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# Biochemical and pharmacological characterization of the venom of the black scorpion *Heterometrus spinifer*

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**Abstract**

The sting of the black scorpion *Heterometrus spinifer*, which can cause intense localized pain, has not been reported to produce lethal cardiovascular complications, which are well known to result from scorpion envenomation as a consequence of a massive release of catecholamines. Therefore, we have undertaken a biochemical and pharmacological characterization of the venom of *H. spinifer*. Pharmacologically, the venom (0.125 $\mu$L/mL) produced a marked, reversible contracture in the chick biventer cervicis muscle that was blocked by *d*-tubocurarine (2 $\mu$M) but not by tetrodotoxin (5 $\mu$M) and $\omega$-conotoxin GVIA (3 $\mu$M). The anticholinesterase neostigmine (1 $\mu$M) potentiated the contracture by 5.3-fold. An ultra-filtrate fraction of MW $<$ 3000 (F3K) of the venom produced a similar contracture in the biventer muscle, whereas the retentate of MW $>$ 3000 did not. In the rat anococcygeus muscle, the venom produced a contractile response that was partially (37.4 $\pm$ 1.6%) blocked by atropine (5 $\mu$M); phentolamine (5 $\mu$M) blocked the remaining response. Tetrodotoxin (5 $\mu$M) did not block the contractile response of the venom on the anococcygeus muscle. Electrospray ionization–mass spectrometry/mass spectrometry confirmed the presence of high concentrations of acetylcholine (79.8 $\pm$ 1.7 $\mu$M) and norepinephrine (146.7 $\pm$ 19.8 $\mu$M) in *H. spinifer* venom, which can fully account for the observed cholinergic and adrenergic effects. In contrast to scorpion venoms that selectively target neuronal ion channels in mediating transmitter release, our data show that *H. spinifer* venom does not possess such activity, which likely explains the apparent lack of lethality of black scorpion envenomation.

*Keywords:* Acetylcholine; Norepinephrine; Scorpion venom; *Heterometrus spinifer*; Electrospray ionization–mass spectrometry/mass spectrometry

## 1. Introduction

Scorpion envenomation is known to cause potentially lethal cardiovascular complications as a result of widespread peripheral sympathetic nerve stimulation and consequent massive outpouring of catecholamines, the so-called "autonomic storm" [1–3] This has been attributed to the activity of potent neurotoxins in scorpion venoms that target sodium and potassium ion channels [4–6]. Although the sting of the morphologically intimidating black scorpion (*Scorpionidae*) is known to cause intense localized pain, lethal envenomation in humans has not been documented [7]. In our preliminary studies to find a likely explanation for this lower order of toxicity, we did not obtain any pharmacological evidence that the venom of the black scorpion *Heterometrus spinifer* modified ion-channel activity to produce the release of neurotransmitters. Interestingly, however, we found evidence for the presence of a cholinoceptor agonist. Although ACh is known to be a constituent of the venoms of some insect species like hornets and wasps [8,9] and of mamba (*Dendroaspis)* snakes [10], the existence in nature of physiologically active choline-esters other than ACh has also been reported [8]. More recently, a novel nicotinic ACh receptor agonist with potent analgesic activity, epibatidine, has been isolated from the skin of the Ecuadorian tree frog *Epipedobates tricolor* [11,12]. Therefore, we have carried out a biochemical and pharmacolog-

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*Abbreviations:* ACh, acetylcholine; AChE, acetylcholinesterase; ACM, anococcygeus muscle; $\alpha$-BTx, $\alpha$-bungarotoxin; CBCM, chick biventer cervicis muscle; $\omega$-CTx/GVIA, $\omega$-conotoxin GVIA; ESI–MS, electrospray ionization–mass spectrometry; ESI–MS/MS, electrospray ionization–mass spectrometry/mass spectrometry; HSV, *Heterometrus spinifer* venom; NE, norepinephrine; *d*-TC, *d*-tubocurarine; and TTx, tetrodotoxin.

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ical characterization of the venom of the Malaysian black scorpion *H. spinifer*. Our current study provides the first report of high concentrations of ACh present together with NE in a scorpion venom. A preliminary report of part of this work has been communicated to the 18th International Congress of Biochemistry and Molecular Biology, Birmingham, UK, July 2000.

## 2. Materials and methods

### 2.1. HSV

*H. spinifer* scorpion colonies maintained in the Venom and Toxin Research Laboratory of the National University of Singapore were used as the source of venom. For extraction of venom, the scorpions were immobilized using a restraining device [13], and the base of the telson (sting) was stimulated electrically (rectangular pulses of 40 V, 1-msec duration at a frequency of 10 Hz) using a Grass stimulator (model S88). For pharmacological experiments, the venom (HSV) ejected by the scorpion was collected in polythene vials, pooled together, and kept frozen at –20°, and when required, thawed slowly at 5° and used immediately for the experiments. For estimation of ACh and NE by mass spectrometry, the pooled venom was centrifuged (4500 *g* for 15 min), and the supernatant was diluted 1:1 with a solution (acetonitrile:methanol:deionized water; 5:4:1, by vol.) and used immediately for the assay.

### 2.2. HSV ultra-filtrate

HSV was subjected to ultra-filtration using Centricon microconcentrators (Amicon Division, WR Grace & Co.) with a 3000 MW cut-off. HSV (300 $\mu$L) was transferred to the sample reservoir of a Centricon tube with a 3000 MW cut-off membrane and centrifuged at 4500 *g* for 90 min at 4°; the filtrates of MW $<$ 3000 (F3K) and retentate (MW $>$ 3000) were collected for pharmacological screening. The procedure was repeated until adequate quantities of both samples were obtained.

### 2.3. Isolated tissue experiments

The rat isolated ACM is a smooth muscle preparation commonly used in pharmacological studies to investigate the effects of drugs on adrenergic and nitrergic transmission as well as the muscarinic response to cholinergic agonists [14]. More recently, the ACM has also been used in our laboratory to study the effects of venoms and toxins on adrenergic and nitrergic neurotransmission [4–6,15,16]. The CBCM has been used to evaluate the cholinergic effects of venoms and toxins on neuromuscular transmission and on post-synaptic nicotinic ACh receptors in skeletal muscle [17]. By using both, the ACM and the CBCM, we were able to carry out a detailed pharmacological characterization of the adrenergic and cholinergic effects produced by HSV.

### 2.4. CBCM

The pair of CBCM were isolated from 7- to 10-day-old chicks [18] and mounted in 8-mL organ baths containing a physiological salt solution (Krebs) of the following composition (mM): NaCl [118]; KCl (4.8); $KH_2PO_4$ (1.2); $CaCl_2$ (2.5); $NaHCO_3$ [25]; $MgSO_4$ (2.4); and d- (+) glucose [11], with a resting tension of approximately 1 g. The solution was maintained at 35° and aerated with 5% carbon dioxide in oxygen. The preparation was allowed to equilibrate for about 45 min with changes of Krebs solution at 15-min intervals. Data were recorded in a Mac Lab system8™ via a Grass force-displacement transducer (model FT03). The responses to exogenously applied ACh (200 $\mu$M/30 sec) and HSV or F3K (1 $\mu$L for a duration until the maximum contracture was evoked) were obtained in the absence and presence of *d*-TC (2 $\mu$M), $\alpha$-BTx (1 $\mu$g/mL), TTx (5 $\mu$M), $\omega$-CTx/GVIA (3 $\mu$M), and neostigmine (1 $\mu$M). Motor responses of the muscle were evoked by electrical field stimulation (7–10 V, 0.1 msec, 0.2 Hz) using a Grass stimulator (model S88).

### 2.5. Rat ACM

The pair of ACM from male Sprague–Dawley rats weighing 280–350 g were isolated [14] and mounted under 1 g resting tension in 4-mL organ baths containing a physiological salt solution (Krebs) of the same composition as for the CBCM preparation. The solution was maintained at 37° and aerated with 5% carbon dioxide in oxygen. The preparation was allowed to equilibrate for about 45 min with changes of Krebs solution at 15-min intervals. Contractile responses were evoked by electrical field stimulation (20–30 V, 1 msec, 10 Hz for 10 sec) using a Grass stimulator (model S88). Data were recorded in a Mac Lab system8™ via a Grass force-displacement transducer (model FT03). The responses to exogenously applied NE (5 $\mu$M/60 sec), ACh (200 $\mu$M/60 sec), and HSV or F3K (3 $\mu$L) (for a duration until the maximum contraction was evoked) were obtained in the absence and presence of phentolamine (5 $\mu$M), atropine (5 $\mu$M), and TTx (2 $\mu$M).

### 2.6. ESI-MS

ACh and NE in HSV were detected by ESI–MS using a Perkin-Elmer Sciex API 300 triple quadrupole LC/MS/MS system (Sciex) equipped with a nebulizer-assisted electrospray (ion spray) source. The mass spectrometer was operated throughout in the positive ion mode. The ion spray voltage was set to 4600 V, and the orifice voltage was set at 30 V. Nitrogen was used as the curtain gas with a flow rate of 0.6 L/min and as the nebulizer gas at a pressure of 30 psi. The samples were introduced by flow injection via a 5-$\mu$L

sample loop into the solvent (acetonitrile:methanol:deionized water; 5:4:1, by vol.) at a flow rate of 50 μL/min, using Shimadzu 10 AD binary pumps as the solvent delivery system.

For the detection of ACh [mass/charge (*m/z*) = 146.2; $M^+$], HSV was introduced into the mass spectrometer by flow injection (50 μL/min), and the intensity of the ion of *m/z* 146.2 was recorded. The concentration of ACh in HSV was determined from an ESI–MS standard curve obtained with various concentrations (50–5000 pmol) of standard ACh (Sigma). NE (*m/z* = 170.2; $M + H^+$) in HSV was detected by ESI–MS using a similar procedure as for ACh. The concentration of NE in HSV was determined from an ESI–MS standard curve obtained with various concentrations (50–5000 pmol) of standard NE (Sigma).

### 2.7. ESI—MS/MS

ESI–MS/MS analysis was carried out to obtain conclusive evidence as to the identity of ACh and NE in HSV. In separate experiments, standard ACh and NE were subjected to ESI-MS/MS. The ion spray voltage was set at 5200 V, the ring voltage at 300 V, and the orifice voltage at 40 V. Ions corresponding to ACh (146.2; $M^+$) and NE (170.2; $M+H^+$) were selectively fragmented in the second quadrupole using nitrogen as the CAD (collision-aided dissociation) gas. The third quadrupole was used to obtain the respective product ion spectra. The ions of *m/z* 146.2 and 170.2 in HSV were then similarly subjected to ESI–MS/MS, and their fragmentation fingerprints were compared with those of standard ACh and NE.

### 2.8. Treatment of HSV with AChE

In another series of experiments, HSV and standard ACh as a positive control were incubated for 30 min with an excess (500 Ellman units) of AChE, and the contents of ACh and choline (*m/z* = 104.2; $M^+$) in HSV were determined using ESI–MS. Choline in HSV was also determined prior to incubation with AChE for comparison.

### 2.9. Drugs and chemicals

Acetylcholine iodide, atropine sulfate, *d*-tubocurarine chloride, norepinephrine (-) bitartrate, neostigmine bromide, phentolamine hydrochloride, AChE, and TTx were obtained from the Sigma Chemical Co. α-BTx and ω-CTx/GVIA were obtained from Latoxan. All chemicals were of analytical grade and were purchased from Sigma. HPLC-grade water was obtained by using a Milli-Q purification system (Millipore).

### 2.10. Statistics

The data from the pharmacological experiments are expressed as the mean ± SEM of at least 6 experiments. Each point on the ESI–MS/MS standard curve for ACh and NE represents the mean ± SEM of 10 estimations by ESI–MS, and the results are expressed as mean ± SEM. Paired *t*-tests (two-tailed) were used to determine significance, and $P < 0.01$ was considered significant.

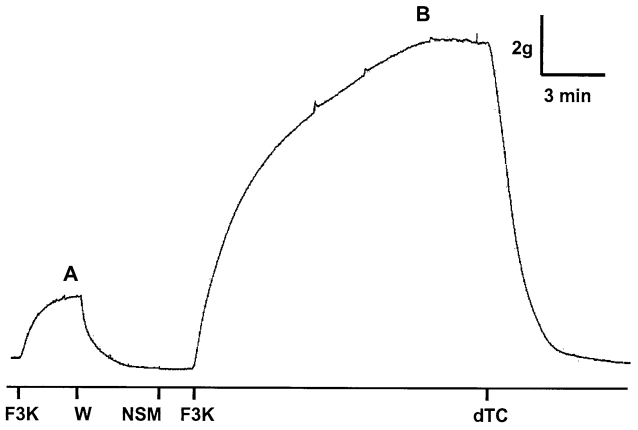


Fig. 1. Contracture produced by the venom ultra-filtrate (F3K; MW < 3000) in the chick biventer cervicis muscle (CBCM) in the absence (A) and presence (B) of the anticholinesterase neostigmine. Abbreviations: F3K, HSV ultra-filtrate, 1 μL; NSM, neostigmine, 1 μM; *d*-TC, *d*-tubocurarine, 2 μM. At point W, the ultra-filtrate was washed out with fresh Krebs solution. This figure is representative of six experiments.

## 3. Results

### 3.1. CBCM

The resting tone of the CBCM remained at baseline after 3 hr in control experiments. HSV (1 μL) produced an immediate and marked contracture of the tissue (1.6 ± 0.1 g tension) that reached a peak within 3 ± 0.1 min and relaxed progressively to control levels within 22.8 ± 1.3 min (data not shown). The ultra-filtrate (F3K) (1 μL), but not the retentate, produced a similar contracture (2.1 ± 0.1 g tension that peaked within 3 ± 0.1 min and lasted for 22 ± 1.6 min (Fig. 1). In the presence of neostigmine (1 μM), the contractile responses to HSV (1 μL) and F3K (1 μL) were

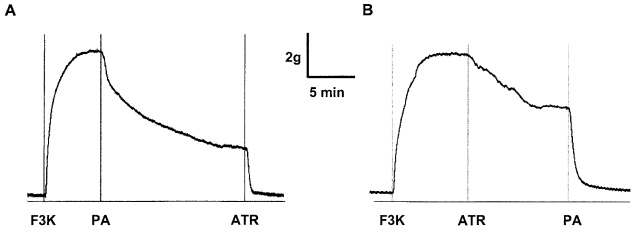


Fig. 2. Contractile response produced by HSV ultra-filtrate (F3K; MW < 3000) in the rat anococcygeus muscle (ACM). Abbreviations: F3K, HSV ultra-filtrate, 3 μL; PA, phentolamine, 5 μM; and ATR, atropine, 5 μM. (A) PA is added prior to the addition of ATR. (B) ATR is added prior to the addition of PA. This figure is representative of six experiments.

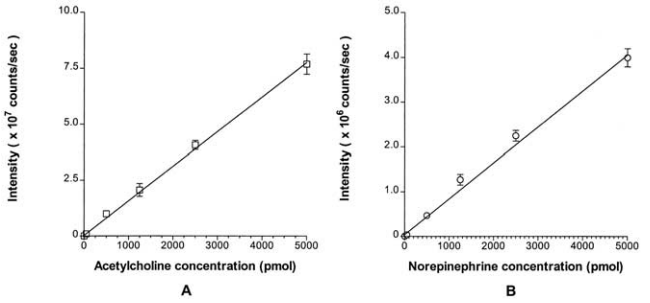


Fig. 3. EIS–MS standard curve for ACh (A) and NE (B). Each point represents the mean of 10 experiments. The vertical bars indicate the standard error where greater than the symbol.

potentiated significantly by 528 ± 30.8 and 632.5 ± 57.4%, respectively (Fig. 1). The contractures produced by HSV, F3K, and exogenous ACh (200 μM/30 sec) were blocked completely by *d*-TC (2 μM) and α-BTx (1 μg/mL), but not by TTx (5 μM) and ω-CTx/GVIA (3 μM), whereas the twitch responses to electrical field stimulation were blocked completely by *d*-TC, α-BTx, TTx, and ω-CTx/GVIA. NE did not produce a contracture in the CBCM.

### 3.2. Rat ACM

In the ACM, HSV (3 μL) produced a marked contractile response. Phentolamine (5 μM) partially (63.8 ± 3.1%, mean ± SEM) blocked the contractile response induced by HSV; the addition of atropine (5 μM) blocked the remaining response (Fig. 2A). Conversely, atropine (5 μM), when added first, blocked the HSV-induced response by 37.4 ± 1.6%, while the subsequent addition of phentolamine (5 μM) completely blocked the remaining response (Fig. 2B). In ACM preparations pretreated with both phentolamine (5 μM) and atropine (5 μM), HSV failed to produce a contractile response (data not shown). In control experiments, phentolamine (5 μM) completely blocked the response to NE (5 μM) but not to ACh (200 μM), whereas atropine (5 μM) completely blocked the response evoked by ACh but not the response to NE (data not shown). TTx (2 μM) failed to block the responses evoked by HSV, NE, or ACh in the ACM, but completely blocked the responses of the muscle to electrical field stimulation (data not shown).

### 3.3. ESI—MS

ESI–MS of pure ACh (MW 146.2) showed a single peak corresponding to an ion with an *m/z* ratio of 146.2 ($M^+$) (see Fig. 4A). A linear standard curve was obtained with various concentrations (50–5000 pmol) of standard ACh (Fig. 3A). When HSV (2.5 μL) was subjected to ESI–MS, an ion with an *m/z* ratio of 146.2, corresponding to the mass of ACh ($M^+$), was observed (data not shown). From the standard curve constructed for ACh, the content of ACh in HSV was determined to be 79.8 ± 1.7 μM. In another series of experiments, the NE standard (MW 169.2) was detected by ESI–MS as a single peak corresponding to an ion with a *m/z* ratio of 170. 2 ($M + H^+ = 170.2$) (see Fig. 5A). When crude HSV (2.5 μL) was subjected to ESI–MS, an ion with a *m/z* ratio of 170.2 corresponding to NE, was observed (data not shown). From a standard curve constructed for the NE standard (Fig. 3B), NE was found to be present in HSV in a concentration of 146.7 ± 19.8 μM.

### 3.4. ESI–MS/MS

ESI–MS/MS of standard ACh yielded a product ion spectrum containing two major ions of *m/z* 87.1 and 60.1 (Fig. 4B). This was identical to the ESI–MS/MS spectrum of the ion of *m/z* 146.2 found in HSV (Fig. 4C). Likewise, the fragmentation pattern of the ion of *m/z* 170.2 present in

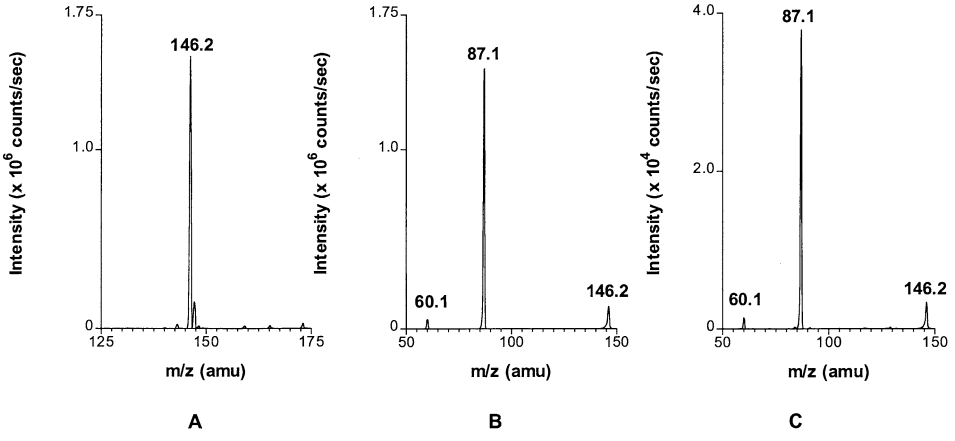


Fig. 4. EIS–MS/MS spectra: (A) parent compound –ACh standard (*m/z* 146.2); (B) fragmentation pattern of ACh standard showing a decrease in intensity of the parent compound (*m/z* 146.2) and the appearance of two daughter ions (*m/z* 87.1 and 60.1); and (C) fragmentation pattern of the ion of *m/z* 146.2 found in HSV showing an identical fingerprint to the MS/MS spectrum of ACh standard. MS/MS was performed twice for ACh standard and three times for the venom.

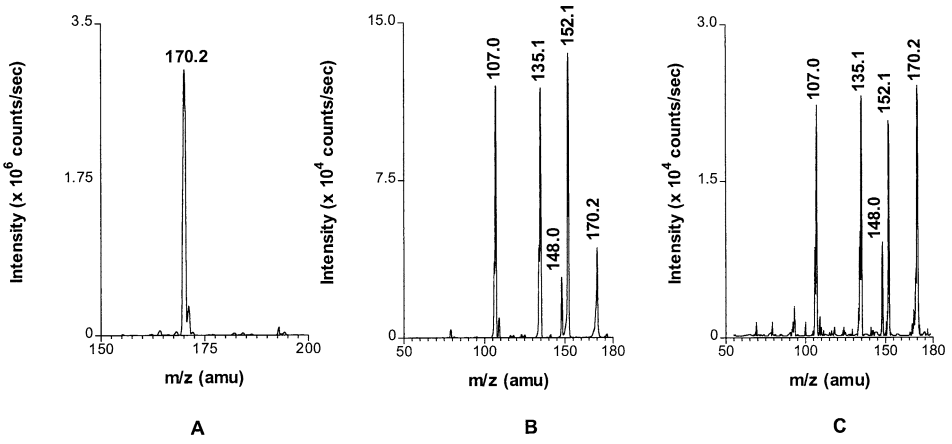


Fig. 5. EIS–MS/MS spectra: (A) parent compound –NE standard (*m/z* 170.2; $M+H^+$); (B) fragmentation pattern of the NE standard showing multiple daughter ions; and (C) fragmentation pattern of the ion of *m/z* 170.2 found in HSV showing an identical fingerprint to the MS/MS spectrum of the NE standard. MS/MS was performed twice for the NE standard and three times for the venom.

HSV matched the MS/MS fingerprint of standard NE (Fig. 5, B and C).

### *3.5. Detection of choline in HSV treated with AChE*

In another series of experiments, to confirm the identity of ACh, HSV and an ACh standard (positive control) were incubated with an excess (500 U) of AChE. AChE cleaves ACh into acetate and choline. In control experiments with standard ACh, choline (MW 104.2) was found to correspond to an ion with an *m/z* ratio of 104.2 ($M^+$) in the ESI–MS spectra (data not shown). HSV treated with AChE showed a significant ($P < 0.01$) reduction in the intensity of the ion of *m/z* 146.2 (corresponding to ACh) and a concomitant appearance of an ion of *m/z* 104.2 (corresponding to choline) (data not shown). The choline content of HSV before treatment with AChE, if any, was not detectable by ESI–MS, whereas the increase in the AChE-treated sample of HSV was found to be highly significant ($P < 0.001$; Fig. 6).

## 4. Discussion

Pharmacological characterization of the venom from the black scorpion *H. spinifer* revealed the presence of a low MW (<3000) cholinoceptor agonist in the venom ultrafiltrate (F3K), since the F3K-induced contracture of the CBCM was blocked by *d*-TC (a selective and competitive antagonist at the nicotinic ACh receptor) and $\alpha$-BTx (a highly selective post-synaptic blocker of nicotinic ACh receptors). Moreover, the F3K-induced contracture was potentiated significantly by the anti-cholinesterase neostigmine, which strongly suggests that the cholinoceptor agonist undergoes hydrolysis by the AChE present in the CBCM. The contracture produced by F3K cannot be attributed to the release of endogenous acetylcholine as a consequence of nerve depolarization since TTx, a selective blocker of voltage-gated sodium channels, did not inhibit the F3K-induced contracture. A pre-synaptic mechanism can be excluded further by the fact that $\omega$-CTx/GVIA (a selective blocker of N-type calcium channels involved in transmitter release) did not inhibit the contracture, whereas the twitches evoked by electrical field stimulation were blocked completely by both TTx and $\omega$-CTx/GVIA.

Our pharmacological studies also showed that the contractile response induced by HSV and F3K on the ACM appears to be due, in part, to stimulation of muscarinic receptors present in the muscle because it was partially (37.4 ± 1.6%) blocked by atropine, a selective muscarinic

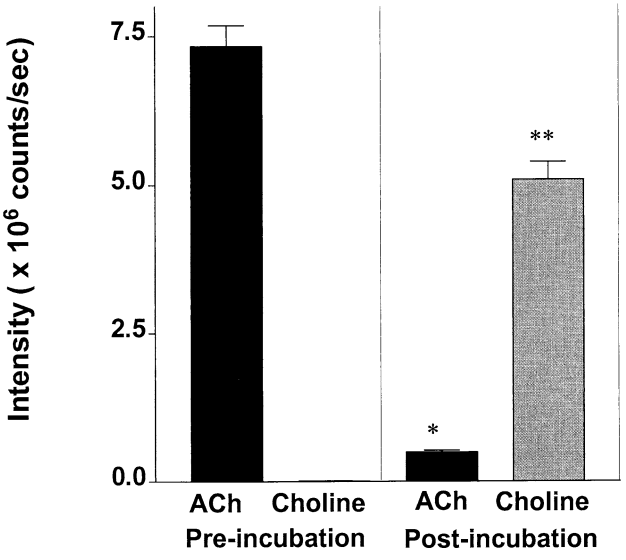


Fig. 6. Electrospray ionization–mass spectrometric quantification of ACh and choline in HSV before and after incubation with 500 Ellman Units of AChE for 30 min (*$P < 0.01$, significant; **$P < 0.001$, highly significant when compared with control values before incubation with AChE). Values are means ± SEM, N = 6.

cholinoceptor antagonist. The partial blockade of the HSV- and F3K-induced contractile responses by atropine suggests that another agonist substance in the venom must also be involved in mediating the residual component of the contractile response. Our pharmacological evidence confirms that an $\alpha$-adrenoceptor agonist is also involved in mediating the contractile response of the ACM to HSV and F3K, since phentolamine, a selective antagonist at post-synaptic $\alpha$-adrenergic receptors, blocked the remaining response. Moreover, in the presence of both atropine and phentolamine, HSV and F3K failed to evoke any contractile response. Although several scorpion venoms and neurotoxins produce potent adrenergic agonist actions in the ACM by modifying neuronal sodium channel activity and releasing NE [4–6], the observed contractile response cannot be attributed to HSV or F3K causing the release of NE via activation of neuronal sodium channels since TTx, a selective blocker of voltage-sensitive sodium channels, failed to block the contractile response to exogenous HSV or F3K but completely blocked the responses to electrical field stimulation. Thus, the component of the contractile response that was partially blocked by phentolamine was mediated via direct stimulation of the post-synaptic $\alpha$-adrenoceptors by an adrenergic agonist. Using ESI–MS and ESI–MS/MS analyses, we have confirmed that ACh ($79.8 \pm 1.7$ $\mu$M) and NE ($146.7 \pm 19.8$ $\mu$M) are, respectively, the cholinergic and adrenergic agonists present in high concentrations in HSV.

There is evidence that ACh may be involved in cholinergic transmission in the nervous system of scorpions [19, 20]. Gwee et al. [21] have already provided evidence that NE is actively synthesized in the usual tyrosine-dopamine pathway in the scorpion *Heterometrus longimanus.* In the spider *Araneus gemma,* it is speculated that NE is associated with venom secretion and, consequently, co-secreted with it [22]. Apart from their possible roles as neurotransmitters, it is difficult to explain why such high concentrations of ACh and NE are present in HSV. Animal venoms generally serve two functions: one defensive, achieved via pain-producing components, including ACh, especially when in combination with compounds like histamine, kinins, and serotonin; the other for paralyzing and immobilizing prey for capture [23–25]. It is possible that NE simply serves as a vasoconstrictor to localize the algesic effect of ACh during envenomation, an action similar to NE restricting the action of local anesthetics at a localized site. This explanation would corroborate the report that envenomation by the black scorpion (*H. scaber)* mainly causes a prolonged, local reaction of a sharp burning sensation around the site of the sting [26]. It has also been postulated that ACh in the venom, as in the case of the mamba (*Dendroaspis*) snakes, could facilitate distribution of more potent neurotoxins in the envenomed prey [10].

The black scorpion *H. spinifer* belongs to the family *Scorpionidae,* members of which are reported to be less lethal than the scorpions belonging to the *Buthidae* family, which include the Indian red scorpion *Mesobuthus tamulus,* the Chinese scorpion *Buthus martensi* Karsch, and the Israeli scorpion *Leiurus quinquestriatus* [7,27]. Scorpions from the *Buthidae* family are well known to contain potent neurotoxins in their venom for offense and defense [24,28]. Lethal envenomation by the physically much smaller scorpions *M. tamulus* and *L. quinquestriatus* has been attributed to the presence of toxins in their venom causing intense nerve depolarization as a consequence of delayed inactivation of sodium channels, resulting in enhanced peripheral sympathetic nerve activity and a massive discharge of catecholamines leading to cardiovascular complications and death [1–3]. In contrast, the major components of the black scorpion (*Heterometrus* species) venom appear to be small organic molecules [7,21,26]. It is likely that the large physical size, powerful pincers, and intense dark hue of the black scorpion are sufficient defense mechanisms for this species [7]. Surprisingly, despite their lower order of toxicity, polypeptide neurotoxins that modify potassium channel activity have also been shown to be present in the venom of scorpions belonging to the *Scorpionidae* family including *Heterometrus, Pandinus,* and *Scorpio maurus* [29–31], although our functional studies did not detect any pharmacological responses that could be attributed to modification of ion channel activity by the black scorpion venom. Moreover, although NE is present in high concentrations in HSV, it is not likely to produce lethal cardiovascular effects in humans upon envenomation by *H. spinifer,* since the entry of NE into the systemic circulation can be expected to be self-limiting due to its inherent vasoconstrictor effects. This probably accounts for the lack of documentation of lethal human envenomation by the black scorpion. It is also likely that the localized pain that follows black scorpion envenomation is mediated by the combined pharmacological effects of the high concentrations of ACh and NE present in its venom.

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