Isolation of a Tarantula Toxin Specific for a Class of Proton-gated Na⁺ Channels

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Acid sensing is associated with nociception, taste transduction, and perception of extracellular pH fluctuations in the brain. Acid sensing is carried out by the simplest class of ligand-gated channels, the family of H⁺-gated Na⁺ channels. These channels have recently been cloned and belong to the acid-sensitive ion channel (ASIC) family. Toxins from animal venoms have been essential for the discovery and characterization of these novel class of acid-sensing ion channels.

This paper describes a novel 40-amino acid toxin from tarantula venom, which potently blocks (IC₅₀ = 0.9 nM) a particular subclass of ASIC channels that are highly expressed in both central nervous system neurons and sensory neurons from dorsal root ganglia. This channel type has properties identical to those described for the homomultimeric assembly of ASIC1a. Homomultimeric assemblies of other members of the ASIC family and heteromultimeric assemblies of ASIC1a with other ASIC subunits are sensitive to the toxin. The new toxin is the first high affinity and highly selective pharmacological agent for this novel class of ion channels. It will be important for future studies of their physiological and physio-pathological roles.

Proton-gated Na⁺-permeable channels are the simplest form of ligand-gated channels. They are present in many neuronal cell types throughout the central nervous system (1–5), suggesting an important function of these channels in signal transduction associated with local pH variations during normal neuronal activity. These channels might also play an important role in pathological situations such as brain ischemia or epilepsy, which produce significant extracellular acidification.

They are also present in nociceptive neurons (1–3, 5, 6) and are thought to be responsible for the sensation of pain that accompanies tissue acidosis in muscle and cardiac ischemia (7, 8), corneal injury (9), and inflammation and local infection (10, 11). It is only very recently that the first proton-gated channel, acid-sensitive ion channel (ASIC) was cloned (12). The ASICs belong to a superfamily that includes amidole-sensitive epithelial Na⁺ channels (13, 14), the FMRFamide-gated Na⁺ channel (15), and the nematode degenerins (DEGs), which probably correspond to mechano-sensitive Na⁺-permeable channels (16).

Several ASIC subunits have now been described: ASIC1a (12), ASIC1b (17), ASIC2a (18–21), ASIC2b (22), and ASIC3 (23–25). The different subunits produce channels with different kinetics, external pH sensitivities, and tissue distribution. They can form functional homomultimers as well as heteromultimers (21, 22, 26). ASIC1a and ASIC1b both mediate rapidly inactivating currents following rapid and modest acidification of the external pH. However, although ASIC1a is present in both brain and afferent sensory neurons, its splice variant ASIC1b is found only in sensory neurons (17). ASIC2a forms an active H⁺-gated channel and is abundant in the brain but essentially absent in sensory neurons, whereas its splice variant ASIC2b is present in both brain and sensory neurons and is inactive as an homomultimer. ASIC2b can form functional heteromultimers with other ASIC subunits and particularly ASIC3 (21). ASIC3 is found exclusively in small sensory neurons that act as nociceptors. Its expression in various heteromultimeric systems generates a biphasic current with a fast inactivating phase followed by a sustained component (22). The association of ASIC2b with ASIC3 forms an heteromultimer with properties (time course and ionic selectivity) similar to those of a native sustained H⁺-sensitive channel, which is present in dorsal root ganglion cells and appears to play a particularly important role in pain sensation (6).

Venoms from snakes, scorpions, sea anemones, marine snails, and spiders are rich sources of peptide toxins that have proven of great value in the functional exploration of voltage-sensitive and ligand-gated ion channels. This report describes the discovery and characterization from the venom of the South American tarantula *Psalmopoeus cambridgei*, of psalmotoxin 1 (PcTX1), the first potent and specific blocker of this new class of ASIC channels.

**EXPERIMENTAL PROCEDURES**

Venom and Toxic Purification—*P. cambridgei* (Araneae Theraphosidae) venom was obtained by electrical stimulation of anesthetized spiders (Invertebrate Biologies). Freeze-dried crude venom was resuspended in distilled water, centrifuged (14,000 rpm, 4 °C, 20 min), filtered on 0.45-μm microfilters (SJT3004S, 4-mm diameter; Millipore), and stored at −20 °C prior to analysis. Crude venom diluted to 10 times the initial volume was fractionated by C8 reversed-phase high pressure liquid chromatography (RP-HPLC) (10 × 250 mm, SCSMS; Nacalai Tesque) using a linear gradient of acetonitrile/water in constant 0.1% trifluoroacetic acid. A second purification step used cation exchange chromatography on a Tosoh SP5PW column (10 × 250 mm, SCSMS; Nacalai Tesque) using a linear gradient of ammonium acetate in water (20 mM to 2 M). A total of 160 μl of venom was purified in two separate batches (10 and 150 μl). All solvents used were of HPLC grade. Separation was conducted on a Hewlett-Packard HP1100 system coupled to a diode array detector and a microcomputer running the Chemstation® software.

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‡‡‡The abbreviations used are: ASIC, acid-sensitive ion channel; PcTX1, psalmotoxin 1; RP, reversed-phase; HPLC, high pressure liquid chromatography; DRG, dorsal root ganglion; Fmoc, N-(9-fluorenyl)-methoxycarbonyl.
Monitoring of the elution was done at 215 and 280 nm.

**Peptide Characterization**—Samples were hydrolyzed in a Waters Pico-Tag station, with 6 h C1 (0.6% phenol) at 110 °C, under vacuum for 20 h. Hydrolyzed peptides were derivatized with phenylisothiocyanate and the derivatized amino acid mixtures were analyzed by C18 RP-HPLC. The purity of chromatographically distributed 50% w/w at 37 °C for 14 h in 100 mM ammonium bicarbonate, 0.1 mM CaCl$_2$ pH 8.1, (b) V$_8$ protease at 37 °C for 24 h in 50 mM ammonium bicarbonate, pH 7.8, in 10% acetonitrile; and (c) BNP-akatope (2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine) at 37 °C for 24 h in 75% acetic acid. Resulting peptides were separated by RP-HPLC using a linear gradient of acetonitrile/water in constant 0.1% trifluoroacetic acid.

Reduced-alkylated toxin was submitted to the following treatments: (a) tosylphenyl chloromethyl ketone-treated Freytag (Sigma) 2% w/w at 37 °C for 14 h in 100 mM ammonium bicarbonate, 0.1 mM CaCl$_2$ pH 8.1, (b) V$_8$ protease at 37 °C for 24 h in 50 mM ammonium bicarbonate, pH 7.8, in 10% acetonitrile; and (c) BNP-akatope (2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine) at 37 °C for 24 h in 75% acetic acid. Resulting peptides were separated by RP-HPLC using a linear gradient of acetonitrile/water in constant 0.1% trifluoroacetic acid.

Mass spectra of native PcTX1 dissolved in $\alpha$-cyano-4-hydroxycinnamic acid matrix were recorded on a MALDI-TOF Perceptive Voyager Elite spectrometer (Perceptive Biosystems), in positive ion linear mode using an internal calibration method with a mixture of $\beta$-insulin (3495.9 Da) and bovine insulin (5733.5 Da). Data were analyzed using the GRAMS386 software. Theoretical molecular masses and pl values were compared with sequence data and results were analyzed with the BLAST software. Synthetic PcTX1 was analyzed on a Micromass Platform II electrospray system (Micromass, Altrincham, UK), in positive mode (cone voltage 20kV, temperature 60 °C).

Sequence homologies were determined using sequences obtained from a search of nonredundant protein data bases via the BLAST server. Sequence alignments and percentages of similarity were calculated with ClustalW.

**Peptide Synthesis and Refolding**—The synthesis of native PcTX1 was performed using the Fmoc/tert-butyl and maximal temporary protection strategy on an Applied Biosystems 4334 synthesizer. The chemical procedure used 0.05 mM of Fmoc-Thr(OtBu)-4-hydroxymethylphenoxy resin (0.39 mmol/g), a 20-fold excess of each amino acid, and dicyclohexylcarbodiimide/1-hydroxy-7-azabenzotriazole activation. Deprotection (1.5 h) and cleavage (200 mg of peptide + resin) were achieved using 10 ml of a mixture trifluoroacetic acid/triisopropylsilane/water (9:5.0:250:0.25, v/v/v/v). The acidic mixture was then precipitated twice in 100 ml of cold diethyl ether. The solid was dissolved in 50 ml of 10% aqueous acetic acid and freeze-dried. The crude reduced toxin was purified by RP-HPLC on a C18 semi-preparative column (21 $\times$ 250 mm, Jupiter) using a 40-min gradient of acetonitrile/water in 0.1% trifluoroacetic acid (0–18% B in 4 min, 30% B in 20 min, and 100% B in 6 min, where B is 90% acetonitrile/H$_2$O/0.1% trifluoroacetic acid).

Oxidation of the reduced toxin was achieved at 0.1 mg/ml in degassed potassium phosphate buffer (100 mM, pH 7.8) using the redox couple reduced glutathione (5 mM)/oxidized glutathione (0.5 mM). The disappearance of the reduced peptide was monitored by RP-HPLC on a C18 (21 $\times$ 250 mm, Jupiter) using a 40-min gradient of acetonitrile/water in 0.1% trifluoroacetic acid (0–18% B in 8 min, 30% B in 18 min, and 60% B in 14 min). Experiments were performed at room temperature (20–24 °C).

**RESULTS**

**Purification**—The screening of several tarantula venoms was carried out against cloned ASIC channels expressed in *Xenopus* oocytes. It singled out *P. cambridgei* venom as containing a potent inhibitor of the ASIC1a proton-gated current. A diluted solution of 1 µl of crude venom (1:1000) applied to the oocyte provoked a 90% block of the ASIC1a current. Bioassay-guided fractionation of the venom by reversed-phase and cation exchange chromatography led to the purification of the minor venom constituent Psmotixin 1 (PcTX1), in a two-step process (Fig. 1, A and B). PcTX1 is a 40-amino acid peptide, possessing 6 cysteines linked by three disulfide bridges. Its full sequence was established by N-terminal Edman degradation of the reduced alkylated toxin and of several cleavage fragments (Fig. 1D). The calculated molecular mass (4689.40 Da average) was in accordance with the measured molecular mass (4689.25 Da) and suggested a free carboxylic acid at the C-terminal extremity.

PcTX1 has limited overall homology to other spider venom toxins identified to date (Fig. 1E). However, it shares a conserved cysteine distribution (Fig. 1F) found both in spider venom and cone snails polypeptide toxins (28, 29). It is a basic polypeptide (pI 10.38 for the native form with disulfide bridges bonded) comprising a large number of basic residues (9 residues, including 4 arginines) but also of acidic residues (6 residues).

**Synthesis**—The chemical synthesis of PcTX1-OH unambiguously confirmed the structure of PcTX1. The purified refolded synthetic toxin (PcTX1 s) and the native form have identical measured molecular mass, and when co-injected in two separate experiments using reversed-phase and cation exchange HPLC, native and synthetic PcTX1 were indistinguishable in their migration and co-eluted in both systems (Fig. 1C). Most electrophysiological experiments were therefore conducted with the synthetic toxin.

**Selective Block of ASIC1a**—The effect of PcTX1 on the activity of ASIC1a, ASIC1b, ASIC2a, and ASIC3 channels expressed in *X. laevis* oocytes is shown in Fig. 2. The natural as well as...
**FIG. 1.** Purification and characterization of PcTX1. 

A, RP-HPLC separation of crude *P. cambridgei* venom (10 μl) with a linear gradient of water/acetonitrile in 0.1% aqueous trifluoroacetic acid. The arrow indicates fraction 10, which containing PcTX1.

B, cation exchange chromatography of fraction 10 with a linear gradient of ammonium acetate (20 mM to 2 M in 88 min).

C, co-elution experiments with the native toxin (PcTX1n, solid trace) injected alone (100 pmol) and co-injected with the synthetic toxin (PcTX1s, dotted trace, 100 pmol each) by cation exchange HPLC. 

D, PcTX1 sequence determination by automated Edman degradation of reduced-alkylated peptide and proteolytic cleavage fragments.

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**EDCIPKWKGVCNHGDCCEGECWKR**

**RRRSFEVCVPKPTK**

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**KRRRSFEVCVPKPTK**

**XXVCVP**

**VCVPKPTK**

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**% Sim Activity**

| Compound | ASIC1a | Kv4.2 | Kv2.1 | VSCC (R) | VSCC (P/N) | Kv4.2/4.3 |
|----------|--------|-------|-------|----------|-----------|----------|
| PcTX1    | 59.5   | 59.8  |       | 60.0     | 68.9      | 55.6     |
| HxTX2    |        |       | 59.8  | 60.0     | 68.9      |          |
| HxTX1    |        |       |       | 60.0     | 68.9      |          |
| SNX482   |        |       |       | 60.0     | 68.9      |          |
| GSTX1A   |        |       |       | 60.0     | 68.9      |          |
| PaTX1    |        |       |       | 60.0     | 68.9      |          |

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**F**

| C | C | C | C | C | C |
|---|---|---|---|---|---|
the synthetic toxin block the ASIC1a current recorded at pH 6, with an IC$_{50}$ of 0.9 nM (Fig. 2, A and B). The blockade is rapid and reversible. PcTX1 at 10 nM also completely blocks the ASIC1a current activated by a pH drop to pH 5 or pH 4 (not shown). PcTX1 is highly selective. Neither the native nor the synthetic PcTX1 (10 nM or 100 nM) blocked ASIC1b currents activated at pH 6 (Fig. 2C). Similarly, the ASIC2a channel activated by a pH drop to pH 5 was insensitive to the action of PcTX1 at 10 nM (Fig. 2D) or 100 nM (not shown). The rapid and slow components of the ASIC3 channel were also insensitive to the perfusion of PcTX1 at 10 nM (Fig. 2E) and 100 nM (not shown). The toxin was also tested on the epithelial Na$^+$ channel formed by the assembly of α, β, and γ subunits (30), and no inhibition occurred with concentrations of 10 nM or 100 nM PcTX1 (n = 3, not shown).

Sequence homologies of PcTX1 with other spider toxins that block different subtypes of voltage-dependent K$^+$ channels such as hanatoxins (Kv2.1) (31), heteropodatoxins (Kv4.2) (32), and phrizotoxins (Kv4.2, Kv4.3) (33) (Fig. 1E) prompted us to test its effects against Kv2.1, Kv2.2, Kv4.2, and Kv4.3 channels expressed in Xenopus oocytes. These channels were not affected by 10 or 100 nM PcTX1 (not shown).

Experiments carried out with the same ASIC channels expressed in COS cells confirmed the results obtained in oocytes. ASIC1a was completely inhibited by 10 nM PcTX1, whereas ASIC1b, ASIC2a, and ASIC3 were insensitive (n = 10 for each channel) to a higher toxin concentration of 50 nM (not shown).

PcTX1 was then assayed on heteromultimers of the ASIC1a subunit (Fig. 3). Co-expression of ASIC1a and ASIC3 in COS cells produces a rapidly inactivating H$^+$-gated current ($\tau$ = 0.19 ± 0.01 s at pH 6, n = 5) that is insensitive to PcTX1 (n = 10) (Fig. 3C), whereas ASIC1a homomultimers produce a current that inactivates more slowly at the same pH (τ = 2.10 ± 0.30 s, n = 10) but that is completely blocked by PcTX1 (10 nM) (Fig. 3A). ASIC1a/ASIC2a heteromultimers were also insensitive to PcTX1 (Fig. 3B).

The ASIC1a channel can also be blocked by amiloride, but the IC$_{50}$ is 10 μM (12), i.e. 10$^4$ times lower in affinity than PcTX1. Moreover, amiloride is not selective. It blocks the transient current generated by ASIC1a (12), ASIC1b (17), ASIC2a (18, 19), and ASIC3 (23).

Activity of PcTX1 on Native Proton-gated Currents—Small DRG neurons isolated from 2-day-old rats were voltage clamped at -60 mV and stimulated by a pH drop from pH 7.3 to pH 6. As previously observed in small sensory neurons from trigeminal ganglia (1), this pH change evoked three different types of responses that are presented in Fig. 4 (A–C). Currents presented in Fig. 4A were blocked by 3–10 nM of the toxin PcTX1, whereas H$^+$-evoked currents in other neurons were insensitive to the toxin (Fig. 4, B–C). DRG neurons express at least two subpopulations of transient currents as judged by their constants of inactivation (Fig. 4, A, B, and D). One population inactivates very rapidly with a time constant of inactivation below 0.5 s, whereas the other one has time constants between 1 and 3 s, the average time constant of inactivation being 1.95 ± 0.14 s (n = 23). The data clearly indicate that the most rapidly inactivating currents with an average time constant of inactivation of 0.24 ± 0.03 s (n = 22) are insensitive to PcTX1. Only the more slowly inactivating H$^+$-gated channels are highly sensitive to PcTX1.

The dose-response curve presented in Fig. 4E was obtained from the PcTX1-sensitive population of neurons. The IC$_{50}$...
PcTX1 blocks ASIC1a homomultimers and is inactive on multimers. COS cells transfected with ASIC1a, ASIC1a + ASIC2a, or ASIC1a + ASIC3 were voltage clamped at −60 mV and subjected to a pH drop as indicated (n = 10). Although ASIC1a homomultimers were inhibited by 10 nM PcTX1 (A), none of the heteromultimers were sensitive to the toxin (B and C). Note that the sustained component produced by ASIC3 homomultimers (Fig. 2E) is absent in the ASIC1a + ASIC3 heteromultimer (C).

value for half-maximum inhibition is 0.7 nM, very similar to the value of 0.9 nM obtained for ASIC1a channels expressed in Xenopus oocytes.

Fig. 4F shows that a change of the extracellular pH from pH 7.3 to pH 6 in neurons that express the channel type shown in Fig. 4A evokes a rapid depolarization resulting in a train of action potentials. This effect is blocked by very low concentrations of PcTX1, and this inhibition is reversible. ASIC channel subunits are highly expressed in cerebellum and particularly in granular cells (12, 34). This is why we have used these cells to analyze the properties of these channels in central nervous system neurons (Fig. 5). Cerebellar granule cells in culture all responded to a pH drop from pH 7.3 to pH 6 with a transient Na⁺ inward current characterized by a time constant of inactivation of 2.06 ± 0.17 s (n = 10) (Fig. 5A). Both the rate of inactivation and the pH dependence of this H⁺-gated Na⁺ channel (pH0.5 6.6 versus pH0.5 6.4) are very similar to those of the ASIC1a channel (Ref. 12 and this work) (Fig. 5B). H⁺-gated Na⁺ channels with the same properties have been recently identified in cortical neurons (35). The transient H⁺-gated Na⁺ channel expressed by granule cells was completely inhibited by 10 nM PcTX1 (n = 10) (Fig. 5A).

**DISCUSSION**

PcTX1 is a novel toxin from tarantula venom that is a potent and specific blocker of one class of H⁺-gated Na⁺ channels. The molecular scaffold of PcTX1 is likely to be similar to that previously described for both cone snail and spider toxins (28, 36). It comprises a triple-stranded antiparallel β-sheet structure reticulated by three disulfide bridges and tightly folded into the “knottin” fold pattern (29). PcTX1 is characterized by the unusual quadruplet Lys25–Arg26–Arg27–Arg28, which probably forms a strongly positive “patch” at the surface of the toxin molecule, constituting an area that is a strong candidate for receptor recognition.

It is particularly intriguing to observe (a) that PcTX1 is absolutely specific for ASIC1a and can distinguish between the two ASIC1 splice variants ASIC1a and ASIC1b, although they only differ in their N-terminal sequence (17); (b) that PcTX1 can also distinguish between ASIC1a, ASIC2 and ASIC3; and (c) that PcTX1 looses its capacity to block ASIC1a as soon as this subunit is associated with another member of the family, be it ASIC2a or ASIC3.

An important site of the interaction of ASIC1a with PcTX1 is probably located in the extracellular stretch of 113 amino acids.
situated immediately after the first transmembrane domain. This is the only extracellular site where the splice variants ASIC1a and ASIC1b are different. They are identical in extracellular regions except for the 113-residue region immediately C-terminal to the first transmembrane domain.

ASIC1a is present in the central nervous system (notably in the hippocampus and the cerebellar granular layer) as well as in DRG neurons (12). Electrophysiological experiments have shown that both cerebellar granule cells and a subpopulation of DRG neurons possess H+–gated currents that inactivate at pH 6 with time constants of 1.95–2.06 s, very similar if not identical to the time constant of inactivation (2.10 ± 0.30 s) of the homomultimeric ASIC1a current expressed in COS cells. The H+–gated currents in these neurons are inhibited by very low concentrations of PcTX1. The resemblance in the inactivation kinetics and pH dependence, in the selective block of the current by PcTX1 and the near identity of the IC50 values for the blockade of ASIC1a channels (IC50 = 0.9 nM) and of native channels (IC50 = 0.7 nM) strongly suggest that the H+–gated current with a τinact of ~2 s in both DRG cells and cerebellar granular cells is mediated by an homomultimeric assembly of ASIC1a. This view is strengthened by the fact that none of the heteromultimeric channels tested (ASIC1a/ASIC2a and ASIC1a/ASIC3) is sensitive to the toxin.

DRG neurons also express H+–gated currents with time constants of inactivation that are either faster or slower than the time constant of inactivation of the homomultimeric ASIC1a current. A class of these proton-sensitive channels inactivates at a fast rate ($\tau_{\text{inact}} = 0.24 \pm 0.03$ s), which turns out, as shown in this work, to be very similar to the rate of inactivation of the ASIC1a/ASIC3 channel expressed in COS cells ($\tau_{\text{inact}} = 0.19 \pm 0.01$ s). This rapidly inactivating current, like the current generated by ASIC1a/ASIC3 heteromultimers, is insensitive to PcTX1.

The ASIC3 channel alone or in association with ASIC2b (22) probably corresponds to the sustained current recorded in DRG cells (6). ASIC3 homomultimers, ASIC3/ASIC2b heteromultimers, and the native noninactivating H+–gated channels are not blocked by PcTX1. It is hoped that further studies will provide other toxins specifically active on these maintained channels that are thought to play an important role in pain (6).

Spider venoms are mixtures of neuroactive peptides capable of incapacitating the prey through a myriad of molecular mechanisms. PcTX1 is a potent tool that now opens the way to a more detailed analysis of the physiological function of the important class of H+–gated Na+ channels.

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