Ouabain attenuates cardiotoxicity induced by other cardiac steroids

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Summary

Background and purpose: All cardiac steroids have a similar structure, bind to and inhibit the ubiquitous transmembrane protein Na^+, K^+-ATPase and increase the force of contraction of heart muscle. However, there are diverse biological responses to different cardiac steroids both at the cellular and at the molecular level. Moreover, we have recently shown that ouabain inhibits digoxin- and bufalin-induced changes in membrane traffic. The present study was designed to test the hypothesis that ouabain also has an inhibitory effect on cardiotoxicity induced by other cardiac steroids.

Experimental approach: The hypothesis was tested in isolated heart muscle preparations and in an *in vivo* model of cardiotoxicity in guinea pigs.

Key results: Ouabain at a low dose attenuated the toxicity induced by bufalin and digoxin in heart muscle preparations. In addition, ouabain at the low dose (91 $ng \cdot kg^{-1} \cdot hr^{-1}$), but not at a higher dose (182 $ng \cdot kg^{-1} \cdot hr^{-1}$), delayed the development of digoxin-induced (500 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) cardiotoxicity in anaesthetized guinea pigs, as manifested by delayed arrhythmia and terminal ventricular fibrillation, as well as a reduced heart rate. In addition, as observed with ouabain, the phosphoinositide 3-kinase inhibitor wortmannin (100 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) delayed the digoxin-induced arrhythmia in anaesthetized guinea pigs.

Conclusions and Implications: The present study demonstrates the inhibitory effect, probably through signal transduction pathways, of ouabain on digoxin- and bufalin-induced cardiotoxicity in guinea pigs. Further understanding of this phenomenon could be beneficial for increasing the therapeutic window for cardiac steroids in the treatment of chronic heart failure.

Keywords: digoxin, bufalin, wortmannin, Na^+, K^+-ATPase, cardiac glycosides, arrhythmias

Abbreviations:

+dT/dt, upward force slope;

-dT/dt, downward force slope;

BP, blood pressure;

gF, gram force;

HR, heart rate;

VT, ventricular tachycardia;

VF, ventricular fibrillation;

Introduction

Cardenolides such as ouabain and digoxin and bufadienolides such as bufalin, as an ingredient of Chan Su extract, were widely used in the Western and Eastern clinical practices for the treatment of atrial fibrillation and heart failure. However,induction of arrhythmia and a narrow therapeutic window limits their therapeutic application (Eichhorn and Gheorghiade, 2002; Wasserstrom and Aistrup, 2005).

Cardiac steroids bind to and inhibit the ubiquitous transmembrane protein Na^+, K^+-ATPase. This enzyme transports three Na^+ out of the cell and two K^+ into the cell, utilizing ATP hydrolysis as the driving force. In addition, the interaction of CS with the Na^+-K^+ATPase elicits the cell-specific activation of several intracellular signaling mechanisms. These include phosphorylation of Src-kinase/MAP-kinase and PKC (Aydemir-Koksoy, Abramowitz *et al.*, 2001; Haas, Wang *et al.*, 2002), Ca^{++} oscillations (Aizman, Uhlen *et al.*, 2001) and changes in intracellular membrane traffic (Rosen, Glukhman *et al.*, 2004).

The usual explanation for the cardiac steroid-induced increase in heart contractility is that the inhibition of Na^+, K^+-ATPase by cardiac steroids causes an increase in intracellular Na^+ which, in turn, attenuates the Na^+/Ca^{++} exchange activity, resulting in an increased intracellular Ca^{++} concentration, and hence increased contractility (Eichhorn *et al.*, 2002). It has been suggested that CS-induced arrhythmia and toxicity result from massive Na^+-K^+ATPase inhibition, leading to intracellular "calcium overload" (Eichhorn *et al.*, 2002; Khatter, Agbanyo *et al.*, 1989). According to these canonical explanations, all cardiac steroids should have similar effects. However, the effects of different cardiac steroids are diverse. For example, in rodents, despite their similar inotropic effects, ouabain and bufalin, but not digoxin, significantly shortened action potential duration (Kieval, Butler *et al.*, 1988; Ruch, Nishio *et al.*, 2003); The LD_{50} of ouabain and digoxin in rats is 14 and 32 mg/kg, respectively (Hovevey-Sion and Kaplanski, 1979; Small, McElroy *et al.*, 1971). However, a decrease in serum K^+ concentration significantly reduced the minimum lethal dose of digoxin but did not affect that of ouabain (Fricke and Klaus, 1981); bufalin produced a significant increase in heart rate (HR) whereas ouabain did not alter it (Pamnani, Chen *et al.*, 1991). Furthermore, digoxin even reduced cardiac rhythm (Segal, McNamara *et al.*, 2000); The infusion of ouabain (Manunta, Hamilton *et al.*,

2000) and bufalin (Pamnani *et al.*, 1991) for several weeks produced hypertension in rats, whereas, digoxin did not exert such an effect (Huang, Kudlac *et al.*, 1999) or even caused a reduction in systemic blood pressure (BP) and prevented ouabain-induced hypertension when given concomitantly (Manunta *et al.*, 2000).

Cardiac steroid-induced responses at the cellular and molecular levels also vary (for review see, Dvela, Rosen *et al.*, 2007). For example, human non-gastric H^+ - and K^+-ATPases are inhibited by bufalin, digoxin, and digitoxin but are virtually resistant to digoxigenin and ouabagenin (Modyanov, Pestov *et al.*, 2003); digoxin and digitoxin, but not ouabain, are substrates for the P-glycoprotein transporter (Pauli-Magnus, Murdter *et al.*, 2001); ouabain and bufalin differentially affected the intracellular signaling protein 14-3-3 in rat lens (McGowan, Russell *et al.*, 1999); Finally, we recently demonstrated that digoxin, bufalin and other cardiac steroids induce the accumulation of endocytosed membrane components and cause alterations in intracellular membrane traffic. Ouabain had no effect on intracellular membrane traffic and even antagonized the changes induced by the other cardiac steroids (Feldmann, Glukmann *et al.*, 2007).

In view of the diversity in the action of the different cardiac steroids, it is reasonable to suggest that their inotropic and/or toxic effects on cardiac muscle may also vary. Since we have observed an antagonistic effect of ouabain on digoxin-induced changes in membrane traffic, a process leading to cellular stress and apoptosis, we hypothesized that ouabain may also protect cardiac cells from toxicity induced by other cardiac steroids. This hypothesis was tested in guinea-pig isolated heart muscles and in an *in vivo* model of cardiotoxicity.

Methods

Animals

All animal care and experimental protocols were approved by the Joint Ethics Committee (IACUC) of the Hebrew University and Hadassah Medical Center.. The Hebrew University is an AAALAC internationally accredited institute. Experiments were performed on 75 male guinea pigs weighing 300-500 g each. The animals were housed according to a 12-hour light/dark cycle and were allowed an acclimatization period of at least 3 days, with normal guinea pig chow and tap water *ad libitum*.

In vivo experimental protocol

The guinea pigs were anaesthetized with urethane (Sigma; 1.5 $g \cdot kg^{-1}$ i.p.), placed in a supine position on a heating pad (CMA 150 – Temperature Controller, CMA/Microdalysis AB, Sweden) and maintained at a constant body temperature of 37.5°-38° C throughout the experiment. Once surgical anaesthesia had been established, tracheotomy was performed and the animals were allowed to breathe spontaneously. Arterial blood pressure (BP) was continuously measured via a millar catheter-transducer (SPC-320, TX, USA) connected to a bridge amplifier (Lablinc, Coulbourn Instruments, PA, USA), placed in the right common carotid artery. A surface electrocardiogram using Leads II and avF from subcutaneous electrodes was made through a resistive bridge (Lablinc, Coulbourn Instruments, PA, USA). Both parameters were recorded via PowerLab (ADIInstruments, Castle Hill, Australia)**.** The right internal jugular vein was canulated for drug administration.

All animals were given an initial i.v. bolus of saline according to the initial BP, in order to avoid metabolic acidosis induced by hypovolemia. The respiratory rate and end-tidal CO_2 were measured in all animals, using a Polaris capnograph (JS-02260, Spegas Industries, Israel). When necessary, mechanical ventilation on 100% O_2 was included, to correct respiratory acidosis. Drugs were administered using a micropump at a rate of $8.3 \mu L \cdot min^{-1}$ (Stoelting, IL, USA).

Due to the highly variable response to cardiac steroids in guinea pigs, all experiments were conducted in pairs, i.e. one animal from the control group and the other from a study group were analyzed simultaneously.

Following at least 60 min equilibrium, the animals were treated with digoxin, ouabain and/or wortmannin, as described below, until terminal ventricular fibrillation (VF) occurred. Three end points were evaluated to test the different effects of the tested solutions: 1) the appearance of the first arrhythmia (as the first ventricular premature beat, or a high grade atrio-ventricular block) both in the ECG and arterial pressure records, 2) the occurrence of ventricular tachycardia (VT) or VF in the ECG and 3) the time of death (cessation of cardiorespiratory activity in the ECG, pressure line and respirator) following the VT/VF was also recorded. The time of onset of the three end points was calculated from the start of infusion. To evaluate the different effects of the tested solutions on heart rate (HR) and BP, the lowest values obtained before the first arrhythmia occurred were calculated as an additional end point.

Ex vivo experimental protocol

The animals were killed by cervical dislocation. The hearts were immediately removed into Krebs−Henseleit bicarbonate buffer (composition in $mmol \cdot L^{-1}$: 118.4 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 2 $CaCl_2$, 1.2 $MgSO_4$, and 5.5 glucose, pH 7.4). The right and left atria and papillary muscles were excised from the heart, secured with silk thread to a polypropylene tissue holder, and mounted vertically in a 15 mL bath. The nutrient solution was aerated with 95% O_2/5% CO_2 and maintained at 37 °C. The left atrium and papillary muscles were driven by a pair of platinum electrodes (field stimulation) with a rectangular current pulse (1 Hz, 0.5 ms, about 1.2 × threshold voltage) generated by an electronic stimulator (Master-8, A.M.P.I., Israel and a custom-made isolated current amplifier). The right atrium beat spontaneously. The developed tension was measured isometrically with a force-displacement transducer (FSG-01, Experimetria, Hungary) connected to a bridge amplifier (Lablinc, Coulbourn Instruments, PA, USA). The data were displayed and recorded on a PC based PowerLab/16sp system interface using a software Chart v4.2 for Windows (ADIInstruments, Castle Hill, Australia).

Following at least 60 min equilibrium at a resting force of 0.2 and 0.4 gF (for papillary muscle and atria, respectively), the muscles were challenged with digoxin, bufalin and/or ouabain as described below until arrhythmia developed or for at least 90 min. The concentrations of bufalin and digoxin that were used have been shown in preliminary

experiments to initiate arrhythmias in more than 90% of the preparations. Ouabain concentrations were chosen as the highest concentration that does not induce increase in muscle contractility. The maximal force amplitude and upward (+dT/dt) and downward force (-dT/dt) development slopes were measured before drug administration (control) and just before the beginning of arrhythmia and calculated as the average of 20 beats.

Membrane preparation and ATPase activity measurements

Crude membranes (P2) from guinea pig heart were prepared as previously described (Haver, Lichtstein *et al.*, 1995), and kept frozen (-70^{o}C) until used. Following thawing, the membranes were incubated in Tris-sodium deoxycholate (0.1%) for 30 min. Membranes were incubated at 37°C with 1 ml of a solution containing 20 mmol·L^{-1} Tris buffer, pH 7.4, 1 mmol·L^{-1} EDTA, 100 mmol·L^{-1} NaCl, 20 mmol·L^{-1} KCl, 1 mmol·L^{-1} $MgCl_2$, 5 mmol·L^{-1} NaN_3, 3 mmol·L^{-1} ATP, and varying concentrations of digoxin and ouabain. The reactions were terminated by the addition of 1 ml of ice-cold 8% TCA. The inorganic phosphate generated by the ATPase (P_i) was measured using Malachite Green assay (Chan, Delfert *et al.*, 1986).

Statistics

The values obtained are expressed as the mean ± S.E. of the number of muscles or animals used in each experiment. Results were analyzed using Student's t-test, the Mann-Whitney test or analysis of variance for comparison between groups, when appropriate, using *SPSS 11.5* for windows (SPSS Inc., Chicago, IL). Differences were considered statistically significant at $p < 0.05$.

Materials

Ouabain, digoxin, bufalin and wortmannin were obtained from Sigma. Other chemicals were also supplied by Sigma or were of the highest quality and purity.

Ouabain and wortmannin were dissolved in Krebs−Henseleit bicarbonate buffer and saline for the *ex vivo* and *in vivo* experiments, respectively. Digoxin and bufalin were dissolved initially in 70% ethanol and then diluted in the appropriate solution. The final

ethanol concentration did not exceed 0.5% and did not affect any of the parameters measured (data not shown).

Results

Reduction of bufalin-induced arrhythmia by ouabain in guinea pig papillary and atrial muscle

The addition of bufalin to guinea pig papillary muscle (0.4 $\mu mol \cdot L^{-1}$) or atrium (225 $nmol \cdot L^{-1}$) preparations caused an immediate and significant increase of about 100% in force amplitude, accompanied by a similar increase in +dT/dt and -dT/dt (Figure 1A-1C). Arrhythmias occurred in all the muscle preparations, although the time to first arrhythmia varied between papillary muscle, left atrium and right atrium, as shown in Figure 1B. In contrast, when ouabain (0.4 $\mu mol \cdot L^{-1}$ or 30 $nmol \cdot L^{-1}$ for papillary muscle and atria, respectively) was added together with bufalin, there was a clear delay in the commencement of arrhythmia in papillary muscle ($P<0.05$) with a trend towards delay in the atria ($P<0.07$ and $P<0.14$, for left atrium and right atrium, respectively, Figure 1B).In the presence of ouabain, most of the papillary muscle preparations did not develop arrhythmia, even after 90 min.

As expected from the delayed arrhythmia, there was also an increase of about 50% in the maximal force amplitude, together with an increase in +dT/dt and -dT/dt, when ouabain and bufalin were applied together, compared with bufalin alone ($P < 0.05$ Figure 1C). There were no significant changes in the chronotropic effect of bufalin on the right atrium when ouabain was added concomitantly (data not shown). Ouabain by itself caused a slight increase of 5-10% in force amplitude, accompanied by a similar increase in +dT/dt and -dT/dt, that continued for more than 90 min without the development of arrhythmias in any preparation (data not shown).

Reduction of digoxin-induced arrhythmia by ouabain in guinea-pig papillary muscle

The addition of digoxin (3.5 $\mu mol \cdot L^{-1}$) to guinea pig papillary muscle preparations caused an immediate and significant increase of about 150% in force amplitude, which was accompanied by a similar increase in +dT/dt and -dT/dt (Figure 1D-1F). Arrhythmias

occurred in all the muscle preparations and the time to first arrhythmia varied (Figure 1E).. The addition of ouabain (0.4μmo•L^{-1}) together with digoxin caused a significant reduction of 30-40% in the inotropic parameters, compared with that obtained using digoxin alone ($P < 0.05$, Figure 1F). This reduction was observed despite the trend of delayed arrhythmia ($P<0.1$, Figure 1E) in the presence of ouabain and the additive effects of ouabain and digoxin on the force of contraction as mentioned above.

Reduction of digoxin-induced arrhythmias in vivo by ouabain in guinea pigs

Guinea pigs were given a constant infusion of digoxin. Digoxin dose and infusion rate (500 μg·kg^{-1}·hr^{-1}) was chosen to induce arrhythmias progressing to terminal VF within 2 h, as shown in Fig 2. To investigate the antagonism by ouabain of digoxin cardiotoxicity, ouabain was infused in concentrations which did not exert any cardiovascular effects on the animals over the same period (2 h). Infusion of ouabain at a relatively low dose (91 ng·kg^{-1}·hr^{-1}), together with digoxin, delayed the occurrence of first arrhythmia and of VT/VF ($P < 0.05$) and time of death, showing the delayed cardiotoxic effects of digoxin. However, infusion of ouabain at a higher dose (182 ng·kg^{-1}·hr^{-1}), which also did not exert any cardiovascular effects when infused alone, did not show any beneficial effect on digoxin-induced toxicity. On the contrary, the time to terminal VF (death) was much shorter ($P < 0.05$), implying an additive cardiotoxic effect of ouabain and digoxin (Figure 2).

Reduction of digoxin-induced changes in blood pressure (BP) and heart rate(HR) by ouabain in guinea pigs

The effect of digoxin infusion (500 μg·kg^{-1}·hr^{-1}) on BP and HR was bi-phasic. As seen in Figure 3, at the beginning of the infusion, digoxin caused a significant reduction in BP and HR of about 15% before the first arrhythmia occurred ($p<0.05$). However, when the plasma concentration increased and the toxic effect began, digoxin caused an elevation in BP and HR by about 20% compared with the values recorded in the control period ($p<0.05$). These elevations continued until VT/VF occurred. Ouabain at the low dose

inhibited the elevation in HR caused by digoxin, showing another aspect of cardiotoxicity inhibition (Figure 3B). Ouabain at the high dose did not significantly affect HR (data not shown). Ouabain did not affect digoxin-induced changes in BP at either dose (Figure 3A and data not shown).

Reduction of digoxin- but not ouabain-induced arrhythmias in vivo by wortmannin in guinea pigs

As described above, we recently demonstrated that ouabain inhibits digoxin-induced changes in intracellular membrane traffic (Feldmann *et al.*, 2007; Rosen *et al.*, 2004). A similar inhibition of digoxin-induced changes in membrane traffic was seen following the addition of wortmannin, an inhibitor of phosphoinositide 3-kinase (PI3K), implying that this kinase is involved in the inhibition by ouabain. We, therefore hypothesized that wortmannin would similarly inhibit digoxin-induced cardiotoxicity. Indeed, as seen in Figure 4A, the simultaneous infusion of digoxin together with wortmannin (100 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) postponed the occurrence of first arrhythmia, of VT/VF and of terminal VF ($P < 0.05$), showing the marked inhibition of digoxin-induced cardiotoxicity. Lower wortmannin infusion rates did not exert significant effects on digoxin-induced toxicity (data not shown). Interestingly, wortmannin (100 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) did not affect ouabain-induced toxicity: Ouabain at a dose of 300 $\mu g \cdot kg^{-1} \cdot hr^{-1}$ caused toxic effects similar to those of digoxin (500 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) but the simultaneous addition of ouabain and wortmannin (100 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) did not delay any of these toxic manifestations (Figure 4B).

Differences between ouabain- and digoxin-induced changes in heart rate and blood pressure in guinea pig

The effects of digoxin infusions (500 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) on BP and HR were biphasic, as described above (Figure 5, see also Figure 3). In contrast to digoxin, ouabain infusion (300 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) caused an elevation in BP only after the first arrhythmia (Figure 5A, $P < 0.05$). Moreover, ouabain did not cause a reduction in HR before the occurrence of the first arrhythmia (Figure 5B, $P < 0.05$). These differences imply a distinct cardiotoxicity pathway of each of the two steroids**.**

Additive inhibition of Na^+, K^+-ATPase activity by digoxin and ouabain.

The unexpected finding that ouabain antagonized digoxin-induced cardiotoxicity, in our *in vivo* and *ex vivo* experimental systems, suggested an opposition in their actions at the molecular level. Inhibition of Na^+, K^+-ATPase activity is considered to underline cardiac steroid-induced toxicity, we determined the inhibition of Na^+, K^+-ATPase activity in guinea pig heart membrane preparations, induced by digoxin, in the presence of different concentrations of ouabain (Figure 6). Basal Na^+, K^+-ATPase activity (values with no digoxin minus the background activity) was found to be about 3.5 μmol P_i·mg protein^{-1}·hr^{-1}. This result is in an agreement with other studies using the same membrane preparation (Liu, Gable *et al.*, 2007). As expected, digoxin inhibited, dose-dependently, Na^+, K^+-ATPase activity at all ouabain concentrations. Notably, these experiments did not reveal any antagonism by ouabain of the inhibitory activity of digoxin.

Discussion and Conclusions

Our study shows for the first time that in the guinea pig, low doses of ouabain effectively inhibited cardiotoxicity induced by other cardiac steroids, such as digoxin and bufalin. This inhibition was manifested by a delay in the start of arrhythmia induced by bufalin and digoxin in isolated heart muscle preparations (Figure 1) and also *in vivo* by a delay in the appearance of arrhythmia, HR elevation and terminal VF induced by digoxin (Figures 2 and 3).

As described in the Introduction, although all cardiac steroids bind to the same receptor, the Na^+, K^+-ATPase, earlier studies have noted differences in the response of the cardiovascular system to treatment with different cardiac steroids as well as in the cellular and molecular mechanisms affected by these compounds (see Dvela *et al.*, 2007). Despite these observations, the vast majority of the literature refers to the two compounds as Digitalis or cardiac steroids, ignoring the dissimilarities between them. We suggest that all cardiac steroids increase the force of contraction of heart muscle via a common mechanism of action. This mechanism presumably includes inhibition of the plasma membrane Na^+, K^+-ATPase, increased [Na^+_{in}], and increased [Ca^{++}_{in}] in discrete intracellular compartments (Eichhorn *et al.*, 2002; Khatter *et al.*, 1989; Ruch *et al.*, 2003). Consequently, as shown in Figure 1C, the increase in the force of contraction by different CS is additive and this may be a consequence of the additive inhibition of Na^+, K^+-ATPase activity (also shown in Figure 6).

The toxic effects of high doses of cardiac steroids on the other hand, can be divided into two types. The first, resulting from a massive inhibition of ion transport by the Na^+, K^+-ATPase, causing intracellular "calcium overload", is common to all cardiac steroids. The second, resulting from an unknown mechanism (see below), is common to all cardiac steroids except ouabain. Hence, in the presence of ouabain, as shown in this study, some toxic manifestations of other cardiac steroids are attenuated. In agreement with this notion is also the observation that digoxin- but not ouabain-induced cardiotoxicity was reduced by wortmannin (Figures 4) and that the two drugs induce different changes in BP and HR (Figure 5).

These findings suggest that experience with ouabain and digoxin treatment in the clinical setting should differ. Indeed, the published reports comparing the beneficial use of

digoxin and ouabain for the treatment of angina pectoris show such differences. Whereas digoxin treatment caused worsening of angina (Ahmed, Rich *et al.*, 2006; Fenn and Gilbert, 1932; Harding, Aronow *et al.*, 1973), ouabain treatment was beneficial (Kern, 1974; Kubicek and Reisner, 1973; Sarre, 1943; Wagenfeld, 1936). The pharmacodynamic characteristics of the two steroids also differ: iv-administration of ouabain caused a maximal effect after 5 minutes, which lasted for 5-7 hours and then rapidly declined (Sarre, 1943). The digoxin effect is slower, starting 5-30 min after injection and reaching a maximum only after 1-4 hrs (Eichhorn *et al.*, 2002). Furthermore, the therapeutic effects of digoxin and ouabain are observed at steady-state plasma concentrations between 0.6 and 2.5 $nmol \cdot L^{-1}$ (Bauman, Didomenico *et al.*, 2006; Selden and Smith, 1972). Digoxin toxicity occurs when the plasma level exceeds 2.5 $nmol \cdot L^{-1}$, illustrating the narrow therapeutic window of the drug (Bauman *et al.*, 2006). Information regarding the toxic concentration threshold for ouabain in humans is not available. However, endogenous ouabain was shown to increase to 86.0 ± 27.2 $nmol \cdot L^{-1}$ in the circulation of athletes following 15 min of exercise, without any toxic manifestations (Bauer, Muller-Ehmsen *et al.*, 2005). The relatively lower toxicity of ouabain and its ability to antagonize the toxic effects of digoxin suggest not only that this steroid should be preferred for clinical use but also that it may serve as an antidote for cardiac steroid intoxication. Intriguingly, as described in the monograph by Kern (1974), already in the 1940-50's, before the availability of Fab fragments for treatment of digoxin intoxication, ouabain was effectively used to treat this condition. Furthermore, it was recommended to use digoxin in combination with oral ouabain to lower the former's toxicity (Kern, 1974). Those recommendations have disappeared with time, but the present study supports their rationale.

The molecular basis for the diversity in action and antagonism between various CS is not known. They may result from differences in steroid structure, rendering differential binding characteristics and consequently diverse activation of signaling pathways in different cells (see Dvela *et al.*, 2007). Our experiments, demonstrating that ouabain did not attenuate digoxin-induced inhibition of Na^+, K^+-ATPase activity (Figure 6) suggest that the antagonism between the steroids is not at the level of the ion transport function of the Na^+, K^+-ATPase. The experiments using wortmannin shed some light on the possible

mechanism involved in the antagonistic effects of ouabain on other cardiac steroid-induced cardiotoxicity. Wortmannin is a fungal metabolite that specifically inhibits PI3K, MAPK, and myosin light-chain kinase. These actions effectively inhibit receptor-mediated endocytosis in several cell types, including myocytes (Charney, Egnor *et al.*, 2004; Nicola and Straus, 2004; Richards, Rizzoli *et al.*, 2004; Yang and Holman, 2005). We previously showed that wortmannin, like ouabain, inhibits cardiac steroid -induced accumulation of endocytosed membranes in NT2 cells (Rosen *et al.*, 2004). The observation that wortmannin inhibits digoxin- but not ouabain-induced cardiotoxicity (Figure 4) suggests that alterations in intracellular membrane traffic are involved in digoxin-induced cardiotoxicity but not in that of ouabain. On the contrary, Nunez-Duran's group showed that direct inhibition of receptor-mediated endocytosis by cooling or wortmannin, delayed the toxic effect of ouabain in isolated guinea pig heart preparations (Nunez-Duran, Atonal *et al.*, 1996; Nunez-Duran, Riboni *et al.*, 1988). This apparent contradiction is probably due to differences between the *ex vivo* and *in vivo* settings. Moreover, as mentioned above, we observed an elevation in BP following wortmannin infusion together with ouabain, compared with ouabain alone, before the onset of the first arrhythmia. This elevation was greater in the *ex vivo* perfused heart lacking the neuronal and hormonal effect of other BP regulatory substances. Taking all the evidence together, we propose that part of the cardiotoxicity induced by cardiac steroids stems from their effect on endocytosed membrane traffic. This effect is antagonized by ouabain, resulting in attenuation of the cardiotoxic manifestations of other cardiac steroids.

This study was designed to investigate the relationship between the cardiotoxicity of different cardiac steroids. Our main finding was the inhibitory effect of ouabain on digoxin- and bufalin-induced cardiotoxicity. Further understanding of this phenomenon could prove beneficial for increasing the therapeutic window of cardiac steroids, in the treatment of chronic heart failure.

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Conflict of interest

None.

References

Ahmed A, Rich MW, Fleg JL, Zile MR, Young JB, Kitzman DW, Love TE, Aronow WS, Adams KF, Jr., Gheorghiade M (2006). Effects of digoxin on morbidity and mortality in diastolic heart failure: the ancillary digitalis investigation group trial. *Circulation* **114:** 397-403.

Aizman O, Uhlen P, Lal M, Brismar H, Aperia A (2001). Ouabain, a steroid hormone that signals with slow calcium oscillations. *Proc Natl Acad Sci U S A* **98:** 13420-13424.

Aydemir-Koksoy A, Abramowitz J, Allen JC (2001). Ouabain-induced signaling and vascular smooth muscle cell proliferation. *J Biol Chem* **276:** 46605-46611.

Bauer N, Muller-Ehmsen J, Kramer U, Hambarchian N, Zobel C, Schwinger RH, Neu H, Kirch U, Grunbaum EG, Schoner W (2005). Ouabain-like compound changes rapidly on physical exercise in humans and dogs: effects of beta-blockade and angiotensin-converting enzyme inhibition. *Hypertension* **45:** 1024-1028.

Bauman JL, Didomenico RJ, Galanter WL (2006). Mechanisms, manifestations, and management of digoxin toxicity in the modern era. *Am J Cardiovasc Drugs* **6:** 77-86.

Chan KM, Delfert D, Junger KD (1986). A direct colorimetric assay for Ca2+ -stimulated ATPase activity. *Anal Biochem* **157:** 375-380.

Charney AN, Egnor RW, Henner D, Rashid H, Cassai N, Sidhu GS (2004). Acid-base effects on intestinal Cl- absorption and vesicular trafficking. *Am J Physiol Cell Physiol* **286:** C1062-1070.

Dvela M, Rosen H, Feldmann T, Nesher M, Lichtstein D (2007). Diverse biological responses to different cardiotonic steroids. *Pathophysiology* **14:** 159-166.

Eichhorn EJ, Gheorghiade M (2002). Digoxin. *Prog Cardiovasc Dis* **44:** 251-266.

Feldmann T, Glukmann V, Medvenev E, Shpolansky U, Galili D, Lichtstein D, Rosen H (2007). Role of endosomal Na+-K+-ATPase and cardiac steroids in the regulation of endocytosis. *Am J Physiol Cell Physiol* **293:** C885-896.

Fenn GK, Gilbert NC (1932). Anginal pain as a result of digitalis administration. *JAMA* **98:** 99-104.

Fricke U, Klaus W (1981). The influence of reduced serum potassium level on the toxicity of some cardenolides in guinea pigs. *Basic Res Cardiol* **76:** 62-78.

Haas M, Wang H, Tian J, Xie Z (2002). Src-mediated inter-receptor cross-talk between the Na+/K+-ATPase and the epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases. *J Biol Chem* **277:** 18694-18702.

Harding PR, Aronow WS, Eisenman J (1973). Digitalis as an antianginal agent. *Chest* **64:** 439-443.

Haver E, Lichtstein D, Munson PJ (1995). Multiple types of binding sites for atrial natriuretic peptide in rat olfactory bulb membranes and synaptosomes. *Brain Res* **681:** 75-83.

Hovevey-Sion D, Kaplanski J (1979). Toxicity of digoxin in acutely and chronically heat-exposed rats. *Res Commun Chem Pathol Pharmacol* **25:** 517-524.

Huang BS, Kudlac M, Kumarathasan R, Leenen FH (1999). Digoxin prevents ouabain and high salt intake-induced hypertension in rats with sinoaortic denervation. *Hypertension* **34:** 733-738.

Kern B (1974). *Der Myokard-Infarkt*. Heidelberg: Haug-Verlag.

Khatter JC, Agbanyo M, Navaratnam S, Nero B, Hoeschen RJ (1989). Digitalis cardiotoxicity: cellular calcium overload a possible mechanism. *Basic Res Cardiol* **84:** 553-563.

Kieval RS, Butler VP, Jr., Derguini F, Bruening RC, Rosen MR (1988). Cellular electrophysiologic effects of vertebrate digitalis-like substances. *J Am Coll Cardiol* **11:** 637-643.

Kubicek F, Reisner T (1973). [Hypoxia tolerance in coronary heart disease as modified by digoxin, beta-methyl-digoxin and g-strophanthin]. *Ther Ggw* **112:** 747-749 passim.

Liu L, Gable ME, Garlid KD, Askari A (2007). Interactions of K+ATP channel blockers with Na+/K+-ATPase. *Mol Cell Biochem* **306:** 231-237.

Manunta P, Hamilton J, Rogowski AC, Hamilton BP, Hamlyn JM (2000). Chronic hypertension induced by ouabain but not digoxin in the rat: antihypertensive effect of digoxin and digitoxin. *Hypertens Res* **23 Suppl:** S77-85.

McGowan MH, Russell P, Carper DA, Lichtstein D (1999). Na+, K+-ATPase inhibitors down-regulate gene expression of the intracellular signaling protein 14-3-3 in rat lens. *J Pharmacol Exp Ther* **289:** 1559-1563.

Modyanov N, Pestov N, Adams G, Crambert G, Tillekeratne M, Zhao H, Korneenko T, Shakhparonov M, Geering K (2003). Nongastric H,K-ATPase: structure and functional properties. *Ann N Y Acad Sci* **986:** 183-187.

Nicola AV, Straus SE (2004). Cellular and viral requirements for rapid endocytic entry of herpes simplex virus. *J Virol* **78:** 7508-7517.

Nunez-Duran H, Atonal F, Contreras P, Melendez E (1996). Endocytosis inhibition protects the isolated guinea pig heart against ouabain toxicity. *Life Sci* **58:** PL193-198.

Nunez-Duran H, Riboni L, Ubaldo E, Kabela E, Barcenas-Ruiz L (1988). Ouabain uptake by endocytosis in isolated guinea pig atria. *Am J Physiol* **255:** C479-485.

Pamnani MB, Chen S, Bryant HJ, Schooley JF, Jr., Eliades DC, Yuan CM, Haddy FJ (1991). Effects of three sodium-potassium adenosine triphosphatase inhibitors. *Hypertension* **18:** 316-324.

Pauli-Magnus C, Murdter T, Godel A, Mettang T, Eichelbaum M, Klotz U, Fromm MF (2001). P-glycoprotein-mediated transport of digitoxin, alpha-methyldigoxin and beta-acetyldigoxin. *Naunyn Schmiedebergs Arch Pharmacol* **363:** 337-343.

Ramirez-Ortega M, Zarco G, Maldonado V, Carrillo JF, Ramos P, Ceballos G, Melendez-Zajgla J, Garcia N, Zazueta C, Chanona J, Suarez J, Pastelin G (2007). Is digitalis compound-induced cardiotoxicity, mediated through guinea-pig cardiomyocytes apoptosis? *Eur J Pharmacol* **566:** 34-42.

Richards DA, Rizzoli SO, Betz WJ (2004). Effects of wortmannin and latrunculin A on slow endocytosis at the frog neuromuscular junction. *J Physiol* **557:** 77-91.

Rosen H, Glukhman V, Feldmann T, Fridman E, Lichtstein D (2004). Cardiac steroids induce changes in recycling of the plasma membrane in human NT2 cells. *Mol Biol Cell* **15:** 1044-1054.

Ruch SR, Nishio M, Wasserstrom JA (2003). Effect of cardiac glycosides on action potential characteristics and contractility in cat ventricular myocytes: role of calcium overload. *J Pharmacol Exp Ther* **307:** 419-428.

Sarre H (1943). Die Ursache der gegensatzlichen Wirkung von Strophanthin and Digitalis auf die Coronarinsuffizienz. *J Mol Med* **22:** 135-141.

Segal JB, McNamara RL, Miller MR, Kim N, Goodman SN, Powe NR, Robinson K, Yu D, Bass EB (2000). The evidence regarding the drugs used for ventricular rate control. *J Fam Pract* **49:** 47-59.

Selden R, Smith TW (1972). Ouabain pharmacokinetics in dog and man. Determination by radioimmunoassay. *Circulation* **45:** 1176-1182.

Small A, McElroy HW, Ide RS (1971). Studies of the electrocardiogram and the toxicity of cardiac glycosides in animals exposed to hyperbaric helium. *Toxicol Appl Pharmacol* **20:** 44-56.

Wagenfeld E (1936). Zur Strophanthinbehandlung der Angina pectoris. *J Mol Med* **15:** 1155-1158.

Wasserstrom JA, Aistrup GL (2005). Digitalis: new actions for an old drug. *Am J Physiol Heart Circ Physiol* **289:** H1781-1793.

Yang J, Holman GD (2005). Insulin and contraction stimulate exocytosis, but increased AMP-activated protein kinase activity resulting from oxidative metabolism stress slows endocytosis of GLUT4 in cardiomyocytes. *J Biol Chem* **280:** 4070-4078.

Figure Legends

Figure 1. Effect of ouabain on bufalin- and digoxin-induced toxicity in heart muscle preparations

Papillary muscles and left atria were electrically stimulated to induce contration; the right atria beat spontaneously. Twitch force was measured (gF). Following a control period, bufalin or digoxin was added to the bath in the presence or absence of ouabain. A. Representative experiment showing delay of arrythmia in papillary muscle in the presence of ouabain (0.4 $\mu mol \cdot L^{-1}$) caused by bufalin (0.4 $\mu mol \cdot L^{-1}$). B. Time elapsed from drug administration to first arrythmia. Bufalin at 0.4 $\mu mol \cdot L^{-1}$ for papillary muscle and 225 $nmol \cdot L^{-1}$ for atria was added in the presence or absence of ouabain at 0.4 $\mu mol \cdot L^{-1}$ and 30 $nmol \cdot L^{-1}$ (for papillary muscle and atria, respectiviely). Preparations which did not develop arrhythmia were given an arbitrary value of 90 min. C. Maximal inotropic effects of papillary muscles (PM), left atria (LA) and right atria (RA) following bufalin (Buf) administration in the presence or absence of ouabain (Oua) at concentrations as in Figure 1B. D. Representative experiment showing the postponement of arrythmia in papillary muscle caused by 3.5 $\mu mol \cdot L^{-1}$ digoxin in the presence of 0.4 $\mu mol \cdot L^{-1}$ ouabain. E. Time elapsed from drug administration to first arrythmia. Digoxin at 0.4 $\mu mol \cdot L^{-1}$ was added in the presene or absence of ouabain at 0.4 $\mu mol \cdot L^{-1}$. Preparations which did not develop arrhythmia were given an arbitrary value of 90 min. F. Maximal inotropic effect on papillary muscle following bufalin or digoxin administration in the presence or absence of ouabain at concentrations as in Figure 1E. The values are expressed as the mean ± S.E (n = 5-12). * = value higer than that obtained in the presence of bufalin alone ($P<0.05$). ** = value lower than that obtained in the presence of digoxin alone ($P<0.05$).

Figure 2. Effect of ouabain on digoxin-induced cardiotoxicity *in vivo*

Digoxin (500 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) was infused into anaesthetized guinea pigs in the presence or absence of ouabain (Oua) at 91 $ng \cdot kg^{-1} \cdot hr^{-1}$ or 182 $ng \cdot kg^{-1} \cdot hr^{-1}$. Time of onset of three end points (time to first arrhythmia (1^{st} arrhythmia), time to first VT-VF and time to death) were calculated from the start of the cardiac steroid infusion. The values are expressed as

the mean ± S.E (n = 4-8). * = value higher than that obtained in the presence of digoxin alone ($P<0.05$). ** = value lower than that obtained in the presence of digoxin alone ($P<0.05$). † = value lower than that obtained in the presence of digoxin with ouabain 91 $ng \cdot kg^{-1} \cdot hr^{-1}$ ($P<0.05$).

Figure 3. Effect of ouabain on digoxin-induced blood pressure and heart rate fluctuations *in vivo*

Digoxin (500 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) was infused into anaesthetized guinea pigs in the presence or absence of ouabain (91 $ng \cdot kg^{-1} \cdot hr^{-1}$). The lowest values of BP and HR, just before onset of first arrhythmia and VT/VF were calculated as the percentage of the values obtained during the control period (before the start of the cardiac steroid infusion). The values are expressed as the mean ± S.E (n = 4-8). * = values higher than control values ($P<0.05$). ** = values lower than control values ($P<0.05$). † = value lower than that obtained for digoxin alone ($P<0.05$) and not different from control values.

Figure 4. Effect of wortmannin on digoxin- and ouabain-induced cardiotoxicity *in vivo*

Digoxin (500 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) or ouabain (300 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) was infused into anaesthetized guinea pigs in the presence or absence of wortmannin (100 $\mu g \cdot kg^{-1} \cdot hr^{-1}$). The time of onset of first arrhythmia and VT/VF and death was calculated from the start of the cardiac steroid infusion. The values are expressed as the mean ± S.E (n = 6). * = value higher than that in the presence of digoxin alone ($P<0.05$).

Figure 5. Differences between ouabain- and digoxin-induced changes in blood pressure (BP) and heart rate (HR) in guinea pigs

Digoxin (500 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) or ouabain (300 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) was infused into anaesthetized guinea-pigs. The lowest BP and HR values, just before the onset of first arrhythmia and VT/VF were calculated as the percentage of the values obtained during the control period

(before the start of the cardiac steroid infusion). The values are expressed as the mean ± S.E (n = 4-8). * = values higher than control values ($P<0.05$). ** = values lower than control values ($P<0.05$). † = value lower than that obtained for digoxin ($P<0.05$) and not different from control values. †† = value higher than that obtained for digoxin ($P<0.05$) and not different from control values.

Figure 6. Effect of ouabain on inhibition of Na^+, K^+-ATPase activity by digoxin.
Guinea pig heart membrane preparations were incubated at 37°C with various concentrations of digoxin in the presence or absence of different ouabain concentrations. Na^+, K^+-ATPase activity was determined by the release of P_i, measured using the Malachite Green assay. Values are expressed as mean ± S.E (n = 5). * = value lower than that obtained in the absence of ouabain ($P<0.05$).

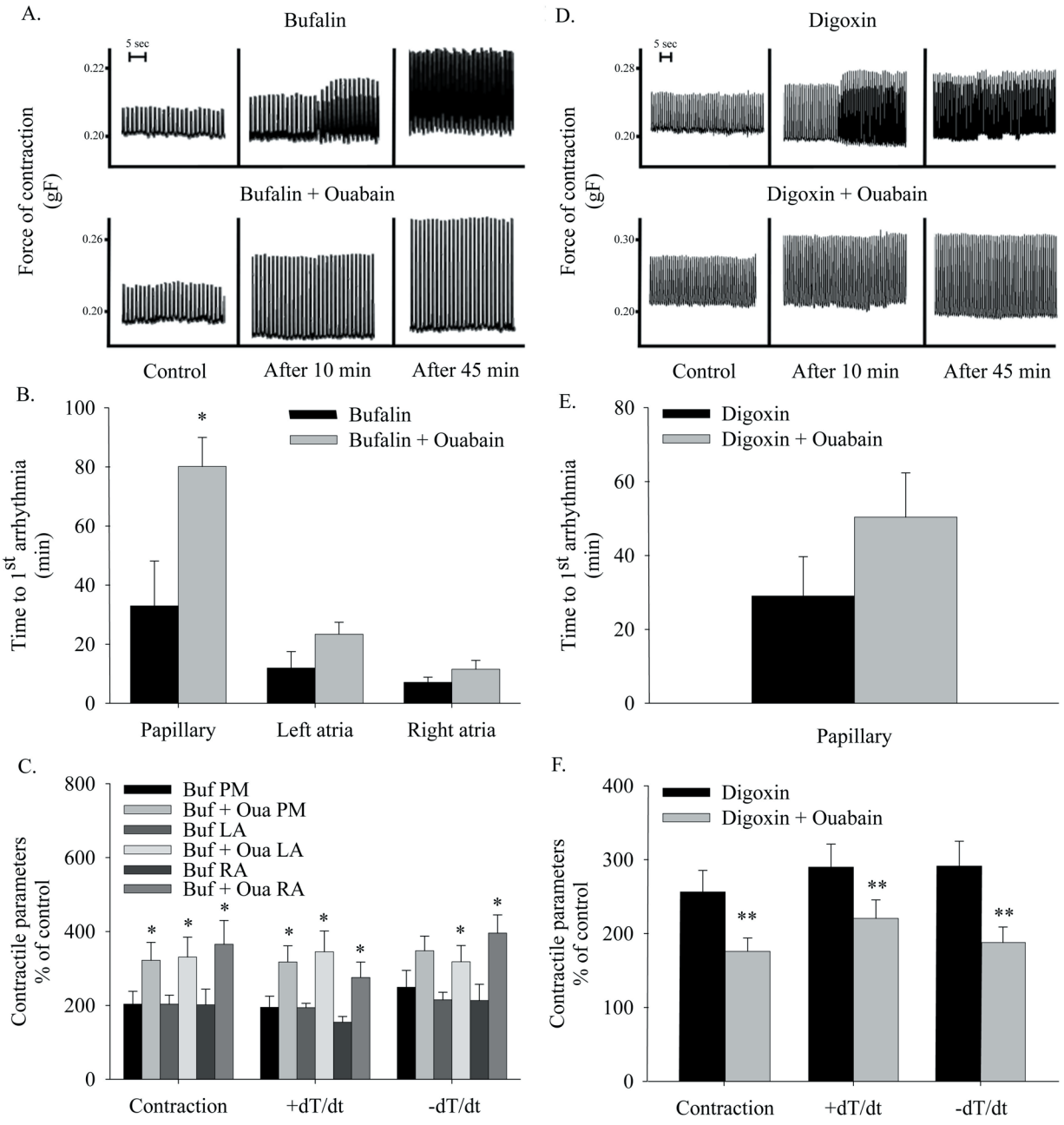
A.
Bufalin
5 sec
0.22
0.20
Force of contraction (gF)
Bufalin + Ouabain
0.26
0.20
Control
After 10 min
After 45 min
D.
Digoxin
5 sec
0.28
0.20
Force of contraction (gF)
Digoxin + Ouabain
0.30
0.20
Control
After 10 min
After 45 min
B.
Time to 1st arrhythmia (min)
100
80
60
40
20
0
*
Bufalin
Bufalin + Ouabain
Papillary
Left atria
Right atria
E.
Time to 1st arrhythmia (min)
80
60
40
20
0
Digoxin
Digoxin + Ouabain
Papillary
C.
Contractile parameters % of control
800
600
400
200
0
Buf PM
Buf + Oua PM
Buf LA
Buf + Oua LA
Buf RA
Buf + Oua RA
*
Contraction
+dT/dt
-dT/dt
F.
Contractile parameters % of control
400
300
200
100
0
Digoxin
Digoxin + Ouabain
**
Contraction
+dT/dt
-dT/dt

Figure1

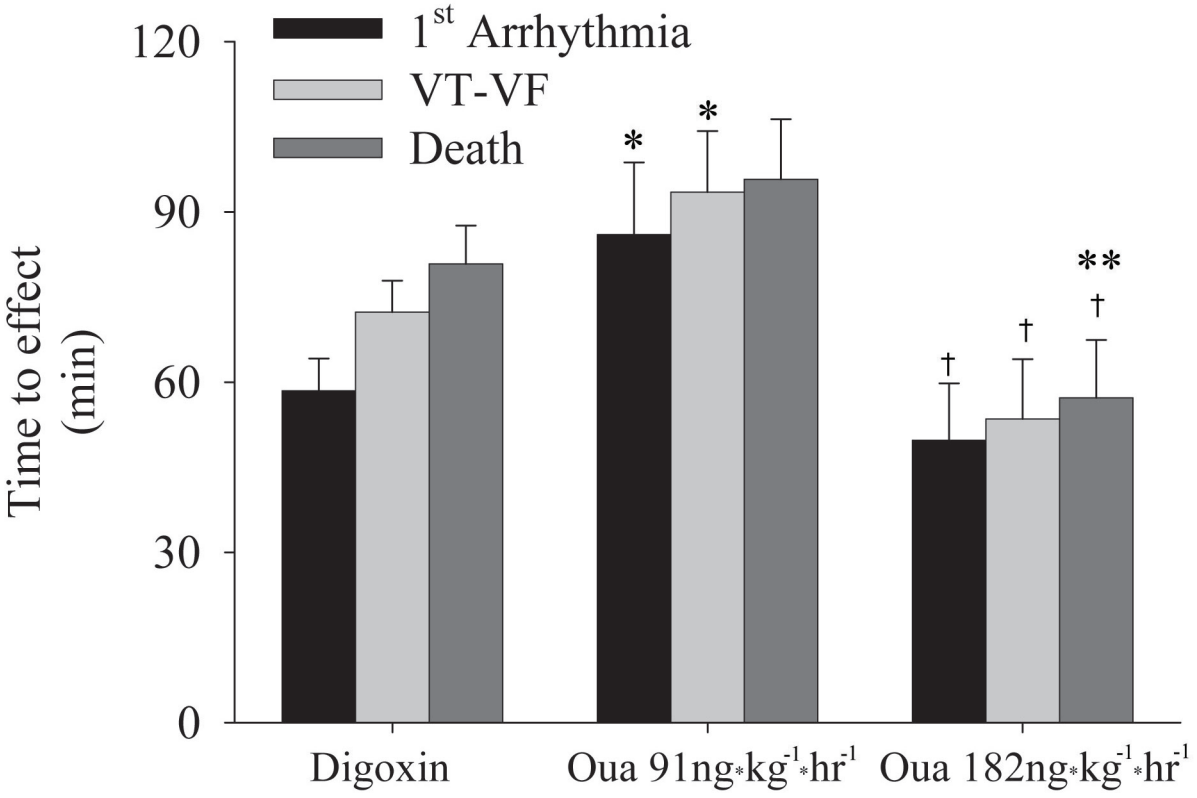
1st Arrhythmia
VT-VF
Death
Time to effect (min)
120
90
60
30
0
*
*
†
†
**
†
Digoxin
Oua 91ng*kg-1*hr-1
Oua 182ng*kg-1*hr-1

Figure2

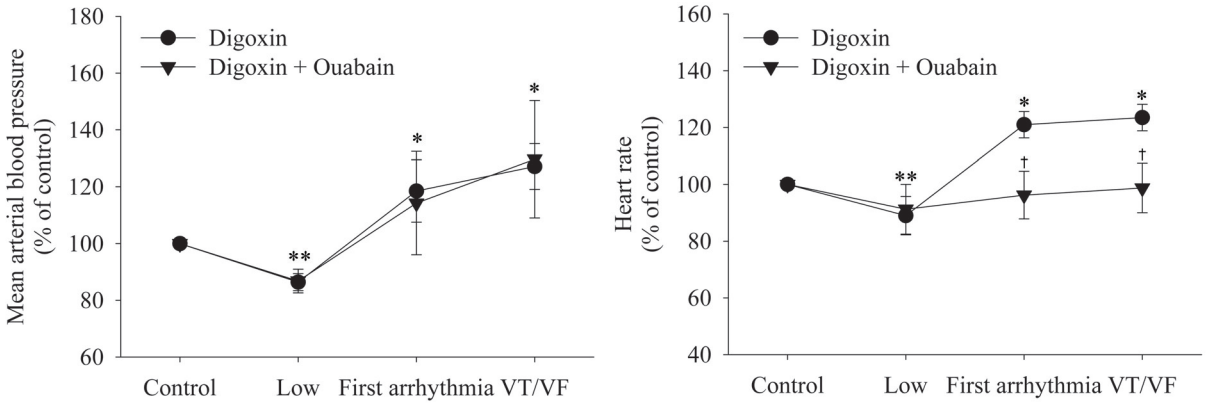
Mean arterial blood pressure
(% of control)
180
160
140
120
100
80
60
Digoxin
Digoxin + Ouabain
**
*
*
Control
Low
First arrhythmia
VT/VF
Heart rate
(% of control)
160
140
120
100
80
60
40
Digoxin
Digoxin + Ouabain
**
*
*
†
†
Control
Low
First arrhythmia
VT/VF

Figure3

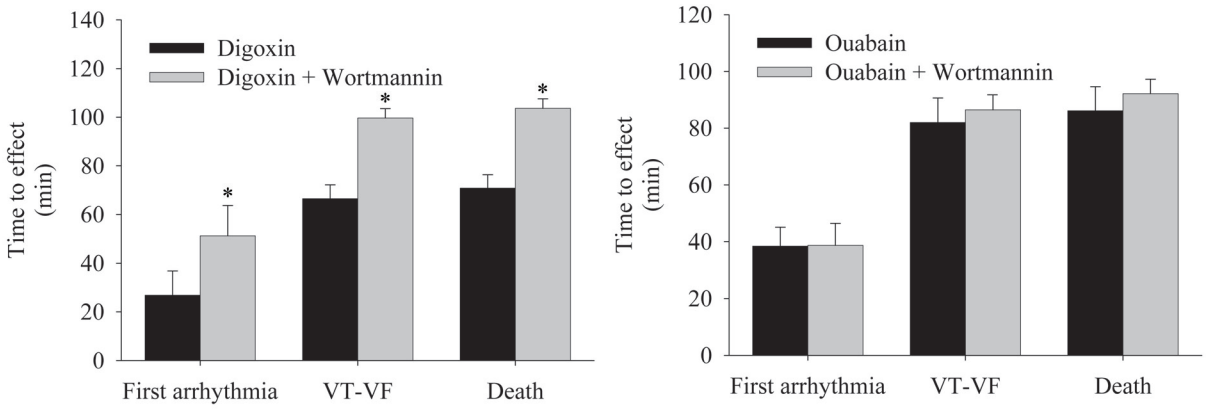
Digoxin
Digoxin + Wortmannin
Time to effect (min)
140
120
100
80
60
40
20
0
First arrhythmia
VT-VF
Death
*
*
*
Ouabain
Ouabain + Wortmannin
Time to effect (min)
120
100
80
60
40
20
0
First arrhythmia
VT-VF
Death

Figure4

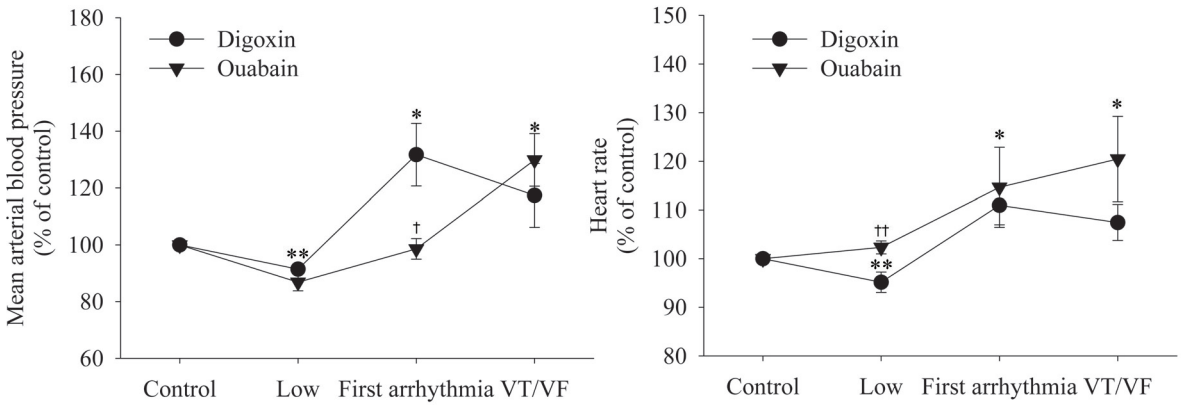
Mean arterial blood pressure
(% of control)
180
160
140
120
100
80
60
Digoxin
Ouabain
*
*
**
†
Control
Low
First arrhythmia
VT/VF
Heart rate
(% of control)
150
140
130
120
110
100
90
80
Digoxin
Ouabain
*
*
††
**
Control
Low
First arrhythmia
VT/VF

Figure5

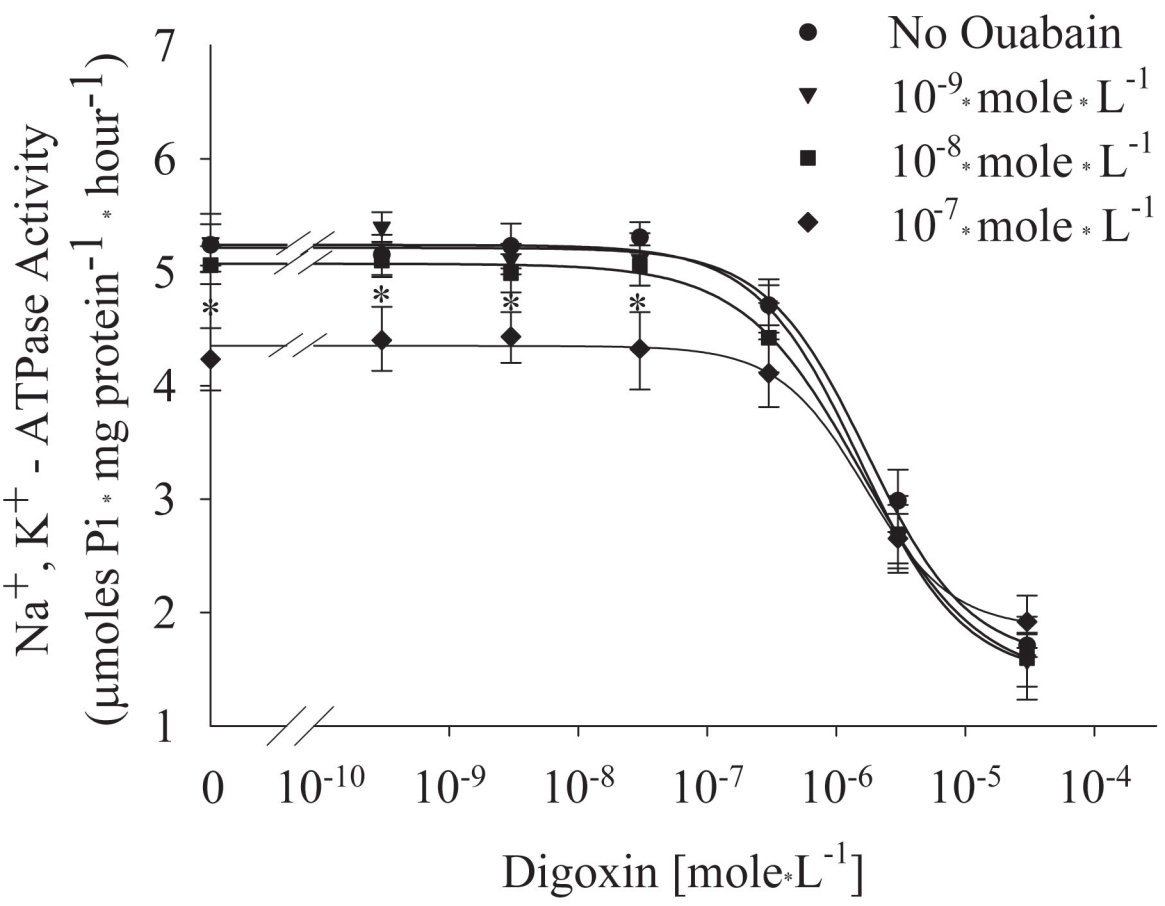
No Ouabain
10-9 * mole * L-1
10-8 * mole * L-1
10-7 * mole * L-1
Na+, K+ - ATPase Activity
(µmoles Pi * mg protein-1 * hour-1)
Digoxin [mole*L-1]
0
10-10
10-9
10-8
10-7
10-6
10-5
10-4
1
2
3
4
5
6
7
*

Figure6