTx1 Slows the Desensitization of ASIC1b Channels

As can be seen from the traces in Fig. 1 A and Fig. 2 A, PcTx1 appeared to slow down the desensitization of ASIC1b. Analyzing the decay time constant from experiments as the one shown in Fig. 2 A, we found that the decay time constant was indeed increased by a factor of ~ 1.4 , independent of the pH used (Fig. 4 A). Opening of ASIC1b channels with pH 6.0 and preincubating the channels with different concentrations of the toxin revealed that the increase of the desensitization time constant was concentration dependent (Fig. 4 B). The concentration dependence could be reasonably well fit with a Hill function assuming the above-determined EC_{50} (100 nM) for the toxin (Fig. 4 B).

At pH 5.0, ASIC1b currents decayed with a time constant τ of 0.6 ± 0.1 s. Preincubation with 500 nM PcTx1 robustly increased the decay time constant τ to 1.5 ± 0.1 s ($n = 6$; $P < 0.01$; Fig. 5). In contrast, the desensitization of ASIC1a was only slightly affected under the

same conditions ($\tau = 1.2 \pm 0.2$ s without toxin and $\tau = 1.3 \pm 0.2$ s with 500 nM PcTx1; $P < 0.01$, $n = 5$; Fig. 5). Similarly, PcTx1 opened ASIC1b more persistently than ASIC1a (Fig. 6 A). ASIC1b currents elicited by 100 nM PcTx1 at pH 6.6 decayed significantly ($P < 0.01$) slower than ASIC1a currents elicited by 100 nM PcTx1 at pH 7.1. The decay time constant was $\tau = 17.6 \pm 3.8$ s ($n = 12$) for ASIC1b and $\tau = 4.3 \pm 0.7$ s ($n = 11$) for ASIC1a currents. The ASIC1b currents were sensitive to amiloride block (100 μ M), several seconds after washing off PcTx1 (Fig. 6 A), indicating that PcTx1 dissociated slowly from the channel with a time constant on the order of several seconds. In contrast, the rising phase of ASIC1b currents was similar to that of ASIC1a currents under these conditions ($P = 0.27$). For both channels, the time constant of the rising phase was around 2 s, much larger than for opening by high H⁺ concentrations. This slow onset of current activation may be explained by the slow association of PcTx1. Slow on and off rates have recently also been shown for the interaction of hanatoxin, which is structurally related to PcTx1 (Escoubas et al., 2003), with Kv2.1 channels (Phillips et al., 2005). Both the time constant describing the rising phase and the time constant describing the decay phase of the PcTx1-induced ASIC1b current decreased with increasing toxin concentrations up to 200 nM (Fig. 6). However, increasing the toxin

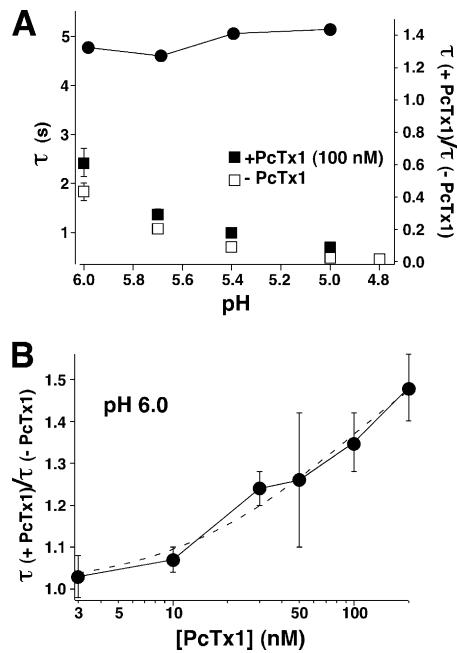


Figure 4. PcTx1 slowed down desensitization of ASIC1b . (A) Diagram showing the time constant of desensitization with and without 100 nM PcTx1 at different pH values (squares). Data are from measurements like the one shown in Fig. 2 A. The ratio of both time constants is shown in the same diagram (filled circles). It was not pH dependent. (B) Diagram showing the dependence of the ratio of the time constant of desensitization with and without PcTx1 at different concentrations of PcTx1 . pH used to activate ASIC1b channels was always pH 6.0. Data are from measurements like the one shown in Fig. 3 A. The dashed line represents a fit to the Hill equation assuming an EC_{50} of 100 nM, as determined in Fig. 3, A and B.

concentration >200 nM did not further decrease the decay time constant ($\tau \sim 12$ s). Such slowing of the desensitization by PcTx1 is expected when PcTx1 stabilized the open state of ASIC1b with respect to the desensitized state.

A Small Region in the Extracellular Loop Determines High PcTx1 Affinity of the Desensitized State

To gain insights, which structural differences between ASIC1a and ASIC1b determine their differential response to PcTx1 , we used a chimeric approach that was based on a series of chimeras that exchange different parts between the two splice variants (Fig. 7 A). Some of these chimeras had already been previously described (Bässler et al., 2001; Babini et al., 2002). As shown in Fig. 7, in these chimeras increasing fragments from ASIC1b replaced the corresponding fragments of ASIC1a , leading to chimeras C43, C61, C92, C98, and C166, with the number denoting the number of amino acids replaced. For example, C166 contained 166 amino acids from ASIC1b at its NH_2 terminus, followed by 19 amino acids from ASIC1a , and finally the common COOH terminus (Fig. 7).

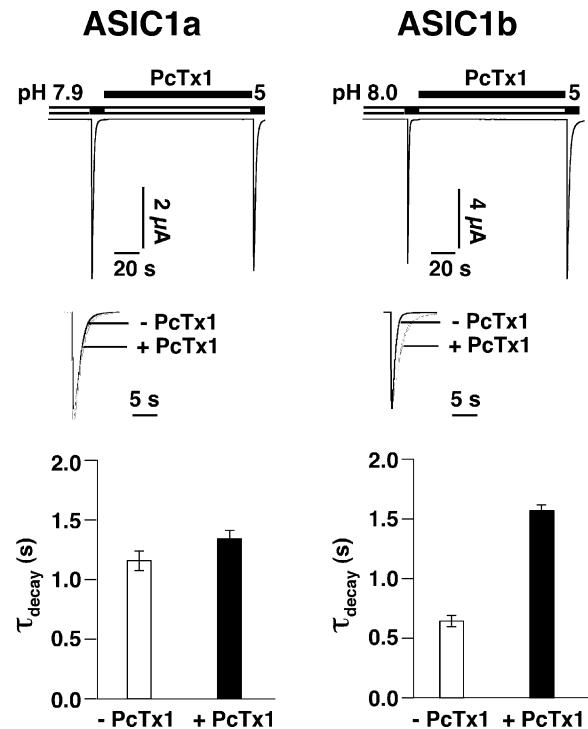


Figure 5. PcTx1 significantly prolonged the desensitization of ASIC1 currents. 500 nM PcTx1 slightly but significantly ($P < 0.01, n = 5$) slowed down desensitization of ASIC1a currents. Desensitization of ASIC1b currents was more strongly slowed down ($P << 0.01, n = 6$). Conditioning pH was pH 7.9 for ASIC1a and pH 8.0 for ASIC1b , respectively. Acidic test pH was always pH 5.0. Top, examples of current traces. Scaled overlaid traces before and after PcTx1 application are shown for better comparison. Bottom, bars representing desensitization time constants of ASIC1 currents before and after PcTx1 application.

First, we looked for inhibition of the chimeras by PcTx1 . We determined for each chimera the pH_{50} of steady-state desensitization (Table I) and applied PcTx1 at a conditioning pH that was ~ 0.2 pH units above this pH_{50} . Fig. 7 illustrates that all five chimeras C43, C62, C92, C98, and C166, like ASIC1a wild type, were almost completely inhibited by 50 nM PcTx1 , while no inhibition was observed on ASIC1b currents under similar conditions. We determined a complete curve for steady-state desensitization in the presence of PcTx1 only for chimera C166, which retained the smallest part of ASIC1a . As expected from the inhibition by PcTx1 of this chimera, 100 nM PcTx1 shifted the curve for steady-state desensitization of chimera C166 significantly ($P < 0.01$) by 0.27 pH units, from $\text{pH } 6.90 \pm 0.01$ ($n = 5$) in the absence of PcTx1 to $\text{pH } 7.17 \pm 0.01$ ($n = 5$) in the presence of 100 nM PcTx1 (Fig. 8 A). This robust shift of the steady-state desensitization curve is in strong contrast to the small shift of the steady-state desensitization curve of ASIC1b wild type (Fig. 1) and suggests that the 19 amino acids of ASIC1a that are retained in C166 are sufficient to confer a high PcTx1 affinity to the

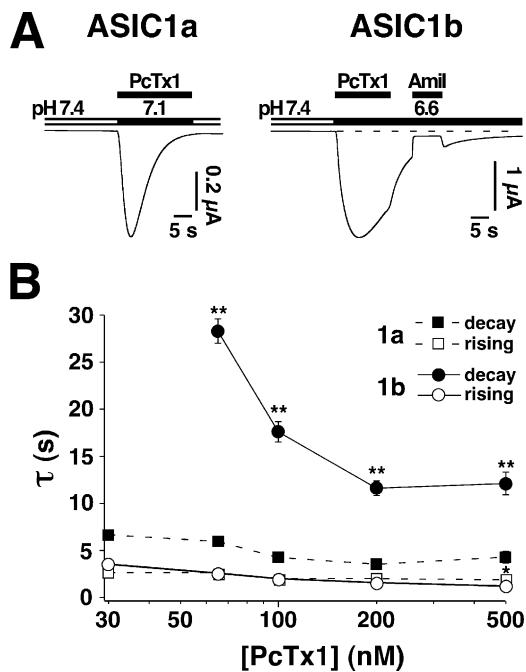


Figure 6. PcTx1 opens ASIC1b more persistently than ASIC1a. (A) When coapplied with pH 7.1, 100 nM PcTx1 induced relatively transient ASIC1a currents (left, $n = 7$). ASIC1b currents that were induced under comparable conditions were of greater relative amplitude and decayed more slowly. They could be blocked by 100 μ M amiloride (right, $n = 6$). (B) Diagram showing the dependence on the PcTx1 concentration of the time constant describing the rising and the decay phase of the PcTx1-elicited currents, determined in the continuous presence of PcTx1 ($n = 4-11$ for ASIC1a; $n = 4-12$ for ASIC1b). The time constants of the rising phase were similar for ASIC1a and ASIC1b currents, while for the decay phase, they were significantly different. For ASIC1b, the decay time constant initially decreased with increasing concentrations of PcTx1 but remained constant around 12 s for high concentrations of PcTx1. Part of the data for ASIC1a have already been published (Chen et al., 2005). **, $P < 0.01$.

desensitized state of the channel. Analyzing inhibition by different concentrations of PcTx1 at pH 7.1 indeed revealed that C166 had an apparent PcTx1 affinity similar to ASIC1a ($IC_{50} = 3.4$ nM, $n = 8-10$; Fig. 8 B). Similar to the steady-state desensitization curve, the activation curve of C166 was shifted by 0.32 pH units by PcTx1 (Fig. 8 A).

Chimeric Channels Reveal Partially Liganded Open Channels
 Interestingly, PcTx1 clearly also opened chimeras C62, C92, C98, and C166 at the conditioning pH in this set of experiments, in addition to inhibit currents evoked by the acidic pulse (Fig. 7). This result suggests an unusually high PcTx1 affinity for the open state of chimeras C62, C92, C98, and C166. We tested this for chimera C92, which was most strongly opened by PcTx1. The apparent affinity for PcTx1, determined by coapplying different PcTx1 concentrations with pH 7.05, was

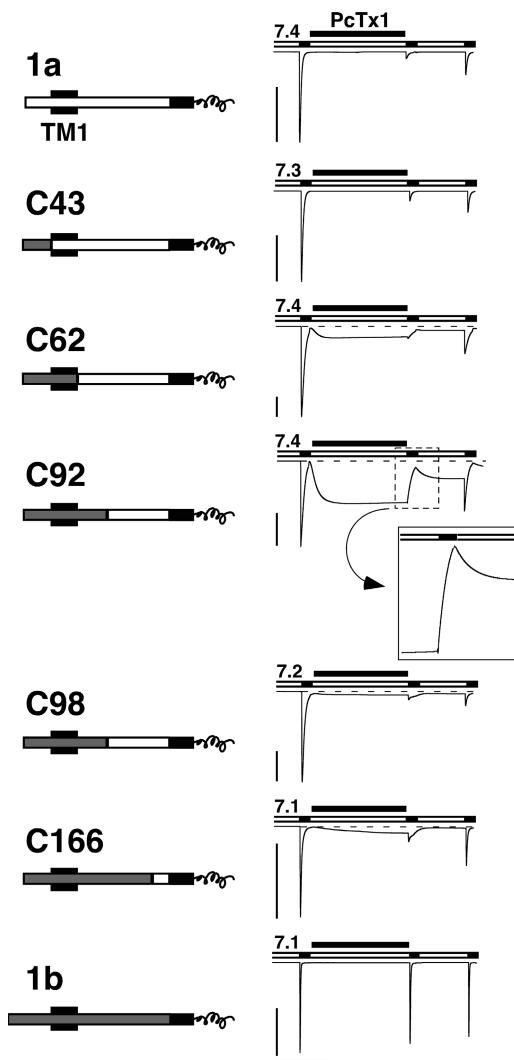


Figure 7. PcTx1 inhibits chimeras between ASIC1a and 1b. Left, chimeras are schematically drawn. NH₂-terminal sequences from ASIC1a are shown as open bars, those from ASIC1b as gray bars. The first transmembrane domain TM1 is indicated as a black box and the common COOH terminus is shown as a black bar; only its first part is shown. The chimeras did have a shorter NH₂ terminus than ASIC1b; this shorter NH₂ terminus corresponds to M3 in Bässler et al. (2001). However, since the missing part of the NH₂ terminus of ASIC1b is supposed to be located in the cytoplasm and since chimera C43 interacted with PcTx1 like ASIC1a, this NH₂-terminal part does not seem to have any strong influence on the interaction with PcTx1. Right, PcTx1 strongly inhibited the currents of ASIC1a and all chimeras, but not of ASIC1b. Conditioning pH was chosen according to the pH₅₀ of steady-state desensitization of each channel. Acidic test pH was always pH 5.0. PcTx1 (50 nM) was applied in the conditioning period for 120 s. Note the opening of chimeras C61, C92, C98, and C166 by application of PcTx1. The transient decrease in current amplitude with C92, after washout of PcTx1 and application of pH 5.0, is enlarged. Scale bars correspond to 5 μ A and 60 s, respectively. Peak current amplitudes (mean \pm SD) were -11.6 ± 6.1 μ A for ASIC1a ($n = 7$), -35.9 ± 18.2 μ A for C43 ($n = 6$), -8.4 ± 9.9 μ A for C61 ($n = 6$), -12.3 ± 4.5 μ A for C91 ($n = 5$), -12.0 ± 4.0 μ A for C98 ($n = 3$), -11.4 ± 3.1 μ A for C166 ($n = 5$), and -11.1 ± 6.0 μ A for ASIC1b ($n = 9$).

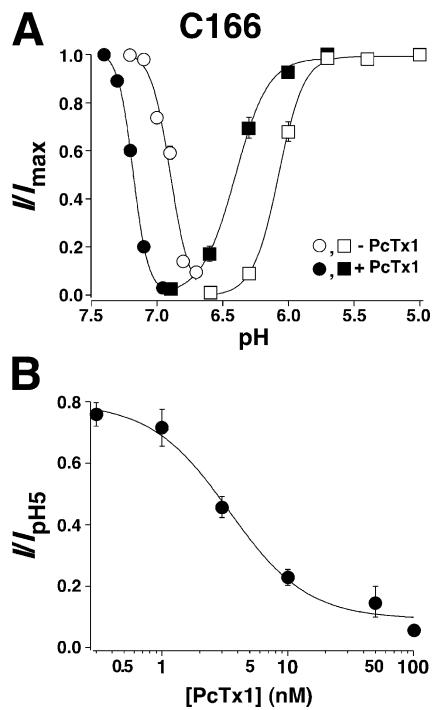


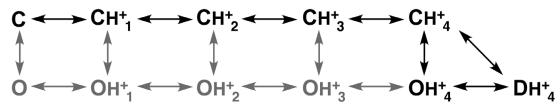
Figure 8. (A) Pctx1 robustly shifted the steady-state desensitization and activation curves of C166. pH_{50} of activation was $pH 6.07 \pm 0.05$ ($n = 7$) in the absence of Pctx1 (open squares) and $pH 6.40 \pm 0.06$ ($n = 5$) in the presence of 100 nM Pctx1 (closed squares). pH_{50} of steady-state desensitization was $pH 6.90 \pm 0.01$ ($n = 5$) in the absence of Pctx1 (open circles) and $pH 7.17 \pm 0.01$ ($n = 5$) in the presence of 100 nM Pctx1 (closed circles). Solid lines are fits of the mean values of each data point to the Hill function (Eq. 1). (B) Concentration-response relationship for inhibition of C166 currents by Pctx1. Different concentrations of the toxin were applied for 120 s during the conditioning period with pH 7.1. The line represents a fit to the Hill equation ($IC_{50} = 3.4$ nM; $n = 8-10$).

indeed about 10-fold higher ($EC_{50} = 11$ nM; Fig. 9 A) than for ASIC1a or ASIC1b. As can be seen from Fig. 7, about 20 s after Pctx1 application, channels in the open state were in equilibrium with channels in the desensitized state, with up to 50% of the channels in the open state. Most likely, Pctx1 transferred part of the channels to the open and part of them to the desensitized state. Application of pH 5.0 immediately after Pctx1 did not open further channels, indicating that indeed no significant fraction of channels remained in the closed state. As mentioned before, such stabilization by Pctx1 of the open state with respect to the desensitized

state would predict a slowing of the desensitization by Pctx1. Indeed, C92 currents elicited by coapplication of pH 7.05 with 100 nM Pctx1 decayed slowly with a time constant of 29.9 ± 8.4 s ($n = 6$; Fig. 9 B).

Interestingly, as illustrated in the inset of Fig. 7, pH 5.0 quickly reduced the current amplitude and after returning to pH 7.4, current amplitude increased again. Such an unusual rebound of the current amplitude can also be seen in Fig. 9 B after returning to pH 7.4. This decrease of the current amplitude by low pH cannot be explained by the reaction Scheme 1 (see INTRODUCTION) and the fact that more than one H^+ is needed for full opening of ASICS (Immke and McCleskey, 2003) has to be taken into account. Extending Scheme 1 to accommodate multiple H^+ binding steps leads to the following scheme:

(SCHEME 2)



According to Scheme 2, which is similar to a Monod-Wyman-Changeux model for cooperativity in allosteric proteins (Monod et al., 1965), there are five closed and open states, with different amounts of the ligand, H^+ , bound. From the fully liganded states, channels could reach also the desensitized state. Binding of H^+ would be highly cooperative, and transition from partially liganded closed states to open and desensitized states would be very infrequent. Thus, usually, channels would open almost exclusively from the fully liganded closed state (black lines). Under these conditions, the more complex Scheme 2 can be condensed to the more simple Scheme 1 (see INTRODUCTION). Our finding that Pctx1 has an unusually high affinity to the open state of chimera C92 predicts that for this chimera, a significant fraction of channels opens also from partially liganded closed states. The decrease of the current amplitude by application of pH 5.0 to open C92 channel is then readily explained by the binding of additional H^+ to these partially liganded open channels. From the fully liganded open state, these channels would then quickly desensitize. After washout of pH 5.0, with some Pctx1 still present, channels would again equilibrate between open states and the desensitized state, leading to the rebound of current. Thus, the effect of Pctx1 on chimera

TABLE I
pH₅₀ of Steady-state Desensitization and Activation

	ASIC1a	C43	C62	C92	C98	C166	ASIC1b
pH₅₀ (SSD)	7.19 ± 0.01 (8)	7.26 ± 0.01 (6)	7.23 ± 0.09 (8)	7.17 ± 0.13 (8)	7.02 ± 0.02 (9)	6.90 ± 0.01 (5)	6.95 ± 0.03 (6)
pH₅₀ (Act)	6.56 ± 0.04 (7)	6.00 ± 0.11 (6)	6.78 ± 0.06 (9)	6.77 ± 0.08 (6)	6.32 ± 0.07 (10)	6.07 ± 0.05 (7)	5.70 ± 0.04 (7)

Data are mean \pm SD for the number n of individual oocytes indicated in brackets. Data for ASIC1a are from Chen et al. (2005) and data for C62, C92, and C98 are from Babini et al. (2002). SSD, steady-state desensitization; Act, activation.

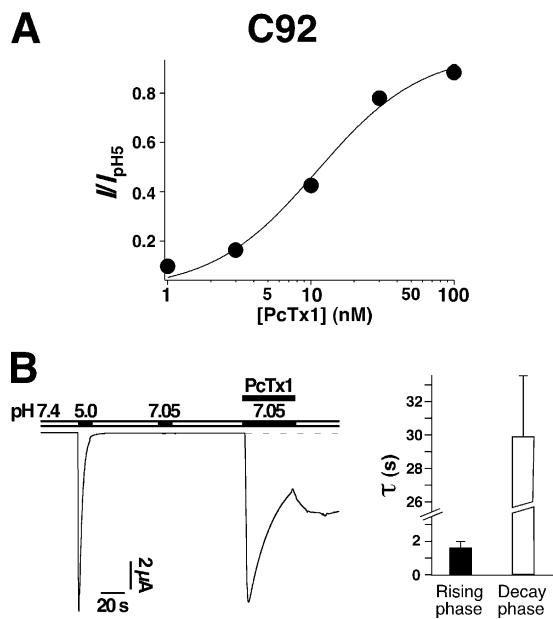


Figure 9. (A) Concentration–response relationship for activation of chimera C92 by Pctx1. Different concentrations of the toxin were coapplied with pH 7.05 for 40 s ($n = 5$ –6). The line represents a fit to the Hill equation ($EC_{50} = 11$ nM). (B) Pctx1 robustly opened chimera C92. Left, 100 nM Pctx1 was coapplied with pH 7.05 ($n = 6$). pH 7.05 alone did not open C92. Note the rebound of the current amplitude after returning to pH 7.4. Right, bars representing time constants of the rising and of the decay phase of the current.

C92 reveals the existence of partially liganded open states, supporting an allosteric reaction scheme, where ligand binding and channel opening are coupled but distinct. Although we cannot exclude more complicated reaction schemes that take into account desensitization from partially liganded open or closed states, Scheme 2 most easily explains our data.

DISCUSSION

Our data show that Pctx1 does not interact specifically with ASIC1a but that it also interacts with ASIC1b. However, ASIC1b is not strongly inhibited by Pctx1 but rather its opening is promoted. Therefore, interaction with ASIC1b shows that facilitated binding of H^+ , an increase in the “true” affinity for H^+ , cannot account for the effect of Pctx1 on ASIC1. Otherwise, according to Scheme 1, one would expect a similar shift of the steady-state desensitization curve and the activation curve. This indicates that Pctx1 binds with different affinities to different states of the channel; Pctx1 binding is state dependent. Although our results do not allow us to directly compare the affinity of Pctx1 for different states of the channels, they suggest that, in the case of ASIC1b, the toxin binds with highest affinity to the open state, and in the case of ASIC1a,

it binds more tightly to the desensitized and open states. The rationale behind this conclusion is as follows. First, the apparent affinity of Pctx1 for the open state is comparable for ASIC1a and ASIC1b; second, the apparent affinity for the desensitized state is much higher for ASIC1a than for ASIC1b; third, desensitization of ASIC1a was not much affected by Pctx1, suggesting that the equilibrium between these two states was not strongly affected; and fourth, desensitization of ASIC1b was slowed down, suggesting that Pctx1 stabilized the open state of ASIC1b relative to the desensitized state. The last interpretation is supported by the strong shift of the equilibrium between open and desensitized states for chimera C92 (Fig. 6), which was characterized by the highest apparent affinity of Pctx1 for the open state.

State dependence of binding of a modifier of channel gating is not surprising because usually channels undergo quite extensive conformational changes upon gating. To turn this argument around, state dependence of Pctx1 binding indicates that ASICs indeed undergo conformational changes upon gating, a view that is not easily compatible with more simple models of ASIC gating (Immke and McCleskey, 2003).

Similar to the inhibition of ASIC1 by Pctx1, tight binding to the inactivated state of voltage-gated Na^+ channels is responsible for the inhibition of these channels by local anesthetics, according to the modulated receptor model (Hille, 1977). Block by local anesthetics is relieved by hyperpolarization (Hille, 1977), much like inhibition by Pctx1 is relieved by increasing the pH. Although local anesthetics bind within and occlude the ion pore, kinetic modeling has shown that their effect is best explained assuming that they also affect channel gating allosterically (Balser et al., 1996). Similarly, inhibition of voltage-gated Cl^- channels by 2-(*p*-chlorophenoxy)butyric acid (CPB), is state dependent (Pusch et al., 2001). CPB binds more tightly to the closed state of the channel and inhibition vanishes at depolarized potentials (Pusch et al., 2001). Inhibition of voltage-gated Ca^{2+} channels by dihydropyridines (DHPs) also has many parallels to the inhibition of ASICs by Pctx1. Nitrendipine, for example, binds more tightly to the inactive state than to the closed state of the channel (Bean, 1984). Moreover, the inhibition by DHPs is antagonized by extracellular Ca^{2+} (Kass and Krafte, 1987), much as the inhibition by Pctx1 is antagonized by extracellular Ca^{2+} (Chen et al., 2005). Strikingly, some DHPs (e.g., BAY K 8644) act as agonists rather than antagonists of Ca^{2+} channels (Hess et al., 1984). Similarly, Pctx1 acts on ASIC1b also like an agonist rather than an antagonist. DHPs potentiate the Ca^{2+} current and shift the activation curve to more negative potentials (Sanguinetti et al., 1986), much like Pctx1 shifts the ASIC1 activation curve to higher pH values. Again, kinetic modeling has shown

that DHP agonists affect channel gating allosterically (Marks and Jones, 1992). Moreover, it has been proposed that DHP agonists allow open states when not all the voltage sensors have moved upon depolarization (Marks and Jones, 1992), similar to our interpretation that, for some chimeras, P_cTx1 allows open states when not all subunits have ligand bound. Given the high similarity to the interaction of local anesthetics with Na^+ channels and of DHPs with Ca^{2+} channels, we think that P_cTx1 most likely is an allosteric modifier of ASIC gating that binds with different affinities to different states of the channel. In this context, it is worthwhile to remember that P_cTx1 is structurally related to other spider toxins (Escoubas et al., 2003) that act as gating modifiers of voltage-gated channels (Swartz and MacKinnon, 1997).

The rates of onset of P_cTx1 inhibition and of recovery from P_cTx1 inhibition are rather large (Chen et al., 2005). We have previously explained this slow process by a membrane-access mechanism for P_cTx1 (Chen et al., 2005). Therefore, even after washout of P_cTx1, P_cTx1 is probably still present in rather high concentrations within the membrane. We can therefore not exclude that the on- and off-reactions of P_cTx1 occur preferentially from the open or, in the case of ASIC1a, desensitized state of the channel, even though P_cTx1 was applied to the closed state of the channel.

We used a series of chimeras between ASIC1a and ASIC1b to identify regions of the two splice variants that determine their differential behavior to P_cTx1. We identified a small region of only 19 amino acids from ASIC1a (amino acids 167–185) that determines a high affinity of P_cTx1 for the desensitized state of ASIC1. This result is in agreement with a very recent study identifying amino acids 157–185 of ASIC1a as conferring a high affinity for P_cTx1 (Salinas et al., 2006). In this study, affinity had been measured with a binding assay using a radiolabeled toxin. Binding was performed at pH 7.25 and, therefore, the affinity that was determined most likely corresponds to the affinity for the desensitized state. Although the authors of this study proposed that amino acids 157–185 constitute part of the P_cTx1 binding site (Salinas et al., 2006), it cannot be excluded that this region allosterically determines the affinity of the desensitized state for P_cTx1.

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