

## Screening of the venoms of two *Parabuthus* scorpion species on isolated cardiomyocytes

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### ABSTRACT

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Crude, diluted venoms of *Parabuthus transvaalicus* and *P. granulatus* were tested on isolated cardiomyocytes. Ventricular myocytes were subjected to hypercontractility tests and whole-cell voltage clamp. Both venoms had a profound influence on the hypercontractility of cardiomyocytes, which was markedly decreased when they were pre-absorbed with commercial antiserum. Whole-cell clamp results showed an increase in the sodium current, and a retardation of the time course of inactivation, implicating the presence of an  $\alpha$  toxin in both venoms.

**Keywords:** Cardiomyocytes, *Parabuthus granulatus*, *Parabuthus transvaalicus*, scorpion, venom

### INTRODUCTION

Although the venoms of Southern African *Parabuthus* species are known to have serious effects on their victims (Müller 1993), these venoms have not yet been investigated. The venoms of *Parabuthus* species are of clinical importance, because envenomation by some members of this genus may be lethal to small domestic animals and man (see Müller 1993).

In recent times, attention has been focused on bioactive substances, because they modulate the physiological processes in the vertebrate body (Bloom & Polak 1981). One of the rich and rewarding sources of bioactive substances is the amphibian skin (Melchiorri & Erspamer 1981). Similarly, animal venoms are also richly endowed with bioactive substances

(Kawai 1991; Strong 1990). In the rapidly expanding field of bioactive substances, considerable effort is being made to screen venoms and isolate novel candidate bioactive substances.

Furthermore, each candidate bioactive substance is carefully tested to ascertain its modulating action on the cellular level of the physiology (Borneman & Hahin 1993; DeBin, Maggio & Strichartz 1993). One of the most rewarding methods for testing the mechanism of the modulating action is the whole-cell clamp method, because it enables accurate pin-pointing of minute alteration in the cellular physiology (Hille 1992).

The whole-cell clamp method has found application in the study of bioactive fractions of animal venoms. These toxins are employed as invaluable selective tools, to block some channels and thus enable specific investigation of the others (Hille 1992).

This study was undertaken to screen the venoms of two *Parabuthus* species in order to test their bioactivity. A subsidiary aim was to gain some insight into the effectiveness of the antivenin at present being employed in the Republic of South Africa.

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## MATERIALS AND METHODS

### Solutions

Calcium-free Tyrode solution: NaCl 130 mM; KCl 5,4 mM;  $\text{KH}_2\text{PO}_4$  1,2 mM;  $\text{MgSO}_4$  1,2 mM; HEPES 6 mM (pH 7,2 adjusted with NaOH).

Tyrode solution: NaCl 137,6 mM; KCl 5,4 mM;  $\text{MgCl}_2$  1,0 mM;  $\text{CaCl}_2$  1,8 mM; HEPES 11,6 mM and glucose 10 mM (pH 7,2 with NaOH).

Low-sodium- Tyrode solution: The same as above, except for NaCl 30 mM, sucrose 227 mM.

Pipette solution:  $\text{CsCl}_2$  125 mM;  $\text{MgCl}_2$  5 mM;  $\text{Na}_2\text{ATP}$  5 mM; ethylene glycol-bis ( $\beta$ -minoethyl ether) N, N', N', N'-tetraacetic acid (EGTA) 15 mM, Tea-Cl 20 mM and HEPES 10 mM (pH 7,2 with NaOH).

### Collection and preparation of venom

Specimens of *Parabuthus transvaalicus* and *P. granulatus* were captured and kept in single cages at room temperature. They were fed on meal worms and received water *ad libitum*. All the specimens were identified by Dr Ansie Dippenaar (Plant Protection Research Institute, Pretoria).

Venom was obtained from each specimen by agitating it until a droplet of venom was visible on the telson. The venom was collected with a hypodermic syringe fitted with a 21-gauge needle. This procedure was repeated several times. Venom from five specimens of each species was pooled and diluted with 5 ml of Tyrode solution, and then divided into four 1-ml aliquots which were rapidly snap-frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$ .

One aliquot of each batch of diluted venom was used to estimate the concentration of protein. This was done indirectly, by employing the Bradford method (Bradford 1976) to assess the total protein content of the dilution. For the sake of simplicity, the total protein content is referred to as the concentration of venom in all the dilutions of venom employed.

Each aliquot of frozen, diluted venom was thawed at  $4^\circ\text{C}$  for 1 h and diluted with Tyrode solution to obtain concentrations of 4  $\mu\text{g/ml}$ , 12  $\mu\text{g/ml}$ , 24  $\mu\text{g/ml}$  and 50  $\mu\text{g/ml}$ , respectively.

Fifty  $\mu\text{g}$  of venom was pre-absorbed with 0,5 ml of South African Institute for Medical Research (SAIMR) commercial antivenin for 12 h at  $4^\circ\text{C}$ .

### Cell isolation

Adult Duncan-Hartley-strain guinea-pigs were decapitated and their hearts promptly removed. Each heart was cannulated, and retrograded perfusion was established via the aorta, by means of Tyrode solution, at  $37^\circ\text{C}$ . The ventricular myocytes were dis-

persed by the employment of a  $\text{Ca}^{2+}$ -free solution containing 6 mg of collagenase (Type II, Sigma) and 10 mg of protease (Type XIV, Sigma) per 40 ml of the calcium-free solution. As soon as a dark area appeared on the outer surface of the ventricle, small pieces were removed and shaken in a beaker containing Tyrode solution with calcium (0,18 mM  $\text{CaCl}_2$ ) to isolate the cells (Mitra & Morad 1985).

### Test for the viability of cardiomyocytes

1-cm<sup>2</sup> Grid chamber (1-mm<sup>2</sup> grid) contained in a perspex block was fitted on a mechanical stage of a microscope. About 60 freshly isolated cardiomyocytes were introduced into the chamber containing 0,5 ml of Tyrode (control), incubated at room temperature for 75 min, and the hypercontracted cardiomyocytes were scored at 25, 50 and 75 min. The above procedure was repeated with venom diluted in Tyrode of each of the species. Dilutions of 4  $\mu\text{g/ml}$ , 24  $\mu\text{g/ml}$  and 50  $\mu\text{g/ml}$  were used, respectively, as well as 50  $\mu\text{g}$  of venom absorbed with antivenin.

### Whole-cell clamp

The whole-cell clamp method (Hamill, Marty, Neher, Sakman & Sigworth 1981) was employed with the use of the isolated myocytes. The cells were perfused through a multi-barrel perfusion system with low sodium Tyrode, in order to obtain  $\text{Na}^+$ -currents ( $I_{\text{Na}}$ ). Voltage clamps were done after giga-ohm seals had been secured with heat-polished borosilicate glass (Jencons Scientific) pipettes (2–4 mega-ohm). Results obtained with a Dagan model 8800 total clamp amplifier were computed and recorded with a clampex (Labmaster) version 5.5 program. The protocol for the whole-cell clamp was as follows: the membrane was clamped from a holding potential of  $-110$  mV with test potentials that varied between  $-60$  mV and  $-35$  mV, with increments of 5 mV. The repeat frequency of the pulse was 0,25 Hz and each pulse duration was 10 ms.

The current-voltage (I-V) relationship curves for sodium were plotted and normalized to 1 nA for both the venom and Tyrode. From these curves the difference in mV between the shift in activation of the venom in relation to the Tyrode was estimated at 0,5  $I_{\text{Na}}$  max.

## RESULTS

### Tests for hypercontraction

At a concentration of 24  $\mu\text{g/ml}$ , the venom of *P. transvaalicus* had a 52% mortality of cardiomyocytes after a 75-min exposure (Fig. 1). When the venom of *P. granulatus* was employed at 50  $\mu\text{g/ml}$  for the same period, 47% of the cells died (Fig. 1). In contrast, when the same concentration of venom from the

above species was employed, but pre-absorbed with antivenin, only 11% and 12% of the cardiomyocytes died, respectively (Fig. 1).

### Whole-cell clamp

The venom of both species tested, had a marked influence on the sodium-current inactivation (Fig. 2). The venom slowed channel inactivation and an influx of sodium ions considerably exceeded the values of the control. The current-voltage relationship revealed that the activation curves had shifted insignificantly towards a more negative potential (about 6 mV) by the venoms of both species.

### DISCUSSION

The test for hypercontraction of cardiomyocytes is a valuable tool for the screening of crude venoms (Van der Walt, Van Rooyen, Oberholzer & Van Aswegen 1992). In this study, the above test showed that the venoms of both species have a marked effect on the viability of isolated cardiomyocytes. Although the venom of *P. transvaalicus* was employed at half the strength of *P. granulatus*, it was nevertheless more potent. This difference in potency may be even more profound in the case of envenomation, as *P. transvaalicus* is known to produce about three times the volume of venom obtained from *P. granulatus* when milked by electrical stimulation (personal communication, P. Hawkins 1995).

At present the commercial antivenin is produced from the venom of *P. transvaalicus* only (Müller 1993). However, this investigation demonstrated that the effects on cardiomyocytes of the venoms of both species are diminished when pre-absorbed with antivenin. Whether or not the antivenin is effective against the neurotoxicity of the venom of *P. granulatus*, is yet to be determined.

The whole-cell-clamp results of this investigation showed an increase in the inward sodium current and a considerable slowing down of inactivation. This is due to the retarding of inactivation (Gonoi & Hille 1987). The latter is typical for  $\alpha$  toxins, known to be present in the venoms of Old World scorpions (Gonoi & Hille 1987), and was also observed in the venom of a New World scorpion *Tityus serrulatus* (Kirsch, Skattebol, Possani & Brown 1989).

The I-V relationship, which indicated a slight negative shift of the activation curve of about 6 mV in the hyperpolarized direction, is in line with the idea of Gonoi & Hille (1987), who argued that any substance that slows inactivation will cause an increase in peak current and a negative shift of the peak conductance curve along the voltage axis (Kirsch *et al.* 1989; Gonoi & Hille 1987). From the latter, it is doubtful whether a  $\beta$ -toxin is present as well because, according to Go-

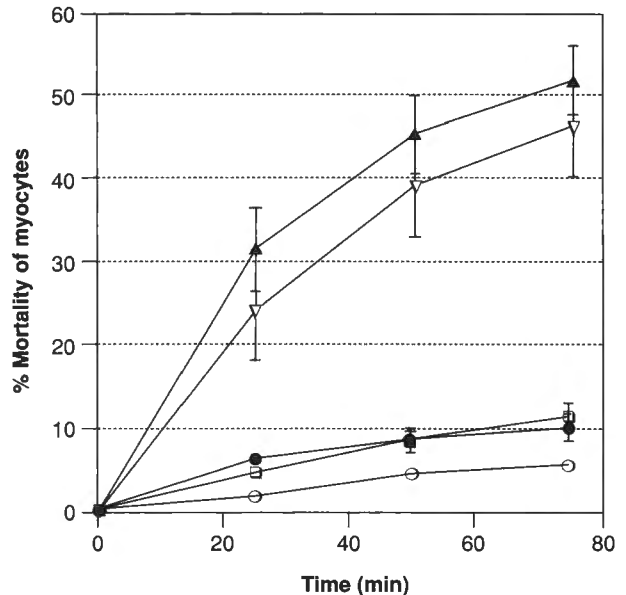


FIG. 1 Percentage mortality of cardiomyocytes versus time after application of the venoms of two scorpion species and the effect of the antivenin ( $n = 6$ )

- ▽ = *P. granulatus* venom (50 µg/ml)
- ▲ = *P. transvaalicus* venom (24 µg/ml)
- = *P. granulatus* venom absorbed with antivenin
- = *P. transvaalicus* venom absorbed with antivenin
- = control (Tyrode)

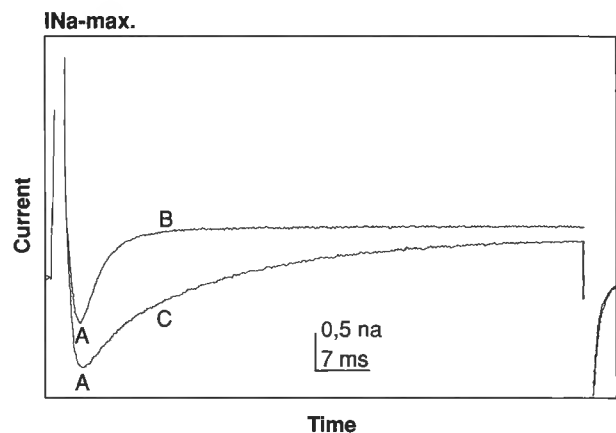


FIG. 2 The influence of venoms on the maximum sodium current and inactivation of myocytes representative of both species ( $n = 7$ )

- A = maximum current
- B = control (Tyrode)
- C = venom

noi & Hille (1987), this shift is more pronounced in scorpion venoms containing it. This phenomenon is also advocated by Yatani, Kirsch, Possani & Brown (1988).

The findings of this investigation clearly showed that the venoms of *P. transvaalicus* and *P. granulatus* do have an effect on cardiomyocytes, and that the form-

er species is, without any doubt, the more potent one. These findings are endorsed by Müller (1993) who demonstrated cardiac involvement of *Parabuthus* envenomation, although they were slight and overwhelmed by the neurotoxic effects. They are also in line with the findings of Gueron, Ilia & Sofer (1993).

Further screening of the above venoms with the use of nerve cells, are under way, and may shed more light on mechanisms which prompt the clinical signs and symptoms.

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## REFERENCES

- BLOOM, S.R. & POLAK, J.M. 1981. Introduction, in *Gut hormones*, 1–9, edited by S.R. Bloom & J.M. Polak. Edinburgh: Churchill Livingstone.
- BORNEMAN, J. & HAHIN, R. 1993. Purification of protein toxins from *Leiurus quinquestriatus* Hebraeus that modify Na channels. *Toxicon*, 31:1019–1038.
- BRADFORD, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72:248–254.
- DEBIN, J.A., MAGGIO, J.E. & STRICHARTZ, G.R. 1993. Purification and characterization of chlorotoxin, a chloride channel ligand and from the venom of the scorpion. *American Journal of Physiology*, 264:C361–C369.
- GONOI, T. & HILLE, B. 1987. Gating of Na channels. *Journal of General Physiology*, 89:253–274.
- GUERON, M., ILIA, R.E. & SOFER, S. 1993. Scorpion venom cardiomyopathy. *American Heart Journal*, 125:1816.
- HAMILL, O.P., MARTY, A., NEHER, E., SAKMAN, B. & SIGWORTH, F.J., 1981. Improved patch-clamp for high resolution current recordings from cells and cell free membrane patches. *Pflügers Archiv*, 391:85–100.
- HILLE, B. 1992. *Ionic channels of excitable membranes*, 2nd ed. Massachusetts: Sinauer.
- KAWAI, N. 1991. Neuroactive toxins of spider venoms. *Journal of Toxicology—Toxin Reviews*, 10:131–167.
- KIRSCH, G.E., SKATTEBOL, A., POSSANI, I.D. & BROWN, A.M. 1989. Modification of Na channel gating by an  $\alpha$  scorpion toxin. *Journal of General Physiology*, 93:67–83.
- MELCHIORRI, P. & ERSPARMER, V. 1981. New amphibian peptides, in *Gut hormones*, edited by S.R. Bloom, & J.M. Polak. Edinburgh: Churchill Livingstone: 39–42.
- MITRA, R. & MORAD, M. 1985. A uniform method for dissociation of myocytes from the heart and stomachs of vertebrates. *American Journal of Physiology*, 249:H1056–H1060.
- MÜLLER, G.J. 1993. Scorpionism in South Africa. *South African Medical Journal*, 83:405–411.
- STRONG, P.N. 1990. Potassium channel toxins. *Pharmacological Therapeutics*, 46:137–162.
- VAN DER WALT, M.P.K., VAN ROOYEN, J.M., OBERHOLZER, G. & VAN ASWEGEN, G. 1992. Cardiotoxicity of the skin of the red-banded rubber frog, *Phrynomerus bifasciatus* (Smith 1847). *Onderstepoort Journal of Veterinary Research*, 59:107–109.
- YATANI, A., KIRSCH, G.E., POSSANI, L.D. & BROWN, A.M. 1988. Effects of New World scorpion toxins on single channel and whole-cell cardiac sodium currents. *American Journal of Physiology*, 254:H443–H452.