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Authors
Yang, Shilong
Yang, Fan
Zhang, Bei
et al.

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A bimodal activation mechanism underlies scorpion toxin–induced pain

Shilong Yang,¹* Fan Yang,²,³* Bei Zhang,⁴ Bo Hyun Lee,⁵ Bowen Li,¹ Lei Luo,¹ Jie Zheng,³† Ren Lai†‡

Venomous animals use peptide toxins for hunting and self-defense. To achieve these goals, toxins need to bind to their targets with high affinity due to the small amount that a single bite or sting can deliver. The scorpion toxin BmP01 is linked to sting-induced excruciating pain; however, the reported minimum concentrations for activating TRPV1 channel or inhibiting voltage-gated potassium (Kv) channels (both in the micromolar range) appear too high to be biologically relevant. We show that the effective concentration of BmP01 is highly pH-dependent—it increases by about 10-fold in inhibiting Kv channels upon a 1-U drop in pH but decreases more than 100-fold in activating TRPV1. Mechanistic investigation revealed that BmP01 binds to one of the two proton-binding sites on TRPV1 and, together with a proton, uses a one-two punch approach to strongly activate the nociceptive channel. Because most animal venoms are acidic, proton-facilitated synergistic action may represent a general strategy for maximizing toxin potency.

INTRODUCTION

Venom has evolved to arm many otherwise defenseless animals with a lethal weapon (1). This effective strategy for survival is beautifully demonstrated by scorpions, spiders, cone snails, and sea anemones (the 5S’s). Most animal venom contains multiple peptide toxins highly selective for their perspective targets, many of which are ion channels (2, 3). For example, charybdotoxin blocks voltage-gated Kv channels with exquisite potency [Ki (inhibitor constant) in the subnanomolar range] (4), causing uncontrollable hyperexcitability of the nervous system (5, 6). Hanatoxins achieve a similar feat by inhibiting voltage sensor movements (7). With each constituent being optimized through evolution to agitate or nullify its target, a tiny drop of venom can paralyze or even kill a large mammal (8). Achieving these feats requires toxins to be effective at extremely low concentrations (picomolar to nanomolar). Mechanistic investigation of animal toxins has revealed many fundamental biological processes, greatly advanced our understanding of toxin target molecules (3), and facilitated drug development (9–11).

Many scorpions can deliver lethal stings. Although legendary for their toxicity, scorpion stings are also known to be extremely painful, apparently to deter larger predators that cannot be overpowered by venom toxicity alone. Like other venomous species, scorpions use a cocktail of toxins to simultaneously manipulate multiple targets. BmP01, a member of the inhibitor cystine knot motif toxins, is a small scorpion peptide toxin known to target the nociceptive TRPV1 ion channel to produce pain (12). Here, we report a mechanistic study of BmP01, which has revealed a novel cooperative action between the toxin and protons, both from the scorpion venom, that yields high potency in activating the toxin’s target.

RESULTS

The Buthidae scorpions, living in East Asian countries, produce more than a hundred peptide toxins, most of which remain poorly understood. BmP01, originally identified from Mesobuthus martensii (Fig. 1A) (4, 14), is one of these less-studied toxins. BmP01 is a short 29–amino acid peptide (Fig. 1B). Like many peptide toxins, BmP01 forms a compact structure using three pairs of disulfide bonds to cross-link a short α-helix and two β-strands (Fig. 1C) (15). The polymodal nociceptive TRPV1 ion channel is a likely target of BmP01 to produce the excruciating pain associated with scorpion sting (12). Similar to the TRPV1-selective agonist capsaicin, BmP01 induced strong calcium influx in TRPV1-expressing human embryonic kidney (HEK) 293 cells but not in untransfected cells (Fig. 1, D and E). Single-channel recording further confirmed direct activation of TRPV1 by BmP01 (Fig. 1F and fig. S1). BmP01-induced TRPV1 activation exhibited two properties characteristic of classic ligand-host interaction. First, BmP01 is highly selective for TRPV1 over its close analogs, such as TRPV3 (Fig. 1G). Second, BmP01-induced TRPV1 activity is strongly concentration-dependent (Fig. 1H). However, it was noticed that TRPV1 activities were observed only when micromolar concentrations of BmP01 were used (median effective concentration [EC50] at 40.4 ± 12.3 μM, n = 10). Similar properties were previously reported in BmP01-mediated inhibition of voltage-gated Kv channels (14, 16). Therefore, given the fact that high concentrations inside the prey are apparently unachievable by a single sting, it appears that BmP01 has evolved to target TRPV1 to produce pain and/or Kv to produce hyperactivity.

In search of an explanation for the apparent paradox regarding the biological role of BmP01, we noticed that scorpions produce venom that is acidic (Fig. 2, A and B). We therefore examined the possibility that under acidic conditions, BmP01 may exhibit higher potencies. This turned out to be the case. Although it took at least 300 μM BmP01 to induce detectable TRPV1 activity under basic conditions (pH 8.0 to 8.5), we observed that the effective concentration dropped substantially at lower pH levels (Fig. 2C). Although there was barely detectable direct activation of TRPV1 by protons alone at pH 6.5, BmP01 induced robust channel currents at 0.1 to 1 μM concentrations. In support of a synergistic action between protons and BmP01, we found that BmP01’s concentration dependence curve exhibited a leftward shift and became shallower at lower pH levels (Fig. 2D). Observations from TRPV1-transfected cells could be reliably reproduced in animal tests. We found that injection of BmP01 in an acidic solution was much more potent in inducing pain behavior in mice, whereas the same increase in acidity did not appreciably potentiate...
capsaicin-evoked pain behavior (Fig. 2E). Lowering pH antagonized the BmP01-mediated inhibition of Kv channels, making the toxin less potent (fig. S2). Our findings thus suggest that TRPV1, but not the Kv channel, is the biological target for BmP01, which exploits the acidity of the venom milieu to boost toxin potency.

To better understand the underlying molecular mechanism for synergetic action between BmP01 and protons, we searched for the channel-interacting site on BmP01. Given that mutations to charged or polar residues in TRPV1 had a strong impact on toxin potency, we screened the charged/polar residues in BmP01. The charged fractions of residue side chains in BmP01 are little affected by lowering pH from 7.0 to 6.5 (table S1). We found that, although most mutant toxins containing an alanine substitution reduced the pre-S6 loop, an important TRPV1 gating element (22), is a key channel structure targeted by BmP01.

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Guided by results from chimeric tests, our follow-up site-directed mutagenesis screening identified three charged or polar residues in the pre-S6 region that likely participate in mediating BmP01 binding. An alanine mutation to each of these residues substantially reduced the toxin’s potency (Fig. 3D and fig. S4). These results revealed that the pre-S6 loop, an important TRPV1 gating element (22), is a key channel structure targeted by BmP01.

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To test the hypothesis that BmP01 and TRPV1 form direct electrostatic interactions via charged/polar residue(s), we carried out a thermodynamic mutant cycle analysis (23, 24) between K23 of BmP01 and each of the three key pre-S6 residues in TRPV1 (Fig. 4A and fig. S6). We found that the coupling energy between K23 and E649 (but not the other two residues) is substantially higher than 1.5 kT, indicating the possibility that a direct salt bridge may exist between these two residues (25).

Using this crucial structural constraint, we conducted Rosetta-based molecular docking (24, 26) of BmP01 onto the TRPV1 outer...
pore. Our predicted toxin-channel complex model suggests that BmP01 binds to the outer pore via direct contact to the pre-S6 loop and its surrounding structures (Fig. 4A). Although many details of the toxin-channel interaction remain to be elucidated, the location of BmP01 binding has important indications. For example, cryo-electron microscopy (cryo-EM) studies suggest that TRPV1 has two activation gates: a lower gate at the S6 bundle crossing and an upper gate at the selectivity filter (27). By interacting with the outer pore, BmP01 may directly affect the closed-open equilibrium of the upper gate.

How does this structural insight help to explain BmP01’s biological function as a potent pain inducer? TRPV1 is a polymodal nociceptor for a wide array of physical and chemical stimuli, including heat, capsaicin, animal toxins, and protons (17, 28–33). Two protonation sites on TRPV1 are thought to mediate proton activation but differ in their functional roles (34). Protonation of E601 at the extracellular end of S5 (Fig. 4A), which is the higher-affinity site for protons, potentiates the channel, whereas protonation of E649—the lower-affinity site for protons and the residue that K23 of BmP01 interacts with—leads to channel activation (34). Strong activation of TRPV1 by protons requires binding of both sites; synergistic proton binding to multiple sites gives rise to the observed steep pH-dependent TRPV1 activation that ensures high sensitivities in detecting tissue damages, such as inflammation (29). Our finding that BmP01 binds to the lower-affinity but high-potency site E649 suggests that BmP01 may act like a proton to promote channel activation. This would allow strong activation of TRPV1 under less acidic conditions that would only yield occupation of E601. Therefore, there is a synergy between protons and BmP01 in activating TRPV1.

The predicted synergistic action by BmP01 and protons was fully supported by functional tests. We found that when protonation of E601 was prevented by the E601A mutation, the potency and pH dependence of BmP01 was substantially reduced (Fig. 4B). At neutral or high pH levels, both the E601A mutant channel and the WT channel responded similarly to BmP01, confirming the mutation’s selective effect on protonation. On the other hand, mimicking protonation by an E601Q mutation substantially enhanced BmP01’s potency at neutral pH (Fig. 4C). Furthermore, consistent with their different functional roles, proton binding to E601 and E649 in sequential order was determined by their pKₐ values (where Kₐ is the acid dissociation constant) values. A point mutation to E601 caused a significant rightward shift in pH-dependent activation (EC₅₀ of 0.43 ± 0.14 µM, n = 7; for WT, EC₅₀ of 0.48 ± 0.06 µM, n = 7) (Fig. 4D), indicating that the pKₐ value for E601 is likely much higher than the pKₐ value for E649.

In summary, our results show that BmP01 is not a potent activator at neutral pH because it occupies only the E649 site. Under acidic conditions that would only yield occupation of E601. Therefore, there is a synergy between protons and BmP01 in activating TRPV1.
conditions, however, when the E601 sites (four per channel) are protonated, occupation of E649 by BmP01 produces strong channel activation (Fig. 4E). The cooperative action of BmP01 and proton causes strong pain locally at the sting site.

**DISCUSSION**

Bimodal activation of TRPV1 by BmP01 and proton reveals an ingenious strategy that scorpions use for self-defense. Taking advantage of the intrinsic acidity of its venom, scorpions produce a peptide toxin that targets TRPV1 with a one-two punch approach, mimicking what protons do to TRPV1 at high concentrations. However, the presence of toxin makes it unnecessary for scorpions to further acidify their venom, which may be incompatible with other aspects of venom biology. Nevertheless, delivering BmP01 in an acidic package can achieve potent TRPV1 activation that is comparable to activation by capsaicin, a pungent irritant in chili peppers (24, 28, 35). Strong boost in potency comes from the cooperative interaction of BmP01 and proton with TRPV1.

BmP01 belongs to the α-KTX8 toxin subfamily (36), whose members share similar topology (16) and sequence features (fig. S5A). For instance, K23, which is critical to BmP01-TRPV1 interaction, is highly conserved. We have observed that other α-KTX8 members, such as Tx203 and OdK1, can also activate TRPV1 at neutral pH (fig. S5B). Increasing the acidity to pH 6.5 largely potentiated TRPV1 activation by either Tx203 or OdK1 just like BmP01 (fig. S5B). These observations indicate that the bimodal activation mechanism may be applicable to other toxins within the α-KTX8 subfamily. However, because protein-protein interactions are affected by many factors, such as size, shape, and hydrophobicity in the microenvironment, case-by-case investigations are needed to determine the exact structural mechanism underlying bimodal activation of TRPV1 by other members of the α-KTX8 subfamily.

Toxins within the α-KTX8 subfamily have long been known as blockers of potassium channels (36). Our observations that some of them can strongly activate TRPV1 channels under acidic conditions are intriguing. Evolutionarily, both Kv and TRP channels belong to the same ion channel superfamily (37), and share a similar topology in the transmembrane domains. The opposite effects of α-KTX8 toxins on these channels reflect important structural differences in the pore regions. Cryo-EM examination of TRPV1 structure reveals that the TRPV1 selectivity filter adopts a “flatter” conformation compared to the similar section of voltage-gated potassium channels, despite almost identical amino acid sequences (19, 27, 38, 39). Structural differences in TRPV1 and potassium channels may cause toxins, such as
BmP01, to use different strategies target these channels. Our study here demonstrates that BmP01 uses K23 to bind to the pore helix—a well-known hotspot for polymodal gating (22)—to open TRPV1 (Fig. 4A), whereas this toxin may use K18 as a "plug" to block the selectivity filter of a potassium channel (16). In light of these findings, it would be attractive to test in vivo whether both ion channels are effectively targeted by BmP01 and other members of the \( \alpha \)-KTX8 subfamily, because activating TRPV1 and inhibiting Kv channels would both contribute to excitation of nociceptive neurons. Being able to simultaneously target both ion channels would give a clear metabolic and biochemical advantage to scorpions, a group of very old animals that have exhibited little anatomic change through evolution.

Virtually all known ion channels are allosteric proteins with a multimeric structural design that incorporates cooperativity in sensing a wide range of physical and chemical stimuli (40, 41). Although BmP01-induced activation of TRPV1 involves highly specific molecular interactions, it is unlikely that this bimodal activation is a unique phenomenon to the \( \alpha \)-KTX8 subfamily of toxins. Animal venoms are generally acidic (42). Scorpion venom is only mildly acidic, whereas those of spider and centipede can reach pH 5.0 to 5.5 (Fig. 2B). In this aspect, venoms are similar to the synaptic vesicle milieu (43–45), Golgi complex, and secretory granules. The acidic environment in membrane-bound cellular compartments is recognized to be crucial for biosynthesis, sorting, and trafficking of proteins (46). Animal toxins delivered in this acidic package must have undergone optimization through evolution to better perform their biological functions. We suggest that, in addition to using a cocktail of toxins, bimodal activation may represent another general survival strategy used by venomous animals.

**MATERIALS AND METHODS**

**Cell transfection**

HEK293T cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin
(100 mg/ml) at 37°C with 5% CO2. Cells were plated on glass coverslips 24 hours before transfection. Transient transfection was conducted by adding 4 µl of Lipofectamine 2000 (Invitrogen) and 4 µg of plasmid DNA into Opti-MEM and then stored for 20 min. The mixer was added in a 35-mm cell culture dish. Electrophysiological experiments and fluorescence imaging recordings were performed between 24 and 48 hours after transfection.

Toxin peptides synthesis and purification

Syntheses of linear Bmp01 and point mutations were carried out on an automatic peptide synthesizer (PerSeptive Biosystems) using a 9-fluorenly methoxycarbonyl/tert-butyl strategy and HOBr/ITBU/NMM coupling method. Crude reduced peptides were purified by reversed-phase high-performance liquid chromatography (HPLC). The purity of peptides was checked to be higher than 95% by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry and HPLC techniques. Linear peptide was refolded under the solution with 10 mM glutathione and 100 mM oxidized glutathione, adjusted to pH 7.0, and placed at room temperature for 24 hours. The successfully refolded peptide was eluted by nearly 30% acetonitrile.

Electrophysiological recordings

Macroscopic and single-channel currents from TRPV1-expressing cells were recorded in whole-cell or outside-out patches using a HEKA EPC10 amplifier controlled with PATCHMASTER software (HEKA). All recordings were performed at room temperature. Patch pipettes were pulled from thin-wall borosilicate glass (A-M Systems) and firepolished to a resistance of ~2 megohms. Both bath and pipette solutions contained 130 mM NaCl, 0.2 mM EDTA, and 3 mM Hepes (pH 7.2). There was no Ca2+ in the solution so as to reduce desensitization to Ca2+.

Activity-related restraint-docking of Bmp01 to the TRPV1 channel

To prepare structures of Bmp01 (PDB ID: 1W7M) and rTRPV1 channel (toxin-bound state; PDB ID: 3J5Q) for molecular docking, they were first relaxed in Rosetta 3.5 (48). For each structure, 10,000 models were generated. The top 10 lowest-energy models converged well, and the lowest-energy model was chosen for docking. To dock the toxin, membrane environment was first set up on the channel model using RosettaScripts (26, 49). On the basis of results of a thermodynamic mutant cycle analysis (Fig. 4A), three distance restraints were set up: because E649 on TRPV1 and K23 on Bmp01 exhibited specific interaction, the distance between their Cα atoms was set to be less than 4.0 Å. On the other hand, the distances between Cα atoms of the T651/K23 pair and the E652/K23 pair were set to be larger than 6.0 Å because coupling energies between these residue pairs indicated no specific interaction between them. A total of 20,000 docking models were generated, from which the top 1000 total energy score models were identified. From this pool, the one model that was in agreement with distance restraints was chosen as the final docking model. All numbering in protein sequence was based on mouse TRPV1.

Animal assay

All mice (C57BL/6j) used in this study were purchased from the Model Animal Research Center of Nanjing University, China. For the paw licking assay, paw was induced in mice by intraplantar injection of capsaicin or Bmp01. Each of the testing materials was dissolved in 100-µl saline. Mice were injected 10 µl of each drug with an appropriate concentration at the plantar surface of the left hind paw. Control mice were injected with the same volume of saline (at pH 7.0 or 6.5). The time of paw licking was recorded immediately after injection by taking videos for 30 min. All efforts were made to reduce the number of animals used and to minimize the suffering of animals.

Data analysis

Experimental data of electrophysiological recordings were acquired and analyzed using the PATCHMASTER program and Igor Pro. Data obtained from experiments involving animals were analyzed by GraphPad Prism 5 (GraphPad Software Inc.). All statistical values are shown as means ± SEM of n repeats of the experiments. Statistical analysis was carried out using Student’s t test; double asterisks were used to indicate the presence of a significant difference at the P < 0.001 level. NS indicates no significance.

For double-mutant cycle analysis, EC50 values of four channel-toxin combinations (WT channel, WT Bmp01: EC50–1–1; mutant channel, WT Bmp01: EC50–2; WT channel, mutant Bmp01: EC50–3; and mutant channel, mutant Bmp01: EC50–4) were determined separately. The strength of coupling was determined by the coupling energy (kT multiplied by lnΩ, where k is the Boltzmann constant and T is temperature in Kelvin) (24, 25). In lnΩ was calculated using the following equation: lnΩ = ln[(EC50–1)/(EC50–4)/(EC50–2)/(EC50–3)]. All values of EC50 [or IC50 (median inhibitory concentration)] and Hill coefficient of concentration-response curves were summarized in tables S2 to S4.
SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/8/e1700810/DC1

fig. S1. BmP01 activates TRPV1 by increasing the open probability.

fig. S2. BmP01 inhibits Kv1.3 channel.

fig. S3. Effects of BmP01 on TRPV1, TRPV3, and their chimeras.

fig. S4. Effects of BmP01 on TRPV1 outlier pore mutants.

fig. S5. Low pH potentiates TRPV1 current response to toxins in the e-KTX8 toxin subfamily.

fig. S6. Concentration-response curves used for the determination of coupling energy between BmP01 and TRPV1.

table S1. Changes in charged fraction of the side chain of tryptophan residues in BmP01.

table S2. BmP01 concentration-response parameters for TRPV1 WT and mutants.

table S3. Proton concentration-response parameters for TRPV1 WT and mutants.

table S4. BmP01 concentration-response parameters for Kv1.3 under different pH values.

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