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CARDIOTOXICITY OF THE SKIN OF THE RED-BANDED RUBBER FROG, *PHRYNOMERUS BIFASCIATUS* (SMITH 1847)

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ABSTRACT

VAN DER WALT, M. P. K., VAN ROOYEN, J. M., OBERHOLZER, G. & VAN ASWEGEN, G., 1991. Cardiotoxicity of the skin of the red-banded rubber frog, *Phrynomerus bifasciatus* (Smith 1847). *Onderstepoort Journal of Veterinary Research*, 59, 107–109 (1992).

The skin of the red-banded rubber frog is said to secrete an unidentified toxin. Whole skin extract as well as HPLC fractions were tested for toxicity on viable, isolated cardiomyocytes. The skin extract and one of its fractions were shown to be cardiotoxic. Whole-cell clamping tests demonstrated that both extract and fraction interfered with the function of the potassium channels of the cardiomyocytes and that the toxic substance has an affinity for the IK_1 channels. Investigations are in progress to isolate and characterize the toxic substance.

INTRODUCTION

Typically the amphibian skin is richly endowed with two types of glands. Many small mucous glands secrete a colourless, watery fluid essential to skin respiration while larger, but scarcer, poison glands provide protection to some degree from predation (Theis, 1932). The secretions of the poison glands contain various toxins including peptides, amines and alkaloids (Lind & Duellman, 1985; Erspamer, Falconier Erspamer, Mazzanti & Endean, 1984; Erspamer, 1971) some of which are potently bioactive (Melchiorri & Erspamer, 1981). Furthermore, some toad species secrete cardiotoxic aglycones in their body fluids [Chen & Kovarikova (1967) and Knowles (1964) as cited by Kellerman, Coetzer & Naudé, 1988].

Although it is known that the skin secretions of the red-banded rubber frog is toxic, irritant (Passmore & Carruthers, 1979) and lethal to a number of other anuran species (Pienaar, Passmore & Carruthers, 1976), the precise nature or specific action of this poison is unknown. Furthermore, toxins have become increasingly important in the study of ion channels (Latorre & Miller, 1983; Moczydlowsky, Lucchesi & Ravindran, 1988) and for these reasons this investigation was launched.

The fraction of the skin extract containing the putative toxin, was tested by direct observation of its ability to kill cardiomyocytes and also by employing a sensitive wholecell clamp method to evaluate its function on ion channels.

MATERIALS AND METHODS

Twenty specimens of *Phrynomerus bifasciatus* were supplied over a period of 6 months by Mr W. D. Haacke of the Transvaal Museum and Dr L. Braack of the National Parks Board. Each batch (consisting of 4–6) specimens were decapitated on arrival, promptly skinned, each skin was mass-measured and the skins pooled for extraction. The pooled skins were extracted with 80 % ethanol at 4 °C for one week, and the extract of twenty specimens was dried by evaporation under a stream of nitrogen. For control, the skins of *Bufo regularis* (common toad) were collected and treated in the same way. A portion of the extracts were employed for HPLC and the rest was used for the hypercontraction tests. The dried extracts was dissolved in a minimum quantity of methanol, filtered and injected into a Beckman System Gold HPLC. Isocratic separation (Guyon-Gruaz, Raulais & Rivaille, 1984) was employed and 21 fractions of 1 mℓ each collected. Each fraction was dried under nitrogen, and stored at −20 °C to be tested for toxicity.

Cell Isolation

Adult Duncan-Hartley strain guinea-pigs were decapitated and their hearts promptly removed. Each heart was cannulated, and retrograded perfusion established via the aorta, employing Tyrode solution at 37 °C. The ventricular myocytes were dispersed by employing a Ca^{2+}-free solution containing 10 mg collagenase[1] and 9 mg protease[2] per 40 mℓ of the calcium free solution. As soon as a dark area appeared on the outer surface of the ventricle small pieces of the ventricle were removed and shaken in a beaker containing Tyrode solution with calcium (0,18 mM $CaCl_2$) to isolate the cells (Mitra & Morad, 1985).

Tests for the hypercontraction of cardiomyocytes

The extract was divided into 20 aliquots, which equals the number of frogs sacrificed, and represent a mean wet skin mass of about 1,43 g for *Phrynomerus bifasciatus* and 5,55 g for *Bufo regularis*. Each aliquot was suspended in 100 μℓ of Tyrode solution, of which 25 μℓ was added to 500 μℓ of Tyrode in the test bath. The dried fractions were diluted in the same way as the extract.

About 100 freshly isolated cardiomyocytes were introduced into different chambers mounted on the stage of an inverted microscope, alternatively containing Tyrode (control), diluted skin extract or each of the diluted fractions. The quiescent cardiomyocytes were observed for changes in shape and eventually death, characterised by hypercontraction and assuming a roundish outline (Borgers, Ver Donck & Vandeplassche, 1988). Viable cells were scored at intervals of 0, 30, 60 and 90 min. In each case the cells that died as a result of the toxin were

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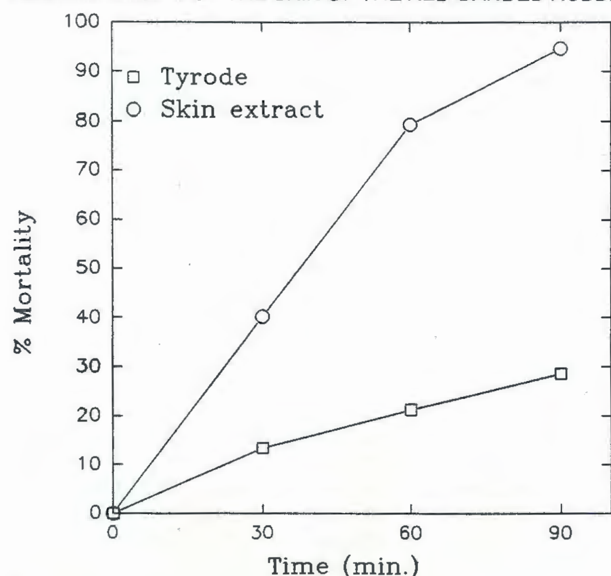

FIG. 1 Percentage mortality of cardiomyocytes versus time after application of the skin extract of the rubber frog. The cells were obtained from one guinea-pig.

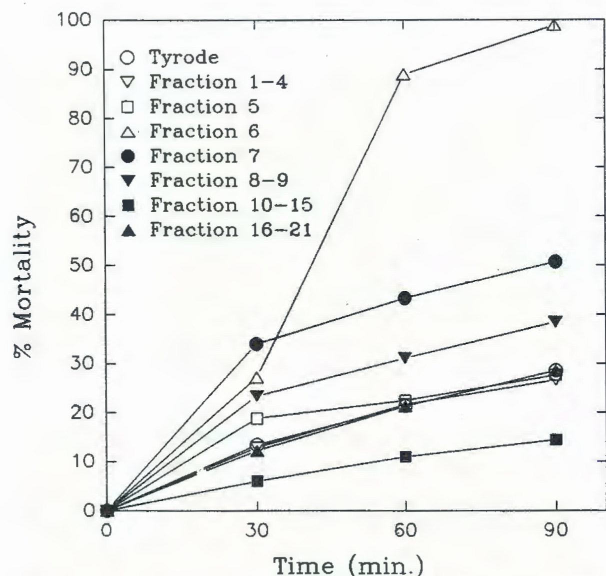

FIG. 2 Percentage mortality of cardiomyocytes versus time after application of the different HPLC fractions.

expressed as percentage of the cells at time 0 and represented in a graph against time.

Electrophysiological recording

The whole-cell clamp method (Hamill, Marty, Neher, Sakman & Sigworth, 1981) was employed utilising the isolated myocytes. The cells were perfused through a multi-barrel perfusion system with Tyrode containing 100 µM $CdCl_2$ in order to eliminate Ca-currents and to obtain IK_1 currents. Voltage clamps were done after gigaohm seals were secured with heat polished borosilicate glass[3]

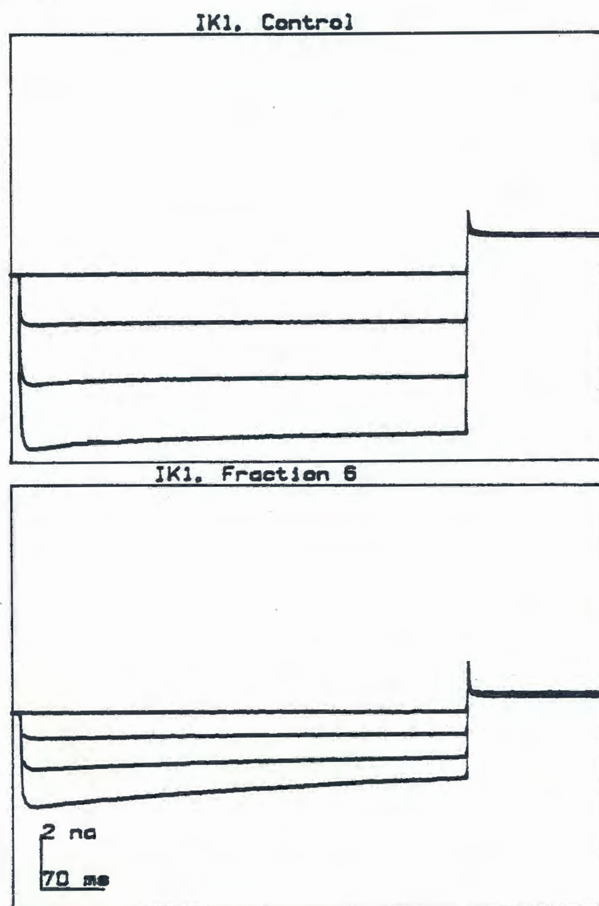

FIG. 3 The influence of the toxin on the inward rectifier potassium channel (IK_1) at −120, −110, −100 and −90 mV, where upper panel is the control and lower panel the toxin. The holding potential was −90 mV.

pipettes (2–4 megaohm). Results obtained with a Dagan model 8800 total clamp amplifier were computed and recorded. The protocol for the whole-cell clamp was as follows: the membrane was clamped from a holding potential of −90 mV with test potentials that varied between −120 mV and + 50 mV with increments of 10 mV. The repeat frequency of the pulse was 0,1 Hz and the IK_1 current measured as the time dependent current 500 ms after the onset of the test pulse.

Solutions

Tyrode solution: 5,4 mM KCl, 1,8 mM $CaCl_2$, 0,5 mM $MgCl_2$, 137,6 mM NaCl, 5 mM glucose, 11,6 mM HEPES, pH adjusted to 7,4 with NaOH.

Pipette solution: 125 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 4 mM ethyleneglycol-bis-(ß-amino-ethylether)N,N,N′,N′-tetra-acetic acid (EGTA), 10 mM HEPES and 0,154 mM $CaCl_2$, pH 7,2 (adjusted with NaOH).

Ca-free solution: 130 mM NaCl, 5,4 mM KCl, 1,2 mM KH_2PO_4, 1,2 mM $MgSO_4$, 6,0 mM HEPES, pH 7,2 (adjusted with NaOH).

Perfusion solution: Normal Tyrode with 100 µM $CdCl_2$. The skin extract and Fraction 6 were dissolved in Tyrode with $CdCl_2$ prior to experimentation.

[3] Jencons (Scientific) Ltd. (H15/10)

Results

The skin extract of *Bufo regularis,* employed as a control had no effect on the viability of the cardiomyocytes, and for this reason it was not employed in the subsequent whole-cell clamp tests.

The skin extract of *Phrynomerus bifasciatus* had a toxic effect on the cardiomyocytes, killing 95 % of the viable cells after 90 min (Fig. 1). Of the 21 fractions tested, only Fraction 6 was notably cardiotoxic (Fig. 2). Ninety-eight per cent of the viable cells died during the test period when exposed to this fraction. With regard to ionic currents, Fraction 6 had no effect on the L-Ca current but caused a marked decrease in the inflow of potassium ions from the cells at hyperpolarised membrane potentials through the inward rectifier potassium channel (IK_1). Fraction 6, after application, markedly decreased the maxsimum inward rectifier current (IK_1) by 46 % (Fig. 3). In 4 experiments with skin extract and 3 with Fraction 6, the same results have been found.

Discussion

To our knowledge this study is the first to employ the test of hypercontraction of cardiomyocytes (Borgers *et al.,* 1988) to screen animal toxins for cardiac toxicity. The results have shown that the hypercontraction test is not only sensitive enough to demonstrate cardiotoxic action in the skin extract, but whole-cell clamping tests further show that it is also able to discriminate between potent and non-potent fractions.

The interest in ion channel toxins is growing rapidly as they are instrumental in the study of these channels and their subtypes (Strong, 1990). While some animal toxins are specific for one subtype of potassium channel only, others may recognise more than 1 (Strong, 1990). Although the latter may be attributed to the concentration at which the toxin has been tested (Moczydlowski *et al.*, 1988), the discriminatory ability is usually inherent to the toxin itself (Strong, 1990). The specificity of this toxic substance from the skin of the rubber frog, together with its marked ability to decrease the flow of potassium ions through the cardiac cell membrane, may in future, prove to be a useful tool in further studies of these channels.

Although peptides and proteins constitute the major group of animal toxins (Strong, 1990), bioactive amines (Erspamer, 1971) and cardiotoxic aglycones have been isolated from the skins of anurans. Should the active component of the rubber frog poison indeed be a cardiac aglycone, it will correspond to the cardiotoxic aglycones found in toads. However, as some polypeptides from animal poisons are known to interfere with the IK_1 channels (Strong, 1990), such a substance may be present in the skin of *Phrynomerus bifasciatus.* Further studies to isolate and characterize the toxic substance reported here, are on the way. Only with the pure toxin in hand, it will be possible to determine its toxicity, and whether or not it may be responsible for the death of other toad species, as reported by Pienaar *et al.* (1976). Likewise, further studies are in progress to identify the putative irritant, the result of which was referred to by Passmore & Carruthers (1979).

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