

# The Tarantula Toxin Psalmotoxin 1 Inhibits Acid-sensing Ion Channel (ASIC) 1a by Increasing Its Apparent H<sup>+</sup> Affinity

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Acid-sensing ion channels (ASICs) are ion channels activated by extracellular protons. They are involved in higher brain functions and perception of pain, taste, and mechanical stimuli. Homomeric ASIC1a is potently inhibited by the tarantula toxin psalmotoxin 1. The mechanism of this inhibition is unknown. Here we show that psalmotoxin 1 inhibits ASIC1a by a unique mechanism: the toxin increases the apparent affinity for H<sup>+</sup> of ASIC1a. Since ASIC1a is activated by H<sup>+</sup> concentrations that are only slightly larger than the resting H<sup>+</sup> concentration, this increase in H<sup>+</sup> affinity is sufficient to shift ASIC1a channels into the desensitized state. As activation of ASIC1a has recently been linked to neurodegeneration associated with stroke, our results suggest chronic desensitization of ASIC1a by a slight increase of its H<sup>+</sup> affinity as a possible way of therapeutic intervention in stroke.

## INTRODUCTION

H<sup>+</sup> is chemically the simplest transmitter. H<sup>+</sup> receptor channels have recently been characterized that open upon an increase in the extracellular concentration of H<sup>+</sup> and desensitize in the continuous presence of H<sup>+</sup> (Waldmann et al., 1997; Waldmann and Lazdunski, 1998). The apparent affinity of these ion channels for H<sup>+</sup>, ~0.3 μM, and their activation and desensitization kinetics is comparable with other ligand-gated channels (Waldmann and Lazdunski, 1998; Bässler et al., 2001), establishing H<sup>+</sup> as the genuine ligand for these channels, the acid-sensing ion channels (ASICs). However, in contrast to other ligands, resting concentrations of H<sup>+</sup> are close to the threshold concentration needed to activate ASICs. Consequently, slightly increased H<sup>+</sup> concentrations, for example during metabolic acidosis, chronically desensitize ASICs (Benson et al., 1999; Alvarez De La Rosa et al., 2002; Babini et al., 2002). This steady-state desensitization of ASICs, induced by ligand concentrations above threshold, is analogous to the steady-state inactivation of voltage-gated channels by continuous depolarization above the threshold potential.

Venomous animals, such as spiders, scorpions, and sea anemones, contain a rich diversity of protein toxins that interact with different classes of ion channels. The main group of toxins that interact with ligand-gated ion channels are the conotoxins produced by cone snails (Terlau and Olivera, 2004). They inhibit channels gated by acetylcholine (McIntosh et al., 1999), by serotonin

(England et al., 1998), and by glutamate (Hammerland et al., 1992), either by competitive or noncompetitive antagonism with the ligand. Toxins that interact with voltage-gated ion channels can be divided in two groups based on the mechanism of interaction. One group produces inhibition by physically occluding the ion pore (Catterall, 1980; Hille, 2001). The other group comprises gating modifiers that interact with the voltage sensor, shifting the voltage dependence of the channels. Some of them (mainly spider toxins) shift the voltage dependence to more positive potentials, inhibiting the channels (McDonough et al., 1997a,b; Swartz and MacKinnon, 1997a; Chuang et al., 1998), whereas others (β-toxins of scorpions) shift the voltage dependence to more negative potentials, leading to increased channel opening at resting membrane potentials (Cestele et al., 1998; Hille, 2001). Still other toxins (α-toxins of scorpions) cause a slowing of inactivation of Na<sup>+</sup> channels (Catterall, 1979; Wang and Strichartz, 1985).

Recently, a novel protein toxin from a tarantula, psalmotoxin 1 (PcTx1), has been isolated that inhibits H<sup>+</sup>-gated ASIC1a (Escoubas et al., 2000) by an unknown mechanism. Interestingly, PcTx1 is structurally unrelated to conotoxins but related to gating modifier toxins of voltage-gated ion channels (Escoubas et al., 2003). Here we show that PcTx1 has a unique mechanism of inhibition: it increases the apparent affinity for H<sup>+</sup> of ASIC1a, leading to chronic desensitization at the resting pH of 7.4.

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

## MATERIALS AND METHODS

### Electrophysiology

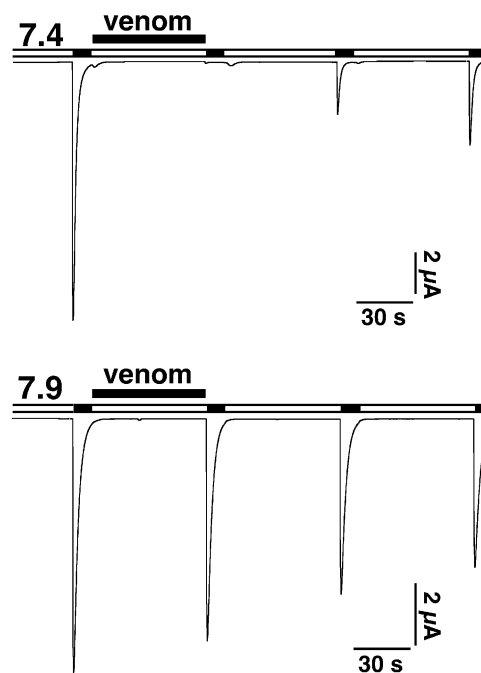
The inhibition of ASIC1a by PcTx1 was investigated by expressing homomeric ASIC1a in *Xenopus laevis* oocytes. Capped ASIC1a cDNA was synthesized by SP6 RNA polymerase from linearized cDNA, using the mMessage mMachine kit (Ambion). Stage V–VI *Xenopus* oocytes were injected with 0.01 ng cRNA and kept in OR-2 medium (concentrations in mM: 82.5 NaCl, 2.5 KCl, 1.0 Na<sub>2</sub>HPO<sub>4</sub>, 5.0 HEPES, 1.0 MgCl<sub>2</sub>, 1.0 CaCl<sub>2</sub>, and 0.5 g/l PVP; pH 7.3) for 2–4 d. Whole cell currents were recorded at 0.1 or 1 kHz and filtered at 20 Hz 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 (npi electronic). Data acquisition and solution exchange were managed using the software CellWorks 5.1.1 (npi electronic). Bath solution contained (in mM) 140 NaCl, 1.8 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 10 HEPES. For the acidic test solutions, HEPES was replaced by MES buffer. Conditioning solution with pH between 6.45 and 7.0 was buffered with 5 mM HEPES/5 mM MES. Solutions containing 0.1 mM Ca<sup>2+</sup> were supplemented with 0.1 mM flufenamic acid to block the large conductance induced in *Xenopus* oocytes by divalent-free extracellular solutions. Holding potential was  $-70$  mV if not otherwise indicated.

Dose–response curves were fit to the Hill equation  $I = a + (I_{\max} - a)/(1 + (EC_{50}/[L])^n)$ , where  $I_{\max}$  is the maximal current,  $a$  is the residual current,  $[L]$  is the concentration of the ligand (PcTx1 or H<sup>+</sup>),  $EC_{50}$  is the concentration at which half-maximal response occurs, and  $n$  is the Hill coefficient. Before fitting, currents from each measurement were normalized to the maximal value measured. Results are reported either, in the text, as mean  $\pm$  SD or, for the figures and amplitude histograms, as mean  $\pm$  SEM. They represent the mean of  $n$  individual measurements on different oocytes. Statistical analysis was done with the unpaired *t*-test. *Psalmopoeus cambridgei* venom was from SpiderPharm.

### Toxin Synthesis and Refolding

The synthesis of native psalmotoxin 1 was performed using the Fmoc/But and maximal temporary protection strategy on a Syro-MultiSyntec peptide synthesizer. The chemical procedure used 0.05 mmol of Fmoc-Thr(OtBu)-2-chlorotrityl resin (0.50 mmol/g), an eightfold excess of each amino acid and 2-(1H-benzotriazole-1-yl)1,1,3,3-tetramethyluronium tetrafluoroborate/1-hydroxybenzotriazole (TBTU/HOBt) activation. Deprotection (2 h) and cleavage (100 mg peptide + resin) were achieved using 5 ml of a mixture of trifluoroacetic acid/thioanisole/ethanedithiole (90/8/2, vol/vol/vol). The acidic mixture was then precipitated three times with diethylether, dissolved in 10% aqueous acetic acid and freeze dried. The crude toxin was purified by RP-HPLC on a C<sub>18</sub> semi-preparative column (10  $\times$  150 mm; Nucleosil) using a 40-min gradient of acetonitrile in 0.055% trifluoroacetic acid (10–80% B in 40 min, where B is 80% acetonitrile/H<sub>2</sub>O/0.05% trifluoroacetic acid).

Oxidation of the reduced toxin was achieved by dissolving the purified peptide with 2 M acetic acid, and diluted to a peptide concentration of 0.015 mM in the presence of reduced/oxidized glutathione (molar ratio of peptide/GSH/GSSG was 1:100:10) and 2 M guanidine hydrochloride. The solution was adjusted to pH 8.0 with aqueous NH<sub>4</sub>OH and stirred slowly at 4°C for 3 d. The folding reaction was monitored by analytical HPLC. The solution was concentrated using a C<sub>18</sub> SepPak (Waters) cartridge and finally lyophilized. Purification of the oxidized product was achieved first by chromatography on a C<sub>8</sub> column using the system above and yielding a purity of  $\sim$ 90%. Finally the product was highly purified on a C<sub>18</sub> column using a 60-min gradient, resulting in a purity of  $\geq$ 95%. The quality of the product was con-



**Figure 1.** The inhibition by PcTx1 is pH dependent. ASIC1a currents were repeatedly activated by pH 5.0. They were completely inhibited by application of the *P. cambridgei* venom (1:20,000 dilution) for 60 s at pH 7.4. In contrast, the venom did not inhibit the current, when it was applied at pH 7.9. Only a linear current rundown can be observed that is usual for ASIC1a and does not depend on the presence of the venom.

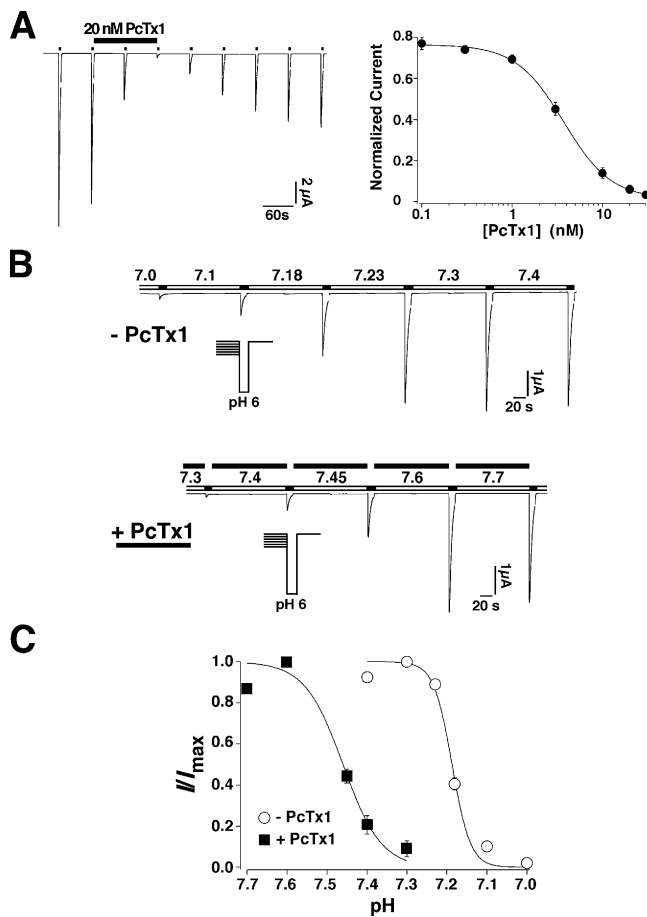
firmed by analytical HPLC, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-MS), and electrospray ionization mass spectrometry (ESI-MS), giving the correct mass of oxidized product.

The affinity of synthetic PcTx1 to ASIC1a was determined as inhibition of ASIC1a current by application of PcTx1 for 125 s in the conditioning period at pH 7.4. Under these conditions,  $K_d$  was 3.7 nM and the Hill coefficient of 1.65, which is in good agreement with previous published data (Escoubas et al., 2000), demonstrating the purity of our preparation. Complete block was achieved with a concentration of 30 nM PcTx1; this concentration was, therefore, used for most of the experiments described in this study. It should be emphasized, however, that the apparent affinity of ASIC1a to PcTx1, as determined above, is pH dependent. Solutions containing PcTx1 were supplemented with 0.05% BSA to avoid absorption by the tubing.

## RESULTS

### Inhibition By PcTx1 Is Sensitive to the Conditioning pH

Fig. 1 shows that the inhibition of ASIC1a by the PcTx1-containing venom of the tarantula *P. cambridgei* was sensitive to the conditioning pH. When a 1:20,000 dilution of the venom was applied to *Xenopus* oocytes expressing homomeric ASIC1a during the conditioning period at pH 7.4, ASIC1a currents were completely blocked, whereas when the same dilution of the venom was applied at pH 7.9, no inhibition could be observed. To avoid unspecific effects by other compounds of the



**Figure 2.** PcTx1 caused a robust shift of the steady-state desensitization curve of ASIC1a to lower  $H^+$  concentrations. (A) Apparent affinity of synthetic PcTx1 to ASIC1a. Left, an example of ASIC1a currents inhibited by 20 nM PcTx1 (applied for 125 s in the conditioning period). Currents were elicited by a pH drop from pH 7.4 to pH 6. Right, dose–response relationship for inhibition of ASIC1a currents by PcTx1. The first test current after PcTx1 application was normalized to the test current just before the toxin application. The line represents a fit to the Hill equation ( $IC_{50} = 3.7$  nM, Hill coefficient = 1.65). Each data point represents mean  $\pm$  SEM ( $n = 5-7$ ). (B) Representative traces of ASIC1a currents evoked by application of pH 6.0 with varying conditioning pH as indicated. Top, without PcTx1, bottom, with PcTx1 (30 nM). Conditioning pH was applied for 120 s. Holding potential was  $-70$  mV. (C)  $H^+$  dependence of steady-state desensitization of ASIC1a currents in the absence (open circles,  $n = 8$ ) or presence (filled squares,  $n = 5$ ) of 30 nM PcTx1.

venom and to apply known concentrations of the toxin, we continued our detailed analysis of the mechanism of inhibition with a pure preparation of the synthetic toxin (see MATERIALS AND METHODS).

#### PcTx1 Inhibits ASIC1a Current By Causing a Shift of the Steady-state Desensitization Curve to Lower $H^+$ Concentrations

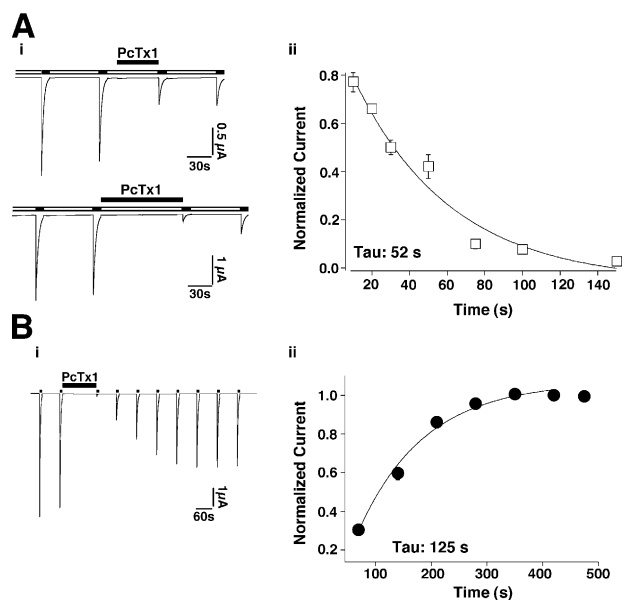
Since the conditioning pH was crucial for the inhibition of ASIC1a currents by PcTx1 (Fig. 1), we asked

whether the toxin affected the steady-state desensitization of ASIC1a. To address this question, we determined the pH dependence of steady-state desensitization for ASIC1a in the absence and in the presence of PcTx1 (30 nM,  $K_d = 3.7$  nM; Fig. 2 A) during the conditioning period. Similar to a previous report (Babini et al., 2002), in the absence of PcTx1, ASIC1a showed half-maximal desensitization at a pH ( $pH_{50}$ ) of  $7.19 \pm 0.01$  ( $n = 8$ ). In strong contrast, in the presence of PcTx1,  $pH_{50}$  of desensitization was  $7.46 \pm 0.02$  (Fig. 2,  $n = 5$ ). Thus, PcTx1 caused a significant ( $P < 0.01$ ) shift of the steady-state desensitization curve of ASIC1a by 0.27 pH units to lower  $H^+$  concentrations. Since steady-state desensitization curves of ASIC1a are very steep (transition from almost 100% to almost 0% available channels in just 0.2 pH units, Fig. 2 C) the PcTx1-induced shift is sufficient to transfer almost all the channels into the desensitized state at pH 7.4, rendering them unavailable for activation. However, at a conditioning pH of 7.9, the PcTx1-induced shift would not be sufficient for desensitization of ASIC1a channels. Thus, the observed shift of the steady-state desensitization curve by PcTx1 can fully account for the inhibition of ASIC1a by PcTx1. These results demonstrate that PcTx1 inhibits ASIC1a currents by drastically shifting the steady-state desensitization curve.

#### The Kinetics of PcTx1 Inhibition Is Slow

We determined the rate of onset of PcTx1 inhibition by applying 30 nM PcTx1 for a varying period of time at pH 7.4 and assessing inhibition of ASIC1a channels by activation with pH 6.0. As shown in Fig. 3, complete inhibition by PcTx1 was achieved only after application for  $\sim 150$  s. The time course of the inhibition could be well described by a single exponential function with a time constant of 52 s. Since PcTx1 induces steady-state desensitization and steady-state desensitization of ASIC1a needs several tens of seconds for completion (Babini et al., 2002), the slow onset of inhibition by PcTx1 is at least in part due to the slow onset of steady-state desensitization and does not directly reflect slow binding of PcTx1. In agreement with this interpretation, application of PcTx1 can, under certain conditions, directly open ASIC1a channels with a time course on the order of 2 s (see Fig. 8).

We determined the rate of recovery from PcTx1 inhibition by applying 30 nM PcTx1 for 150 s to achieve complete inhibition. After washout of PcTx1, available ASIC1a channels were assessed every 70 s by application of pH 6.0. As illustrated in Fig. 3, ASIC1a slowly recovered from the PcTx1 inhibition and reached maximal current amplitude only  $\sim 350$  s after washout of PcTx1. Fit to a mono-exponential function revealed a time constant of 125 s ( $K_{off} = 8.0 \times 10^{-3} s^{-1}$ ). Since recovery of ASIC1a from desensitization is complete

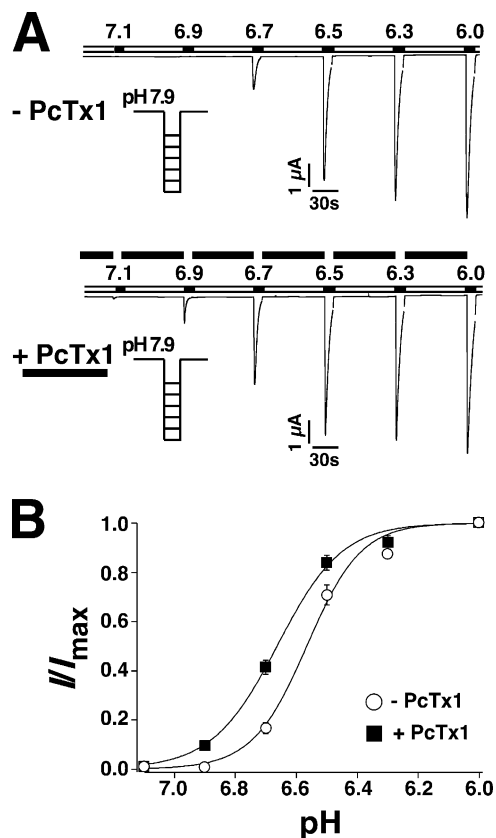


**Figure 3.** Kinetics of inhibition by PcTx1. (A, i) ASIC1a current traces illustrating inhibition by PcTx1. PcTx1 (30 nM) was applied for 50 and 100 s, respectively. (A, ii) Current peak amplitudes (normalized to the current amplitude before applying the toxin) are plotted against the corresponding time period of PcTx1 application. Each data point represents the mean  $\pm$  SEM of 5–7 individual measurements. The time constant for onset of inhibition, obtained by fitting the mean data to a single exponential function, was 52 s. (B, i) Representative current traces of ASIC1a channels recovering from inhibition by PcTx1 (30 nM, applied for 150 s). ASIC1a channels that had recovered from inhibition were assessed every 70 s by application of pH 6.0. The conditioning period between channel activation was 60 s, allowing ASIC1a to fully recover from low pH-induced desensitization. (B, ii) Current peak amplitudes (normalized to the maximal current amplitude after toxin washout) are plotted against the corresponding time after washout. Each data point represents the mean  $\pm$  SEM of 10 individual measurements. The time constant for recovery from inhibition was  $125 \pm 56$  s.

within a few seconds (Babini et al., 2002), slow recovery from PcTx1 inhibition cannot be explained by slow recovery from desensitization, suggesting slow unbinding of PcTx1.

#### PcTx1 Increases the Apparent Affinity for H<sup>+</sup> of ASIC1a

The shift of the steady-state desensitization curve to lower H<sup>+</sup> concentrations can be explained by an increased apparent affinity for H<sup>+</sup>. To further address the H<sup>+</sup> affinity of ASIC1a in the presence of PcTx1, we examined the pH dependence of ASIC1a activation in the absence and in the presence of PcTx1. We used a conditioning pH of 7.9 in order to avoid inhibition by PcTx1 (Fig. 2) and varied the pH to activate ASIC1a channels. PcTx1 was applied only during the conditioning period. Similar to previous results (Babini et al., 2002), in the absence of PcTx1, pH<sub>50</sub> of activation was  $6.56 \pm 0.04$  ( $n = 7$ ). In contrast, in the presence of



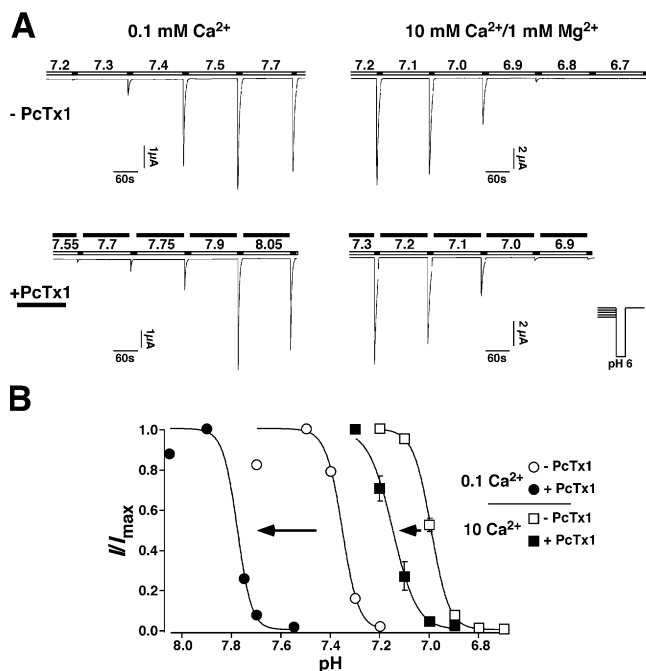
**Figure 4.** PcTx1 increased the apparent affinity for H<sup>+</sup> of ASIC1a. (A) Representative current traces elicited by pH ranging from 7.1 to 6.0 with conditioning pH 7.9 (applied for 70 s). Top, without PcTx1, bottom, with PcTx1 (30 nM). (B) H<sup>+</sup> dependence of ASIC1a activation in the absence ( $n = 7$ , open circles) or in the presence ( $n = 7$ , filled squares) of 30 nM PcTx1. PcTx1 significantly ( $P < 0.01$ ) shifted the pH activation curve to lower H<sup>+</sup> concentrations.

PcTx1 (30 nM), pH<sub>50</sub> of activation was  $6.66 \pm 0.04$  (Fig. 4,  $n = 7$ ). Thus, PcTx1 also slightly, but significantly ( $P < 0.01$ ), shifted the activation curves to lower H<sup>+</sup> concentrations, confirming that PcTx1 increases the apparent H<sup>+</sup> affinity of ASIC1a. This shift of the activation curve by PcTx1 directly demonstrates that PcTx1 bound to ASIC1a at pH 7.9 and that channels with PcTx1 bound can open. In contrast to the apparent affinity to H<sup>+</sup>, the time constant of desensitization ( $\tau_{des}$ ) of ASIC1a currents was not significantly affected by PcTx1 (at pH 6.0,  $\tau_{des} = 4.0 \pm 0.7$  s,  $n = 7$ , without PcTx1, and  $\tau_{des} = 4.6 \pm 1.5$  s,  $n = 7$ , with PcTx1,  $P = 0.4$ ).

#### PcTx1 Competes with Ca<sup>2+</sup> in Binding To ASIC1a Channels

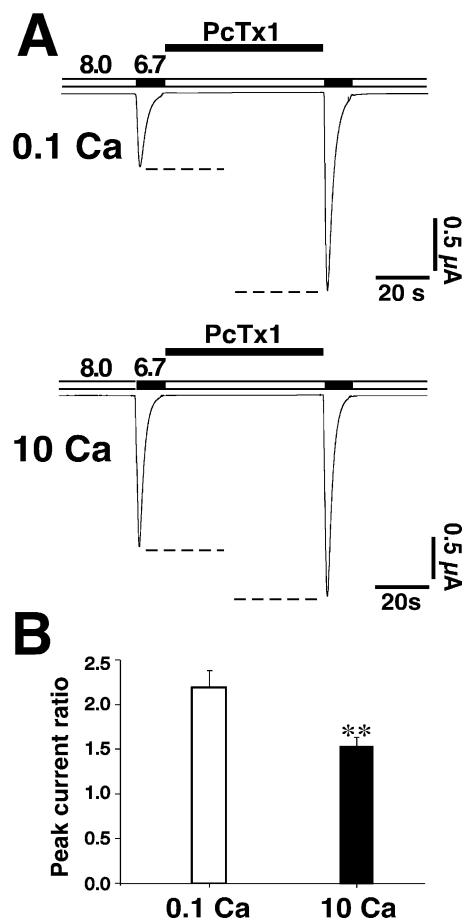
We previously reported that Ca<sup>2+</sup> stabilizes the closed state of ASIC1a and that it competes with H<sup>+</sup> in the binding to ASIC1a (Babini et al., 2002). An increased apparent affinity for H<sup>+</sup> can therefore be explained by a reduced binding of Ca<sup>2+</sup>. Therefore, we next ad-





**Figure 5.**  $\text{Ca}^{2+}$  dependence of the shift of the steady-state desensitization curve. (A) Representative traces of ASIC1a currents evoked by application of pH 6.0 with varying conditioning pH as indicated. Top, without PcTx1, bottom, with PcTx1 (30 nM). Conditioning solution contained 0.1 mM  $\text{Ca}^{2+}$  (left) or 10 mM  $\text{Ca}^{2+}$ /1.0 mM  $\text{Mg}^{2+}$  (right) and was applied for 120 s. Solution of pH 6.0 always contained 1.8 mM  $\text{Ca}^{2+}$ /1.0 mM  $\text{Mg}^{2+}$ . (B)  $\text{H}^+$  dependence of steady-state desensitization of ASIC1a currents in the absence (open circles, 0.1 mM  $\text{Ca}^{2+}$ ,  $n = 7$ ; open squares, 10 mM  $\text{Ca}^{2+}$ /1.0 mM  $\text{Mg}^{2+}$ ,  $n = 8$ ) or presence (filled circles, 0.1 mM  $\text{Ca}^{2+}$ ,  $n = 6$ ; filled squares, 10 mM  $\text{Ca}^{2+}$ /1.0 mM  $\text{Mg}^{2+}$ ,  $n = 5$ ) of 30 nM PcTx1. At low  $\text{Ca}^{2+}$  concentrations, PcTx1 caused a more robust shift (by 0.42 pH units) of the steady-state desensitization curve than at high  $\text{Ca}^{2+}$  concentrations (by 0.16 pH units). Note that  $\text{Ca}^{2+}$  itself also shifts the steady-state desensitization curves.

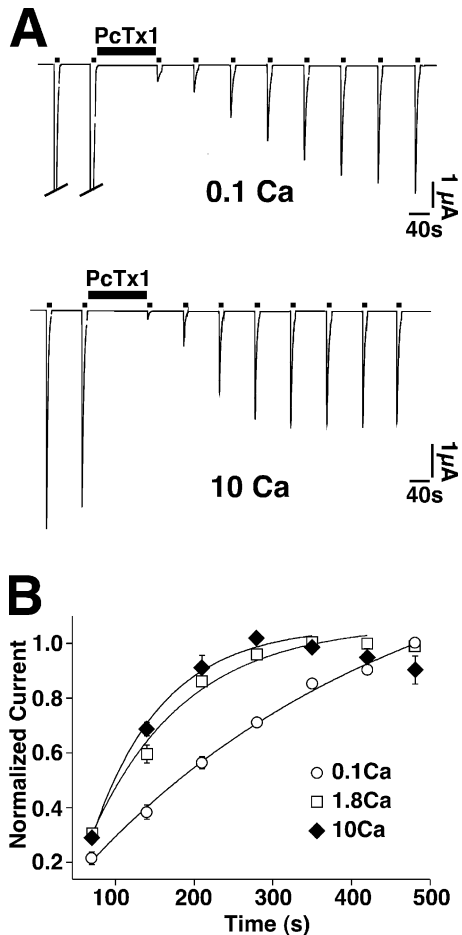
ressed the role of  $\text{Ca}^{2+}$  in the inhibition by PcTx1. First, we determined the steady-state desensitization curve in the presence and in the absence of PcTx1, with either a low concentration (0.1 mM) or a high concentration (10 mM) of  $\text{Ca}^{2+}$  in the conditioning solution. Fig. 5 shows that with 0.1 mM conditioning  $\text{Ca}^{2+}$ , PcTx1 shifted the steady-state desensitization curve by 0.42 pH units to lower  $\text{H}^+$  concentrations ( $\text{pH}_{50}$  was  $7.36 \pm 0.01$ ,  $n = 7$ , in the absence of PcTx1, and  $7.78 \pm 0.01$ ,  $n = 6$ , in the presence of PcTx1), while with 10 mM conditioning  $\text{Ca}^{2+}$ , PcTx1 shifted the steady-state desensitization curve by only 0.16 pH units ( $\text{pH}_{50}$  was  $6.99 \pm 0.02$ ,  $n = 8$ , in the absence of PcTx1, and  $7.15 \pm 0.04$ ,  $n = 6$ , in the presence of PcTx1). As shown above (Fig. 2), with a physiological  $\text{Ca}^{2+}$  concentration (1.8 mM), PcTx1 shifted the steady-state desensitization curve by 0.27 pH units, an intermediate value to 0.42 and 0.16. Thus, the degree of the PcTx1-induced shift of the steady-state desensitization curve decreased



**Figure 6.**  $\text{Ca}^{2+}$  inhibits the binding of PcTx1 to ASIC1a channels. (A) 30 nM PcTx1 was applied together with either 0.1 mM  $\text{Ca}^{2+}$  (top) or 10 mM  $\text{Ca}^{2+}$ /1.0 mM  $\text{Mg}^{2+}$  (bottom), in the conditioning solution (pH 8.0). Test solution always contained 1.8 mM  $\text{Ca}^{2+}$ /1.0 mM  $\text{Mg}^{2+}$ , pH 6.7. (B) The potentiation of the ASIC1a current, due to increased affinity to  $\text{H}^+$ , is expressed as the ratio of peak currents after and before application of PcTx1. White bar, 0.1  $\text{Ca}^{2+}$  ( $n = 6$ ); black bar, 10 mM  $\text{Ca}^{2+}$ /1.0 mM  $\text{Mg}^{2+}$  ( $n = 6$ ). High  $\text{Ca}^{2+}$  significantly ( $P < 0.01$ ) reduced the potentiation of ASIC1a currents by PcTx1.

with increasing  $\text{Ca}^{2+}$  concentrations, suggesting competition between  $\text{Ca}^{2+}$  and PcTx1.

We next addressed the question of whether  $\text{Ca}^{2+}$  and PcTx1 compete directly for binding to the channel. To do so, we used conditioning pH 8.0 and activated channels with pH 6.7. As can be deduced from Figs. 2 and 4, under these conditions, PcTx1 will not inhibit ASIC1a but rather potentiate ASIC1a currents, due to the increased affinity for  $\text{H}^+$ . We used this protocol with low and high concentrations of  $\text{Ca}^{2+}$  (0.1 and 10 mM) in the conditioning solution. At 0.1 mM  $\text{Ca}^{2+}$ , potentiation of the ASIC1a current amplitude was significantly ( $P < 0.01$ ) stronger than at 10 mM  $\text{Ca}^{2+}$  (Fig. 6; ratio of peak current amplitudes with and without PcTx1 was  $2.2 \pm 0.4$  with 0.1 mM  $\text{Ca}^{2+}$ ,  $n = 6$ , and  $1.5 \pm 0.2$  with 10 mM  $\text{Ca}^{2+}$ ,  $n = 6$ ). Since the off rate of  $\text{Ca}^{2+}$  is large,



**Figure 7.**  $\text{Ca}^{2+}$  speeds recovery from inhibition by PcTx1. (A) Representative traces of ASIC1a currents recovering from the inhibition by PcTx1 (30 nM). Protocol as in Fig. 3 B, but with 0.1 mM  $\text{Ca}^{2+}$  and pH 7.55 (top) or 10 mM  $\text{Ca}^{2+}$ /1.0 mM  $\text{Mg}^{2+}$  and pH 7.06 (bottom) during the conditioning period. The different pH values were chosen to account for the influence of  $\text{Ca}^{2+}$  on the speed of recovery from desensitization. (B) Current peak amplitudes (normalized to the maximal current amplitude) are plotted against the corresponding time after toxin washout. The time constants, obtained by fitting the data to a mono-exponential function, were  $441 \pm 141$  s (0.1 Ca, open circles,  $n = 7$ ),  $125 \pm 56$  s (1.8 Ca, open squares,  $n = 10$ ), and  $118 \pm 38$  s (10 Ca, filled diamonds,  $n = 6$ ). Data for 1.8  $\text{Ca}^{2+}$  are from Fig. 3.

the different concentrations of  $\text{Ca}^{2+}$  in the conditioning solution per se do not change the current amplitude (Babini et al., 2002). Since moreover during the activation by pH 6.7 the toxin was no longer applied, this result shows that during the conditioning period, more toxin bound to the channel at low  $\text{Ca}^{2+}$  concentrations than at high  $\text{Ca}^{2+}$  concentrations. Thus,  $\text{Ca}^{2+}$  competes with PcTx1 by preventing PcTx1 from binding to the channel.

$\text{Ca}^{2+}$  and PcTx1 could compete by binding to the same site or to two different sites on the channel. Binding to the same site would predict that the unbinding of PcTx1 should be independent from the  $\text{Ca}^{2+}$  con-

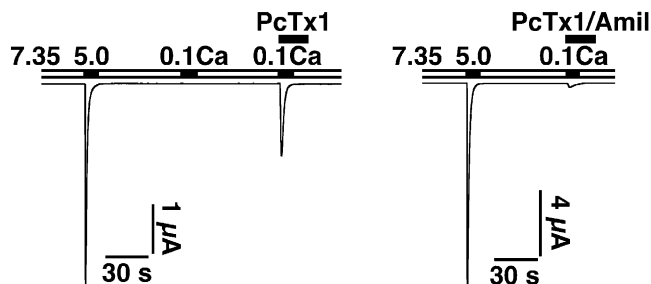
centration, whereas binding to two different sites would predict that the unbinding of PcTx1 should be facilitated at higher  $\text{Ca}^{2+}$  concentrations. To address this point, we monitored recovery from PcTx1 inhibition using conditioning solutions containing either 10 mM  $\text{Ca}^{2+}$  or 0.1 mM  $\text{Ca}^{2+}$ . Fig. 7 shows that the recovery from PcTx1 inhibition became faster with increasing  $\text{Ca}^{2+}$  concentrations (time constant  $\tau = 441 \pm 141$  s with 0.1 mM  $\text{Ca}^{2+}$ ,  $n = 7$ , and  $\tau = 118 \pm 38$  s with 10 mM  $\text{Ca}^{2+}$ ,  $n = 6$ ,  $P < 0.01$ ). This result is in agreement with the idea that  $\text{Ca}^{2+}$  promotes the unbinding of PcTx1 and that  $\text{Ca}^{2+}$  binds to a different site than does PcTx1. However, there are other possible explanations for this result and it does not prove allosteric binding.

### PcTx1 Directly Opens ASIC1a Channels

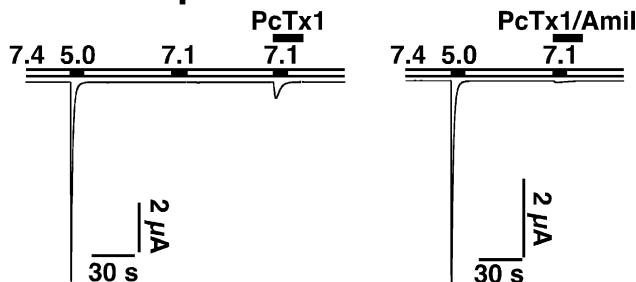
The basic features of ASIC gating can be described by a kinetic scheme where  $\text{H}^+$  binds to the closed state of the channel and closed channels with  $\text{H}^+$  bound can either desensitize or open.  $\text{H}^+$ -bound closed, open, and desensitized states would be cyclically connected (see DISCUSSION). Since PcTx1 shifts channels into the desensitized state, this kinetic scheme predicts that upon PcTx1 binding, some ASIC1a channels briefly open before desensitizing. At low  $\text{H}^+$  concentrations, the opening of ASIC1a channels by PcTx1 would not lead to a macroscopic current because the rate of opening of individual channels is much smaller than the rate of desensitization and the open state is never significantly populated. Therefore, to address if PcTx1 is indeed able to open ASIC1a channels, we choose conditions under which ASIC1a channels more readily open, such as increasing the conditioning  $\text{H}^+$  concentration or decreasing the  $\text{Ca}^{2+}$  concentration. These conditions should increase the rate of opening of ASIC1a channels. First, we choose a conditioning pH of 7.35 (Fig. 2 C), the lowest pH value where steady-state desensitization of ASIC1a is negligible. Switching from 1.8 mM  $\text{Ca}^{2+}$  to 0.1 mM  $\text{Ca}^{2+}$  did not open ASIC1a channels (Fig. 8). In contrast, simultaneously switching from 1.8 mM to 0.1 mM  $\text{Ca}^{2+}$  and applying PcTx1 (100 nM) induced a transient inward current, which was sensitive to 100  $\mu\text{M}$  amiloride (Fig. 8). Similarly, simultaneously lowering the pH from 7.4 to 7.1 and applying PcTx1 (100 nM) induced a transient amiloride-sensitive inward current, whereas just lowering the pH from 7.4 to 7.1 did not induce any current. These data allow us to watch ASIC1a channels that bound PcTx1 on their way from the closed to the desensitized state, illustrating that PcTx1 inhibits ASIC1a by increasing its apparent affinity to  $\text{H}^+$ .

The rising phase of the PcTx1-induced current could be fit to a single exponential function with a time constant of  $1.8 \pm 0.3$  s (0.1  $\text{Ca}^{2+}$ ,  $n = 5$ ) and  $2.8 \pm 0.8$  s (pH 7.1,  $n = 5$ ), demonstrating that under these condi-

## A Low Calcium



## B Low pH



**Figure 8.** PcTx1 directly opens ASIC1a at certain conditions. (A) At pH 7.35, 100 nM PcTx1 elicited transient inward currents when coapplied with low  $\text{Ca}^{2+}$  concentrations (0.1 mM, left,  $n = 5$ ). These PcTx1-elicited currents could be blocked by 100  $\mu\text{M}$  amiloride (right,  $n = 4$ ). (B) When coapplied with pH 7.1, 100 nM PcTx1 elicited transient inward currents (left,  $n = 5$ ), which could be blocked by 100  $\mu\text{M}$  amiloride (right,  $n = 3$ ).

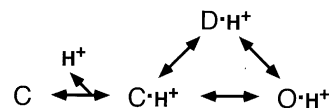
tions, binding of PcTx1 to ASIC1a happened on the order of seconds rather than tens of seconds as suggested by the slow onset of inhibition.

## DISCUSSION

In this study, we have shown that the spider toxin PcTx1 inhibits ASIC1a by increasing its apparent affinity for its natural ligand,  $\text{H}^+$ . Evidence for the increased apparent affinity is the shift of the steady-state desensitization curve and the  $\text{H}^+$  activation curve to lower  $\text{H}^+$  concentrations. A shift of the steady-state desensitization curve by 0.27 pH units corresponds to an approximately twofold increase in the apparent affinity for  $\text{H}^+$ . This relatively modest increase in affinity can account for inhibition only in conjunction with the extraordinarily steep steady-state desensitization curves and the  $\text{H}^+$  affinity of ASIC1a being very close to the resting concentration of  $\text{H}^+$ . Resting concentrations of  $\text{H}^+$  are 40–50 nM (pH 7.4–pH 7.3), and 65 nM of  $\text{H}^+$  (pH 7.2) will already desensitize 50% of ASIC1a channels at equilibrium (Fig. 2). Hence a considerable fraction of ASIC1a channels will be already desensitized under resting conditions and the twofold increase of the apparent  $\text{H}^+$  affinity will shift most channels into the de-

sensitized state and can fully account for block by PcTx1. This result also shows that ASIC1a is an extremely sensitive receptor for  $\text{H}^+$ , with an apparent affinity for its ligand as high as it can be without leading to significant channel desensitization. It should be emphasized that the PcTx1-induced shift of the steady-state desensitization curve is concentration dependent; that means that lower PcTx1 concentrations will induce a smaller shift and higher concentrations will induce a larger shift.

Different mechanisms could explain the apparent increase in  $\text{H}^+$  affinity of ASIC1a by PcTx1. Assuming the above-mentioned kinetic scheme:



PcTx1 could either modify the energetics of the  $\text{H}^+$  binding step, increasing the affinity of ASIC1a for  $\text{H}^+$  and thus shifting the equilibrium between the closed state without  $\text{H}^+$  (C) and the closed state with  $\text{H}^+$  bound ( $\text{C}\cdot\text{H}^+$ ) toward the bound state. Or it could modify the energetics of the gating step, shifting the equilibrium between the bound closed state ( $\text{C}\cdot\text{H}^+$ ) and the open state ( $\text{O}\cdot\text{H}^+$ ) toward the open state, analogous to the interaction proposed for the tarantula toxin hanatoxin and the voltage-dependent  $\text{K}^+$  channel Kv2.1 (Swartz and MacKinnon, 1997a). And finally, PcTx1 could have a higher affinity to the open and/or the desensitized state than to the closed state, which would be an example for the modulated receptor concept that has been proposed for the interaction of local anesthetics with voltage-gated  $\text{Na}^+$  channels (Hille, 1977) and of dihydropyridines with L-type  $\text{Ca}^{2+}$  channels (Bean, 1984). Our results do not allow us to unequivocally decide between these three possibilities.

On and off rates for PcTx1 were strikingly slow. We determined an off rate  $K_{\text{off}}$  of  $8.0 \times 10^{-3} \text{ s}^{-1}$ . For the same pH, we determined an apparent  $K_{\text{d}}$  of  $3.7 \times 10^{-9} \text{ M}$  (see MATERIALS AND METHODS). Assuming that  $K_{\text{d}} = K_{\text{off}}/K_{\text{on}}$ , one can calculate an apparent on rate  $K_{\text{on}}$  of  $2.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ , which is smaller than expected from a simply diffusion-limited process. Similarly slow on and off rates are characteristic for toxins that are structurally related to PcTx1 (Swartz and MacKinnon, 1995; Oswald et al., 2002). Recently, it has been proposed that these slow rates may be due to a membrane-access mechanism for the toxins (Lee and MacKinnon, 2004; Suchyna et al., 2004). According to this hypothesis, these toxins partition into the membrane, leading to their accumulation in high concentrations around the channel (Lee and MacKinnon, 2004; Suchyna et al., 2004). Thus, binding energy between toxin and channel need not be as high as would be expected for an interaction with nanomolar affinity, and slow inhibi-

tion kinetics could be due to a slow rate of membrane partitioning. The highly similar structure of PcTx1 to gating modifier toxins (Escoubas et al., 2003) suggests that PcTx1 also partitions into the membrane, providing a clue to the slow kinetics and implying that the toxin-binding site on ASIC1a is close to or within the membrane-spanning parts of the channel protein.

We also show that  $\text{Ca}^{2+}$  competes with PcTx1, probably by inhibiting binding of PcTx1 to the channel. Previously, we and others have shown that  $\text{Ca}^{2+}$  is crucial for the activation of ASICs (Babini et al., 2002; Immke and McCleskey, 2003; Paukert et al., 2004).  $\text{Ca}^{2+}$  competes with  $\text{H}^+$ , reducing the apparent affinity for  $\text{H}^+$  (Babini et al., 2002; Immke and McCleskey, 2003). Thus, at least part of the effect of PcTx1 on ASIC1a can be explained in terms of a competition with  $\text{Ca}^{2+}$ . It is likely that the  $\text{Ca}^{2+}$  binding site on the channel contains negatively charged side chains of amino acids and that protonation of these side chains leads to  $\text{Ca}^{2+}$  unbinding. Hence the  $\text{Ca}^{2+}$  binding site may be identical to the "H<sup>+</sup> receptor" of ASICs. Moreover, gating modifier toxins that cause a shift of the voltage dependence of voltage-gated channels interact directly with the voltage sensor (Swartz and MacKinnon, 1997b; Ruta and MacKinnon, 2004). Thus, it is attractive to speculate that PcTx1 interacts with the  $\text{Ca}^{2+}/\text{H}^+$  binding site of ASIC1a. In agreement with this hypothesis is the clustering of several positively charged amino acids in the putative interaction domain of PcTx1 (Escoubas et al., 2003) that are likely to interact with negatively charged amino acids on the channel protein. However, ASIC1a can open with PcTx1 bound, whereas  $\text{Ca}^{2+}$  probably has to unbind for channel opening (Immke and McCleskey, 2003; Paukert et al., 2004), arguing against an identical binding site for  $\text{Ca}^{2+}$  and PcTx1. Moreover and as pointed out previously, the  $\text{Ca}^{2+}$  dependence of the off rate of PcTx1 (Fig. 7) suggests that  $\text{Ca}^{2+}$  binds to a different site than PcTx1. Finally, it cannot be excluded that  $\text{Ca}^{2+}$  exerts its effects via screening of surface charges; the competition between PcTx1 and  $\text{Ca}^{2+}$  could then be much similar to the competition between dihydropyridines (the  $\text{Ca}^{2+}$  antagonists) and  $\text{Ca}^{2+}$  on heart  $\text{Ca}^{2+}$  channels (Kass and Krafte, 1987). Irrespective of the relation to the  $\text{Ca}^{2+}$  binding site, we anticipate that identification of the PcTx1 binding site will increase our understanding of activation of ASIC1a by  $\text{H}^+$ .

Within the ASIC family, increased apparent ligand affinity may be an inhibitory mechanism that applies to other toxin/channel interactions than only PcTx1/ASIC1a. Recently, a protein toxin from a sea anemone, APETx2, has been identified that inhibits ASIC3 (Diochot et al., 2004); APETx2 does not inhibit ASIC1a. The interaction of ASIC3 with APETx2 is characterized by inhibition during steady state (at pH 7.4), nanomo-

lar affinity, slow kinetics, and unchanged single channel conductance (Diochot et al., 2004). These characteristics suggest that APETx2 may inhibit ASIC3 by an increased apparent  $\text{H}^+$  affinity.

Understanding the PcTx1/ASIC1a interaction may be of therapeutic value. Very recently, it has been shown that activation of ASIC1a during the acidosis accompanying brain ischemia leads to significant  $\text{Ca}^{2+}$  influx that contributes to ischemic cell death in the brain (Xiong et al., 2004). Inhibition of ASIC1a by PcTx1 significantly decreased neuronal death during ischemia (Xiong et al., 2004). Therefore, our results suggest that a modest increase in the apparent affinity of ASIC1a by pharmacological intervention could desensitize ASIC1a, being of potential benefit in patients at risk of stroke.

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