



# Mast cell chymase reduces the toxicity of Gila monster venom, scorpion venom, and vasoactive intestinal polypeptide in mice

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**Mast cell degranulation is important in the pathogenesis of anaphylaxis and allergic disorders. Many animal venoms contain components that can induce mast cell degranulation, and this has been thought to contribute to the pathology and mortality caused by envenomation. However, we recently reported evidence that mast cells can enhance the resistance of mice to the venoms of certain snakes and that mouse mast cell–derived carboxypeptidase A3 (CPA3) can contribute to this effect. Here, we investigated whether mast cells can enhance resistance to the venom of the Gila monster, a toxic component of that venom (helodermin), and the structurally similar mammalian peptide, vasoactive intestinal polypeptide (VIP). Using 2 types of mast cell–deficient mice, as well as mice selectively lacking CPA3 activity or the chymase mouse mast cell protease-4 (MCPT4), we found that mast cells and MCPT4, which can degrade helodermin, can enhance host resistance to the toxicity of Gila monster venom. Mast cells and MCPT4 also can limit the toxicity associated with high concentrations of VIP and can reduce the morbidity and mortality induced by venoms from 2 species of scorpions. Our findings support the notion that mast cells can enhance innate defense by degradation of diverse animal toxins and that release of MCPT4, in addition to CPA3, can contribute to this mast cell function.**

## Introduction

In addition to their roles as effector cells in anaphylaxis and allergic disorders, there is evidence that mast cells can enhance innate host defense through functions such as directly killing pathogens or augmenting pathogen-induced inflammatory responses (1–3) or by degrading potentially toxic endogenous peptides generated in such settings (4, 5). We recently reported that mast cells also can enhance resistance in mice to the morbidity and mortality induced by the whole venoms of 3 species of snakes and the honeybee (6). In the case of the Israeli mole viper, *Atractaspis engaddensis*, pharmacological evidence and studies in mice containing mast cells treated with shRNA to reduce expression of carboxypeptidase A3 (CPA3, which also has been designated mouse mast cell–derived carboxypeptidase A3) activity suggested that CPA3 is important for reducing the toxicity of both the whole venom and its major toxin, sarafotoxin 6b (6). The essential role for CPA3 in degrading and enhancing resistance to sarafotoxin 6b was demonstrated by showing that mutant mice selectively lacking CPA3 activity exhibited impaired resistance to the lethal effects of that venom peptide (7). How-

ever, the extent to which mast cells and their specific products might be able to enhance resistance to other animal venoms or exogenous toxins remained to be determined.

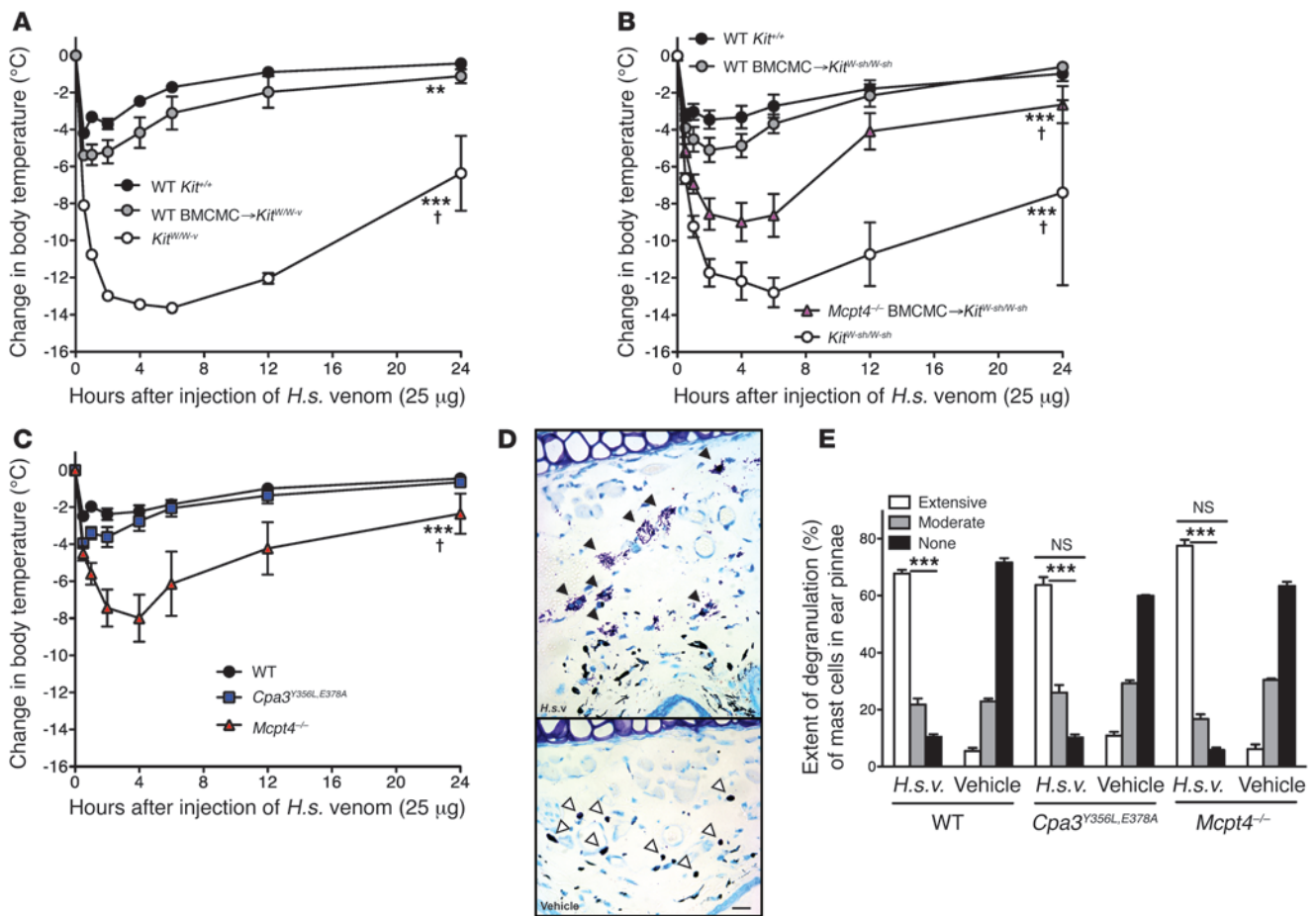
Notably, both sarafotoxin 6b (6) and its structurally related mammalian molecule, the vasoconstrictor peptide endothelin-1 (ET-1) (8), can induce mast cell degranulation in mice, and mast cells (4, 7) and mast cell–derived CPA3 (6, 7) can enhance resistance to the morbidity and mortality associated with ET-1 or sarafotoxin 6b. We therefore searched for other examples of reptile venoms that contain peptides that are structurally similar to mammalian peptides known to activate mast cells. We decided to investigate the venom of the poisonous lizard the Gila monster (*Heloderma suspectum*) because its venom contains the toxin helodermin, which has structural and functional similarities to mammalian vasoactive intestinal polypeptide (VIP) (9–12).

The Gila monster is native to the southwestern United States and northern Mexico. In contrast to snake envenomation, human bites by lizards are relatively rare but appear to be increasing in parallel with improvements in captive-breeding techniques (13). Envenomation by the Gila monster is not ordinarily fatal to adult humans, but results in intense pain, edema, weakness, and nausea associated with hypotension or tachycardia and can even cause anaphylactic shock or myocardial infarction (13–17). These signs and symptoms probably reflect powerful physiological effects of the kallikrein-like peptides and other bioactive peptides in *Heloderma*.

**Authorship note:** Mitsuteru Akahoshi and Chang Ho Song contributed equally to this work.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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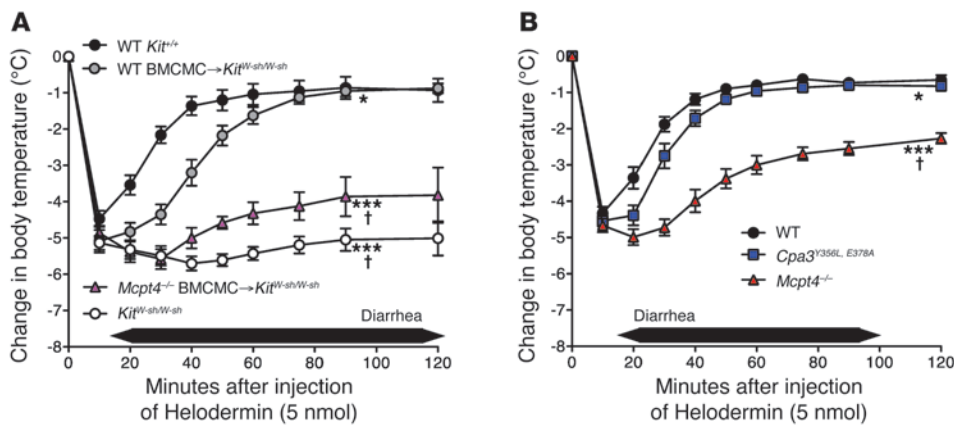


**Figure 1**

Mast cells can diminish *H. susceptor* venom-induced (*H.s. venom*-induced) hypothermia and mortality through MCPT4-dependent mechanisms. Changes in rectal temperatures after i.d. injection of *H. susceptor* venom (25 µg in 20 µl DMEM solution) into ear pinnae (1 ear pinna of each mouse). (A) WT WBB6F1-Kit<sup>+/+</sup>, mast cell-deficient WBB6F1-Kit<sup>W/W-v</sup>, and WT BMCMC→Kit<sup>W/W-v</sup> mice. Death rates of Kit<sup>+/+</sup>, WT BMCMC→Kit<sup>W/W-v</sup>, and Kit<sup>W/W-v</sup> mice within 24 hours after *H. susceptor* venom injection were 0% (0/21), 7% (1/15; *P* = 0.42 versus Kit<sup>+/+</sup> mice), and 65% (13/20; *P* < 0.0001 versus Kit<sup>+/+</sup> mice), respectively. (B) WT C57BL/6-Kit<sup>+/+</sup>, mast cell-deficient C57BL/6-Kit<sup>W-sh/W-sh</sup>, WT BMCMC→Kit<sup>W-sh/W-sh</sup>, and Mcpt4<sup>-/-</sup> BMCMC→Kit<sup>W-sh/W-sh</sup> mice. Death rates of Kit<sup>+/+</sup>, WT BMCMC→Kit<sup>W-sh/W-sh</sup>, Mcpt4<sup>-/-</sup> BMCMC→Kit<sup>W-sh/W-sh</sup>, and Kit<sup>W-sh/W-sh</sup> mice within 24 hours after *H. susceptor* venom injection were 5% (1/19), 11% (2/18, *P* = 0.48 versus Kit<sup>+/+</sup> mice), 43% (6/14; *P* = 0.01 versus Kit<sup>+/+</sup> mice), and 50% (10/20; *P* = 0.006 versus Kit<sup>+/+</sup> mice), respectively. (C) WT C57BL/6-Kit<sup>+/+</sup>, C57BL/6-Cpa3<sup>Y356L,E378A</sup>, and C57BL/6-Mcpt4<sup>-/-</sup> mice. Death rates of Kit<sup>+/+</sup>, Cpa3<sup>Y356L,E378A</sup>, and Mcpt4<sup>-/-</sup> mice within 24 hours after *H. susceptor* venom injection were 7% (1/15), 0% (0/14; *P* = 0.52 versus Kit<sup>+/+</sup> mice), and 40% (6/15, *P* = 0.007 versus Kit<sup>+/+</sup> mice), respectively. Each panel shows data pooled from at least 3 independent experiments with each group of mice (*n* = 2–5 mice per group per experiment). \*\**P* < 0.01; \*\*\**P* < 0.001 versus WT WBB6F1-Kit<sup>+/+</sup> or WT C57BL/6-Kit<sup>+/+</sup> mice; †*P* < 0.01–0.001 versus each of the other groups (A–C). (D) Extensive degranulation of mast cells (some indicated by black arrowheads) 1 hour after i.d. injection of *H. susceptor* venom (25 µg in 20 µl DMEM), but not vehicle (DMEM) alone (mast cells without evidence of degranulation are indicated by white arrowheads) in WT C57BL/6 mice (toluidine blue stain). Scale bar: 50 µm. (E) Degranulation of mast cells 60 minutes after i.d. injection of *H. susceptor* venom (25 µg in 20 µl DMEM) or vehicle (DMEM) alone in WT C57BL/6, Mcpt4<sup>-/-</sup>, or Cpa3<sup>Y356L,E378A</sup> mice (injection was into 1 ear pinna of each mouse). \*\*\**P* < 0.001 versus corresponding vehicle-injected groups; NS (*P* > 0.05) versus values for WT mice. Data are presented as mean ± SEM (A–C) or mean + SEM (E).

derma venom (17, 18). In the most severe reported case of Gila monster envenomation (19), the victim's signs and symptoms were reported to be similar to those seen in pancreatic cholera, a condition produced by VIP-secreting tumors (also known as VIPomas), which is also called the watery diarrhea, hypokalemia, and achlorhydria (WDHA) syndrome (14, 17). The signs and symptoms observed in that case are consistent with the results of recent studies of molecular evolution in suggesting an important role for VIP-like bioactive peptides in the pathology of Gila monster envenomation (14, 17, 20).

Of the 2 VIP-related peptides that have been isolated from *Heloderma* venoms, helospectin (exendin-1) and helodermin (exendin-2), the 35-amino-acid peptide helodermin is the major VIP-like peptide in Gila monster venom (21). Helodermin, the first VIP-related peptide identified in an animal other than a mammal or bird (9–11), is a hypotensive toxin that can bind to mammalian VIP receptors (10, 22), a property that is likely to have developed as a result of convergent evolution (12). In addition to its vasodepressor activity, helodermin is thought to be responsible, at least in part, for the tachycardia seen in human Gila monster enven-



**Figure 2** Mast cells and MCPT4 can diminish helodermin-induced hypothermia and diarrhea in mice. Changes in rectal temperatures after i.d. injection of helodermin (5 nmol in 20  $\mu$ l DMEM solution) in ear pinnae (1 ear pinna of each mouse). (A) WT C57BL/6-*Kit*<sup>+/+</sup>, C57BL/6-*Kit*<sup>W-sh/W-sh</sup>, WT BMCMC → *Kit*<sup>W-sh/W-sh</sup>, and *Mcpt4*<sup>-/-</sup> BMCMC → *Kit*<sup>W-sh/W-sh</sup> mice. The rates of diarrhea within 2 hours after helodermin injection in *Kit*<sup>+/+</sup>, WT BMCMC → *Kit*<sup>W-sh/W-sh</sup>, *Mcpt4*<sup>-/-</sup> BMCMC → *Kit*<sup>W-sh/W-sh</sup>, and *Kit*<sup>W-sh/W-sh</sup> mice were 20% (2/10), 50% (4/8,  $P = 0.2$  versus *Kit*<sup>+/+</sup> mice), 100% (5/5,  $P = 0.007$  versus *Kit*<sup>+/+</sup> mice), and 100% (11/11,  $P = 0.0002$  versus *Kit*<sup>+/+</sup> mice), respectively. (B) C57BL/6 WT, *Cpa3*<sup>Y356L,E378A</sup>, and *Mcpt4*<sup>-/-</sup> mice. The rates of diarrhea within 2 hours after helodermin injection in *Kit*<sup>+/+</sup>, *Cpa3*<sup>Y356L,E378A</sup>, and *Mcpt4*<sup>-/-</sup> mice were 22% (2/9), 50% (4/8,  $P = 0.25$  versus *Kit*<sup>+/+</sup> mice), and 100% (9/9,  $P = 0.001$  versus *Kit*<sup>+/+</sup> mice), respectively. \* $P < 0.05$ ; \*\*\* $P < 0.001$  versus WT *Kit*<sup>+/+</sup> mice; † $P < 0.05$ –0.001 versus each of the other groups (A and B). Each panel shows data pooled from at least 3 independent experiments with each group of mice except for *Mcpt4*<sup>-/-</sup> BMCMC → *Kit*<sup>W-sh/W-sh</sup> mice, with which 2 independent experiments were performed ( $n = 1$ –3 mice per group per experiment). Data are presented as mean  $\pm$  SEM.

omation (18, 23). Previous reports have provided evidence for the existence of complex interactions between mast cells and VIP, in that mast cells can produce VIP (24, 25) as well as express VIP receptors (26–29) and degranulate in response to VIP (27, 29), but mast cell-derived proteases can degrade VIP (30, 31). However, to our knowledge, the possible interactions between mast cells and helodermin have not yet been studied.

We investigated whether mast cells and the mast cell-derived proteases mouse mast cell protease-4 (MCPT4, which has also been designated mMCP-4), MCPT5 (which has also been designated mMCP-5), and CPA3 might influence the morbidity and mortality induced by *H. suspectum* venom, by helodermin, or by mammalian VIP. Venoms derived from several animal species other than reptiles and honeybees, including those from scorpions (32), also have been shown to activate mast cells. We therefore also tested venoms from 2 species of medically important scorpions, the deathstalker (yellow) scorpion (*Leiurus quinquestriatus hebraeus*) (33–35) and the Arizona bark scorpion (*Centruroides exilicauda*) (35–37). By using mast cell-engrafted genetically mast cell-deficient WBB6F1-*Kit*<sup>W/W<sup>v</sup></sup> and C57BL/6-*Kit*<sup>W-sh/W-sh</sup> mice as well as mice either selectively lacking MCPT4 or CPA3 plus MCPT5 or bearing an enzymatically inactive form of CPA3, we demonstrate that mast cells can enhance host resistance to the toxicity of Gila monster and scorpion venoms, and VIP, predominantly through MCPT4-dependent mechanisms.

**Results**

*Mast cells and MCPT4 can enhance resistance to H. suspectum venom-induced morbidity and mortality.* We first administered 2 different amounts of *H. suspectum* venom (5 and 50  $\mu$ g) intradermally (i.d.) in the ear pinnae of WT and genetically mast cell-deficient mice. We used the ear

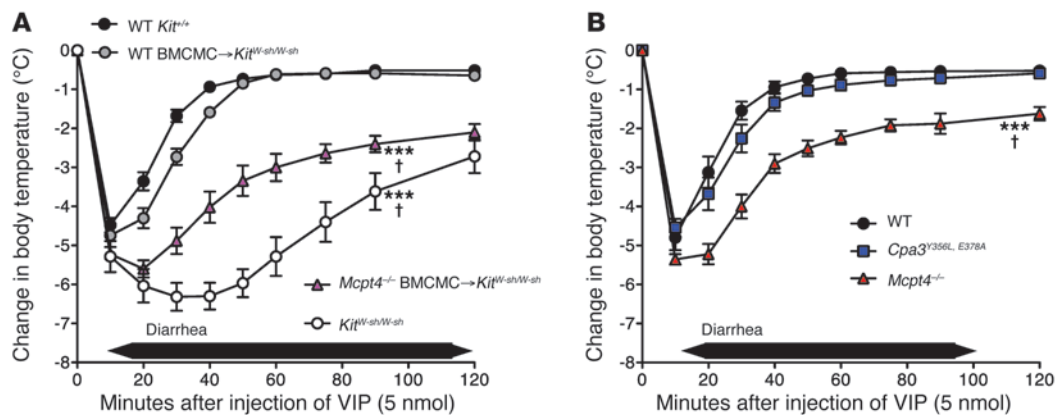
pinna as the site of venom injection because most reptile bites involve the skin and subcutaneous tissue and also because this relatively hairless area is useful for local mast cell-engraftment experiments. All mice survived challenge with 5  $\mu$ g of *H. suspectum* venom (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI46139DS1). Most WT *Kit*<sup>+/+</sup> mice also survived challenge with 50  $\mu$ g of venom but some exhibited hypothermia, whereas 50  $\mu$ g of *H. suspectum* venom induced severe hypothermia and death in all of the mast cell-deficient WBB6F1-*Kit*<sup>W/W<sup>v</sup></sup> mice or C57BL/6-*Kit*<sup>W-sh/W-sh</sup> mice tested (Supplemental Figure 1). In this and all subsequent experiments, mice that survived for 24 hours after injection of venom appeared to recover fully. Based on these results, we decided to use 25  $\mu$ g of *H. suspectum* venom for subsequent experiments.

When challenged with 25  $\mu$ g of *H. suspectum* venom i.d. in 1 ear, mast cell-deficient *Kit*<sup>W/W<sup>v</sup></sup> mice developed severe hypothermia and

65% of mice died within 24 hours, whereas the corresponding WT *Kit*<sup>+/+</sup> mice exhibited only a slight drop in body temperature and all of the mice survived and fully recovered (Figure 1A). We then examined mast cell-deficient mice that had been engrafted with bone marrow-derived cultured mast cells (BMCMCs) from WBB6F1-*Kit*<sup>+/+</sup> mice (WT BMCMC → *Kit*<sup>W/W<sup>v</sup></sup> mice) (38, 39). The local engraftment of mast cell-deficient mice with WT BMCMCs in 1 ear pinna resulted in levels of resistance to the mortality induced by 25  $\mu$ g of *H. suspectum* venom that were statistically indistinguishable from those in the corresponding WT mice (Figure 1A). Similar results were obtained when these experiments were repeated with WT C57BL/6-*Kit*<sup>+/+</sup> mice, C57BL/6-*Kit*<sup>W-sh/W-sh</sup> mice, and WT BMCMC-engrafted *Kit*<sup>W-sh/W-sh</sup> mice (Figure 1B).

MCPT4 is considered to be the mouse functional counterpart of human chymase (40), and CPA3 is the only carboxypeptidase expressed in mast cells. To assess whether MCPT4 or CPA3 might contribute to the mast cell’s ability to reduce the toxicity of *H. suspectum* venom, we tested mice that either selectively lacked MCPT4 (*Mcpt4*<sup>-/-</sup> mice) (41) or that contained only a catalytically inactive form of CPA3 (*Cpa3*<sup>Y356L,E378A</sup> mice) (7). We found that WT, *Mcpt4*<sup>-/-</sup>, and *Cpa3*<sup>Y356L,E378A</sup> mice contained nearly equal numbers of mast cells in the ear pinnae (Supplemental Figure 2). Moreover, intradermal injection of *H. suspectum* venom induced levels of mild hypothermia in *Cpa3*<sup>Y356L,E378A</sup> mice that were similar to those in the corresponding WT control mice, and none of the *Cpa3*<sup>Y356L,E378A</sup> mice and only 1 out of 15 WT mice died (Figure 1C). In contrast, identically challenged *Mcpt4*<sup>-/-</sup> mice developed severe hypothermia and 6 of 15 (40%) died (Figure 1C).

To determine whether mast cells and mast cell MCPT4 could enhance resistance to *H. suspectum* venom even when such mast



**Figure 3**

Mast cells and MCPT4 can diminish VIP-induced hypothermia and diarrhea in mice. Changes in rectal temperatures after i.d. injection of VIP (5 nmol in 20  $\mu$ l DMEM solution) in ear pinnae (1 ear pinna of each mouse). (A) WT C57BL/6-*Kit*<sup>+/+</sup>, C57BL/6-*Kit*<sup>W-sh/W-sh</sup>, WT BMCMC $\rightarrow$ *Kit*<sup>W-sh/W-sh</sup>, and *Mcpt4*<sup>-/-</sup> BMCMC $\rightarrow$ *Kit*<sup>W-sh/W-sh</sup> mice. Rates of diarrhea within 2 hours after VIP injection in *Kit*<sup>+/+</sup>, WT BMCMC $\rightarrow$ *Kit*<sup>W-sh/W-sh</sup>, *Mcpt4*<sup>-/-</sup> BMCMC $\rightarrow$ *Kit*<sup>W-sh/W-sh</sup>, and *Kit*<sup>W-sh/W-sh</sup> mice were 13% (1/9), 29% (2/7;  $P = 0.25$  versus *Kit*<sup>+/+</sup> mice), 100% (5/5;  $P = 0.003$  versus *Kit*<sup>+/+</sup> mice), and 100% (9/9;  $P = 0.0002$  versus *Kit*<sup>+/+</sup> mice), respectively. (B) C57BL/6 WT, *Cpa3*<sup>Y356L,E378A</sup>, and *Mcpt4*<sup>-/-</sup> mice. Rates of diarrhea within 2 hours after VIP injection in *Kit*<sup>+/+</sup>, *Cpa3*<sup>Y356L,E378A</sup>, and *Mcpt4*<sup>-/-</sup> mice were 22% (2/9), 56% (5/9;  $P = 0.17$  versus *Kit*<sup>+/+</sup> mice), and 100% (10/10;  $P = 0.0007$  versus *Kit*<sup>+/+</sup> mice), respectively. \*\*\* $P < 0.001$  versus WT *Kit*<sup>+/+</sup> mice; † $P < 0.05$ –0.001 versus each of the other groups (A and B). Each panel shows data pooled from at least 3 independent experiments with each group of mice except for *Mcpt4*<sup>-/-</sup> BMCMC $\rightarrow$ *Kit*<sup>W-sh/W-sh</sup> mice, with which 2 independent experiments were performed ( $n = 1$ –3 mice per group per experiment). Data are presented as mean  $\pm$  SEM.

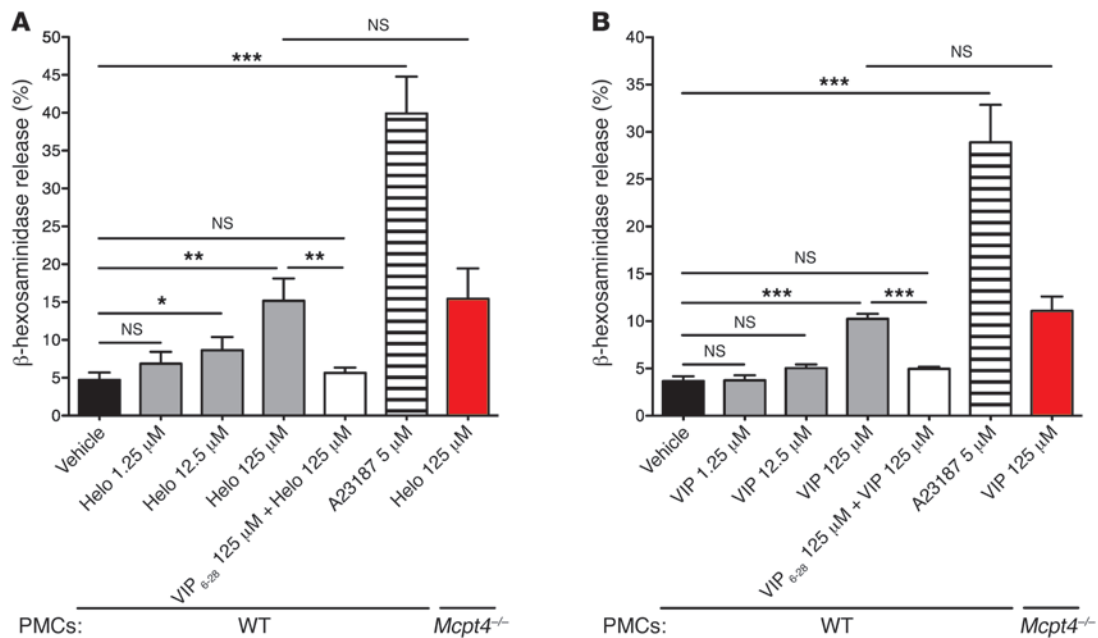
cells were engrafted only in 1 ear pinna of genetically mast cell-deficient *Kit*<sup>W-sh/W-sh</sup> mice, we injected 25  $\mu$ g of *H. suspectum* venom i.d. into the ear pinnae of *Kit*<sup>W-sh/W-sh</sup> mice that had been engrafted at that site with BMCMCs derived from either WT or *Mcpt4*<sup>-/-</sup> mice. Although WT and *Mcpt4*<sup>-/-</sup> BMCMC-engrafted mice showed similar numbers of mast cells in ear pinnae (Supplemental Figure 2), those that had received *Mcpt4*<sup>-/-</sup> BMCMCs developed more severe hypothermia and 6 of 14 (43%) died, versus only 2 of 18 WT BMCMC $\rightarrow$ *Kit*<sup>W-sh/W-sh</sup> mice (Figure 1B). It is likely that MCPT4 expression is restricted largely or even exclusively to mast cells (40, 42, 43), and the data in Figure 1B support this conclusion by showing that a defect in host resistance to *H. suspectum* venom can be observed in mice in which only the engrafted mast cells were genetically unable to produce MCPT4. We next quantified to what extent *H. suspectum* venom can induce degranulation of skin mast cells in the various mice used in our experiments, as this will result in the local secretion of proteases and other constituents of the mast cells' granules. Intradermal injection of *H. suspectum* venom, but not vehicle, induced extensive degranulation of mast cells in the ear pinnae, as assessed 1 hour after the injection (Figure 1D), and the extent of such mast cell degranulation did not differ in WT, *Mcpt4*<sup>-/-</sup>, and *Cpa3*<sup>Y356L,E378A</sup> mice (Figure 1E).

Taken together, our results indicate that *H. suspectum* venom can induce extensive degranulation of dermal mast cells in mice, that dermal mast cells can enhance the resistance of mice to the morbidity and mortality induced by i.d. injection of *H. suspectum* venom, and that MCPT4, but not CPA3, can contribute substantially to the ability of mast cells to limit the toxicity of *H. suspectum* venom. These findings are particularly interesting in that mast cell- and mast cell-MCPT4-dependent enhancement of host resistance to *H. suspectum* venom was observed in mice in which mast cells had been engrafted only in the ear pinna challenged with the venom. Thus, such mast cell-dependent enhancement of host resistance did not require systemic engraftment of mast cells. However, we noted that both *Mcpt4*<sup>-/-</sup> mice (Figure 1C)

and *Mcpt4*<sup>-/-</sup> BMCMC $\rightarrow$ *Kit*<sup>W-sh/W-sh</sup> mice (Figure 1B) developed less hypothermia than did *Kit*<sup>W-sh/W-sh</sup> mice (Figure 1B). This might reflect the contribution of a mast cell-dependent but MCPT4-independent mechanism that can contribute to host resistance in this model and/or the presence of phenotypic abnormalities in *Kit*<sup>W-sh/W-sh</sup> mice that reduce their resistance to this venom independently of their mast cell deficiency.

*Mast cells and MCPT4 can enhance resistance to helodermin-induced morbidity.* While Gila monster venom contains many different toxins, we were particularly interested in whether the toxicity of helodermin, the major VIP-like peptide in Gila monster venom, also might be diminished in a mast cell-dependent manner. All mast cell-deficient *Kit*<sup>W-sh/W-sh</sup> mice injected i.d. with 5 nmol (19.2  $\mu$ g) of helodermin developed sustained hypothermia and diarrhea (a finding also observed in the most severe case of Gila monster envenomation in humans; ref. 19), whereas identically challenged WT mice developed only mild, transient hypothermia and exhibited low rates of diarrhea (20%) (Figure 2A). Engraftment of mast cell-deficient mice with WT BMCMCs at the site later used for i.d. injection of helodermin largely restored WT levels of resistance against helodermin-induced hypothermia and diarrhea (Figure 2A).

To assess the extent to which the differences in the responses of mast cell-deficient and WT mice might reflect the lack of mast cell proteases in mast cell-deficient mice, we examined *Mcpt4*<sup>-/-</sup> and *Cpa3*<sup>Y356L,E378A</sup> mice. *Cpa3*<sup>Y356L,E378A</sup> mice responded to i.d. injection of 5 nmol of helodermin with mild hypothermia similar to (albeit significantly more than) that of WT mice, and diarrhea developed in a larger percentage of *Cpa3*<sup>Y356L,E378A</sup> than WT mice; however, with the numbers of mice tested, this result was not statistically significant (Figure 2B). In contrast, *Mcpt4*<sup>-/-</sup> mice exhibited a more substantial drop in temperature and all developed diarrhea ( $P < 0.005$  vs. WT mice) (Figure 2B). Indeed, *Mcpt4*<sup>-/-</sup> BMCMC $\rightarrow$ *Kit*<sup>W-sh/W-sh</sup> mice, like mast cell-deficient *Kit*<sup>W-sh/W-sh</sup> mice, exhibited sustained hypothermia and diarrhea (Figure 2A).



**Figure 4**

Helodermin (Helo) and VIP can activate mast cells at least partly through VIP receptors. Purified PMCs from WT or *Mcpt4*<sup>-/-</sup> mice were incubated with vehicle (Tyrode’s buffer) alone or with the indicated concentrations of helodermin (A), VIP (B), or A23187 calcium ionophore (A23187) (A and B) for 30 minutes at 37°C. Some cells were pretreated with the VIP receptor antagonist VIP<sub>6-28</sub>. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; NS (*P* > 0.05) for the comparison shown. Each panel shows data pooled from the 4 or more independent experiments we performed, each of which gave similar results (in A and B, *n* = 4–14 determinations per group). Data are presented as mean + SEM.

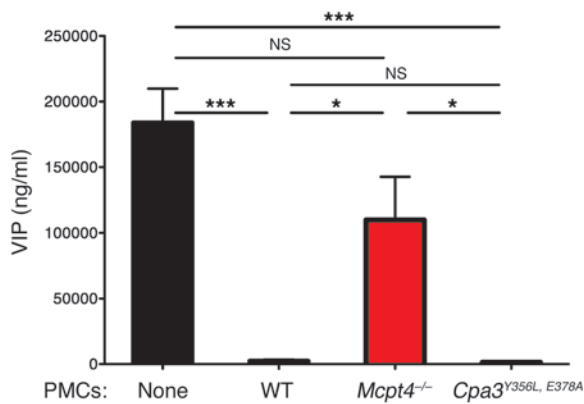
*Mast cells and MCPT4 can limit the toxicity of VIP in vivo.* We previously reported evidence that, in mice, mast cells can enhance resistance to the morbidity and mortality of both ET-1 (4, 7) and sarafotoxin 6b, the structurally similar peptide found in the venom of the poisonous snake, *A. engaddensis* (6, 7), and that the major protease responsible for degrading ET-1 and sarafotoxin 6b is CPA3 (6, 7). Other mast cell-derived proteases, including chymase, have been reported to degrade VIP in vitro (30, 31). We therefore examined whether mast cells can enhance host resistance to VIP, a mammalian peptide that is structurally similar to helodermin.

When challenged i.d. with 5 nmol (16.6 μg) of VIP, mast cell-deficient *Kit*<sup>W-sh/W-sh</sup> mice developed prolonged hypothermia and diarrhea (a finding also observed in patients with VIPoma; refs. 44, 45), whereas the corresponding WT *Kit*<sup>+/+</sup> mice quickly recovered from their hypothermia and few exhibited diarrhea (Figure 3A). *Kit*<sup>W-sh/W-sh</sup> and mice engrafted with WT mast cells exhibited significantly enhanced resistance to VIP-induced hypothermia and diarrhea (Figure 3A). In response to challenge with 5 nmol of VIP, hypothermia and rates of diarrhea in *Cpa3*<sup>Y356L,E378A</sup> mice were not statistically different from those in WT mice, although the rate of diarrhea was higher than in WT mice. In contrast, compared with WT mice, *Mcpt4*<sup>-/-</sup> mice developed a more substantial reduction in body temperature and a higher rate of diarrhea (Figure 3B). Furthermore, *Mcpt4*<sup>-/-</sup> BMCMC-engrafted *Kit*<sup>W-sh/W-sh</sup> mice exhibited hypothermia responses that were intermediate between those of the WT BMCMC-engrafted mice and *Kit*<sup>W-sh/W-sh</sup> mice, and 100% of the *Mcpt4*<sup>-/-</sup> BMCMC→*Kit*<sup>W-sh/W-sh</sup> mice, like the mast cell-deficient *Kit*<sup>W-sh/W-sh</sup> mice, developed diarrhea (Figure 3A). Like i.d. injection of *H. suspectum* venom, i.d. injection of helodermin or VIP also induced extensive degranulation of mast cells in the ear pinnae,

and the extent of such mast cell degranulation did not differ among the groups of mice tested (Supplemental Figure 3). Thus, our experiments in genetically mast cell-deficient mice indicate that mast cells can limit the magnitude of the systemic toxicity induced by either helodermin or VIP. Moreover, our work with mice deficient in MCPT4 or lacking CPA3 protease activity indicates that MCPT4, but not CPA3, significantly contributes to the mast cell’s ability to enhance resistance to helodermin or VIP.

*Activation of mast cells by helodermin or VIP via VIP receptors.* To assess how helodermin or VIP interacts with mast cells, we used a pharmaceutical approach employing the specific VIP receptor antagonist VIP<sub>6-28</sub> (46). Consistent with the results of our assessment of mast cell degranulation in vivo (Supplemental Figure 3), we found that helodermin (Figure 4A) and VIP (Figure 4B) can induce β-hexosaminidase release from purified mouse peritoneal mast cells (PMCs; purity > 75%) in a dose-dependent manner and that the extent of such mast cell activation did not differ between PMCs from WT and *Mcpt4*<sup>-/-</sup> mice. Moreover, pretreatment with VIP<sub>6-28</sub> significantly inhibited helodermin- or VIP-induced β-hexosaminidase release from PMCs, indicating that these toxins can activate mast cells at least partly through VIP receptors. Our findings are consistent with the results of prior work indicating that various mast cell populations can express receptors for VIP, which, depending on the type of mast cell population, may be VPAC<sub>1</sub> (formerly designated VIP receptor type I/PACAP receptor type II) (26, 27) or VPAC<sub>2</sub> (formerly designated VIP receptor type II/PACAP receptor type III) (28, 29).

*Evidence that MCPT4 contributes to mast cell-dependent degradation of VIP in vitro and in vivo.* To evaluate directly the importance of mast cell-derived MCPT4 in degrading VIP, VIP (125 μM) was incubated ex vivo with medium alone or with medium contain-



**Figure 5**

Mast cell chymase (MCPT4) contributes to mast cell-dependent degradation of VIP. VIP (125  $\mu$ M in 150  $\mu$ l) was incubated ex vivo at 37°C for 30 minutes with medium (DMEM) alone (no mast cells) or with medium containing purified PMCs ( $8 \times 10^5$ ) from WT C57BL/6 mice (WT PMCs), C57BL/6-*Mcpt4*<sup>-/-</sup> mice (*Mcpt4*<sup>-/-</sup> PMCs), or C57BL/6-*Cpa3*<sup>Y356L, E378A</sup> mice (*Cpa3*<sup>Y356L, E378A</sup> PMCs). The remaining amount of VIP was then measured by ELISA. \**P* < 0.015; \*\*\**P* < 0.001; NS (*P* > 0.05) for the comparisons shown (Mann-Whitney *U* test). The panel shows data pooled from the 3 or more independent experiments we performed, each of which gave similar results (*n* = 3–6 determinations per group). Data are presented as mean + SEM.

ing purified PMCs from WT mice (WT PMCs), *Mcpt4*<sup>-/-</sup> mice (*Mcpt4*<sup>-/-</sup> PMCs), or *Cpa3*<sup>Y356L, E378A</sup> mice (*Cpa3*<sup>Y356L, E378A</sup> PMCs), and the amount of remaining VIP was measured by ELISA. WT PMCs and *Cpa3*<sup>Y356L, E378A</sup> PMCs almost completely degraded VIP under these conditions, whereas *Mcpt4*<sup>-/-</sup> PMCs exhibited little or no ability to degrade VIP (Figure 5).

To assess the role of MCPT4 in the degradation of VIP in vivo, we measured the levels of VIP in blood and ear tissue after i.d. injection of 5 nmol of helodermin versus VIP in the ear pinnae of WT mice, mast cell-deficient *Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice, WT BMCMC-engrafted *Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice, and *Mcpt4*<sup>-/-</sup> mice (Supplemental Figure 4). After injection of VIP, the amount of VIP in either sera or ears was significantly higher in *Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice than in WT mice or WT BMCMC→*Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice, whereas *Mcpt4*<sup>-/-</sup> mice exhibited significantly higher concentrations of VIP in the sera and injected ears than did WT mice, but the levels were not as high as those in *Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice. The latter result suggests that mast cell functions other than those related to the cell's content of MCPT4 may contribute to the ability of mast cells to reduce levels of VIP in vivo. We also found that the concentrations of VIP in the sera and ears did not differ between WT mice injected with helodermin or control mice not injected with peptide. These results indicate that MCPT4 contributes, at least in part, to the mast cell-dependent degradation of VIP in vivo as well as in vitro, and that i.d. injection of helodermin does not detectably increase local or systemic concentrations of endogenous VIP.

*Mass spectrometry evidence that MCPT4 can degrade VIP or helodermin.* Since we could not detect helodermin using our ELISA for VIP, we performed mass spectrometry on samples prepared in the same way as described above for analysis by ELISA. We identified 4 cleavage sites in VIP (Figure 6A) and 5 in helodermin (Figure 6B) that were detected after incubation of the peptides with purified WT PMCs but not *Mcpt4*<sup>-/-</sup> PMCs. Two of the cleavage sites we

identified, 1 in VIP and 2 in helodermin (at a Phe-Thr and a Tyr-Leu), were previously predicted to be susceptible to cleavage by chymase (47), and 1 of these (at Tyr-Leu) is a previously reported chymase cleavage site in VIP (30). We also found a single cleavage site in VIP that was observed after incubation of the peptide with PMCs from either WT or *Mcpt4*<sup>-/-</sup> mice (Figure 6A), probably reflecting the action of a mast cell-associated protease other than MCPT4, perhaps tryptase, which has been reported to cleave VIP at Arg-Lys (30, 31).

*Evidence that MCPT5 does not contribute significantly to the mast cell's ability to detoxify helodermin or VIP.* By mass spectrometry, we found Val and Ala cleavage sites in VIP or helodermin that would be predicted to be susceptible to MCPT5 (which has elastolytic activity) (48) but not to the chymase MCPT4 (47). We previously showed that the absence of MCPT4 in *Mcpt4*<sup>-/-</sup> mice does not result in reduced expression or storage of other mast cell proteases (including MCPT5) (41, 49, 50). However, to investigate the potential role of MCPT5 in degrading VIP or helodermin, we tested the ability of PMCs from *Cpa3*-deficient (*Cpa3*<sup>-/-</sup>) mice, which lack both CPA3 and MCPT5 proteins (51), to degrade VIP in vitro. We found that *Cpa3*<sup>-/-</sup> PMCs, like WT PMCs, almost completely degraded VIP under the conditions tested (Figure 7A). We then challenged *Cpa3*<sup>-/-</sup> versus WT mice i.d. with 5 nmol (19.2  $\mu$ g) of helodermin in vivo and found that the body temperature change and the rate of diarrhea in *Cpa3*<sup>-/-</sup> mice injected with helodermin were not statistically different from those in the identically treated WT mice (Figure 7B). We also confirmed that the number of mast cells, as well as the extent of helodermin-induced mast cell degranulation in the ear pinnae, in *Cpa3*<sup>-/-</sup> mice were similar to those in the other groups of mice tested (Supplemental Figures 2 and 3). These results indicate that the contributions of MCPT5 to the degradation of helodermin or VIP are likely to be relatively minor compared with the effects of MCPT4. However, as we have speculated previously (40), it is possible that MCPT5 (and perhaps other proteases) can gain access to these substrates once MCPT4 has performed initial cleavages of the peptides.

*Mast cells and MCPT4 can limit the toxicity of the venoms of 2 species of scorpion.* To assess the potential roles of mast cells and their proteases in the pathology and mortality induced by venoms from animals other than reptiles, we next tested venoms derived from 2 medically important scorpions, 1 from the Old World, the deathstalker (yellow) scorpion (*L. quinquestriatus hebraeus*) (33–35), and 1 from the New World, the Arizona bark scorpion (*C. exilicauda*) (35–37). Based on results obtained in preliminary experiments to assess the responses of WT or mast cell-deficient mice to various amounts of each type of venom (Supplemental Figure 5), we elected to inject mice with 7.5  $\mu$ g of each type of venom in subsequent experiments.

Mast cell-deficient *Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice injected i.d. with 7.5  $\mu$ g of *L. quinquestriatus hebraeus* venom exhibited significantly lower body temperatures than WT mice, and all of them died approximately 1 hour after *L. quinquestriatus hebraeus* venom injection (Figure 8A). In contrast, all WT *Kit*<sup>+/+</sup> mice survived and fully recovered. Moreover, engraftment of *Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice with WT BMCMCs resulted in levels of protection against hypothermia and death that were statistically indistinguishable from those in WT mice (Figure 8A). Similar results were obtained when the same experiments were performed in C57BL/6-*Kit*<sup>+/+</sup>, C57BL/6-*Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup>, and WT BMCMC-engrafted *Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice (Figure 8C). Like WT mice, all *Cpa3*<sup>Y356L, E378A</sup> mice survived after i.d. injection of 7.5  $\mu$ g of *L. quinquestriatus hebraeus*



**Figure 6**

Mast cell chymase can degrade either VIP or helodermin. (A) VIP (125 μM in 150 μl) or (B) helodermin (125 μM in 150 μl) was incubated *ex vivo* at 37°C for 30 minutes with medium (DMEM) alone (no mast cells) or with medium containing purified PMCs (8 × 10<sup>5</sup>) from WT C57BL/6 mice (WT PMCs), C57BL/6-*Mcpt4*<sup>-/-</sup> mice (*Mcpt4*<sup>-/-</sup> PMCs), or C57BL/6-*Cpa3*<sup>Y356L,E378A</sup> mice (*Cpa3*<sup>Y356L,E378A</sup> PMCs); supernatants were then analyzed by mass spectrometry. Red arrows indicate cleavage sites found in (A) VIP or (B) helodermin after incubation with WT PMCs but not *Mcpt4*<sup>-/-</sup> PMCs. The long blue arrow indicates the single cleavage site in VIP that was found after incubation with either WT or *Mcpt4*<sup>-/-</sup> PMCs. White arrows indicate sites predicted to be susceptible to cleavage, and the dashed arrow indicates a previously reported cleavage site. The figure depicts results obtained in 2 independent experiments that gave similar results.

venom, whereas 7 of 16 *Mcpt4*<sup>-/-</sup> mice died (Figure 8B). Roughly half (8 of 15) of the *Mcpt4*<sup>-/-</sup> BMCMC-engrafted *Kit*<sup>W-sh/W-sh</sup> mice also died upon injection of *L. quinquestriatus hebraeus* venom, versus 0 of 12 WT mice and 2 of 15 WT BMCMC-engrafted *Kit*<sup>W-sh/W-sh</sup> mice (Figure 8C). Similar results were obtained when the various groups of mice were injected *i.d.* with 7.5 μg of *C. exilicauda* venom, except that roughly half of the mast cell-deficient mice survived this dose of venom, whereas all mast cell-deficient mice injected with the same amount of *L. quinquestriatus hebraeus* venom died (Figure 9). We also found that, like Gila monster venom, venom from either scorpion induced extensive degranulation of mast cells upon injection into the ear pinnae of WT, *Mcpt4*<sup>-/-</sup>, or *Cpa3*<sup>Y356L,E378A</sup> mice (Supplemental Figure 6).

**Discussion**

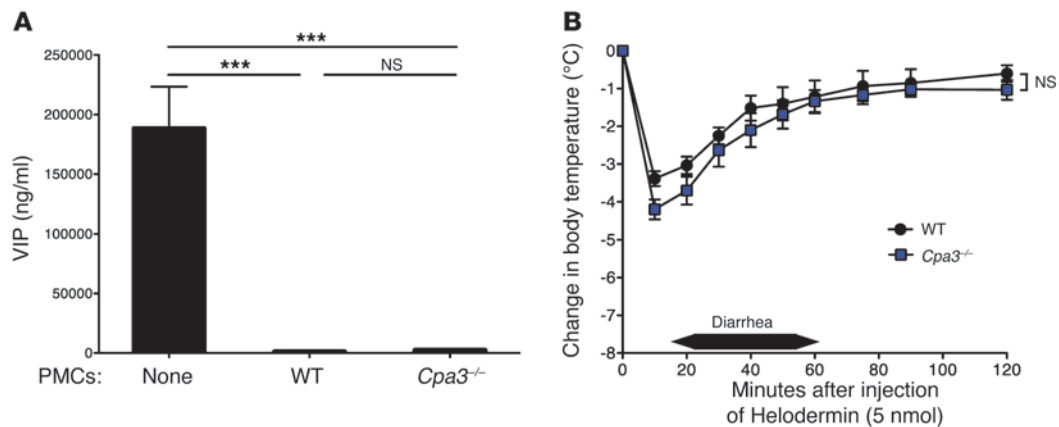
We found that 2 types of genetically mast cell-deficient mice, WBB6F1-*Kit*<sup>W/W-v</sup> and C57BL/6-*Kit*<sup>W-sh/W-sh</sup> mice, and C57BL/6-*Mcpt4*<sup>-/-</sup> mice, were more susceptible than the corresponding WT mice to the morbidity and mortality induced by the *i.d.* injection of the venoms of the Gila monster or of 2 species of scorpion. Our evidence indicates that mast cell-associated MCPT4 contributes to the ability of mast cells to enhance resistance to the toxicity of both helodermin, the major VIP-like peptide in Gila monster venom, and mammalian VIP, the structurally similar endogenous peptide.

Local engraftment of mast cell-deficient mice with WT mast cells only at the site of venom or peptide injection enhanced resistance to the toxicity induced by Gila monster or scorpion venoms, helodermin, or VIP to a greater extent than did engraftment with *Mcpt4*<sup>-/-</sup> BMCMCs. Thus, a relatively small population of mast cells containing MCPT4 appears to be sufficient, at least if these cells are present at the site of envenomation, to increase the mouse's ability to withstand the toxic effects of such venoms. Moreover, each of the venoms or toxic peptides tested induced extensive degranulation of skin mast cells at the site of injection. Finally, we found that WT mast cells were much more effective than *Mcpt4*<sup>-/-</sup> mast cells in degrading helodermin or VIP *in vitro*.

Together, these findings indicate that mast cells can enhance host resistance to the toxicity of these lizard and scorpion venoms, or to helodermin or VIP, at least partly through the local release of MCPT4, which can then degrade venom components. In contrast, our experiments in mice whose CPA3 lacked catalytic activity, or in *Cpa3*<sup>-/-</sup> mice, which lack both CPA3 and MCPT5 proteins, indicate that CPA3 and MCPT5 make substantially less or no contribution to resistance to these particular venoms or toxic peptides.

We think that these findings help to illuminate how mast cells can influence 2 different areas of biology: (a) innate host resistance to animal venoms and (b) regulation of the toxicity of endogenous biologically active peptides. Host resistance to the toxicity of animal venoms probably reflects the contribution of many different factors (52, 53); however, the mast cell has only recently been shown to deserve a place on that list (6, 7). Indeed, the induction of mast cell degranulation at sites of envenomation until recently has been thought to contribute to the pathology induced by the venom (6, 54). Notably, Higginbotham proposed in 1965 that mast cell heparin might increase resistance to Russell's viper venom (55) and in 1971 that mast cell heparin might also confer resistance to honeybee stings (56). Unfortunately, mast cell-deficient mice had not yet been reported when those studies were published, and the idea that mast cells conferred more benefit than harm in snake or honeybee envenomation only recently was tested (and confirmed) using mast cell-engrafted genetically mast cell-deficient mice (6). In the case of mice injected with *A. engaddensis* venom, several lines of evidence indicate that CPA3 is the major mast cell protease that enhances host resistance (6, 7). Pharmacological evidence suggests that CPA3 also contributes to the mouse mast cell-dependent enhancement of resistance to the venoms of the Western diamond-back rattlesnake and the Southern copperhead (6).

In contrast, the evidence presented herein indicates that MCPT4 is more important than CPA3 in the mast cell-dependent enhancement of the resistance of mice to the venoms of the Gila monster or of 2 medically important scorpions, the deathstalker (yellow) scorpion (*L. quinquestriatus hebraeus*) and the Arizona bark



**Figure 7**

Evidence that MCPT5 does not contribute significantly to the mast cell's ability to degrade and detoxify helodermin or VIP. **(A)** VIP (125  $\mu$ M in 150  $\mu$ l) was incubated *ex vivo* at 37°C for 30 minutes with medium (DMEM) alone (no mast cells), or with medium containing purified PMCs ( $8 \times 10^5$ ) from WT mice (WT PMCs) or *Cpa3*<sup>-/-</sup> mice, which lack both CPA3 and MCPT5 (*Cpa3*<sup>-/-</sup> PMCs). The remaining amount of VIP was then measured by ELISA. \*\*\* $P < 0.001$ ; NS ( $P > 0.05$ ) for the comparisons shown (Mann-Whitney *U* test). The figure shows data pooled from the 4 independent experiments we performed, each of which gave similar results ( $n = 4$  determinations per group). Data are presented as mean + SEM. **(B)** Changes in rectal temperatures after *i.d.* injection of helodermin (5 nmol in 20  $\mu$ l DMEM solution) in the ear pinnae (1 ear pinna of each mouse) of WT and *Cpa3*<sup>-/-</sup> mice. NS ( $P > 0.05$ ) for the comparisons shown. Rates of diarrhea in *Kit*<sup>+/+</sup> and *Cpa3*<sup>-/-</sup> mice within 2 hours after helodermin injection were 17% (1/6) and 33% (2/6), respectively.  $P = 0.5$ . Data are presented as mean  $\pm$  SEM. Each panel shows data pooled from 3 independent experiments with each group of mice, each of which gave similar results ( $n = 2$  mice per group per experiment).

scorpion (*C. exilicauda*). Taken together, our studies of the ability of mast cells to enhance resistance to the venoms of 3 poisonous snakes and 1 venomous lizard, as well as 3 venomous arthropods, suggest that the ability of mast cells to store and rapidly release large amounts of proteases with distinct profiles of substrate specificity may permit these cells to contribute to innate host defense against a variety of animal venoms containing diverse toxic substances. This, of course, does not rule out the possibility that the induction of mast cell degranulation by the venoms of some species of poisonous animals can increase the pathology associated with such envenomation. However, no such example has yet been reported based on studies in mice deficient in mast cells or individual mast cell-associated proteases.

Many animal venoms contain peptides that have structural and functional similarities to endogenous mammalian peptides (12, 57), and many of these venom-associated peptides can bind to the receptors for the corresponding mammalian peptides (58). It appears likely that convergent evolution, rather than an origin from a common ancestral gene, explains the presence of some of these peptides in reptile venoms, including helodermin (12) and sarafotoxin 6b (59). Indeed, helodermin has been detected only in the salivary glands of the Gila monster, whereas a VIP-like peptide that may represent a true ortholog of mammalian VIP can be detected in other Gila monster tissues (12). Thus, the ability of the Gila monster to concentrate large amounts of helodermin in its salivary secretions may have developed in large part so that the lizard's venom can activate VIP receptors in cells of the lizard's prey, with effects that induce disability or death in the prey animal.

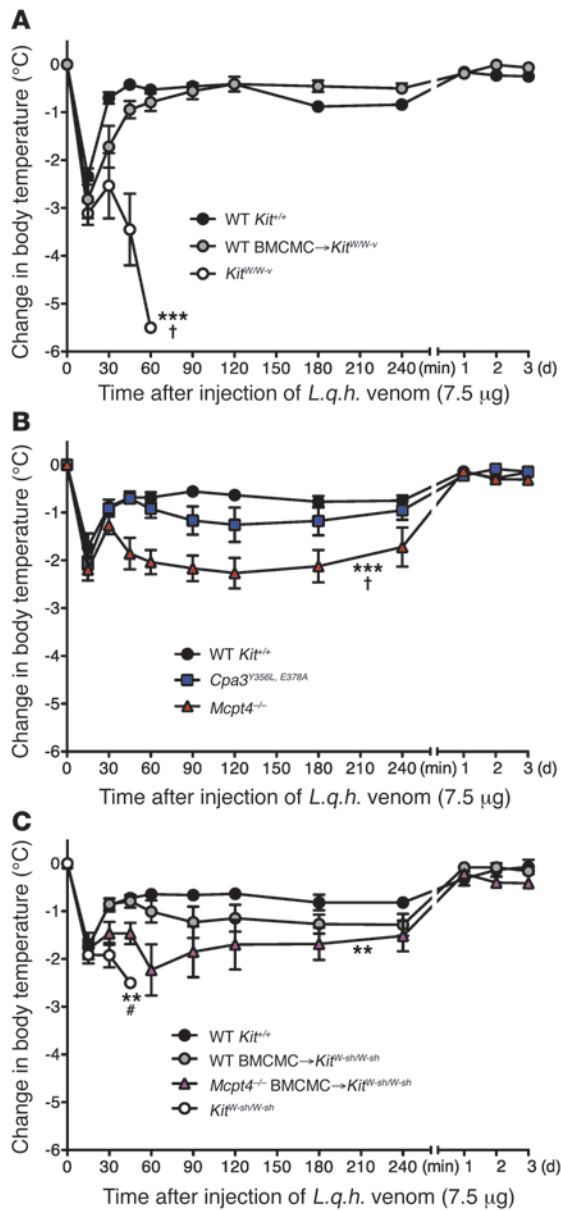
Endogenous peptides such as VIP and ET-1 also can induce pathology when produced inappropriately and/or in excessively high amounts, and therefore it is important to have regulatory mechanisms to diminish the concentrations of such peptides or to counteract their effects. We previously reported evidence that mast cells can reduce the pathology and mortality associated with

high levels of ET-1 (4, 6, 7) or neurotensin (5) by mechanisms that involve CPA3 (6, 7) or neurolysin (5), respectively. In the present study, we found that mast cell-associated MCPT4 can degrade VIP and reduce the hypothermia and diarrhea observed in mice injected with large amounts of this peptide.

There are at least 2 implications of these findings. First, they support the general hypothesis that the ability of mast cells to produce a diverse complement of enzymes may be useful in permitting these cells to reduce the potential toxicity of a variety of endogenous peptides that can activate mast cell degranulation when present in high enough concentrations, as well as to degrade and limit the toxicity of structurally and functionally similar peptides that are present in animal venoms. More specifically, our findings suggest that mast cell-dependent degradation of VIP may either help to limit the pathology associated with excessive levels of this peptide, such as when it is produced by a tumor (44, 45) and perhaps in other settings, and/or may contribute to pathology associated with abnormally low levels of this peptide. For example, reduced levels of VIP have been reported in the airways of patients with severe asthma (60), and VIP-deficient mice exhibit airway hyperresponsiveness and inflammation that can be partially rescued by administration of VIP (61). Taken together, these findings raise the possibility that mast cell-dependent degradation of VIP might contribute to pathology in some settings, including asthma.

Mast cells have long been viewed as important contributors to disease, both in the context of anaphylaxis and allergic diseases and in many other disorders (62–65). The findings presented herein and in our prior work (4–7) indicate that the expression by mast cells of receptors for VIP, ET-1, neurotensin, and other endogenous peptides that can trigger mast cell degranulation, when combined with the mast cell's ability to produce enzymes that can degrade these and related peptides, permits mast cells to contribute to health in 2 different contexts: reducing the toxicity associated with high concentrations of the endogenous peptides





**Figure 8**

Mast cells and MCPT4 can limit the toxicity of deathstalker (yellow) scorpion (*L. q.h.*). Changes in rectal temperature after i.d. injection of *L. quinquestriatus hebraeus* venom (7.5 µg in 50 µl of PBS) into a single ear pinna. **(A)** WT WBB6F<sub>1</sub>-*Kit*<sup>+/+</sup>, mast cell-deficient WBB6F<sub>1</sub>-*Kit*<sup>W/W-v</sup>, and WT BMCMC→*Kit*<sup>W/W-v</sup> mice. Death rates of *Kit*<sup>+/+</sup>, WT BMCMC→*Kit*<sup>W/W-v</sup>, and *Kit*<sup>W/W-v</sup> mice within 3 days after *L. quinquestriatus hebraeus* venom injection were 0% (0/10), 10% (1/10; *P* = 0.5 versus *Kit*<sup>+/+</sup> mice), and 100% (10/10, *P* < 0.0001 versus *Kit*<sup>+/+</sup> mice), respectively. **(B)** C57BL/6 WT, *Cpa3*<sup>Y356L,E378A</sup>, and *Mcpt4*<sup>-/-</sup> mice. Death rates of *Kit*<sup>+/+</sup>, *Cpa3*<sup>Y356L,E378A</sup>, and *Mcpt4*<sup>-/-</sup> mice within 3 days after *L. quinquestriatus hebraeus* venom injection were 0% (0/12), 0% (0/15; *P* = 1.0 versus *Kit*<sup>+/+</sup> mice), and 44% (7/16; *P* = 0.01 versus *Kit*<sup>+/+</sup> mice), respectively. **(C)** WT C57BL/6-*Kit*<sup>+/+</sup>, mast cell-deficient C57BL/6-*Kit*<sup>W-sh/W-sh</sup>, WT BMCMC→*Kit*<sup>W-sh/W-sh</sup>, and *Mcpt4*<sup>-/-</sup> BMCMC→*Kit*<sup>W-sh/W-sh</sup> mice. Death rates of *Kit*<sup>+/+</sup>, WT BMCMC→*Kit*<sup>W-sh/W-sh</sup>, *Mcpt4*<sup>-/-</sup> BMCMC→*Kit*<sup>W-sh/W-sh</sup>, and *Kit*<sup>W-sh/W-sh</sup> mice within 3 days after *L. quinquestriatus hebraeus* venom injection were 0% (0/11), 13% (2/15; *P* = 0.32 versus *Kit*<sup>+/+</sup> mice), 57% (8/15, *P* = 0.004 versus *Kit*<sup>+/+</sup> mice), and 100% (15/15, *P* < 0.0001 versus *Kit*<sup>+/+</sup> mice), respectively. \*\**P* < 0.01; \*\*\**P* < 0.001 versus WT WBB6F<sub>1</sub>-*Kit*<sup>+/+</sup> or WT C57BL/6-*Kit*<sup>+/+</sup> mice; †*P* < 0.01 versus each of the other groups (A and B); #*P* < 0.01 versus WT BMCMC→*Kit*<sup>W-sh/W-sh</sup> mice (C). Each panel shows data pooled from at least 3 independent experiments with each group of mice (*n* = 2–5 mice per group per experiment). Data are presented as mean ± SEM.

highly sensitized subjects to such venoms can result in anaphylaxis (68, 73, 75). However, the possibility should be considered that the presence of anti-venom IgE may further increase the ability of mast cell degranulation to enhance resistance to such venoms, at least in those subjects whose antibody-dependent reactions to such venoms stop short of anaphylaxis.

**Methods**

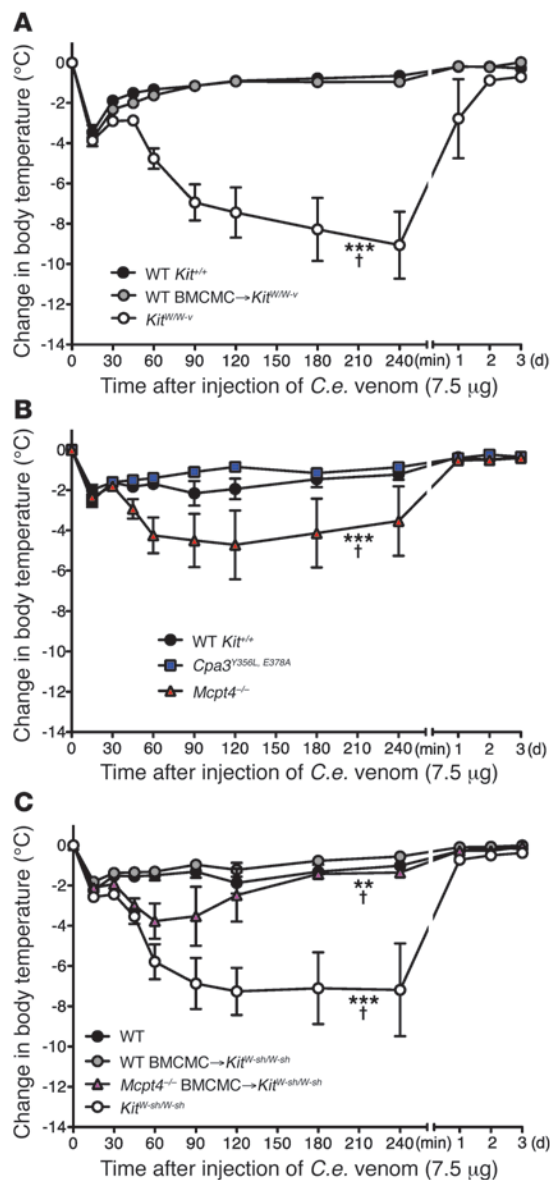
**Mice.** *c-Kit* mutant genetically mast cell-deficient (WB/ReJ-*Kit*<sup>W/W</sup> × C57BL/6J-*Kit*<sup>W/W-v</sup>)F<sub>1</sub>-*Kit*<sup>W/W-v</sup> (WBB6F<sub>1</sub>-*Kit*<sup>W/W-v</sup>) mice, congenic normal WBB6F<sub>1</sub>-*Kit*<sup>+/+</sup> (*Kit*<sup>+/+</sup>) mice, and C57BL/6J mice were purchased from Jackson Laboratory. C57BL/6-*Kit*<sup>W-sh/W-sh</sup> mice were originally provided by Peter Besmer (Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York, USA); these mice were then backcrossed to C57BL/6J mice for more than 11 generations. *Mcpt4*<sup>-/-</sup> mice (41), *Cpa3*<sup>-/-</sup> mice (51), and *Cpa3*<sup>Y356L,E378A</sup> mice (7) on the C57BL/6 background were bred and maintained at the Stanford University Research Animal Facility. Both adult *Kit*<sup>W/W-v</sup> mice and adult *Kit*<sup>W-sh/W-sh</sup> mice have a profound deficiency of mast cells, including less than 1% WT levels of mast cells in the skin (39, 76, 77). All animal care and experimentation were conducted in compliance with the guidelines of the NIH and with the specific approval of the Institutional Animal Care and Use Committee of Stanford University.

**Mast cell engraftment.** Selective engraftment of mast cells in mast cell-deficient WBB6F<sub>1</sub>-*Kit*<sup>W/W-v</sup> or C57BL/6-*Kit*<sup>W-sh/W-sh</sup> mice was performed as described (39). Bone marrow cells derived from WBB6F<sub>1</sub>-*Kit*<sup>+/+</sup>, C57BL/6-*Kit*<sup>+/+</sup>, or C57BL/6-*Mcpt4*<sup>-/-</sup> mice (female, 4–6-week-old) were cultured in IL-3-containing medium for 4–6 weeks to generate cell populations that contained more than 95% immature mast cells. 2.0 × 10<sup>6</sup> BMCMCs were injected i.d. into each mouse (2 injections into 1 ear pinna; 1.0 × 10<sup>6</sup> cells in 25 µl DMEM per injection), and the recipients were used for experiments 6–8 weeks later.

**Chemicals and venoms.** We purchased VIP and VIP<sub>6-28</sub> (Bachem), *H. suspectum* venom (Sigma-Aldrich), helodermin (Abgent), *L. quinquestriatus hebraeus* venom (Latoxan), and *C. exilicauda* venom (Spider Pharm Inc.) from the manufacturers.

**Measurement of VIP.** VIP levels in the supernatants were measured by ELISA in accordance with the manufacturer’s instructions (Bachem).

and limiting the pathology induced by structurally and functionally similar peptides contained in animal venoms. Indeed, we find it appealing to speculate that a mast cell-dependent mechanism that may have developed primarily to help to restore homeostasis in the face of conditions associated with elevated levels of peptides such as VIP and ET-1 (namely, the activation of mast cells by such peptides, inducing the release of proteases that can degrade the peptide) may also provide a selective advantage by enhancing host resistance to structurally and functionally similar peptides in animal venoms. It is tempting to speculate further that the occurrence of large numbers of mast cells in the skin, a frequent site of envenomation, in part reflects evolutionary pressure to position these cells where they can rapidly respond to, and thereby help to limit the toxicity of, the venoms of poisonous invertebrates and reptiles. Finally, the development of IgE antibodies to venom components has been reported for snake (66–68), honeybee (69–71), and scorpion (72–74) venoms. It is well known that exposure of



**Histology and quantification of mast cells and mast cell degranulation.** Mice were sacrificed by CO<sub>2</sub> inhalation, and samples of ear pinna were fixed in 10% (v/v) buffered formalin, embedded in paraffin (ensuring a cross-sectional orientation), cut into 4-μm-thick sections, and stained with 0.1% (v/v) toluidine blue, pH 1, for the detection of mast cells (cytoplasmic granules appeared purple). Sections were “coded” so the evaluator was not aware of their identity. Ear pinna mast cells were counted in 5–8 consecutive fields, each with dimensions of 760 × 1020 μm, with a ×10 microscope objective (final magnification, ×100), and mast cells per horizontal ear cartilage field length (mm) were counted by computer-generated image analysis (NIH ImageJ software, version 1.29x). Quantification was performed on the entire length of a strip of skin extending from the base to the tip of the ear pinna (about 8–13 mm). After intradermal engraftment of BMCMCs, *Kit*<sup>W-sh/hW-sh</sup> or *Kit*<sup>W/W-v</sup> mice had mast cells from the base to the tip of the ear pinnae in an anatomical distribution similar to that of the native mast cell populations in the corresponding WT mice. Degranulation of mast cells in the ear pinna was quantified as previously described (78) in 1-μm-thick, Giemsa-stained, Epon-embedded sections examined at a final magnifica-

### Figure 9

Mast cells and MCPT4 can limit the toxicity of Arizona bark scorpion (*C.e.*). Changes in rectal temperature after i.d. injection of *C. exilicauda* venom (7.5 μg in 50 μl of PBS) into a single ear pinna. (A) WT WBB6F<sub>1</sub>-*Kit*<sup>+/+</sup>, mast cell-deficient WBB6F<sub>1</sub>-*Kit*<sup>W/W-v</sup>, and WT BMCMC→*Kit*<sup>W/W-v</sup> mice. Death rates of *Kit*<sup>+/+</sup>, WT BMCMC→*Kit*<sup>W/W-v</sup>, and *Kit*<sup>W/W-v</sup> mice within 3 days after *C. exilicauda* venom injection were 0% (0/11), 0% (0/10;  $P = 0.1$  versus *Kit*<sup>+/+</sup> mice), and 50% (5/10;  $P = 0.01$  versus *Kit*<sup>+/+</sup> mice), respectively. (B) C57BL/6 WT, *Cpa3*<sup>Y356L,E378A</sup>, and *Mcpt4*<sup>-/-</sup> mice. Death rates of *Kit*<sup>+/+</sup>, *Cpa3*<sup>Y356L,E378A</sup>, and *Mcpt4*<sup>-/-</sup> mice within 3 days after *C. exilicauda* venom injection were 0% (0/11), 0% (0/13;  $P = 1.0$  versus *Kit*<sup>+/+</sup> mice), and 42% (5/12,  $P = 0.01$  versus *Kit*<sup>+/+</sup> mice), respectively. (C) WT C57BL/6-*Kit*<sup>+/+</sup>, mast cell-deficient C57BL/6-*Kit*<sup>W-sh/W-sh</sup>, WT BMCMC→*Kit*<sup>W-sh/W-sh</sup>, and *Mcpt4*<sup>-/-</sup> BMCMC→*Kit*<sup>W-sh/W-sh</sup> mice. Death rates of *Kit*<sup>+/+</sup>, WT BMCMC→*Kit*<sup>W-sh/W-sh</sup>, *Mcpt4*<sup>-/-</sup> BMCMC→*Kit*<sup>W-sh/W-sh</sup>, and *Kit*<sup>W-sh/W-sh</sup> mice within 3 days after *C. exilicauda* venom injection were 0% (0/12), 0% (0/12;  $P = 1.0$  versus *Kit*<sup>+/+</sup> mice), 50% (6/12,  $P = 0.007$  versus *Kit*<sup>+/+</sup> mice), and 58% (7/12,  $P = 0.002$  versus *Kit*<sup>+/+</sup> mice), respectively. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  versus WT WBB6F<sub>1</sub>-*Kit*<sup>+/+</sup> or WT C57BL/6-*Kit*<sup>+/+</sup> mice; † $P < 0.01$  to 0.001 versus each of the other groups (A–C). Each panel shows data pooled from at least 3 independent experiments with each group of mice ( $n = 2–5$  mice per group per experiment). Data are presented as mean ± SEM.

tion of ×1000, with the results expressed as percentage of mast cells exhibiting evidence of degranulation scored as follows: extensive (>50% of granules in that cell exhibiting evidence of degranulation); moderate (10%–50% of granules in that cell exhibiting evidence of degranulation); and none (<10% of granules in that cell exhibiting evidence of degranulation).

**Purification of PMCs.** Mice were sacrificed by CO<sub>2</sub> inhalation. To harvest peritoneal cells, 10 ml of HBSS buffer containing 10 U/ml heparin and 10% FCS were injected into the peritoneal cavity, and the abdomen was massaged gently for 30–60 seconds. Peritoneal cells were layered onto 1.5 ml of 23% HistoDenz (Sigma-Aldrich) in HBSS buffer containing 10 U/ml heparin and 10% FCS and centrifuged at 469 g for 15 minutes at room temperature. After washing, cells were stained with toluidine blue, and PMCs represented 70%–90% of the cells recovered.

**β-Hexosaminidase release assay.** We stimulated a total of  $1 \times 10^6$  PMCs (from WT or *Mcpt4*<sup>-/-</sup> mice)/ml in Tyrode's buffer (10 mM HEPES buffer [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.1% glucose, and 0.1% BSA [fraction V; Sigma-Aldrich]) with the indicated concentrations of helodermin, VIP, or A23187 calcium ionophore (Sigma-Aldrich) for 30 minutes at 37°C with or without pretreatment (10 minutes before) with the VIP receptor antagonist VIP<sub>6–28</sub>; supernatants were collected after centrifugation. We used supernatants from nonstimulated PMCs treated with 10 μl of 0.5% (v/v) Triton X-100 (Sigma-Aldrich) to determine the maximal (100%) cellular β-hexosaminidase content, to which the experimental samples were normalized. We calculated the percentage of β-hexosaminidase release using an enzyme immunoassay with p-nitrophenyl-N-acetyl-β-D-glucosamine (p-NAG; Sigma-Aldrich) substrate as follows: 10 μl of culture supernatant was added to the wells of a 96-well flat-bottomed plate. 50 μl of 1.3 mg/ml p-NAG solution in 100 mM sodium citrate, pH 4.5, was added, and the plate was incubated for 60 minutes at 37°C. We then added 150 μl of 200 mM glycine, pH 10.7, to stop the reaction and measured the OD<sub>405</sub>.

**Measurement of VIP in sera and ear skin lysates.** Blood and ear tissue samples were obtained 30 minutes after i.d. injection into the right ear pinnae of 5 nmol of helodermin or VIP. We prepared ear skin lysates by sonicating finely chopped ear tissue in 400 μl T-PER EDTA-free lysis buffer (Pierce) containing protease inhibitors (Roche). Samples were frozen at -80°C overnight, then thawed and centrifuged at 16000 g for 20 minutes at 4°C; supernatants were collected. We measured VIP concentrations in the supernatants by ELISA (Bachem).



**Fourier transform mass spectrometry.** Cleavage reactions of VIP and helodermin peptides were separated on a 150 mm × 0.075 mm Zorbax C18 reversed phase column (Agilent), with a 5 μM particle size and 300 Å pore size, run at a flow rate of 300 nl/min in 0.1% trifluoroacetic acid/water (A):0.1% trifluoroacetic acid/acetonitrile (B). The column was run on a gradient of 3% B to 30% B for 35 minutes, 35% B to 90% B for 10 minutes, held at 90% B for 2 minutes, then rapidly decreased to 3% B over 0.1 minute, and reequilibrated at 3% for 20 minutes. Eluted peptides were run on a Thermo LTQ-FT mass spectrometer using Fourier transform mass spectrometry (FTMS) + p ESI Full MS scanning mode, mass range 400–1800; FT resolution was set to 100,000. The monoisotopic masses of the major peaks were observed and then searched against the known sequence of VIP and helodermin peptides to identify the cleavage products. The identity of the cleavage products was confirmed with MS/MS fragmentation and spectral matching. To compare the experimental and control samples, extracted ion chromatograms were prepared for the major peaks in the experimental runs; then the same extracted ion chromatograms were used to compare to the control sample.

**Statistics.** The differences in the percentages of groups of mice that died within 24 hours after injection with helodermin, VIP, or various venoms were compared by Fisher's exact test, whereas ANOVA for repeated measures was used to assess differences in body temperature responses. Unless

otherwise specified, all other data were tested for statistical significance using the unpaired 2-tailed Student's *t* test or, to compare values for the extent of mast cell degranulation, the  $\chi^2$  test. *P* < 0.05 was considered statistically significant.

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