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Pharmacological evidence that neuropeptides mediate part of the actions of scorpion venom on the guinea pig ileum

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Abstract

Severe human scorpion envenoming is characterised by instability of several physiological systems and death. These manifestations are explained by the ability of the venom toxins to activate sodium channels in nerve terminals with the subsequent release of neurotransmitters, specially acetylcholine and noradrenaline. However, there is evidence to suggest that other neurotransmitters are also released. We now have sought evidence for a role of the substance P receptor, the tachykinin NK_1 receptor, in mediating part of the contractile actions of *Tityus serrulatus* venom on the isolated guinea pig ileum. Scorpion venom induced a significant elevation of baseline tension with frequent and periodic superimposed contractions on the elevated baseline. Pretreatment with atropine partially blocked the elevation in baseline and in the number of superimposed contractions. These responses were also partially inhibited by the tachykinin NK_1 receptor antagonist, CP96,345 (the dihydrochloride salt of (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)methyl)-1-azabicycol[2.2.2]octan-3-amine), but not by its inactive enantiomer, CP96,344 (the 2*R*–3*R* enantiomer of CP96,345). Pretreatment with the combination of atropine and CP96,345 completely inhibited the effects of the venom. Moreover, pretreatment with the combined drugs abolished the effects of toxin gamma, a toxin purified from the venom. Finally, another tachykinin NK_1 receptor antagonist, RP67,580 ((3aR, 7ar)-7,7-diphenyl-2-[1-imino-2-(2-methoxy-phenyl)ethyl]perhydroisoindol-4-one), significantly inhibited the venom-induced contractions. These results demonstrate an important role for NK_1 receptors in mediating part of the contractile effects of the venom on guinea pig ileum. The release of neuropeptides may play an important role in the systemic manifestations of severe envenoming.

Keywords: (*Tityus serrulatus*); Tachykinin NK_1 receptor; Substance P; Toxin gamma; Envenoming; Scorpion venom

1. Introduction

In tropical and subtropical countries, human scorpion poisoning is frequent and fatal accidents are commonly reported, specially amongst children (Freire-Maia et al., 1994). In Brazil, *Tityus serrulatus* is the most important scorpion species, causes most accidents, and induces the most severe forms of poisoning. The clinical manifestations of severe envenoming include unremitting nausea and vomiting, pain, diarrhoea, cardiac arrhythmias and arterial hypertension followed by hypotension and shock. In the most severe cases, pulmonary oedema is a frequent finding and usually the cause of death (Amaral et al., 1993). In animals, the intravenous injection of scorpion toxins induces physiological manifestations similar to those of human envenoming. Thus, there are reports of increased bowel function, hypertension, cardiac arrhythmias and pulmonary oedema following the systemic injection of scorpion toxins (Freire-Maia et al., 1978; Azevedo et al., 1983; Freire-Maia and Campos, 1989). These manifestations are explained by the ability of the venom toxins to act on sodium channels on neuronal terminals, leading to depolarization of axonal membranes and consequent release of neuromediators which will stimulate various organs, including the gut, heart and vascular tissue (Freire-Maia and Campos, 1989). There is much experimental evidence that noradrenaline and acetylcholine mediate most of the physiological actions of scorpion venom in animal models both in vitro and in vivo. However, there is some experimental evidence to suggest that other neurotransmitters are also involved in the actions of these toxins (Diniz and Torres, 1968; Cunha-Melo et al., 1973; Romano-Silva et al., 1994; Teixeira et al., 1998). The release of these

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non-adrenergic, non-cholinergic neurotransmitters may play an important role in the pathophysiology of scorpion envenoming.

A few studies have evaluated the ability of scorpion toxins to contract the isolated rat or guinea pig ileum (reviewed in Freire-Maia and Campos, 1989). These studies have repeatedly demonstrated that the venom induces the contraction of the ileum indirectly via the release of neurotransmitters, specially acethylcholine. However, the acetylcholine receptor antagonist, atropine, only inhibits the venom-induced contraction by approximately 50–60% suggesting that other neurotransmitters are released locally (Cunha-Melo et al., 1973). Previous studies have provided indirect evidence that scorpion toxins have the ability of releasing the neuropeptide, substance P, from the gut. Tafuri et al. (1974) showed that pretreatment of the gut with scorpion toxins reduced the amount of electron dense vesicles (in which substance P is thought to be stored) from Auerbach's plexus. Moreover, Hial and Diniz (1971) showed that the gut content of substance P was diminished 24 h after toxin injection. Inasmuch as substance P induces the contraction of both the circular and longitudinal muscle layers of the intestine (Barthó and Holzer, 1985; Costa et al., 1985; Nguyen-Le et al., 1996), the present study was undertaken to evaluate whether the tachykinin NK_1 receptor, of which substance P is the main ligand, is involved in the contraction of the isolated guinea-pig ileum produced by *T. serrulatus* scorpion venom.

2. Material and methods

2.1. Animals

Guinea-pigs weighing 400–500 g were purchased from Fundação Ezequiel Dias (Belo Horizonte, Brazil) and were kept under standard conditions in our bioscience unit.

2.2. Preparation of ileum strips

The guinea pigs were killed by cervical dislocation and exsanguinated. After laparotomy, the distal ileum was removed and 1-cm strips were suspended in 10 ml aerated Tyrode's solution (136.8 mM NaCl; 2.7 mM KCl; 1.4 mM $CaCl_2$; 12.0 mM $NaHCO_3$; 5.5 mM glucose; 1.0 mM $MgCl_2$; 0.4 mM NaH_2PO_4) at 37°C and attached to a tension transducer (Grass, USA) connected to a data acquisition device (CWE, Akron, OH, USA). All strips were put under an initial tension of 1 g. After 30 min to allow stabilisation of the preparation, contractions were recorded in the presence of various substances or venom toxin. The following agents were used to induce contraction of the preparation: acetylcholine (5×10^{-7} M), histamine (10^{-6} M) and substance P (10^{-8} M). After the contraction reached a peak, the preparation was washed thrice and allowed to rest for 5 min between each addition. Then, scorpion venom was used at the concentration of 5 μg/ml and allowed to act for periods of up to 5 min. The use of this concentration of venom was based on results of preliminary studies demonstrating optimal contraction of guinea pig ileum at 5 μg/ml. After the addition of the venom, the preparation was washed four times and allowed to rest for at least 20 min. Atropine (1.5×10^{-7} M), the tachykinin NK_1 receptor antagonist CP96,345 (5.0×10^{-8} M and 5.0×10^{-7} M), its inactive enantiomer CP96,344 (5.0×10^{-7} M), RP67,580 (10^{-7} M to 10^{-6} M) or atropine plus CP96,345 or RP67,580 was then added to the Tyrode's solution prior to addition of acetylcholine, substance P, histamine or scorpion venom. Previous studies have demonstrated that the guinea pig ileum does not undergo tachyphylaxis following the addition of repeated doses of the venom (Cunha-Melo et al., 1973). In some experiments, toxin gamma (2.5 μg/ml, see Section 3) was used to contract the guinea pig ileum alone or in the presence of atropine, CP96,345 (5×10^{-7} M) or atropine plus CP96,345.

2.3. Purification of toxin gamma

Toxin γ was purified from *T. serrulatus* venom through gel filtration on Sephadex G-25 and chromatography on CM-cellulose-52 as previously described (Sampaio et al., 1983). This purification procedure yields electrophoretically pure toxins of which toxin γ is the most widely studied (Sampaio et al., 1983).

2.4. Determination of superimposed contractions

Superimposed contractions are defined here as a contraction of at least 1.0 g amplitude and of short duration (≤ 2 s) which was superimposed on a contraction of longer duration (elevation of baseline). The number of superimposed contractions was measured for a period of 5 min following the addition of scorpion venom.

2.5. Drugs

Acethylcholine, atropine, histamine and substance P were purchased from Sigma (St. Louis, USA). The NK_1 antagonist, CP96,345 (the dihydrochloride salt of (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)methyl)-1-azabicycol[2.2.2]octan-3-amine) and CP96,344 (the 2*R*–3*R* enantiomer of CP96,345) were kind gifts of Pfizer (Groton, USA). RP67,580 ((3aR, 7ar)-7,7-diphenyl-2-[1-imino-2-(2-methoxy-phenyl)ethyl]perhydroisoindol-4-one) was a gift of Rhône-Poulenc Rorer (France). The activity of these drugs as antagonists of NK_1 receptors has been previously characterised on the guinea pig ileum (Nguyen-Le et al., 1996). *T. serrulatus* scorpion venom was kindly provided by Fundação Ezequiel Dias (Belo Horizonte, Brazil).

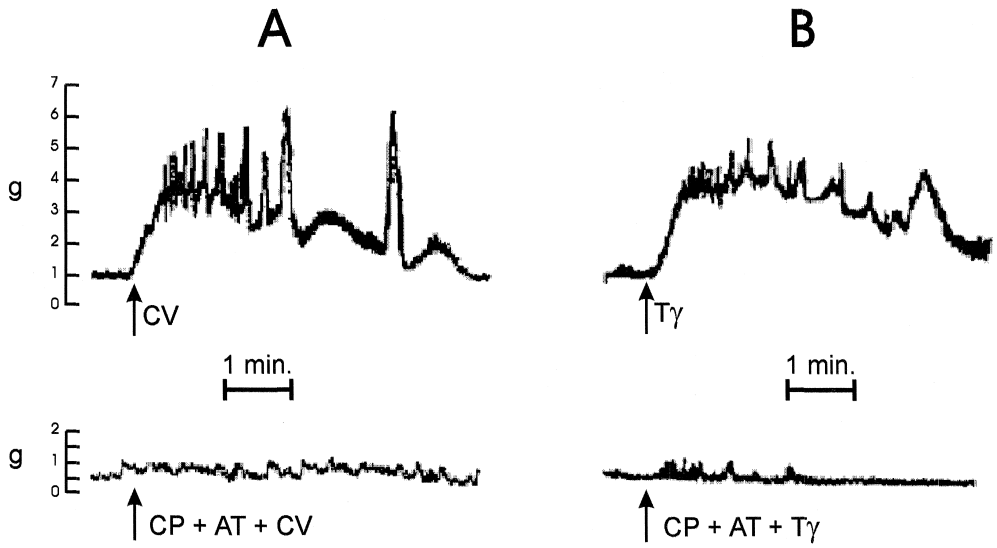

Fig. 1. Representative traces for the contractile effects of scorpion venom (A) and one of its purified toxins, toxin γ, (B) on the guinea pig ileum. In the top traces we observe the contraction of strips in the presence of (A) scorpion venom (CV, 5 μg/ml) and (B) toxin γ (Tγ, 2.5 μg/ml) alone. In the lower traces, the NK_1 antagonist, CP96,345 (CP, 5×10^{-7} M), and atropine (AT, 1.5×10^{-7} M) were added to the preparation prior to the venom or toxin γ. Strips from guinea pig ileum were immersed in 10 ml aerated Tyrode's solution at 37°C and put under an initial tension of 1.0 g.

2.6. Statistical analysis

The results were analysed by means of an analysis of variance (ANOVA) on normally distributed data. *P*-values were assigned using the Newman–Keuls procedure and values of $P < 0.05$ were considered significant. Results are presented as the means ± S.E.M.

3. Results

3.1. Effect of scorpion venom on the guinea-pig ileum

Fig. 1A shows the effects of the addition of crude scorpion venom to the guinea pig ileum preparation. There was a significant elevation of the baseline tension (baseline, 1.0 ± 0.2 g; venom, 3.34 ± 0.15 g, $n = 12$). In addition, frequent and periodic contractions superimposed on the elevated baseline were noticed throughout the observation period (see Fig. 1A). Two additions of the venom separated by a 30-min period did not induce tachyphylaxis (First addition of venom, 2.4 ± 0.3 g over baseline; second addition of venom, 2.2 ± 0.4 g, $n = 6$). This is in agreement with our previous observation that the venom-induced contraction does not undergo tachyphylaxis following an initial addition of the same concentration of venom (Cunha-Melo et al., 1973). For the experiments evaluating the effects of drug treatment on the venom-induced contraction, we compared the elevation of baseline in the absence or presence of an acetylcholine muscarinic receptor antagonist (atropine), tachykinin NK_1 receptor antagonists (CP96,345 and RP67,580) or a combination of both

Table 1
Effects of the NK_1 antagonist, CP96,345, alone or in combination with atropine on the contractile responses induced by acetylcholine, histamine or substance P in the guinea pig ileum

	Control	+ CP96,345 (5×10^{-8} M)	+ CP96,345 (5×10^{-7} M)	+ Atropine	+ Atropine + CP96,345 (5×10^{-8} M)	+ Atropine + CP96,345 (5×10^{-7} M)
Elevation of baseline (g)						
Acetylcholine	3.42 ± 0.19	3.24 ± 0.41	3.22 ± 0.38	−0.09 ± 0.07[b]	−0.11 ± 0.09[b]	−0.47 ± 0.27[b]
Histamine	4.58 ± 0.20	3.69 ± 0.43	3.90 ± 0.78	4.47 ± 0.37	4.12 ± 0.43	4.39 ± 0.65
Substance P	3.06 ± 0.20	1.10 ± 0.54[a]	0.44 ± 0.09[b]	2.70 ± 0.21	0.68 ± 0.50	0.09 ± 0.08[c]

Acetylcholine, histamine and substance P were used at the concentrations of 5×10^{-7} M, 10^{-6} M and 10^{-8} M, respectively. Atropine was used at the concentration of 1.5×10^{-7} M. Atropine and/or CP96,345 were added 60 s prior to the addition of the stimuli.
Results are means ± S.E.M. for at least six animals in each group.
Strips from guinea pig ileum were immersed in 10 ml aerated Tyrode's solution at 37°C and put under an initial tension of 1.0 g.
[a] $P < 0.05$ and [b] $P < 0.01$ when compared to control and [c] $P < 0.05$ when comparing the effects of CP96,345 in the presence or absence of atropine.

classes of drugs. The effects of these drugs on the occurrence of the superimposed contractions were also noted.

3.2. Partial blockade by atropine of scorpion venom-induced contraction of the isolated guinea-pig ileum

Atropine at a concentration of 1.5×10^{-7} M which completely blocked the contraction induced by acetylcholine (5×10^{-7} M; Table 1) decreased the venom-induced contraction by 56% (Fig. 2). Moreover, atropine significantly reduced the occurrence of superimposed contractions following the addition of scorpion venom (Fig. 3). In contrast, atropine had no significant effect on histamine- or substance P-induced contraction of the guinea pig ileum (Table 1).

3.3. Effects of NK_1 receptor antagonists alone or in combination with atropine on scorpion venom-induced contraction of the isolated guinea-pig ileum

At the concentration of 5×10^{-8} M, the NK_1 receptor antagonist, CP96,345, blocked substance P-induced contraction by 64% (Table 1) but had no significant effect on the venom-induced contraction (Fig. 2). However, at a concentration 10 times higher (5×10^{-7} M), CP96,345 inhibited the substance P- and the venom-induced contraction by 86% and 42%, respectively (Fig. 2 and Table 1). Similarly, CP96,345 (5×10^{-7} M) significantly suppressed the frequency of superimposed contractions induced by the venom (Fig. 3). At the concentrations used, CP96,345 had no significant effects on contraction of the guinea pig ileum induced by histamine or acetylcholine (Table 1). Pretreatment of the preparation with CP96,344 (5×10^{-7} M), an inactive enantiomer of CP96,345, had no inhibitory effect on the venom-induced contraction (venom, 2.3 ± 0.3 g over baseline; CP 96,344 + venom, 2.1 ± 0.1, $n = 4$). Moreover, CP96,344 failed to alter the frequency of superimposed contractions following the addition of the venom (data not shown).

Next, we evaluated whether CP96,345 and atropine had additive effects for inhibition of the venom-induced contractions. After addition of both antagonists, the guinea pig ileum virtually did not respond to stimulation with scorpion venom (Fig. 1A). The combined pretreatment with atropine and 5×10^{-7} M CP96,345 inhibited the venom-induced contraction by 94% (see Fig. 2). Similarly, no superimposed contractions were observed following venom

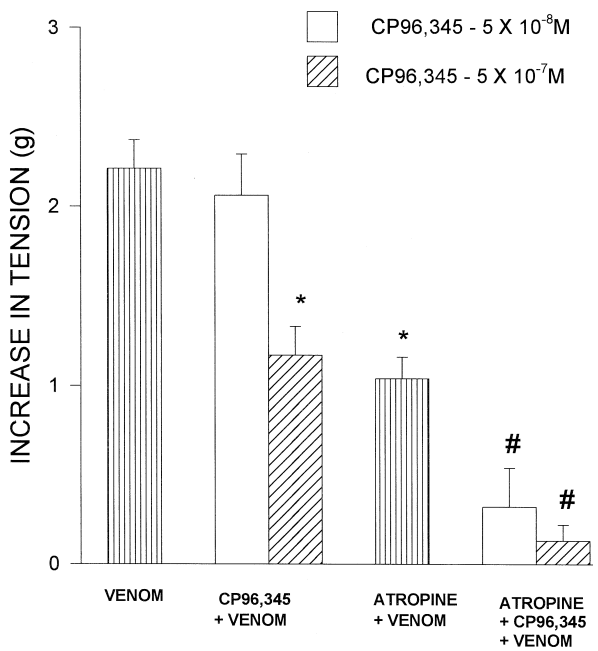

Fig. 2. Effects of the NK_1 antagonist, CP96,345, alone or in combination with atropine on the contractile responses induced by scorpion venom on the guinea pig ileum. Scorpion venom was used at the concentration of 5 μg/ml. CP96,345 (5×10^{-8} M and 5×10^{-7} M) and/or atropine (1.5×10^{-7} M) were added 60 s prior to the addition of the venom. Results are the means ± S.E.M. for 5–6 animals in each group. Strips from guinea pig ileum were immersed in 10 ml aerated Tyrode's solution at 37°C and put under an initial tension of 1.0 g. $^{*}P < 0.05$ in comparison with venom alone and $^{\#}P < 0.05$ in comparison with venom-induced contractions in the presence of atropine only.

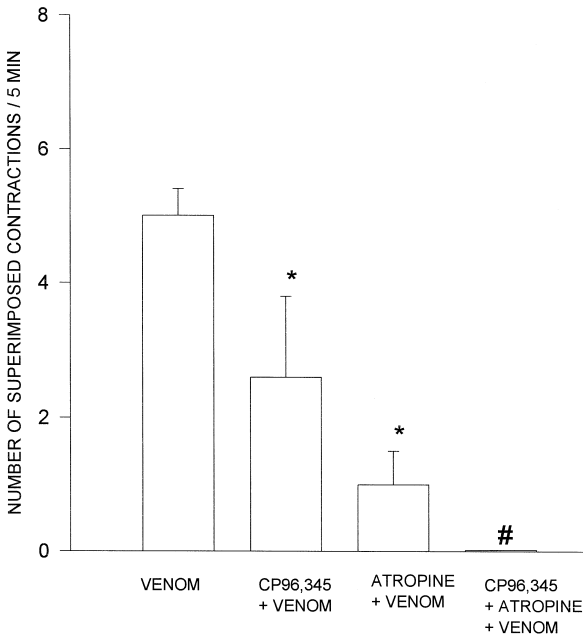

Fig. 3. Effects of the NK_1 antagonist, CP96,345, alone or in combination with atropine on the number of superimposed contractions induced by scorpion venom on the guinea pig ileum. Scorpion venom was used at the concentration of 5 μg/ml. CP96,345 (5×10^{-7} M) and/or atropine (1.5×10^{-7} M) were added 60 s prior to the addition of the venom. Results are the means ± S.E.M. for 5–6 animals in each group. Strips from guinea pig ileum were immersed in 10 ml aerated Tyrode's solution at 37°C and put under an initial tension of 1.0 g. The number of superimposed contractions was measured in 5-min periods. $^{*}P < 0.05$ in comparison with venom alone and $^{\#}P < 0.05$ in comparison with venom-induced superimposed contractions in the presence of atropine or CP96,345.

addition in the presence of atropine and CP96,345 (Fig. 3). Moreover, the combination pretreatment had no significant effect on the contractions induced by histamine (Table 1). The addition of atropine to the organ bath containing CP96,345 appeared to induce a significantly greater inhibition of substance P-induced contraction than did CP96,345 alone (Table 1). In contrast to the effects of CP96,345, pretreatment of the preparations with a combination of CP96,344 (5×10^{-7} M) and atropine was not more effective on the contraction induced by the venom (5 μg/ml) than was atropine alone (atropine + venom, 1.2 ± 0.2 g over baseline; CP 96,344 + atropine + venom, 1.6 ± 0.3, $n = 4$).

In order to confirm that the inhibitory effects of CP96,345 were related to inhibition of NK_1 receptors, we used another structurally unrelated NK_1 receptor antagonist, RP67,580. When used at concentrations of up to 10^{-6} M, RP67,580 effectively blocked the contraction induced by 5×10^{-8} M substance P (over 85% inhibition) and inhibited by approximately 70% the contraction elicited by 5 μg/ml of scorpion venom (data not shown). Moreover, when RP67,580 (10^{-6} M) was used together with atropine, the venom-induced responses were virtually abolished (venom, 2.4 ± 0.3 g over baseline; RP67,580 + atropine + venom, 0.2 ± 0.1 g, $n = 4$, $P < 0.01$).

3.4. Effects of the NK_1 receptor antagonist, CP96,345, alone or in combination with atropine on the contraction of the isolated guinea-pig ileum induced by a toxin purified from scorpion venom

Fig. 1B shows the effects of the addition of a toxin purified from *T. serrulatus* scorpion venom, toxin γ. Similarly to the effects observed after addition of the crude venom, toxin γ induced a significant elevation of baseline and frequent superimposed contractions in the isolated guinea pig ileum (Fig. 1B, Table 2). Combined treatment with atropine and CP96,345 (5×10^{-7} M) virtually abolished the γ-toxin-induced elevation of baseline and the superimposed contractions (Fig. 1B, Table 2).

Table 2
Effects of the NK_1 antagonist, CP96,345, alone or in combination with atropine on the contractile responses induced by toxin γ on the guinea pig ileum

	Elevation of the baseline (g)	Number of superimposed contractions (per 5 min)
Control (Toxin γ)	2.36 ± 0.85	6 ± 3
+ CP96,345	0.819 ± 0.32	6 ± 3
+ Atropine	1.75 ± 0.09	2 ± 2
+ CP96,345 + Atropine	-0.09 ± 0.06[a]	0 ± 0[a]

Toxin γ, CP96,345 and atropine were used at the concentrations of 2.5 μg/ml, 5×10^{-7} M and 1.5×10^{-7} M, respectively. Atropine and/or CP96,345 were added 60 s prior to the addition of toxin γ.
Results are the means ± S.E.M. for three animals in each group.
Strips from guinea pig ileum were immersed in 10 ml aerated Tyrode's solution at 37°C and submitted to an initial tension of 1.0 g.
[a] $P < 0.05$ in comparison with control.

4. Discussion

Scorpion sting is still an important cause of envenoming in tropical and subtropical regions. In the most severe cases, scorpion envenoming is characterised by instability of several physiological systems, pulmonary oedema and death. The ability of purified scorpion toxins to activate sodium channels in nerve terminals with the subsequent release of acetylcholine and noradrenaline accounts for most of the physiological actions of these toxins (Freire-Maia and Campos, 1989). However, there is evidence to suggest that other neurotransmitters may also play a role in mediating the physiological actions of scorpion toxins (Cunha-Melo et al., 1973; Romano-Silva et al., 1994; Teixeira et al., 1998). We now sought evidence for a role of the substance P receptor, the tachykinin NK_1 receptor in mediating part of the actions of *T. serrulatus* toxin on the isolated guinea pig ileum.

The addition of scorpion venom to the isolated guinea pig ileum induced a significant elevation of baseline followed by the occurrence of contractions of small duration superimposed on this elevation (Fig. 1). At a concentration that abolished the contraction induced by acetylcholine, atropine blocked the venom-induced contraction by approximately 60%. This is in agreement with our previous results demonstrating the importance of the release of acetylcholine for the actions of the venom on the guinea pig ileum and other preparations (Cunha-Melo et al., 1973; Drumond et al., 1995). However, it seems likely that the release of acetylcholine alone is not able to explain the contractile effects of the venom on the guinea pig ileum.

With guinea pig ileum, the tachykinins, which include substance P, appear to act as excitatory transmitters for both the circular and longitudinal muscle layers and also to affect motility indirectly via activation of enteric neurons (Nguyen-Le et al., 1996). The effects of substance P on the motility of the guinea-pig ileum are mostly direct (mostly via NK_1 receptors, but also via NK_2 receptors) and partly neurogenic (via NK_3 receptors) (Nguyen-Le et al., 1996; Holzer and Holzer-Petsche, 1997). We have previously shown that *T. serrulatus* toxin decreases the number of electron-dense vesicles containing substance P from Auerbach's plexus (Tafuri et al., 1974) and this is consistent with the ability of scorpion toxins to decrease the substance P content in the gut (Hial and Diniz, 1971). Because the NK_1 receptor mediates most of the contractile actions of substance P on the guinea pig ileum, we investigated the possible involvement of an antagonist of the NK_1 receptor, CP-96,345 (Nguyen-Le et al., 1996), on the venom-induced contraction. When used alone, CP-96,345 and RP67,580 inhibited the venom-induced contraction by ap-

proximately 40% and 70%, respectively. More important, when CP-96,345 or RP67,580 were used with atropine, the combination abolished both the elevation of the baseline and the superimposed contractions induced by the scorpion venom on the guinea pig ileum. In addition, CP96,344, an enantiomer of CP96,345 inactive to inhibit tachykinin NK_1 receptors, failed to suppress the venom-induced contractions. The latter results suggest that the inhibitory effects of CP96,345 are due to its ability to block tachykinin NK_1 receptors and not to other, unrelated, effects, such as inhibition of ion channels (Guard et al., 1993). This suggestion is corroborated by the finding that another tachykinin NK_1 receptor antagonist, RP67,580, also blocked the contractile effects of the venom.

Interestingly, the combined treatment with CP96,345 (or RP67,580) and atropine was significantly more effective to block the contractile effects of substance P than was the NK_1 receptor antagonist alone. These results suggest that part of the contractile actions of substance P on the guinea pig ileum are indirect, via the release of acetylcholine. In agreement with this suggestion, Nguyen-Le et al. (1996) have shown that substance P is capable of releasing acetylcholine from nerve terminals on the guinea pig ileum via activation of NK_3 receptors.

We also examined the effects of muscarinic and NK_1 receptor blockade on the contractile effects of toxin γ, a toxin purified from crude venom of *T. serrulatus*. We chose to use this toxin because it appears to be relatively selective for the release of acetylcholine in some preparations in vitro (Drumond et al., 1995). As did the crude venom, the toxin induced a significant elevation of baseline and frequent superimposed contractions of the ileum. Moreover, pretreatment with atropine and CP96,345 combined completely inhibited the toxin γ-induced effects. These results suggest that the contractile effects of *T. serrulatus* venom or one of its purified toxins in the isolated guinea pig ileum are mediated by acetylcholine acting on muscarinic receptors, and a neuropeptide, presumably substance P, acting on tachykinin NK_1 receptors.

Palframan et al. (1996) have previously demonstrated that oedema formation evoked by the intradermal administration of *Phoneutria nigriventer* spider venom in rat skin was mediated, at least in part, via a mechanism involving tachykinin NK_1 receptors. The ability of animal toxins to release neuropeptides could be a link between activation of neurons and the inflammatory manifestations of envenoming. In this context, the ability of scorpion toxins to release neuropeptides in vivo could be a link between envenoming and lung oedema, a significant cause of death in patients thus afflicted (Amaral et al., 1993). We are presently investigating this possibility.

Acknowledgements

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