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Spider venom: enhancement of venom efficacy mediated by different synergistic strategies in *Cupiennius salei*

Benno Wullschleger, Wolfgang Nentwig and Lucia Kuhn-Nentwig*
Zoological Institute, University of Bern, Baltzerstrasse 6, CH-3012 Bern, Switzerland
*Author for correspondence (e-mail: lucia.kuhn@zos.unibe.ch)

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Summary

Besides the power of the chelicerae, synergistic interactions between different components in the venom of *Cupiennius salei* ensure the hunting success of this spider. The main components of the venom were tested alone or in combination according to their physiological venom concentrations in *Drosophila* bioassays. The high K^+ ion content of the venom synergistically increases the insecticidal activity of the neurotoxins CSTX-1, CSTX-9 and CSTX-13 by 20% but does not influence the insecticidal effectiveness of the antimicrobially and cytolytically acting cupiennin 1a. Histamine only enhances the activity of the main neurotoxin CSTX-1. An important role in the envenomation process is exhibited by cupiennin 1a, which increases the insecticidal activity of the above-mentioned neurotoxins by up to 65%. Additionally, the highly synergistic effect of the enhancer CSTX-13 on CSTX-1, provoked in non-toxic physiological concentrations, could be verified for CSTX-9, but not for cupiennin 1a. CSTX-1 and CSTX-9 show positive interactions only when both are injected in toxic non-physiological concentrations.

Key words: neurotoxin, *Drosophila melanogaster*, bioassay, synergism, multicomponent venom.

Introduction

Spider venoms are complex mixtures of components used to paralyse prey items and defend against predators. Rapid paralysis results from the modifications of various ion channel targets by low molecular mass compounds, neurotoxic peptides and proteins. However, much less is known about the interactions of venom components within a prey organism (Adams, 2004; Kuhn-Nentwig et al., 2004).

The Central American spider *Cupiennius salei* (Keyserling 1877) uses its venom as economically as possible. The amount of venom injected varies depending on size, activity, defense behaviour and venom sensitivity of a prey item (Malli et al., 1999; Wigger et al., 2002; Wullschleger and Nentwig, 2002). This optimal venom dosage is continued on the biochemical level through positive interactions among various venom components (Kuhn-Nentwig et al., 1998; Wullschleger et al., 2004). In *C. salei* venom, proteins with molecular masses above 10 kDa have been identified. Additionally, disulfide-rich neurotoxins, highly cationic peptides with molecular masses between 3–10 kDa, and low molecular mass compounds, such as ions, biogenic amines, polyamines and neurotransmitters, are present (Kuhn-Nentwig et al., 2004).

To date, toxicological information and sequence data for the neurotoxins CSTX-1 and CSTX-9, and the neurotoxic two-chain enhancer peptide CSTX-13 have been reported (Kuhn-Nentwig et al., 2004; Wullschleger et al., 2004). Furthermore, antimicrobially and cytolytically acting cupiennins have been identified. These highly cationic, α-helical, cysteine-free peptides may play a dual role in the venom: protection of the venomous apparatus against microbial invaders and, after venom injection into prey, an enhancement of the paralytic effect of the neurotoxins (Kuhn-Nentwig, 2003). Insecticidal activities of similar cytolytically acting peptides have also been reported for the spider *Lycosa carolinensis* (Yan and Adams, 1998) and the ant *Pachycondyla goeldii* (Orivel et al., 2001). Beyond these insecticidal activities, additional synergistic interactions with neurotoxins have been demonstrated for the spiders *Oxyopes kitabensis* (Corzo et al., 2002) and *C. salei* (Kuhn-Nentwig et al., 2004).

Only limited information is available about possible synergistic effects of low molecular mass substances with neurotoxins immediately after venom injection into a prey item (Chan et al., 1975; Inceoglu et al., 2003; Wullschleger et al., 2004). It was previously shown that histamine and taurine facilitate the neurotoxic activity of CSTX-1 from *C. salei* (Kuhn-Nentwig et al., 1998). Accordingly, it was also hypothesised that μ-agatoxins, which are disulfide-bridged short peptides modifying Na^+ channels, enhance the short-term action of α-agatoxins (acylpolyamines). Both agatoxins have been identified in the venom of the spider *Agelenopsis aperta.* Furthermore, additive interactions among different ω-agatoxins, which are disulfide-bridge-rich voltage activated Ca^{2+} channel inhibitors, have been reported for *A. aperta*

(McDonough et al., 2002; Adams, 2004). However, these findings were mainly obtained through neurophysiological investigations and bioassays of different venom components without considering their physiological ratios as they occur in the venom.

In the study reported here, we analysed interactions among the main low molecular mass components and the most important identified peptides (Kuhn-Nentwig et al., 2004) as well as interactions between these peptides. Finally, we compared the paralytic activity of crude venom to the synergistic activity caused by defined venom components. The findings show multiple interactions of venom components in different molar ratios and help us to understand the complex nature of spider venom.

Materials and methods

Peptide isolation

Maintenance of *Cupiennius salei* in our laboratory, venom collection and isolation of CSTX-1, CSTX-9, CSTX-13 and cupiennin 1a were carried out as previously described (Kuhn-Nentwig et al., 1994, 2002). Chemicals were of analytical grade and purchased from Merck (Germany), and histamine and taurine from Sigma (St Louis, MO, USA).

Bioassays

The LD_{50} bioassays were performed according to Escoubas et al. (1995) using 1–3 day old *Drosophila melanogaster* female flies. The injection was always applied into the mesothorax laterally and the injected volume was 0.05 µl of 0.1 mol l^{-1} ammonium acetate, pH 6.1. As control, for each assay 20 flies were injected with this solution. All further injections with different components were also carried out in 0.05 µl of 0.1 mol l^{-1} ammonium acetate, pH 6.1 (four concentrations, 20 flies each). Mortality rates were recorded 24 h after injection. LD_{50} bioassays were performed with (1) crude venom, (2) CSTX-1, and (3) CSTX-1 in combination with CSTX-9, CSTX-13, cupiennin 1a, histamine and KCl in their physiological venom concentrations. The physiological venom concentrations and the LD_{50} values of the main low molecular components as well as of the most important identified peptides in the venom of *C. salei* are given in Tables 1 and 2.

Interactions between low molecular mass components and peptides

Interactions between venom peptides and low molecular mass components were investigated in bioassays with 0.315 pmol CSTX-1 mg^{-1} fly, 7.95 pmol CSTX-9 mg^{-1} fly and 5.0 pmol cupiennin 1a mg^{-1} fly. We tested the peptides alone or in combination with histamine (5.7 mmol l^{-1}), taurine (0.07 mmol l^{-1}), and KCl (215 mmol l^{-1}) in their physiological venom concentrations. For statistical analyses the flies were divided in two independent series of 15 groups each with five

Table 1. *The physiological venom concentrations and the LD_{50} values of tested venom components of the spider* C. salei *on* D. melanogaster

	KCl	Histamine	Taurine	CSTX-1	CSTX-9	CSTX-13	Cupiennin 1a
LD_{50} values (95% confidence interval)	102 nmol[§] (91.7–118.4)	51 nmol[§] (44.2–61.2)	>8.9 nmol[§]	0.45 pmol (0.40–0.55)	10.6 pmol (9.5–13.7)	16.3 pmol[§] (14.5–27.5)	5.9 pmol[‡] (4.2–8.3)
Physiological venom concentration	215 mmol l^{-1}*	5.7 mmol l^{-1}*	0.07 mmol l^{-1}*	1.4–3.3 mmol l^{-1}*	0.2–1.1 mmol l^{-1}[†]	0.2–0.4 mmol l^{-1}[§]	≈1.2 mmol l^{-1}[‡]

The physiological concentrations and the LD_{50} values (nmol or pmol mg^{-1} fly) had been reported previously in *Kuhn-Nentwig et al. (1994), [†]Schaller et al. (2001), [‡]Kuhn-Nentwig et al. (2002) and [§]Wullschleger et al. (2004).

Table 2. *Bioassay combinations of various venom peptides of* C. salei *on* D. melanogaster

	Testing the effect of peptide (A)				on the activity of peptide (B)	
Series of experiment			pmol mg^{-1} fly	Molar ratio		pmol mg^{-1} fly
1		Cupiennin 1a	0.53	1:0.6 1:15 1:23.8	CSTX-1 CSTX-9 CSTX-13	0.315 7.95 12.6
2	(i)	CSTX-9	0.06	1:5.7	CSTX-1	0.315
	(ii)	CSTX-9	7.95	1:0.06	CSTX-1	0.47
3	(i)	CSTX-13	0.035	1:9	CSTX-1	0.315
	(ii)	CSTX-13	0.035	1:227	CSTX-9	7.95
	(iii)	CSTX-13	4.97	1:1.6	CSTX-9	7.95
	(iv)	CSTX-13 CSTX-9	1.25 2.78	1:4 1:1.8	Cupiennin 1a	5.00

Different amounts of peptides (A and B) were dissolved in 0.1 mol l^{-1} ammonium acetate, at pH 6.1 to test the effect of peptide (A) on the activity of peptide (B). The injected volume was 0.05 µl per fly. Physiological venom peptide molar ratios are shaded grey.

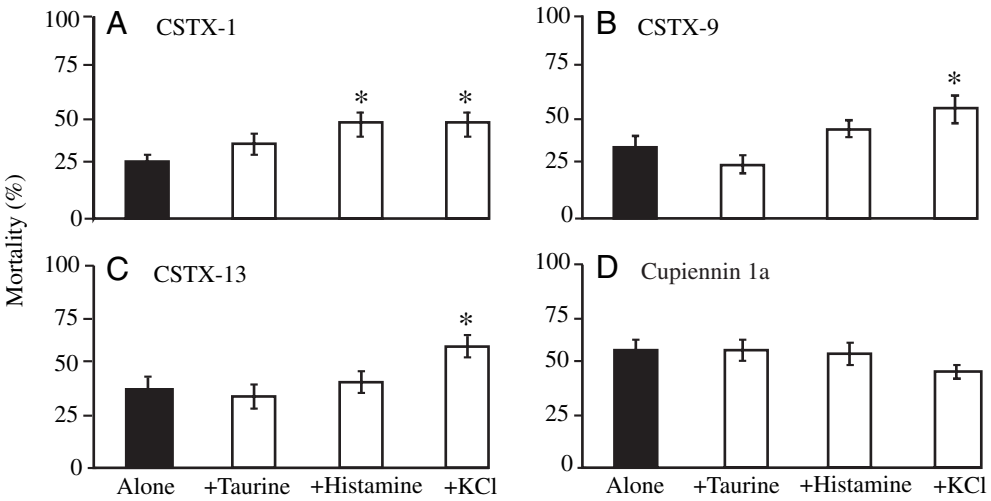

Fig. 1. Synergistic effects between low molecular mass components and CSTX-1, CSTX-9, CSTX-13 or cupiennin 1a as measured in a *Drosophila* bioassay. (A) The mortality caused by CSTX-1 alone (filled bar) (5.99 μmol l^{-1}) was compared with the mortality due to co-injection (open bars) with taurine (0.07 mmol l^{-1}; n.s.), histamine (5.7 mmol l^{-1}; **P*<0.01), or KCl (215 mmol l^{-1}; **P*<0.01). (B) The mortality caused by CSTX-9 alone (143.14 μmol l^{-1}) was compared with the mortality due to co-injection with taurine (0.07 mmol l^{-1}; n.s.), histamine (5.7 mmol l^{-1}; n.s.), or KCl (215 mmol l^{-1}; **P*<0.01). (C) The mortality caused by CSTX-13 alone (239.4 μmol l^{-1}) was compared with the mortality due to co-injection with taurine (0.07 mmol l^{-1}; n.s.), histamine (5.7 mmol l^{-1}; n.s.), or KCl (215 mmol l^{-1}; **P*<0.01) (data from Wullschleger et al., 2004). (D) The mortality caused by cupiennin 1a alone (90 μmol l^{-1}) was compared with the mortality due to co-injection with taurine (0.07 mmol l^{-1}; n.s.), histamine (5.7 mmol l^{-1}; n.s.) or KCl (215 mmol l^{-1}; n.s.). Taurine, histamine or KCl injected in physiological venom concentrations showed no toxic effect when administered alone as controls (not shown). All data with mean ±S.E.M. (*N*=30 groups); Fisher LSD test.

flies (*N*=30 groups). For every series 20 flies were injected as control.

Interactions between peptides

In a first series of experiments (Table 2), we analysed possible synergistic effects between the cytolytic cupiennin 1a and the neurotoxic CSTX-1 or CSTX-9. Cupiennin 1a (0.53 pmol mg^{-1} fly) was applied in a non-toxic concentration alone as well as in combination with the CSTX-peptides (0.315 pmol CSTX-1 mg^{-1} fly or 7.95 pmol CSTX-9 mg^{-1} fly).

In a second series of experiments, we analysed possible interactions between the neurotoxic peptides CSTX-1 and CSTX-9. (i) 0.315 pmol CSTX-1 mg^{-1} fly alone and in combination with 0.06 pmol CSTX-9 mg^{-1} fly were injected, corresponding to their molar ratio in the crude venom (5.7:1). (ii) CSTX-1 (0.47 pmol mg^{-1} fly) and CSTX-9 (7.95 pmol mg^{-1} fly) were injected separately and in combination, both in a toxic concentration (Table 2).

In a third series of experiments we have analysed the synergistic activity of CSTX-13 on the neurotoxins CSTX-1 and CSTX-9 as well as on the cytolytic cupiennin 1a. (i) Bioassays were performed with 0.315 pmol CSTX-1 mg^{-1} fly alone and in combination with 0.035 pmol CSTX-13 mg^{-1} fly (non-toxic concentration) corresponding to their molar ratio in the crude venom (9:1) (repetition of Wullschleger et al., 2004). (ii) Next, the mortality of 7.95 pmol CSTX-9 mg^{-1} fly was compared with the mortality of 7.95 pmol CSTX-9 mg^{-1} fly combined with either 0.035 pmol CSTX-13 mg^{-1} fly (molar ratio of 227:1) or (iii) 4.97 pmol CSTX-13 mg^{-1} fly (physiological molar ratio 1.6:1), respectively. (iv) Finally, the mortality of 5.0 pmol cupiennin 1a mg^{-1} fly was compared with the mortality of 5.0 pmol cupiennin 1a mg^{-1} fly combined with either 1.25 pmol CSTX-13 mg^{-1} fly (physiological molar ratio 4:1) or 2.78 pmol CSTX-9 mg^{-1} fly (physiological molar ratio 1.8:1), respectively (Table 2). For statistical analyses the flies were divided in two independent series of 12 groups each with five flies (*N*=24 groups). Twenty flies were used as control for each series of bioassay.

Calculations and statistics

Mortality rates corresponds to the number of dead flies out of a total of *N*=150 flies [2 × (15 × 5)] for interactions between low molecular mass components and peptides and *N*=120 flies [2 × (12 × 5)] for interactions between peptides.

LD_{50} calculations were done using Proban software (Version 1.1, Jedrychowski, 1991, shareware). The relative mortality of *D. melanogaster* was arcsinus square root-transformed and treated as the dependent variable, whereas the venom components or the co-injected peptides were treated as nominal independent variables. The experiments were analysed using generalised linear models. The means of the nominal independent variables venom components or co-injected peptides, respectively, were compared pairwise by the Fisher LSD method. Fulfilment of model assumptions was checked by visual inspection of the residuals distribution for every statistical test conducted. Statistics were done with S-PLUS 6.0 Professional software.

Results

Interactions between low molecular mass components and peptides

Control injections of taurine (3.88 pmol mg^{-1} fly) or histamine (316.67 pmol mg^{-1} fly) alone, corresponding to their venom concentrations, showed no paralytic effects in the *Drosophila* bioassays. By contrast, KCl (11.94 nmol mg^{-1} fly)

caused a short paralytic effect for 4–5 min of all tested flies, as reported previously (Wullschleger et al., 2004). The concentrations of the peptides co-injected with these low molecular mass components were chosen to produce mortalities below their LD_{50} values. The injection of 0.315 pmol CSTX-1 mg^{-1} fly generated a mortality rate of 27% (41 dead out of 150). No statistically significant difference in mortality increase was observed when taurine was co-injected (36% mortality; 54 dead out of 150). However, co-injection of CSTX-1 with histamine (47% mortality; 71 dead out of 150; $P<0.01$) or KCl (47% mortality; 71 dead out of 150; $P<0.01$), significantly increased the mortality (Fig. 1A). Injection of 7.95 pmol CSTX-9 mg^{-1} fly resulted in mortality rate of 35% (53 dead out of 150). No significant increase was observed for co-injection with taurine (34% mortality; 51 dead out of 150) or with histamine (44% mortality; 66 dead out of 150). However, co-injection with KCl (54% mortality; 81 dead out of 150; $P<0.01$) significantly increased the mortality (Fig. 1B). When injected alone, CSTX-13 caused a mortality of 39% (59 dead out of 150) in a concentration of 12.6 pmol mg^{-1} fly. No significant increase was observed for co-injection with taurine (34% mortality; 51 dead out of 150) or with histamine (42% mortality; 63 dead of 150). However, co-injection with KCl (59% mortality; 89 dead out of 150; $P<0.01$) significantly increased the mortality (Fig. 1C, data from Wullschleger et al., 2004). Injection of 5.0 pmol cupiennin 1a mg^{-1} fly caused a mortality rate of 58% (87 dead out of 150). No significant increase was observed for co-injection with taurine (57% mortality; 86 dead out of 150), histamine (56% mortality; 84 dead out of 150) or KCl (47% mortality; 71 dead out of 150) (Fig. 1D). All injected control flies showed no symptoms and survived.

Interactions between peptides

In a concentration of 0.53 pmol mg^{-1} fly, cupiennin 1a caused no insecticidal activities in the bioassay. Control injection of CSTX-1 (32 dead out of 120), CSTX-9 (42 dead out of 120) and CSTX-13 (47 dead out of 120) alone caused mortalities below 50%. Co-injection of cupiennin 1a with CSTX-1 increased the mortality from 27% to 92% (32 to 110 dead out of 120; $P<0.001$), co-injection with CSTX-9 increased the mortality from 35% to 78% (42 to 94 dead out of 120; $P<0.001$) and co-injection with CSTX-13 increased the mortality from 39% to 97% (47 to 116 dead out of 120; $P<0.001$) (Fig. 2, left, middle and right).

In a second series of experiments, a possible cooperativity between CSTX-1 and CSTX-9 was analysed first according to their molar ratio in the venom of 5.7:1 (0.315 pmol CSTX-1 mg^{-1} fly: 0.06 pmol CSTX-9 mg^{-1} fly). Injection of 0.06 pmol CSTX-9 mg^{-1} fly had no effect on the flies. No enhanced mortality was observed between the injection of 0.315 pmol CSTX-1 mg^{-1} fly alone (16% mortality; 19 dead out of 120) and in combination with CSTX-9 (17% mortality; 20 dead out of 120). Secondly, cupiennin 1a and CSTX-9 appear in the venom in a molar ratio of 1.8:1 (5.0 pmol cupiennin 1a mg^{-1} fly : 2.78 pmol CSTX-9 mg^{-1} fly). Injection of CSTX-9 in this concentration is non-toxic. Furthermore, a mortality rate of 56% (67 dead out of 120) by injection of cupiennin 1a alone is not increased by co-injection with CSTX-9 (mortality of 61%; 73 dead out of 120) (Fig. 3A).

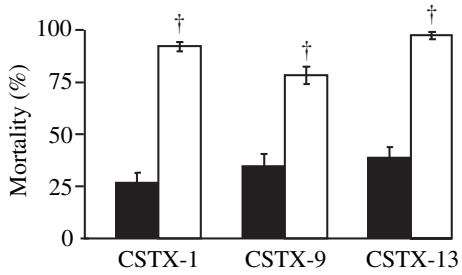

Fig. 2. Synergistic effects between cupiennin 1a and CSTX-1, CSTX-9 or CSTX-13 as measured in a *Drosophila* bioassay. (Left) The mortality caused by CSTX-1 alone (filled bar; 5.99 μmol l^{-1}) was compared with the mortality due to co-injection with cupiennin 1a (open bar; 0.96 μmol l^{-1}; $^{\dagger}P<0.001$). (Middle) The mortality caused by CSTX-9 alone (filled bar; 143.14 μmol l^{-1}) was compared with the mortality due to co-injection with cupiennin 1a (open bar; 0.96 μmol l^{-1}; $^{\dagger}P<0.001$). (Right) the mortality of CSTX-13 alone (filled bar; 239.4 μmol l^{-1}) was compared with the mortality due to co-injection with cupiennin 1a (open bar; 0.96 μmol l^{-1}; $^{\dagger}P<0.001$) (data from Wullschleger et al., 2004). Cupiennin 1a showed no toxic effect when administered alone as control (not shown). All data with mean ±S.E.M. (N=24 groups); Fisher LSD test.

Control injection of CSTX-1 (0.47 pmol CSTX-1 mg^{-1} fly) or CSTX-9 (7.95 pmol CSTX-9 mg^{-1} fly) alone generated mortalities of 33% (40 dead out of 120, for CSTX-1) or of 12% (14 dead out of 120, for CSTX-9). Interestingly, co-injection of both toxins resulted in a mortality of 98% (118 dead out of 120) and differed significantly from the theoretical sum of both toxins (45%; 54 dead out of 120; $P<0.001$) (Fig. 3B).

In a third series of experiments possible enhancer effects of CSTX-13 on the insecticidal activity of CSTX-9 and cupiennin 1a, corresponding to their venom concentrations, were investigated. The molar ratio of CSTX-9 and CSTX-13 in the venom is 1.6:1 (7.95 pmol CSTX-9 mg^{-1} fly: 4.97 pmol CSTX-13 mg^{-1} fly). CSTX-13 alone was used in the above-mentioned concentration and was non-toxic. Co-injection of both enhanced the mortality caused by CSTX-9 alone from 37% to 98% (44 to 118 dead out of 120; $P<0.001$). Surprisingly, co-injection of CSTX-9 and CSTX-13 even in a molar ratio of 227:1 (7.95 pmol CSTX-9 mg^{-1} fly: 0.035 pmol CSTX-13 mg^{-1} fly) increased the mortality nearly as much (91%; 109 dead out of 120; $P<0.001$, not shown). By contrast, co-injection of cupiennin 1a (5.0 pmol mg^{-1} fly) and CSTX-13 (1.25 pmol mg^{-1} fly) in their physiological venom ratio of 4:1 failed to increase the mortality rate significantly (50%; 60 dead out of 120), above that of cupiennin 1a alone (56%; 67 dead out of 120) (Fig. 4).

LD_{50} bioassays

The main neurotoxin CSTX-1 shows an LD_{50} value of 0.45 pmol mg^{-1} fly (95% confidence limits: 0.40–0.55). A

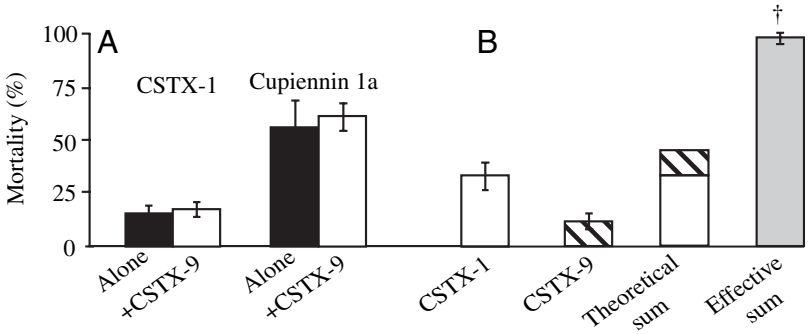

Fig. 3. Interactions between CSTX-9 and CSTX-1, or cupiennin 1a as measured in a *Drosophila* bioassay. (A) The mortality caused by CSTX-1 alone (filled bar; 5.99 μmol l^{-1}) was compared with the mortality due to co-injection with CSTX-9 (open bar; 1.05 μmol l^{-1}; n.s.) corresponding to their physiological venom ratio. The mortality caused by cupiennin 1a alone (filled bar; 90 μmol l^{-1}) was compared with the mortality due to co-injection with CSTX-9 (filled bar; 50 μmol l^{-1}; n.s.). CSTX-9 showed no toxic effect when administered alone (control, not shown). (B) The mortality caused by CSTX-1 alone (open bar; 8.99 μmol l^{-1}) or CSTX-9 alone (hatched bar; 143.14 μmol l^{-1}), or their theoretical sum respectively, was compared with the mortality due to the co-injection of CSTX-1 (8.99 μmol l^{-1}) and CSTX-9 (effective sum; 143.14 μmol l^{-1}; $^{\dagger}P<0.001$). All data with mean ±S.E.M. (N=24 groups); Fisher LSD test.

combined injection of CSTX-1 with CSTX-9, CSTX-13, cupiennin 1a, histamine and KCl, corresponding to their physiological venom concentrations (Wullschleger et al., 2004), resulted in a decreased LD_{50} value of 0.10 pmol mg^{-1} fly (95% confidence limits: 0.09–1.06). The LD_{50} of crude venom amounts to 0.017 nl mg^{-1} fly (95% confidence limits: 0.016–0.021), corresponding to 0.02–0.06 pmol CSTX-1 mg^{-1} fly.

Discussion

Interactions between low molecular mass components and peptides

The combined injections of KCl and the neurotoxins CSTX-1 or CSTX-9 resulted in an increase of approximately 20% mortality, a finding which confirms our previous results with CSTX-13 (Wullschleger et al., 2004) and indicates a general synergistic effect of potassium ions on the paralytic activity of diverse neurotoxins.

The strategy of combining a high K^+ concentration with specific neurotoxins in the prevenom, thus enhancing paralytic activity, was first reported for the scorpion *Parabuthus transvaalicus* (Inceoglu et al., 2003). It was hypothesised that the potassium equilibrium potential is locally shifted and the resulting paralytic effects are further amplified through the presence of peptide toxins, inhibiting ion channels that are responsible for the regeneration of the K^+ equilibrium potential. *C. salei* exhibits a comparable strategy but uses a 2.7-fold higher concentration of potassium ions in its venom than *P. transvaalicus* to enhance its venom efficacy. This high K^+ ion concentration can also provoke a nerve depolarisation thus affecting Ca^{2+} channels, which are in turn inhibited by CSTX-1, a known L-type calcium channel inhibitor (Mafra et al., 2001).

As for cupiennin 1a, no synergistic effect of KCl on its insecticidal activity was detected. In contrast to the neurotoxins CSTX-1, CSTX-9 and CSTX-13, the cytolytically active cupiennin 1a adopts an α-helical conformation in the presence of negatively charged membranes, accumulates at the membrane surface and inserts itself into the lipid bilayer resulting in a destruction of cell membranes (Kuhn-Nentwig et al., 2002). Histamine, a neurotransmitter in insect nerve systems (Nässel, 1999) and present in the spider venom, caused a significant mortality increase of 20% when co-injected with CSTX-1, but was less effective in combination with CSTX-9 or CSTX-13 in *Drosophila* flies (Wullschleger et al., 2004). Taurine, a neuromodulator in insects (Bicker, 1991) and also present in the spider venom had no paralytic effect when co-injected with CSTX-1, CSTX-9 or CSTX-13. By contrast, we were previously able to show in a blow fly bioassay that the neurotoxicity of CSTX-1 was enhanced by both taurine and histamine when injected in its physiological venom concentrations (Kuhn-Nentwig et al., 1998). This could indicate that synergistic interactions are highly species and neurotoxin specific, despite the close relationship between both fly families.

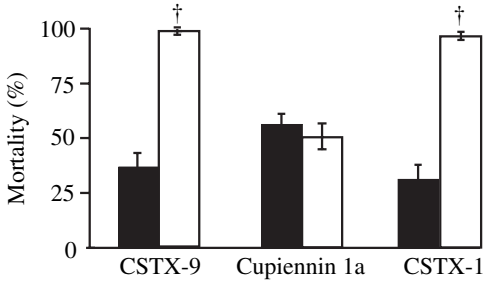

Fig. 4. Synergistic effects between CSTX-13 and CSTX-9, cupiennin 1a or CSTX-1, corresponding to their molar concentrations in the venom, as measured in a *Drosophila* bioassay. (Left) The mortality caused by CSTX-9 alone (filled bar; 143.14 μmol l^{-1}) was compared with the mortality due to co-injection with CSTX-13 (open bar; 95.14 μmol l^{-1}, $^{\dagger}P<0.001$) in a molar ratio of 1.6:1. (Middle) The mortality caused by cupiennin 1a (filled bar; 90 μmol l^{-1}) was compared with the mortality due to co-injection with CSTX-13 (open bar; 22.5 μmol l^{-1}, n.s.) in a molar ratio of 4:1. (Right) The mortality caused by CSTX-1 (filled bar; 5.99 μmol l^{-1}) was compared with the mortality due to co-injection with CSTX-13 (open bar; 0.67 μmol l^{-1}, $^{\dagger}P<0.001$) in a molar ratio of 9:1. CSTX-13 showed no toxic effect when administered alone as control (not shown). All data with mean ±S.E.M. (N=24 groups); Fisher LSD test.

Interactions between peptides

As shown previously, cupiennin 1a dramatically enhances the efficacy of the neurotoxins CSTX-1 and CSTX-13 although it is applied in a completely non-toxic concentration, or even 20-fold below its LD_{50} (Kuhn-Nentwig et al., 2004; Wullschleger et al., 2004). This synergistic effect was additionally proven for the neurotoxin CSTX-9 (Fig. 2). Positive insecticidal cooperativity between the cytolytically active oxyopinins and the neurotoxin Oxytoxin 1 is also reported for the spider *Oxyopes kitabensis* (Corzo et al., 2002). There is evidence that these highly cationic peptides, found in the venom of *O. kitabensis* and *C. salei*, afford diverse neurotoxins better access to their targets through their cytolytic activities. By contrast, the insecticidal activity of cupiennin 1a was definitely not enhanced when administered with the neurotoxins CSTX-9 or CSTX-13. The dramatic enhancer effect of the two-chain peptide CSTX-13 on the insecticidal activity of CSTX-1 in a concentration 440-fold below its LD_{50} value as reported recently (Wullschleger et al., 2004) could also be enlarged on the neurotoxin CSTX-9. Furthermore, the synergistic activity of CSTX-13 and the neurotoxins CSTX-1 and CSTX-9 is highly specific.

The strong synergistic activities generated by cupiennin 1a and CSTX-13 are based on their physiological venom concentrations, which implies that both components were applied in non-toxic concentrations together with the neurotoxins CSTX-1 or CSTX-9. In contrast to these results, CSTX-9 did not enhance the neurotoxic activity of CSTX-1 when co-injected corresponding to its molar ratio in the venom. However, co-injection of CSTX-1 and CSTX-9, both in toxic concentrations, increased the toxicity by more than 50% when compared with the theoretical toxicity sum of CSTX-1 and CSTX-9 injected alone, thus exhibiting a positive cooperativity. These findings are in agreement with other reports in which positive cooperativities between different neurotoxins were demonstrated by applying the components in a 1:1 molar ratio or in concentrations in which both components alone cause intoxications, or by using toxins from different sources (Bindokas et al., 1991; Herrmann et al., 1995; Shu and Liang, 1999; Regev et al., 2003; Adams, 2004).

LD_{50} bioassays

Corresponding to their venom concentrations, injection of a combination of CSTX-1, CSTX-9, CSTX-13, cupiennin 1a, histamine and KCl into *Drosophila* flies resulted in an LD_{50} value which is 4.5-fold lower than a single injection of CSTX-1. Taking this and the LD_{50} value obtained by injection of native venom into account, the venom components mentioned above are responsible for up to 57% of the crude venom efficacy. Obviously, still other unknown components are important in the envenomation process and cause at least 43% of the toxicity of *C. salei* venom.

The interactions of different venom components presented here are extremely complex: histamine and taurine seem to enhance the activity of CSTX-1 highly specifically, and their effects are, in part, species specific. The high K^+ ion concentration in the venom facilitates the neurotoxin activity, but not that of cupiennin 1a. However, this group of cytolytic peptides dramatically enhances the activity of the neurotoxins. In addition, the neurotoxins are further amplified by the two-chain enhancer CSTX-13. Differences in LD_{50} values obtained by injection of crude venom into various arthropods (Kuhn-Nentwig et al., 1998) lead us to assume that the interactions presented here cannot be generalised and are only validated for *Drosophila melanogaster*.

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