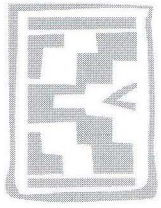

The effect of three bufadienolide cardiac glycosides on contraction of isolated rat jejunum

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ABSTRACT

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Three cardiac glycosides were screened for pharmacological effects on isolated rat jejunum. The contraction of rat jejunum with epoxyscillirosidin, a non-cumulative bufadienolide, and cotyledoside and tyledoside D, both cumulative neurotoxic bufadienolides were compared with methacholine. The results indicate that all three bufadienolides cause contraction of jejunal smooth muscle. When combined with atropine (1×10^{-6} M) the response of epoxyscillirosidin and tyledoside D decreased, indicating suppression of a cholinergic response caused by the cardiac glycosides.

Keywords: Bufadienolide, cardiac glycosides, cotyledoside, epoxyscillirosidin, methacholine, rat jejunum, tyledoside D

INTRODUCTION

Poisoning of livestock by cardiac glycoside-containing plants is collectively the most important plant-associated poisoning in southern Africa (Kellerman, Naudé & Fourie 1996). Chemically, two major groups of cardiac glycosides *viz.* the cardenolides and bufadienolides are recognised. Poisoning by bufadienolide-containing plants surpasses cardenolide-induced poisonings in importance and may be either acute or chronic. Tulp poisoning (induced by various *Homeria* and *Moraea* spp.) is an acute toxicity caused by non-cumulative bufadienolides such as 1α, 2α-epoxyscillirosidin (Kellerman, Coetzer & Naudé 1988; Naudé & Potgieter 1971). In livestock the respiratory, cardiovascular, gastrointestinal and nervous systems are affected. The nervous signs of acute bufadienolide poisoning manifest as tremor and posterior paresis (Kellerman *et al.* 1988; Kellerman *et al.* 1996).

Three different genera of the Crassulaceae (*Cotyledon*, *Tylecodon* and *Kalanchoe* spp.), often referred to as plakkies, may on the other hand, cause either acute or chronic poisoning. The chronic form of cardiac glycoside poisoning is referred to as krimpsiekte which is a paretic/paralytic syndrome primarily affecting small stock. The term krimpsiekte refers to the tucked-in, shrunken posture of the affected animal. Despite the fact that this poisoning has caused severe livestock losses to farmers since the turn of the nineteenth century major gaps in our knowledge of krimpsiekte still exist. This toxicosis is generally believed to be caused by the ingestion of cumulative bufadienolides which have neurotoxic properties unique to the compounds encountered

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in these members of the Crassulaceae (Anderson, Joubert, Prozesky, Kellerman, Schultz, Procos, & Olivier 1983; Botha, Van der Lugt, Erasmus, Kellerman, Schultz & Vleggaar 1997; Van der Walt, Van Rooyen, Kellerman, Carmeliet & Verdonck 1997).

Cotyledoside, a neurotoxic bufadienolide isolated from *Tylecodon wallichii*, induced pronounced vacuolation of the white matter of thalamic nuclei in sheep (Botha *et al.* 1997). Tyledoside D, another cumulative neurotoxic bufadienolide, has recently been isolated from *Tylecodon ventricosus* (Botha, Kellerman, Schultz, Erasmus, Vleggaar & Retief 1998).

Acute bufadienolide poisoning is ascribed to inhibition of Na^+/K^+-ATPase, but the mechanism of toxicity of krimpsiekte is still obscure (Kellerman *et al.* 1988). The thalamic lesions previously described are meaningful and might explain some of the motor function deficiencies (Botha *et al.* 1997). However, during a trial where krimpsiekte was experimentally reproduced, sheep also showed signs of neuromuscular dysfunction (Botha *et al.* 1997). Sheep receiving consecutive daily cotyledoside injections could only stand for short periods before becoming fatigued and severe muscle trembling ensued (Botha *et al.* 1997). Guinea-pigs were used to screen for toxic fractions during the isolation of cotyledoside and tyledoside D (Botha *et al.* 1997; 1998). Signs of toxicity observed in guinea-pigs included tremors, neck paresis and paralysis. Guinea-pigs tired rapidly after light exercise, but recovered following a short rest period, a phenomenon which somewhat resembles myasthenia gravis (Botha *et al.* 1997; 1998; Dewey 1997). Naudé & Potgieter (1971) also observed a curare-like paralysis in guinea-pigs while isolating epoxyscillirosidin from *Homeria pallida*.

It was postulated that the neuromuscular signs observed with cardiac glycoside poisoning could result from binding of these bufadienolides to the nicotinic receptors at the neuromuscular junction. The objective of this experiment was to ascertain whether cotyledoside, tyledoside D and epoxyscillirosidin have cholinergic activity using an isolated rat jejunum model (Van Rossum 1963).

MATERIALS AND METHODS

Adult Sprague-Dawley rats (n = 14) of both sexes were obtained from the Experimental Animal Centre of the Potchefstroom University, and sacrificed by placing them in a CO_2 chamber. The jejunum was removed and placed in Tyrode solution (135 mM NaCl, 2.6 mM KCl, 1.4 mM $CaCl_2$, 0.49 mM $MgSO_4$, 0.32 mM $NaH_2PO_4.2H_2O$, 11.9 mM $NaHCO_3$ and 11.2 mM glucose; all reagents were analytical grade, Merck, South Africa) at 37 °C. A segment of jejunum about 15 cm distal to the stomach was removed, flushed with Tyrode solution and dissected free of fat and connective tissue. Sections of jejunum (20–30 mm long) were suspended vertically in an organ bath filled with Tyrode solution; the chambers were maintained at 37 °C and bubbled with carbogen (95 % O_2 and 5 % CO_2). One end of the jejunum strip was attached to a fixed hook, whereas the other end was connected to a force transducer. The jejunum was allowed to stabilise for 1 h before commencing the experiment.

The contractile responses of the test substance were recorded via a Statham UL5 microscale accessory to a Statham UL2 strain gauge transducer (Statham, USA) which, in turn, was coupled to a Metrohm Labograph potentiometric recorder (Model E478; Metrohm Incorporated, Switzerland). Cumulative concentration-effect curves of the contractile responses to the test substance were obtained (Van Rossum 1963). Test substances included: cotyledoside (isolated from *T. wallichii* [Botha *et al.* 1997]), tyledoside D (isolated from *T. grandiflorus* by Anderson and co-workers, 1983) and epoxyscillirosidin (isolated from *H. pallida* and obtained from the Toxicology Division, Onderstepoort Veterinary Institute). All three bufadienolides used were more than 95 % pure and were dissolved in dimethyl sulphoxide (DMSO) to the appropriate concentrations. The contraction of rat jejunum with epoxyscillirosidin, cotyledoside and tyledoside D was compared to that obtained with methacholine (Aldrich Chemical Company, USA). The isolated rat jejunum was also incubated with $1x10^{-6}$ M atropine (Atropine sulphate crystals, Merck, South Africa) prior to administration of increasing doses of the three relevant cardiac glycosides. The number of replicates was restricted due to the difficult and cumbersome processes of extraction, isolation and purification of these cardiac glycosides from plant material.

RESULTS

The log dose-response curves of methacholine, tyledoside D, cotyledoside and epoxyscillirosidin are depicted in Fig. 1–3. All three cardiac glycosides caused contraction of the isolated rat jejunum when administered alone. The maximal effects were

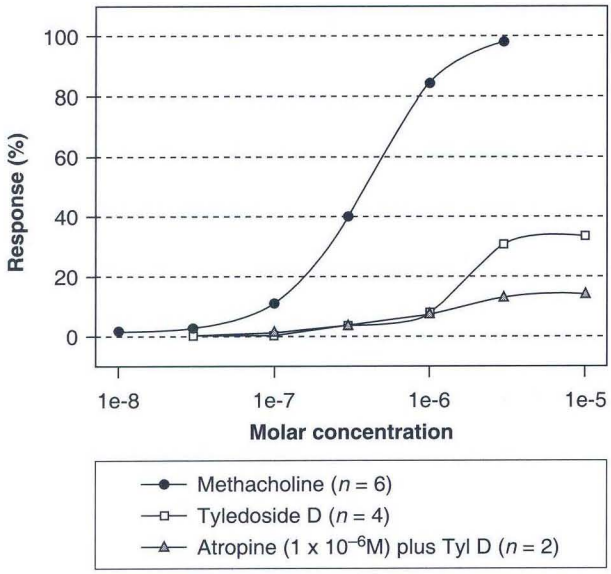

FIG. 1 Log dose-response curves of methacholine and tyledoside D (without and with $1x10^{-6}$ M atropine)

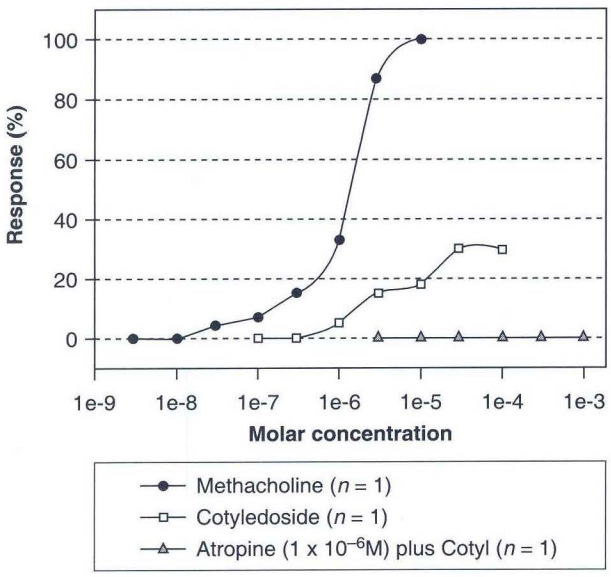

FIG. 2 Log dose-response curves of methacholine and cotyledoside (without and with 1 x 10^{-6} M atropine)

approximately two-thirds lower, the curves were shifted to the right and were of a different shape when compared to the standard methacholine curves. Incubation with atropine (1 x 10^{-6} M) prior to administration of increasing doses of the relevant cardiac glycosides decreased the maximal effect of tyledoside D and epoxyscillirosidin. No response was elicited when the atropine and cotyledoside combination was evaluated. The EC_{50} of the different cardiac glycosides (without and with 1 x 10^{-6} M atropine) were: tyledoside D 1.51 x 10^{-6} M; atropine

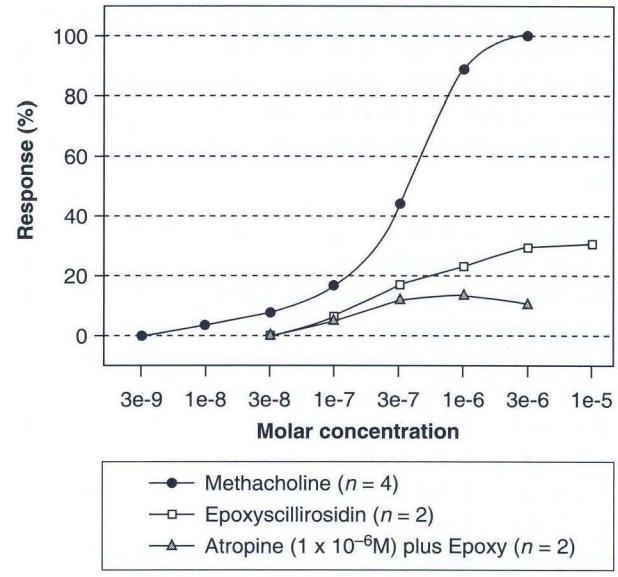

FIG. 3 Log dose-response curves of methacholine and epoxyscillirosidin (without and with 1 x 10^{-6} M atropine)

plus tyledoside D 9.38 x 10^{-7} M; epoxyscillirosidin 2.21 x 10^{-7} M; atropine plus epoxyscillirosidin 1.06 x 10^{-7} M and cotyledoside 3.49 x 10^{-6} M. An EC_{50} for methacholine (n = 6) of 4.5 x 10^{-7} M was determined.

DISCUSSION

These results indicate that epoxyscillirosidin, cotyledoside and tyledoside D are potential agonists at cholinergic receptors. They cause smooth muscle contraction in the isolated small intestine, but are less effective than methacholine in producing this effect. When administered together with atropine (a competitive, pharmacological muscarinic antagonist), the curve is shifted, although not parallel, to the right and the maximal effect is lowered indicating suppression of the cholinergic response caused by the cardiac glycosides. The lack of response when the atropine and cotyledoside combination was evaluated is probably due to the isolated small intestine being non-viable at that stage.

It is, therefore, possible that these bufadienolides also have an indirect muscarinic effect on the small intestine through an agonistic effect on the post-synaptic nicotinic receptors in the parasympathetic ganglia. This would result in the release of acetylcholine from the post-synaptic neuron stimulating the muscarinic receptors in the gastro-intestinal smooth muscle, causing contraction. This hypothesis helps to explain the lowering of the maximal effect by relatively high doses of atropine. The an-

tagonism is thus not overcome by increasing concentrations of the bufadienolide, since the total amount of acetylcholine that can be released by the post-synaptic neurons is lower than the concentration required to overcome the antagonism of atropine.

Although statistical significance of these results could not be verified, this is the first indication that the neuromuscular signs of cardiac glycoside poisoning could be due to cholinergic effects. It is postulated that the neurotoxic cumulative bufadienolides have an affinity for the nicotinic receptors at the neuromuscular junction, but with much lower intrinsic activity. During intoxication a number of these post-synaptic receptors might thus be occupied resulting in a decreased number of functional nicotinic receptors. With repetitive firing of the motor nerve terminal the acetylcholine stores are depleted (rundown) and the few remaining unoccupied nicotinic receptors are soon bound with acetylcholine and consequently desensitised to further stimulation and fatigue sets in (Dewey 1997). It is surmised that following a rest period the paretic condition of the sheep and guinea-pigs in the aforementioned trials (Botha *et al.* 1997; 1998) improved as a result of the build-up of acetylcholine reversing the muscle paresis. However, this is only a hypothesis and a trial should be designed to investigate the effect of these bufadienolides on nicotinic receptors at the neuromuscular junction.

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