Cardiovascular excitatory effect on rats of a fraction isolated from the eyestalk of shrimp: *Peneaus vanameii*.

Efecto cardiovascular excitatorio en la rata, de una fracción aislada del tallo óptico del camarón: *Peneaus vanameii*.

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Abstract

The crustacean nervous system is an important source of substances with diverse biological activities, particularly affecting invertebrate cardiocirculatory physiology. However, the effects of these substances on the cardiovascular system of higher vertebrates are not very well documented. The purpose of this study was to evaluate the effects of a cardioexcitatory substance (CES) isolated from the eyestalk of the shrimp *Peneaus vanameii* on rat cardiovascular function. The administration of a purified fraction of this substance raised mean arterial pressure by 37.33 ± 5.00 mm Hg, pulse pressure 35.00 ± 4.93 mm Hg and heart rate 80.00 ± 12.83 beats/min over basal values ($p < 0.01$). Evaluation of the possible underlying mechanisms of this hypertensive and tachycardic effect reveled that dihydroergotamine pretreatment (20 µg/0.2 mL) reduced the effect of CES on mean blood pressure, but not on heart rate. Propranolol pretreatment (4 µg/0.2 mL) reduced the tachycardia, but not the hypertensive response. Enalapril pretreatment (5 µg/0.2 mL) did not modify the effects induced by CES on heart rate or blood pressure, and the verapamil pretreatment

(1 μg/0.2 mL) reduced both cardiovascular changes by 85% (p < 0.01). These results indicate that CES isolated from the shrimp eyestalk produces hypertension and tachycardia mediated by adrenergic receptors in association to calcium channels activation.

Key words: *Peneaus vanameii*, peptide fraction, blood pressure, heart rate.

Resumen

El sistema nervioso de crustáceos es una fuente importante de sustancias con actividad biológica diversa, particularmente la que afecta la fisiología cardiocirculatoria de los invertebrados. Sin embargo, los efectos de estas sustancias sobre el sistema cardiovascular de mamíferos no están bien documentados. El objetivo de este estudio, fue evaluar los efectos de una sustancia cardioexcitatoria (SCE) aislada del tallo óptico del camarón *Peneaus vanameii* sobre la función cardiovascular de la rata. La administración de una fracción purificada de esta sustancia incrementó la presión arterial media en 37,33 ± 5,0 mm de Hg, la presión arterial diferencial en 35,00 ± 4,93 mm de Hg, así como la frecuencia cardiaca 80,00 ± 12,83 lat/min sobre los valores basales (p < 0,01). La evaluación del mecanismo por el cual este efecto hipertensor y taquicardizante se produjo indicó que el tratamiento con dihidroergotamina (20 μg/0,2 mL) redujo los efectos del SCE sobre la presión arterial media, pero no sobre la frecuencia cardiaca. El pretratamiento con propranolol (4 μg/0,2 mL) redujo la taquicardia pero no la respuesta hipertensiva. El pretratamiento con enalapril (5 μg/0,2 mL) no modificó los efectos inducidos por SCE sobre el corazón o los vasos sanguíneos, el pretratamiento con verapamil (1 μg/0,2 mL) redujo ambos cambios cardiovasculares en un 85% (p < 0,01). Estos resultados indican que el SCE aislado del tallo óptico del camarón produce hipertensión y taquicardia mediada a través de receptores adrenérgicos, en asociación con una activación de los canales de calcio.

Palabras clave: *Peneaus vanameii*; fracción peptídica, presión arterial, frecuencia cardiaca.

INTRODUCTION

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Cardiovascular excitatory effect

The crustacean nervous system is an important source of neurohormones, neurotransmitters and neuromodulators with diverse biological activities, exhibiting important effects on the cardiocirculatory physiology of both vertebrates and invertebrates. The neuronal groups and neurosecretory cells responsive to these substances have been identified in vertebrates and invertebrates by autoradiographic, immunocytochemical and histochemical techniques, which have allowed to establish their biochemical homology in different animal species[1,2,3,4,5,6,7].

Extensive research on crustacean neuropeptides confirmed the physiological role of these substances; however their effects as neurohormones or neurotransmitters in vertebrate and invertebrate species raised the possibility of some phylogenetic relationship between related species, and their role in neuroendocrine integration[8,9,10,11,12,13,14].

Among the peptides identified in invertebrates, particularly in crustaceans, are worthy of mention: the hyperglycemic hormone, some peptides associated to circadian rhythm control, several hormones related to water-mineral balance, and a group of substances with important stimulating actions on the cardiovascular system[15,16,17,18,19,20,21].

These cardioexcitatory substances exert important effects on the heart function of several animal species, particularly related to environmental adaptation and the circadian rhythm. Preliminary studies carried out with substances isolated from mollusks and crustaceans suggest their coexistence with other biogenic amines such as catecholamines, serotonin and octopamin, in the same nerve terminal. Biochemical studies and purification techniques allowed the isolation and characterization of different peptidic compounds with specific biological activities, such as the peptide FMRFamide, which has the property of stimulating cardiac activity in mollusks and crustaceans[22,23,24,25,24]. Furthermore, an additional group of low molecular weight peptides were identified in crustaceans, exhibiting remarkable chemical homology, and physiological effects similar to the ones found in mammalian neuropeptides. Autoradiographic and immunocytochemical studies have shown the presence of specific receptors for these peptides in the rat dorsal root and primary afferent fibers of the spinal cord. The presence of these peptides was also established in autonomic fibers innervating cardiac muscle, pericardium and ganglionic tissue in

crustaceans, gastropods and higher vertebrates[25],[26],[27],[28],[29],[30],[31],[32]

Experimental evidence indicates that these neuropeptide families are associated to cardiocirculatory control in invertebrates; and their chemical resemblance to the ones present in vertebrate cardiac and neural tissues suggests that they represent an evolutionary step in vertebrate cardiovascular regulation. Because the cardiovascular activity of neuropeptides present in the eyestalk of the shrimp *Peneaus vanameii* has not been explored, the objective of this study was to evaluate the cardiovascular activity of a purified peptide fraction from the eyestalk of *Peneaus vanameii* on blood pressure and heart rate of anesthetized rats.

MATERIALS AND METHODS

Penaeus vanameii shrimps (n = 1900) 100 g average weight were provided by Siembramar, C.A., Barcelona, Anzoátegui State, Venezuela.

Cardiovascular tests were performed on 60 male Sprague-Dawley rats, 300 g average weight, from the Venezuelan Institute of Scientific Research (IVIC). These animals were kept in the animal room of the Health Sciences School of the Universidad de Oriente Venezuela, maintained with *ad libitum* access to water and food pellets (Ratarina®, Purina C.A.).

Preparation and fractionation of crude eyestalk fraction

Out from 1900 crude shrimps, 300 g of crustacean eyestalks were obtained by a cut at the point of attachment to the shrimp cephalothorax. The eyestalks were maintained in reagent grade acetone at 12°C and later homogenized in a Sorvall® homogenizer at 3500 rpm for 15 min; afterwards, acetone was evaporated at room temperature. The resulting 300 mg powder was treated with chloroform under the same condition as above, and dried. The eyestalk powder obtained was then extracted with 400 mL of distilled water. The yellow supernatant was decanted after centrifuging at 4°C at 12000 rpm for 10 min, and dried under reduced pressure at 40°C, yielding a 180 mg residue and was termed "crude extract", which was then fractionated on Sephadex G-25 (Pharmacia Fine Chemicals) column, using methanol as eluent to give five fractions termed I-V.

Bioassay with the purified fractions

Bioassays on motor neurons from the third abdominal ganglion of *Penaeus vanameii* were performed to each of the five obtained fractions, in order to establish its biological activity, according to methods published elsewhere which briefly, consists of recording the spontaneous activity of the superficial branch oh the third root of this ganglion with a suction electrode. The measurement of firing rate corresponds to the activity of motoneuron f-5)[32]. The highest activity level was observed in fraction V. All cardiovascular assays, made on rats, were performed with this particular fraction. The purified fraction V corresponds to extracted peptidic compound (CES) according to already published procedures carried out in samples of invertebrate nervous tissues The percentage of recovery at the end of this procedure was (w/w = 0.06%)[1, 2, 11, 34].

Evaluation of Fraction V Cardioexcitatory substance (CES) on blood pressure and heart rate

In order to test the cardiovascular activity of CES, 50 rats were anesthetized with sodium pentobarbital (35 mg/Kg i.p.) and its femoral vein catheterized with an Intramedic® PE 50 polyethylene catheter; this venous line was used to administer CES or its vehicle; the femoral artery was catheterized, for blood pressure recording with a Statham® Strain-Gauge transducer, connected to a Grass® 7E Polygraph via a $7DA_1$ preamplifier. Heart rate was monitored from the electrocardiography recordings obtained from four subcutaneous platinum electrodes placed on the animal's limbs, and connected to the Polygraph EKG leads I and II preamplifier.

Bolues of 0.2 mL intravenous injections containing increasing doses of CES, with a range of 10-800 µg were administered to rats in order to establish the dose evoking the maximal response. Blood pressure and heart rate were continuously recorded; the maximal effect was achieved at the 800 µg dose. This dose was used in all subsequent experiments before and after pharmacological blockade. The control group consisted of 10 rats intravenously injected with a 0.2 mL bolus of 0.9% NaCl, under the same experimental conditions. Mean arterial pressure was calculated as diastolic blood pressure + 1/3 [systolic (SBP)-diastolic blood pressure (DBP)].

Evaluation of pharmacological blockade on CES cardiovascular responses

In order to establish the role of a-adrenergic receptors on the response, a group of 10 rats received 20 µg/0.2 mL dose of intravenous dihydroergotamine. b-adrenoceptor cardiovascular stimulation was evaluated through 4 µg/0.2 mL propranolol pretreatment. Pretreatment with enalapril (5 µg/0.2 mL) and verapamil (1 µg/0.2 mL) was also evaluated to rule out the role of the renin-angiotensin system and calcium channels, respectively, on CES cardiovascular induced changes. In all cases, after 15 minutes of drug pretreatment, 800 µg of CES was injected, with continuous blood pressure and heart rate recordings.

Statistical analysis

All results are expressed as mean ± standard error of the mean. Statistical significance was tested by non-paired t tests for intergroup mean comparisons. All other comparisons were made with two-way ANOVA. Statistical significance was considered when $p < 0.05$. Data was computed with SPSS program version 11.0.

RESULTS

Intravenous administration of 800µg of CES increased mean arterial pressure by 37.33 ± 5.00 mmHg (41%), and pulse pressure (SBP minus DBP) by 35.00 ± 4.93 mmHg (113%). Values were significantly higher than the ones observed after vehicle injection. $F_{(19)} = 38.30$, $p < 0.0001$ for mean arterial pressure, $F_{(13)} = 23.46$, $p = 0.0004$ for pulse pressure (Figs. 1 and 2).

CES increased heart rate by 80.00 ± 12.83 beats/min, representing an increase of 27. 46% over basal values $F_{(19)} = 21.97$, $p = 0.0002$ (Fig. 3). Effects on blood pressure began approximately 10 seconds after CES injection and lasted for 15-20 minutes, after which all parameters returned to their original levels. The injection of 0.2 mL vehicle did not change heart rate.

Effect of pharmacological pretreatment on CES effect

Dihydroergotamine (DHE) pretreatment produced a mild 10.0 mm Hg initial reduction of both SBP and DBP basal blood pressure. After injection of DHE, there was a 26.13 ± 1.26 mmHg reduction of control response induced by CES on blood pressure (Fig. 4). However, DHE did not significantly modify the CES-induced tachycardia by CES. $F(14) = 40.52$, $p < 0.0001$ (Fig. 5).

Intravenous pretreatment with propranolol produced a 60 beats/min (–17%) heart rate decrease below baseline, which was considered evidence of b-adrenergic blockade. Propranolol did not significantly reduce the changes in blood pressure induced by CES ($p > 0.05$) (Fig. 4). However, propranolol reduced the CES induced tachycardia by 46.25 ± 3.39 beats/min (–66%) $F(13) = 79.89$, $p < 0.0001$ (Fig. 5).

Enalapril pretreatment did not modify the cardiovascular changes induced by CES on blood pressure or heart rate ($p > 0.05$) (Figs. 4 and 5).

Verapamil significantly reduced the cardiovascular responses to CES in both mean blood pressure and heart rate by 28.7 ± 2.10 mmHg and 68.5 ± 2.50 beats/min, respectively, this verapamil blockade was highly significant $F(13) = 38.02$, $p < 0.0001$) (Figs. 4 and 5).

DISCUSSION

Experimental evidence indicates that neuropeptides of the crustacean nervous system, including the eyestalk, have biological activity on many organs from diverse animal species; as it was demonstrated on neural and cardiovascular tissues from invertebrates and vertebrates[1, 10, 11, 29, 36]. On the other hand biochemical experiments indicate that some substances isolated from crustaceans nervous system show a structural homology with other cardioexcitatory peptides obtained from mollusks, gastropods and insects; and the presence of both, neural circuits and specific receptors for these peptides

have also been found in mammalian cardiovascular tissues[2, 37, 38].

The employed extraction procedure eliminated proteins, low molecular weight substances and lipids: allowing isolation of peptides into the CES. Intravenous administration of CES in all our experimental animals produced a significant increase in systolic and diastolic blood pressure, with a concomitant increase in heart rate, which indicates an important stimulation of the rat cardiovascular system. This effect had an average duration of 20 minutes, which means there is some deactivating mechanism which could be operating at plasma such as peptidases, kidney excretion or liver biotransformation into inactive compounds.

There is no previous experimental report about the effect of cardiovascular excitatory shrimp neural extracted CES on the rat cardiovascular system of the effect of these neuropeptides enzimatic deactivationat cardiovascular activity.ood pres. Previous *in vitro* studies performed in our laboratory showed CES contains peptides having a positive inotropic and chronotropic effects on isolated rat atria and significant vasopressor effects on isolated thoracic aortic rings, which means CES contains substances having a stimulatory action on these organs.

Our results coincide with other researcher's findings that have demonstrated a cardioaccelerating effect on mollusks and snails hearts induced by cardioactive neuropeptides obtained from crustacean and insect nervous systems, and biochemical studies have suggested some degree of structural homology between them[36, 37, 38].

Dihydroergotamine produced an important reduction of the hypertensive response to CES, injection but did not change the tachycardia induced by assayed CES. These findings suggest a role of vasopressor a-adrenergic receptors in the hypertensive response induced by CES. However, this experiment does not ruled out some facilitatory effect of CES on noradrenaline release from sympathetic nerve terminals. Dihydroergotamine is not a selective á adrenergic blocker, it can antagonize serotonin at $5HT_{1A}$, $5HT_{2B}$ and $5HT_{2C}$ receptors; so, we can not disregard a serotonin agonist effect of CES. Previous administration of propranolol significantly reduced the tachycardia induced by CES without any modification on blood pressure, suggesting the b adrenergic receptors mediate this tachycardic response. The effects of CES are similar to those produced by catecholamines such as adrenaline but the presence of this

catecholamine can be ruled out because it is not found in the eyestalk of *Peneaus vanameii.*

Pretreatment with enalapril did not modify CES-induced cardiovascular changes, suggesting that angiotensin II synthesis does not play a role in the cardiovascular response produced by this neural fraction.

Previous administration of verapamil produced a highly significant decrease of both the hypertensive and tachycardic response to CES, indicating an important role of calcium channels in the cardiovascular response to this substance. These findings have not been previously reported, but are consistent with former results obtained; showing "in vitro" that verapamil significantly reduces the effects of CES on isolated atrial contraction strength and aortic contractile response. There is a report in invertebrates showing that exposure of heart cells of *Lymnaea* snail to these cardioactive peptides changes the opening and closing kinetics of calcium channels[6].

In conclusion, CES extracted from shrimp eyestalk Peneaus *vanameii* contains a cardioaccelerating and pressor substance of peptidic nature, and whose effects were antagonized by verapamil and dihydroergotamine, suggesting an á-adrenergic, calcium-ediated action; without any involvement of angiotensin II receptors because enalapril did not introduce any change on its actions.

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