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THE TOXIC EFFECTS ON CARDIAC MYOCYTES OF TYLEDOSIDE F, A CUMULATIVE NEUROTOXIC CARDIAC GLYCOSIDE ISOLATED FROM *TYLECODON GRANDIFLORUS*

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ABSTRACT

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To investigate the cardiac cellular effects of tyledoside F, a cumulative neurotoxic bufadienolide and ouabain, a non-cumulative cardenolide, the whole-cell clamp method was used to measure the Na-K pump current after the Na-K pump had been activated by high intracellular Na^{2+}. The toxic effects of tyledoside F and ouabain on cardiac myocytes were also investigated by observing the effect of the Ca^{2+} overload on the viability of myocytes during a period of 75 min.

From the results it is clear that there are similarities in the direct effects of tyledoside F and ouabain on the Na-K pump. It was found that ouabain inhibited the Na-K pump current more than that of tyledoside F. With regard to Ca^{2+} overload, there are differences in their mode of production of Ca^{2+} overload because cinnarizine protects the myocytes against ouabain-induced Ca^{2+} overload but not against tyledoside-induced Ca^{2+} overload. This study shows that with the whole-cell clamp technique tyledoside F inhibited the Na-K pump in a manner similar to inhibition of the pump by ouabain. Viability studies with myocytes indicated that tyledoside F also has other effects which are different from these of ouabain.

INTRODUCTION

Poisoning of animals by plants in the genera Tylecodon, Cotyledon and Kalanchoe (Kellerman, Coetzer & Naude, 1988) is of economic importance to agriculture in southern Africa. The tyledosides, a series of bufadienolide cardiac glycosides were isolated from *Tylecodon grandiflorus*. Some of these namely tyledoside F have cumulative neurotoxic effects in addition to the known cardiac effects and can lead to krimpsiekte: a paralytic syndrome in sheep (Anderson, Joubert, Prozesky, Kellerman, Schultz, Procos & Olivier, 1983; Kellerman *et al.*, 1988).

The differences between the paretic krimpsiekte syndrome and typical acute cardiac glycoside poisoning induced by the plant material or bufadienolides from inter alia *Tylecodon grandiflorus* (Anderson *et al.*, 1983), is of scientific importance and may have practical implications to agriculture. We therefore decided to extend the research on the toxic effects of these bufadienolides to the effects on isolated cardiac myocytes and to investigate whether the cardiac cellular effects differ electrophysiologically from that of a known non-neurotoxic cardenolide glycoside, ouabain.

Cardiac glycosides such as ouabain cause augmentation of intracellular sodium concentration as a result of inhibition of Na-K-ATPase in cardiac muscle. The increased $[Na]_i$ then leads to an increased accumulation of calcium via Na-Ca exchange, causing a toxic effect in heart cells (Kim, Cragoe & Smith, 1987).

The electrogenic Na-K pump usually pumps more Na^+ out of the cell than K^+ into the cell, and so generates an outward component of membrane current, the pump current (Gadsby, Kimura & Noma, 1985; Mogul, Rasmussen, Singer & Ten Eick, 1989). It is technically difficult to measure pump current accurately over a sufficiently wide voltage range, but with the whole-cell clamp technique effective control of both extra- and intracellular solutions, as well as membrane voltage, is obtained. Applying this technique on isolated guinea-pig myocytes, it was possible to measure Na-K pump current between −100 mV and +60 mV, after eliminating passive currents flowing through the potassium and calcium channels.

The aim of this study was to evaluate the effects of a neorotoxic bufadienolide glycoside, tyledoside F from *Tylecodon grandiflorus* on the Na-K pump current by making use of the whole-cell clamp technique and to compare the cellular effects with the effects of ouabain which inhibits the Na-K-ATPase and therefore the Na-K pump.

MATERIALS AND METHODS

Isolation of myocytes

Adult guinea-pigs were used and after the animal was stunned with a blow behind the head and killed by severing the carotid artery, the heart was removed and perfused retrogradely through an aortic cannula with tyrode solution. After 5 min of perfusion at 37 °C on a Langendorff apparatus, the tyrode solution was changed for a Ca-free solution for 5 min, after which the heart was perfused with a 40 mℓ of Ca-free solution, containing 9 mg Collagenase[1] and 10 mg Protease[2]. Perfusion with the solution containing enzymes was usually stopped within 5 min or as soon as dark areas appeared on the ventricles. The heart was then perfused with a tyrode solution, containing 0,18 mM Ca for a few minutes, after which the ventricles were cut in small pieces. The isolated cells were shaken free in a 50 mℓ beaker containing tyrode solution. The above-mentioned method is a method for cell isolation related to that employed by Mitra & Morad (1985). The rod-shaped, quiescent ventricular myocytes were used for experimentation.

Solutions

Tyrode solution contained (in mM): 5,4 KCl; 1,8 $CaCl_2$; 0,5 $MgCl_2$; 137,6 NaCl; 5 glucose and 11,6 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES). The pH was adjusted to 7,4 with NaOH. During the experiments the temperature was kept constant at 37 °C. The solution for the suction pipettes contained (in mM): 91 $CsCl_2$; 34 NaCl;

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[1] Type II, Sigma

[2] Type XIV, Sigma

5 $MgCl_2$; 5 Na_2ATP; 15 ethyleneglycol-bis-(β-amino-ethylether)N,N,N′,N′-tetra-acetic acid (EGTA); 10 HEPES; 20 tetra-ethylammonium-chloridmonohydrat (TEA-Cl). The pH was adjusted to 7,2 with NaOH. The $CsCl_2$ as well as TEA-Cl was used to eliminate the potassium currents, and the cells were loaded with 34 mM NaCl to activate the Na-K-pump (Gadsby *et al.*, 1985). Normal tyrode solution (see above) with 1 mM $BaCl_2$ and 100 mM $CdCl_2$ was used for perfusing the isolated myocytes with experimentation. The Ba^{2+} and Cd^{2+} were added to eliminate inward currents through K^+ and Ca^{2+} channels respectively. Under these conditions membrane currents were small and largely time-independent, confirming that other channel currents were depressed. In certain experiments the KCl in tyrode was omitted to inactivate the Na-K pump.

Recording techniques

The whole-cell clamp technique described by Hamil, Marty, Neher, Sakman, Sigworth (1981) was used on the isolated myocytes. Suction pipettes with resistance values between 3 and 4 MΩ were drawn from borosilicate glass[3] and fire-polished. After formation of a giga ohm seal with the cell membrane, the membrane was ruptured and at least 15 min was allowed for internal dialysis to reach equilibrium. Internal dialysis was indicated by a relatively rapid depolarization after rupture of the membrane. Voltage clamps were done by using the chopped clamp method with a Dagan[4] patch clamp amplifier. To construct a current-voltage relationship, the pump current was recorded by clamping the membrane for 1 200 ms from a holding potential of −50 mV to test potentials between −100 mV and +60 mV with 10 mV increments. The time-independent Na-K pump current was measured 400 ms after onset of the activating pulse. Pre-drug controls were performed and, after application of 10^{-5} M ouabain[5] or 10^{-5} M tyledoside F, the same procedures were repeated to evaluate the influence of ouabain or tyledoside on the Na-K pump currents. The results were recorded with a personal computer and processed with a special computer programme.[6]

Induction of hypercontracture by pathological stimuli

Upon exposure to pathological stimuli, isolated quiescent cardiomyocytes started to contract vigorously, followed by sarcomere shortening and bleb formation. Eventually, cells rounded up as a consequence of irreversible contracture. These shape changes in the isolated myocytes indicate a loss of viability and represent a state of Ca^{2+} overloading (Borgers, Verdonck & Vandeplassche, 1988). In this study, use was made of ouabain and tyledoside F to induce a Ca^{2+} overload for the study of the shape changes of isolated intact myocytes. The cells that remained after the pathological stimulus were counted under an inverted microscope within different time intervals (t = 0, before the pathological stimulus, t = 25, 50 and 75 min after the pathological stimulus). The number of cells that died off was calculated for the different time intervals.

Statistical analysis

Data presented in this study are means ± standard error of the mean.

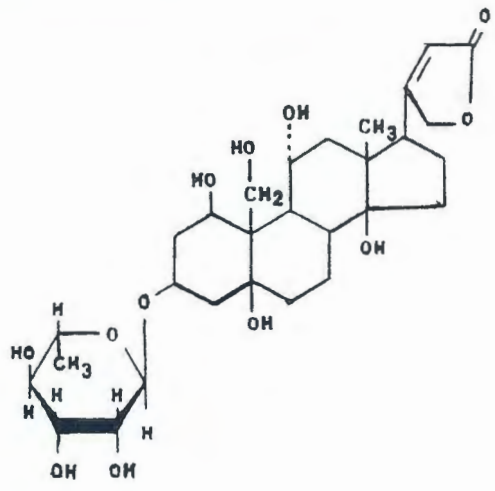
[3] Jencons, H15/10

[4] Dagan, Model 8800 Total clamp

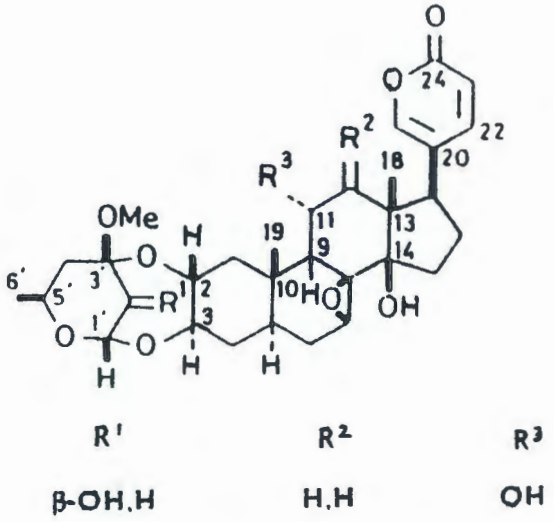
[5] "G-strophanthin", Merck

[6] Clampex (Labmaster TM40) Version 5.5

Structure formulas of glycosides used



Ouabain



Tyledoside F

RESULTS

The steady state current voltage relationships for the membrane current were measured at the end of 1 200 ms voltage steps from a holding potential of −50 mV and test potentials ranging from −100 mV to +60 mV (Fig. 1). The magnitude of the time-independent outward current (Na-K pump current) averaged 310 pA ± 8,2 pA (n = 17) at a membrane potential of 0 mV. The mean cell capacitance was 212,8 pF ± 75 pF (n = 11). Pump currents with amplitudes of approximately 250 pA at membrane voltage of 0 mV and holding potential of −40 mV are found in the literature (Gadsby & Nakao, 1989).

To test that the currents induced were indeed Na-K pump currents, KCl was omitted from the perfusion solution. This resulted in a decrease in outward (positive) current (Fig. 1) and confirmed that the current measured is a Na-K pump current. When 10^{-5} M ouabain is applied, the curve is shifted more towards the zero current line, which shows that the Na-K pump current is further inhibited by ouabain.

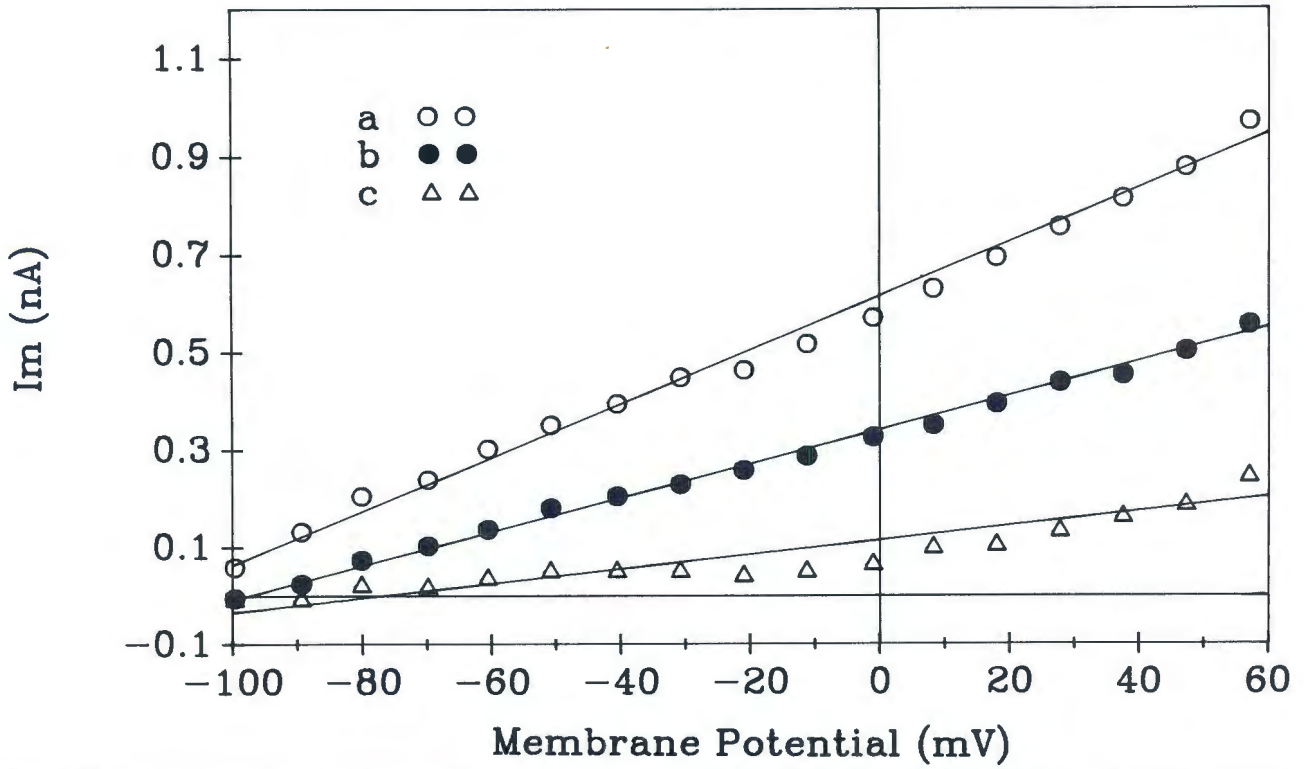


FIG. 1 The effect of ouabain and potassium on the membrane pump current where $[K^+]_o$ = 5,4 mM (curve a); $[K^+]_o$ = 0 mM (curve b) and (curve c) = 0 mM K^+ + 10^{-5} M ouabain. The pump currents were activated by 30 mM Na intracellular

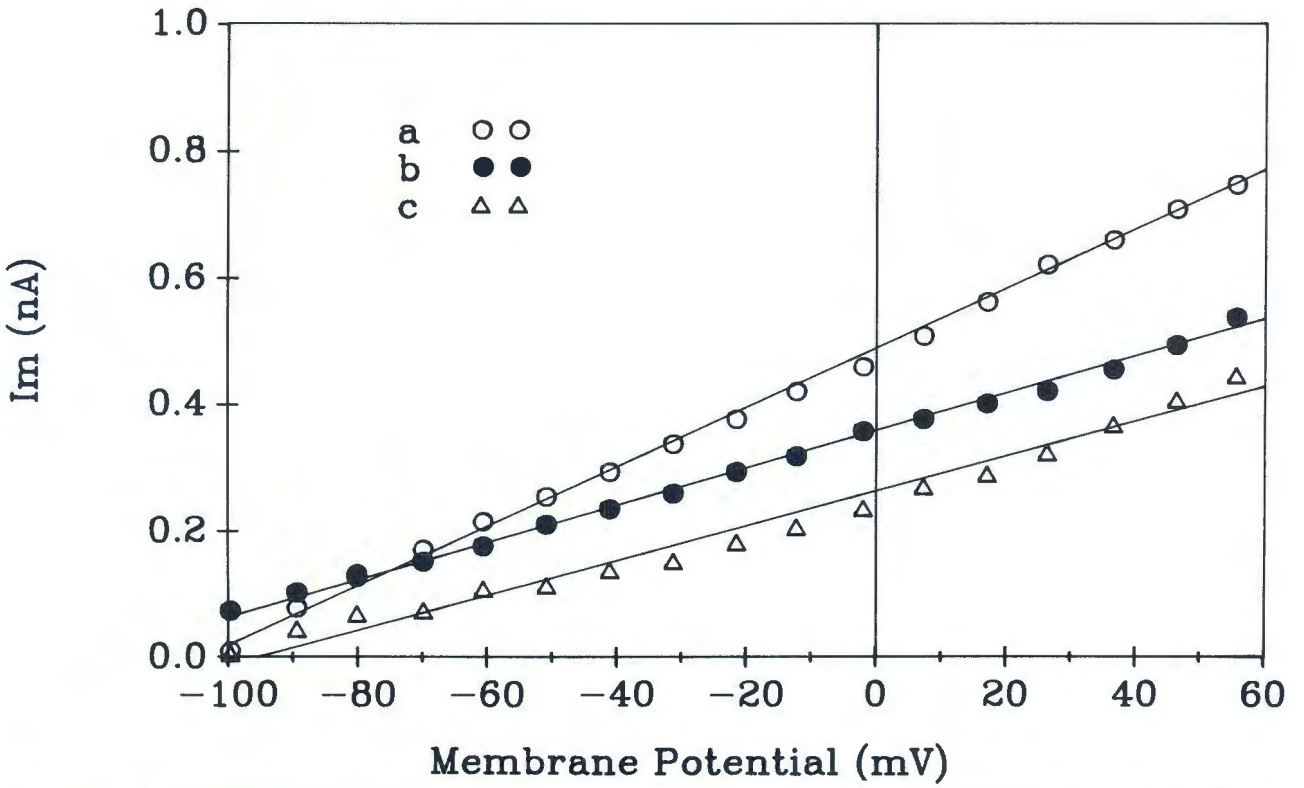


FIG. 2 The effect of tyledoside F and potassium on the membrane pump current where (a) is the control curve, $[K^+]_o$ = 5,4 mM; (b) $[K^+]_o$ = 0 mM and (c) 10^{-5} M tyledoside F + 0 mM K^+. The pump currents were activated by 30 mM Na intracellular

It is evident from the literature that the Na-K pump is inactivated by removing the extracellular potassium (Mogul *et al.*, 1989) and the pump current is further inhibited by ouabain application (Mogul *et al.*, 1989; Gadsby *et al.*, 1985).

The experimental procedure was repeated with another myocyte, the current-voltage relationship recorded (Fig. 2), but instead of ouabain, tyledoside F (10^{-5} M) was applied. Tyledoside F also inhibited the outward membrane current and consequently

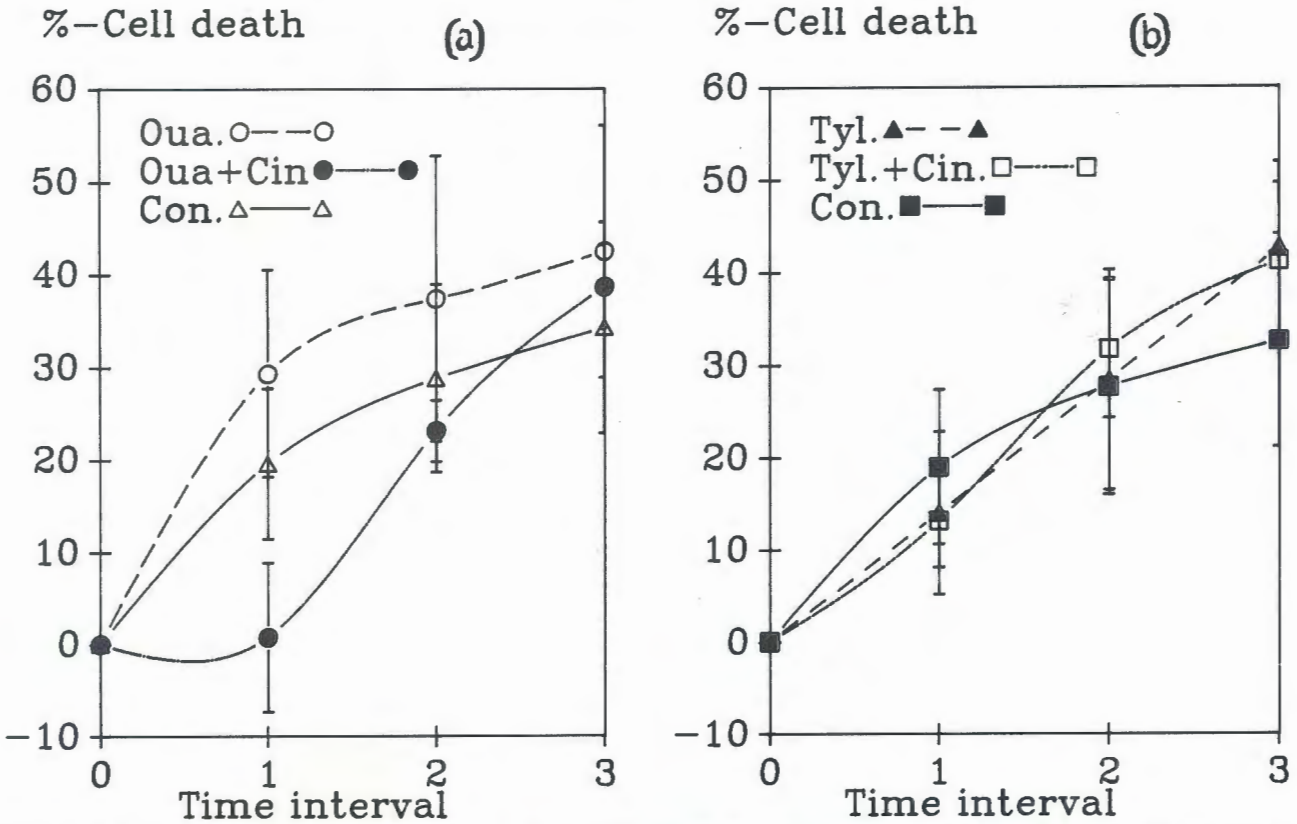


FIG. 3a & b The influence of 10^{-5} M cinnarizine on the mortality of isolated cardiac myocytes. 10^{-4} M ouabain (Fig. 3a) and 10^{-4} M tyledoside F (Fig. 3b) were used to induce the pathological stimulus of Ca^{2+}-overload. In each instance cinnarizine was used as a protective agent against Ca^{2+} overload. Time interval t = 0, before the pathological stimulus and t, 1 = 25; 2 = 50 and 3 = 75 min after the pathological stimulus respectively

inhibited the Na-K pump. In Tables 1a & 1b the results of the magnitude of the decreases in membrane pump currents are summarized. The magnitude of the decrease in membrane pump currents for tyledoside F were 172,3 ± 12,8 pA (n = 6) and 141,2 ± 10,5 pA (n = 6) at membrane potentials of 0 mV and −30 mV, respectively (holding potential was −50 mV). The magnitude of the decrease in membrane pump currents for ouabain was 286,4 ± 61,2 pA and 242,0 ± 53,1 pA (n = 3) for the same experimental conditions. The magnitude of the decrease in membrane pump current is much smaller after application of tyledoside F than after ouabain application, however, both glycosides, tyledoside F and ouabain, inhibit the outward membrane current and therefore the Na-K pump.

TABLE 1a Magnitude of the decrease in time-independent membrane pump currents for a neurotoxic cardiac glycoside namely tyledoside F, isolated from *Tylecodon grandiflorus*

Experiment No	Bufadienolide	Membrane potential	Membrane current Im (400 ms) (pA)
V9630	Tyledoside F	0 mV	255,6
V9630	Tyledoside F	−30 mV	222,2
V9808	Tyledoside F	0 mV	82,7
V9808	Tyledoside F	−30 mV	63,5
$V9905S_1$	Tyledoside F	0 mV	89,5
$V9905S_1$	Tyledoside F	−30 mV	97,4
$V9905S_2$	Tyledoside F	0 mV	144,4
$V9905S_2$	Tyledoside F	−30 mV	100,0
V9928	Tyledoside F	0 mV	234,8
V9928	Tyledoside F	−30 mV	191,3
V9116	Tyledoside F	0 mV	227,0
V9116	Tyledoside F	−30 mV	172,7
		0 mV	172,3 ± 12,8 (n=6)
		−30 mV	141,2 ± 10,5 (n=6)

TABLE 1b Magnitude of the decrease in time-independent membrane pump currents for a cardiac glycoside namely ouabain

Experiment No	Cardiac glycoside	Membrane potential	Membrane current Im (400 ms) (pA)
V9801	Ouabain	0 mV	106,5
V9801	Ouabain	−30 mV	63,0
V9012	Ouabain	0 mV	473,7
V9012	Ouabain	−30 mV	368,4
V9017	Ouabain	0 mV	278,9
V9017	Ouabain	−30 mV	294,7
		0 mV	286,4 ± 61,2 (n=3)
		−30 mV	242,0 ± 53,1 (n=3)

Shape changes in isolated myocytes were induced with 10^{-4} M ouabain and 10^{-4} M tyledoside F (Fig. 3). The increase in the number of myocytes that died as a result of a Ca^{2+} overload are nearly the same for both ouabain and tyledoside F. When cinnarizine[7], a calcium antagonist, is used as a protective agent against the glycoside induced Ca^{2+} overload, it is evident from Fig. 3a that cinnarizine has a protective effect, at least for time interval 1 and 2, if the Ca^{2+} overload is induced by ouabain. This result is in agreement with the observations of Jonkman, Boddeke & Van Zwieten (1986). The myocytes could not be protected by cinnarizine against Ca^{2+} overload as induced by tyledoside F application (Fig. 3b). It seems likely that Ca^{2+} antagonists, such as cinnarizine, that are devoid of slow channel affinity can prevent intracellular Ca^{2+} overload caused by ouabain intoxication but not by tyledoside F intoxication.

[7] "Cinnarizine", Jansen Pharmaceutica, Beerse, Belgium

DISCUSSION

In this study, we investigated the cellular effects of a cumulative bufadienolide glycoside, tyledoside F and a known cardenolide cardiac glycoside, ouabain, that inhibits the Na-K pump. Both tyledoside F and ouabain inhibit the Na-K pump, but for the same concentration the decrease in pump current is much smaller, probably because the affinity of the Na-K-ATPase differs for ouabain and tyledoside F. The basic ring structures of the 2 glycosides are also different and, if the structure of the glycoside determines its specific function or cellular effects, this may explain why different effects on the Na-K pump were detected. In a few other experiments under the same experimental procedures as in this study, (unpublished data) we have used proscillaridine[8], which is also a bufadienolide and concluded that the differences found can be ascribed to the fact that tyledoside F and proscillaridine are bufadienolides and ouabain a cardenolide. With our experiments on isolated myocytes we determined the cardiac effects and excluded the neurotoxic and cumulative effects found in sheep (Anderson *et al.*, 1983; Kellerman *et al.*, 1988).

In 1 experiment on rat myocytes, using the whole-cell clamp technique (unpublished data), it is confirmed that the sensitivity of cardiac Na-K-ATPase for cardiac glycosides also differs in different animal species, as proposed by Godfraind (1984).

Cinnarizine, a Ca^{2+} antagonist, devoid of slow channel affinity, can prevent intracellular Ca^{2+} overload in myocytes induced by ouabain, but in tyledoside F-induced Ca^{2+} overload it does not show any protective effects. With 2 experiments, under the same experimental conditions for production of Ca^{2+} overload (unpublished data), we have demonstrated that blockade of the L-type calcium channel with 100 μM Cd^{2+} and blockade of the T-type calcium channel with 100 μM Ni^{2+} have no protective effect against Ca^{2+} overload, as induced with tyledoside F. This lead us to the assumption that the increase in flow of Ca^{2+} ions through ion channels after application of tyledoside F does not flow through the L- or T-type calcium channels but rather through some other routes to produce the Ca^{2+} overload. The Ca^{2+} ions in the cytosol, after application of tyledoside F or ouabain, may probably come from different intracellular sites to increase the intracellular free Ca^{2+} concentration with a subsequent Ca^{2+} overload and resultant cell death. Structural changes in the sarcolemma as a cause of tyledoside F application may cause the sarcolemma to be devoid of Ca^{2+} deposits and this subsequently lead to an increase in cytosolic Ca^{2+} (Borgers, Thoné & Verdonck, 1985).

In conclusion, although both ouabain and tyledoside F suppress the Na-K pump, it seems that there are differences in their mode of production of the Ca^{2+} overload. Further research on the mechanism of production of Ca^{2+} overload may therefore lead to a different therapeutic approach in the treatment of krimpsiekte in sheep. This study provides new information regarding the effects of cumulative bufadienolides on isolated cardiac myocytes. The cardiac cellular effects resemble the acute phase of krimpsiekte in sheep where ECG changes are evident (Anderson *et al.*, 1983). This report also illustrates the new possibilities of the whole-cell clamp technique to study the activity of the Na-K-ATPase, and therefore the Na-K pump, and to use shape changes of cardiac myocytes to study toxic effects and pathological stimuli on the cardiac system.

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[8] "Talusin", Holpro Pharmaceuticals, Jhb