

Effect of Cardiac Glycosides on Action Potential Characteristics and Contractility in Cat Ventricular Myocytes: Role of Calcium Overload

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ABSTRACT

There is increasing evidence that cardiac glycosides act through mechanisms distinct from inhibition of the sodium pump but which may contribute to their cardiac actions. To more fully define differences between agents indicative of multiple sites of action, we studied changes in contractility and action potential (AP) configuration in cat ventricular myocytes produced by six cardiac glycosides (ouabain, ouabagenin, dihydroouabain, actodigin, digoxin, and resibufogenin). AP shortening was observed only with ouabain and actodigin. There was extensive inotropic variability between agents, with some giving full inotropic effects before automaticity occurred whereas others produced minimal inotropy before toxicity. AP shortening was not a result of alterations in calcium current or the inward rectifier potassium current, but correlated with an increase in steady-state outward current (I_{ss}), which was sensitive to KB-R7943, a Na^+ - Ca^{2+} exchange (NCX) inhibitor.

Interestingly, I_{ss} was observed following exposure to ouabain and dihydroouabain, suggesting that an additional mechanism is operative with dihydroouabain that prevents AP shortening. Further investigation into differences in inotropy between ouabagenin, dihydroouabain and ouabain revealed almost identical responses under AP voltage clamp. Thus all agents appear to act on the sodium pump and thereby secondarily increase the outward reverse mode NCX current, but the extent of AP duration shortening and positive inotropy elicited by each agent is limited by development of their toxic actions. The quantitative differences between cardiac glycosides suggest that mechanisms independent of sodium pump inhibition may result from an altered threshold for calcium overload possibly involving direct or indirect effects on calcium release from the sarcoplasmic reticulum.

The well described action of cardiac glycosides to inhibit the Na^+ , K^+ -ATPase (Na^+ pump, sodium pump) is believed by many investigators to be the mechanism of central importance in both inotropic and toxic effects of these agents (Akeria et al., 1970; Lee and Dagostino, 1982; Gadsby et al., 1985; Steimers et al., 1990). Indeed, studies supporting the sodium pump inhibition hypothesis are so frequent in the literature that the implication is that the action of cardiac glycosides on the sodium pump is the only significant effect mediated by cardiac glycosides in the myocardium. However, there is also substantial evidence that cardiac glycosides may act at sites other than the sodium pump, and that these

alternative sites may account for important differences between agents. Support for alternative mechanisms underlying inotropic and/or toxic effects of cardiac glycosides includes findings that cardiac glycoside analogs differ significantly in their effects on intracellular $[\text{Na}^+]$, action and resting membrane potentials (Wasserstrom et al., 1991), and toxic to therapeutic ratios in both isolated preparations (Karagueuzian and Katzung, 1981) and whole animals (Mendez et al., 1974). In addition, studies directed at defining alternative cardiac glycoside mechanisms have revealed intracellular sites of action independent of the sodium pump, including the sarcoplasmic reticulum (Fujino and Fujino, 1982; Isenberg, 1984) and its calcium release channel (Rardon and Wasserstrom, 1990; McGarry and Williams, 1993; Sagawa et al., 2002).

Despite growing evidence in support of alternative mechanisms and their role in determining differences in the effects of cardiac glycoside analogs, few studies have compared

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ABBREVIATIONS: AP, action potential; APD, action potential duration; KHB, Krebs-Henseleit buffer; DHO, dihydroouabain; NCX, sodium-calcium exchanger; RMP, resting membrane potential; I_{ss} , outward steady-state current; I_{Ca} , calcium current (L-type); I_{K1} , inward rectifying potassium current; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid.

cardiac glycoside analog actions using isolated cardiac myocytes. In contrast to multicellular preparations, the use of single cardiac cells provides considerable advantages in evaluating cardiac glycoside effects. Of key importance is the elimination of extracellular diffusion barriers that confound measurement of membrane currents and other cellular processes dependent on ion gradients. In addition, nearly all previous studies have used a variety of multicellular preparations under an equally varied number of experimental conditions, making comparisons between agents extremely difficult. The purpose of this study was to characterize the electrophysiological and inotropic effects of different cardiac glycosides in isolated cardiac myocytes in a standardized manner to determine whether there are indeed differences in cellular actions of these agents. Six cardiac glycoside analogs were chosen for study based on specific differences in their molecular structures. The study was designed first to characterize differences between these six cardiac glycoside analogs on action potential (AP) configuration and contractility in isolated cat ventricular myocytes, and then to define the underlying mechanisms that account for the differences in their actions.

Materials and Methods

Isolation of Cat Ventricular Myocytes. After anesthesia by sodium pentobarbital (approximately 45 mg/kg, i.p.), the hearts from adult cats were excised and perfused through the aorta and coronary arteries with Ca^{2+} -free modified Krebs-Henseleit buffer (KHB) for 5 min. The heart was then perfused with 0.08% collagenase in KHB solution (Worthington CLS II) for 8 to 10 min at 36°C. Ventricular tissue was minced and incubated in a shaker bath for 5 min in KHB-collagenase solution at 36°C. Cells were then filtered (200- μm nylon sieve), washed free of collagenase and placed in KHB containing 1% albumin and 1 mM Ca^{2+} for 15 min. Cells were stored in 1.8 mM Ca^{2+} Tyrode's solution at room temperature and used up to 36 h after isolation. Typical cell yields are 60 to 70% with this technique. Only rod-shaped, Ca^{2+} -tolerant myocytes with visible cross-striations were used for study.

Solutions and Drugs. (Modified) Tyrode's solution: 140 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl_2 , 5 mM HEPES, 0.4 mM NaH_2PO_4 , 11 mM glucose, 1.8 mM CaCl_2 , pH = 7.4 (NaOH). I_{Ca} extracellular solution: 0.2 mM BaCl_2 added to Tyrode's solution to block I_{K1} . Ramp protocol extracellular solution: 0.1 mM CdCl_2 added to Tyrode's solution to block I_{Ca} . High resistance microelectrode solution: 500 mM KCl. Whole-cell pipette solution: 10 mM NaCl, 120 mM potassium-aspartate, 25 mM KCl, 20 mM HEPES, 0.5 mM MgCl_2 , 4 mM K_2ATP , 0.056 or 1.0 mM EGTA. Cardiac glycosides were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of resibufogenin, which was obtained from Tientsin First Central Pharmaceutical (Tientsin, China). Cardiac glycoside stock solutions of 1 to 10

mM were made in distilled water [dihydroouabain (DHO), resibufogenin], or 50% EtOH/water (ouabain, ouabagenin, actodigin, digoxin). KB-R7943 was a gift from Kanebo, Ltd. (Osaka, Japan). In the experiments in which I_{Ca} was measured, a low concentration of Ba^{2+} (0.2 mM) was sufficient to block background K^+ current but had little effect on magnitude and kinetics of I_{Ca} in the presence of 1.8 mM CaCl_2 . We have used this approach previously (Nishio et al., 2002) and found it to be a useful means to measure I_{Ca} under conditions in which normal physiological conditions are otherwise maintained (physiological $[\text{Ca}^{2+}]$, temperature, K^+ gradient).

Molecular Structures of the Six Cardiac Glycosides Studied. To more accurately define differences between cardiac glycoside agents and further evaluate cardiac glycoside structure-activity relationships, six cardiac glycosides (ouabain, ouabagenin, DHO, actodigin, digoxin, and resibufogenin) were chosen for electrophysiology and contractility studies in single cat ventricular myocytes. Figure 1 shows the molecular structure of these agents. Of particular note, DHO and ouabagenin differ from the prototypical analog ouabain by saturation of the lactone ring and omission of the carbohydrate moiety, respectively. Actodigin differs from ouabain in the site of attachment of the lactone ring at C-17 of the steroid nucleus. Resibufogenin, which unlike the other analogs is isolated from an animal source (frog skin), has a unique 6-membered lactone ring at C-17, and also lacks the sugar at C-3. Digoxin, chosen primarily for its importance in clinical medicine, shares many similarities with ouabain but differs in the type of carbohydrate at C-3, and the number and position of hydroxyl groups attached to the steroid nucleus, thereby making it the most hydrophobic of the group.

Measurement of Voltage, Current, and Fractional Shortening in Single Ventricular Myocytes. Several drops of cell suspension were placed in an experimental chamber (0.5 ml volume) mounted on the stage of an inverted microscope (Nikon Diaphot; Nikon, Tokyo, Japan). After allowing 10 min for the cells to adhere to the chamber bottom, they were superfused (2–3 ml/min) with Tyrode's solution maintained at $36 \pm 1^\circ\text{C}$ using a Peltier device.

For experiments that included measurement of fractional shortening, the video image of the long axis of the cell was aligned with the rasters of the video edge detector (Crescent Electronics, Salt Lake City, UT), which continuously monitored cell length along a single line. Fractional shortening was defined as the ratio of cell shortening to resting cell length. Intracellular access was then obtained using either high-resistance microelectrodes or whole-cell patch pipettes.

High-Resistance Microelectrode Method. A microelectrode (15–30 M Ω resistance) was pressed gently against the cell surface forming a relatively low-resistance seal. Access was then obtained by briefly turning up the capacitance compensation, causing the amplifier circuitry to ring. Immediately following access, negative holding current (~1 nA) was applied to maintain a resting membrane potential (RMP) of -70 to -80 mV. A high-resistance seal spontaneously formed within a few minutes, and RMP became more negative, indicating successful impalement. Background holding current was then turned off. The primary advantage of

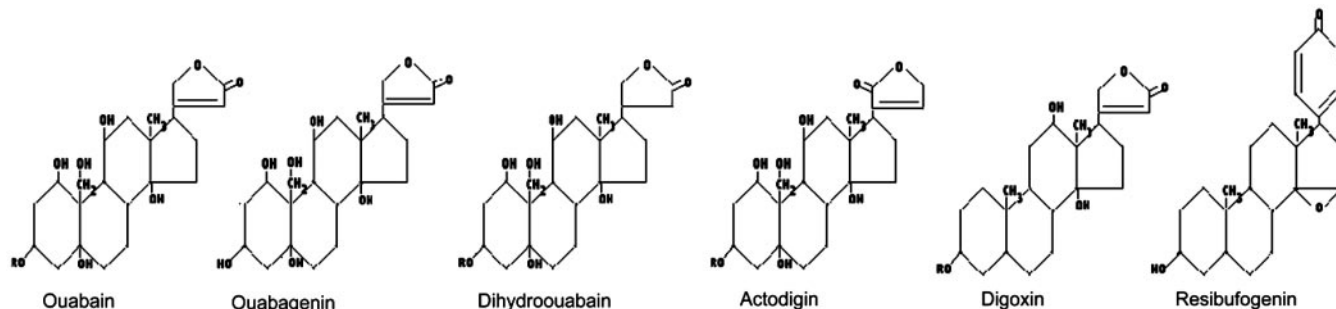


Fig. 1. Molecular structure of the six cardiac glycosides used in the study.

this technique is that it allows electrical access of the cell without appreciable diffusion between the cytoplasm and the electrode solution. The result is greater stability in measurement of I_{Ca} and contractility.

Whole-Cell Suction Pipette Method. A 2 to 3 M Ω pipette was pressed gently against the cell surface, and suction was applied (50–100 cm of H₂O), allowing formation of a gigaohm seal. Application of a brief suction pulse then ruptured the membrane patch giving electrical and physical access to the cell interior. Advantages of this technique include low-resistance access, and the ability to control the ionic composition of the cytoplasm (diffusion through a relatively wide-bore pipette). Disadvantages include I_{Ca} “run-down” that interferes with stable measurement of I_{Ca} and contractility.

After obtaining access to the cell interior, voltage- and current-clamp protocols were directed by pCLAMP6 software (Axon Instruments Inc., Union City, CA). For voltage-clamp experiments, discontinuous single-electrode voltage-clamp mode of the Axoclamp-2A was used, which allowed simultaneous measurement of both current and actual (not command) membrane potential. In addition, series resistance compensation is unnecessary because current passage does not occur during voltage measurement since each task is performed during alternate phases of every duty cycle. Voltage, current, and cell-length signals were digitized by a TL-1 DMA interface (Axon Instruments Inc.) at 7 to 10 kHz and channeled into the Clampex data acquisition program, for later analysis using the Clampfit program (pCLAMP6). Additional details of the use of the switch clamp with high-resistance electrodes can be found in previous publications (see Salata and Wasserstrom, 1988; Wasserstrom and Salata, 1988).

Statistical Analysis. Concentration-response curves were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test if criteria for significance were met. Data sets that did not fit parametric criteria were analyzed using nonparametric rank analysis, followed by Dunn’s test where appropriate. All means represent the results of five to eleven experiments, unless stated otherwise. Data were considered significant when $p < 0.05$.

Results

Effect of Cardiac Glycosides on Contractility and Action Potential Characteristics in Cat Ventricular Myocytes. Using high-resistance microelectrodes, single cat ventricular myocytes were paced at 1 Hz while APs and contractility (unloaded cell shortening) were recorded simultaneously. Figure 2 shows data from three separate experiments, demonstrating the effects of 3 μ M ouabain (left), 10 μ M DHO (middle), and 10 μ M ouabagenin (right). The high inotropic concentrations of all three glycosides presented here produce maximal contractile responses. Spontaneous contractions and delayed after-depolarizations indicative of cardiotoxicity were observed immediately after these recordings were made. Note that despite similar inotropic responses elicited by ouabain and DHO, only ouabain shortens the AP before the onset of spontaneous contractions. Shortening of the AP observed with ouabain and acotidigin was statistically significant for both AP duration at 50% (AP_{50}) and 90% (AP_{90}) of repolarization, as shown in Fig. 3. Ouabagenin, resibufogenin, and digoxin had no significant effect on AP duration. DHO was unique in that it induced slight AP prolongation at concentrations approaching toxicity.

Concentration-effect curves for inotropy for the six cardiac glycoside analogs are illustrated in Fig. 4. Calculated values for effective concentration at 50% of maximal effect (EC_{50}) are shown in the tables below. The only significant difference in maximal inotropic response lies between ouabain and ouabagenin. Comparison of maximal inotropy (before the onset of toxicity) indicates that ouabain produces a significantly greater increase in cell shortening than its aglycone ouabagenin (2.16 ± 0.14 versus 1.43 ± 0.05 , respectively; $n = 11$ and 7, respectively, $p < 0.05$).

Additional electrophysiology data are presented in Table 1. Control AP parameters (Table 1) did not differ between experimental groups. In general, 1 to 3 mV of RMP depolariza-

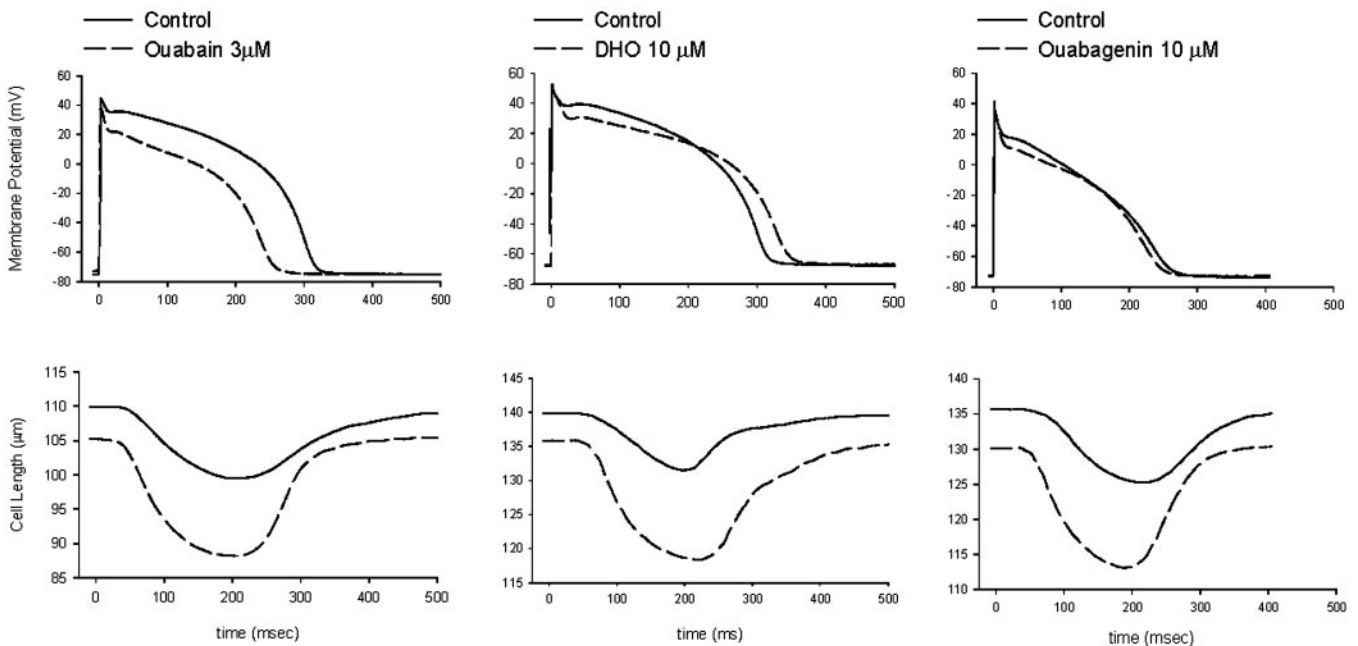


Fig. 2. Differential effects of ouabain and DHO on cat ventricular myocytes: action potentials and contractions. High inotropic concentration of ouabain (3 μ M; top left), but not DHO (10 μ M; top right), elicits action potential shortening. Effect of cardiac glycoside is indicated by the broken line trace in each figure. Both ouabain (left) and DHO (middle) are capable of producing equivalent inotropic responses, as indicated by comparable increases in active cell shortening upon addition of the agents. In contrast, maximal inotropic concentration of ouabagenin has less inotropic effect than either ouabain or DHO (right). Similar to DHO, ouabagenin has minimal effect on APD.

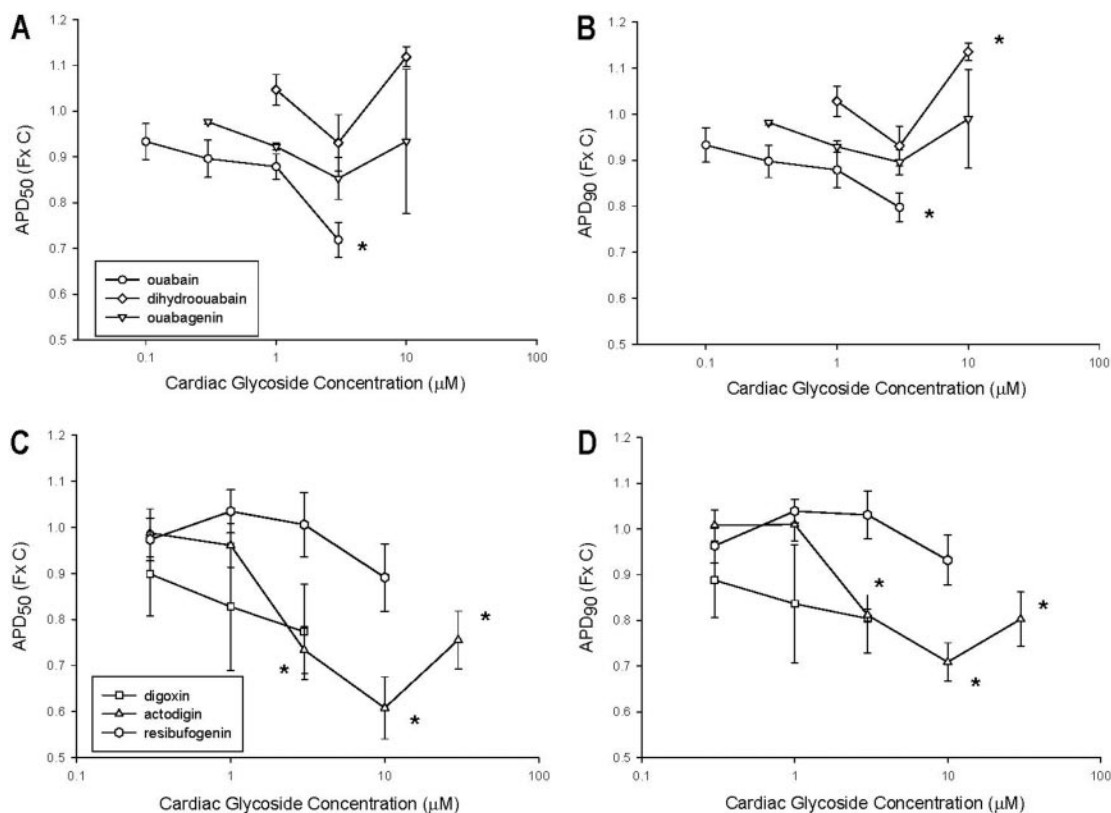


Fig. 3. Concentration-APD response curves for six cardiac glycosides. Effect of increasing cardiac glycoside concentration on APD₅₀ (panels A and C) and APD₉₀ (panels B and D) is shown. Ouabain, DHO, and ouabagenin concentration-response curves are presented in the upper panels (A and B). Digoxin, actodigin, and resibufogenin are presented in lower panels (C and D). APD values are given as a fraction of control values (error bars = S.E.M.). As noted, ouabain and actodigin elicit action potential shortening, whereas DHO elicits a small but significant prolongation of the AP at high inotropic concentrations. *, $p < 0.05$ indicates significant difference from control.

tion and a 5 to 10 mV decrease in AP amplitude was observed at maximal cardiac glycoside concentrations, with no significant differences noted between agents.

Effect of Ouabain on I_{Ca} . Finding significant differences between cardiac glycosides on AP shortening prompted an investigation into membrane currents that might explain these observed differences. Voltage-clamp studies were conducted to evaluate effects of ouabain on I_{Ca} as a possible mechanism for AP shortening using high-resistance microelectrodes. As demonstrated in Fig. 5A, exposure to 3 μ M ouabain has little effect on the magnitude of I_{Ca} , although in this example the rate of inactivation appears to be increased. With longer exposure times (10 min), there is a small decrease in I_{Ca} ; however, this decrease was not statistically significant (Fig. 5B). DHO shows a similar effect on I_{Ca} (Fig. 5C), with a small time-dependent decrease in magnitude. Thus the difference in action potential changes induced by these agents cannot be explained on the basis of alterations in I_{Ca} . Further experiments evaluating the stability of I_{Ca} measurements under control conditions (without cardiac glycoside) showed no time-dependent diminution of I_{Ca} (Fig. 5D) demonstrating that the slight decrease in current magnitude is in fact a result of actions of both drugs.

Effect of Ouabain on I_{K1} . Further voltage-clamp studies were conducted to evaluate effects of ouabain on the inward rectifier current (I_{K1}) as a possible mechanism for action potential shortening. Figure 6A shows representative I_{K1} data obtained with high-resistance electrodes. The voltage-clamp protocol is presented in the top trace. From a holding

potential of -40 mV, hyperpolarizing and depolarizing voltage steps were used to assess both the inward and outward I_{K1} currents, measured at the end of the test voltage step. Although I_{Ca} was not blocked in these experiments, values for I_{K1} at voltages positive to -40 mV were assumed to be fairly accurate at the end of the voltage-clamp steps after I_{Ca} was mostly inactivated (300 ms). Current recordings at hyperpolarizing potentials show large inward currents; depolarizing pulses yielded progressively diminishing outward currents typical of cat ventricular I_{K1} . Exposure to 3 μ M ouabain caused no change in inward currents and a slowly developing outward current during depolarization. This outward current component increased with exposure time and was investigated further using a ramp protocol, discussed below. Composite voltage-current data (Fig. 6B) again shows no change in steady-state I_{K1} over the voltage range of -140 to 30 mV, with a small increase in outward current at voltage steps above 40 mV, which did not achieve statistical significance.

Effect of Ouabain and DHO on the Steady-State Outward Current (I_{ss}). A ramp protocol was used to evaluate the late outward current component noted above using high-resistance electrodes. The voltage protocol and representative data are presented in Fig. 7. Cd^{2+} (0.1 mM) was added to block I_{Ca} . As illustrated in Fig. 7A, 10 min of exposure to 3 μ M ouabain induced a pronounced increase in outward current (previously termed "outward steady-state current", I_{ss} ; Levi, 1993) that is observed at voltages above -20 mV. However, pretreatment of cells with 10 μ M KB-R7943, a blocker

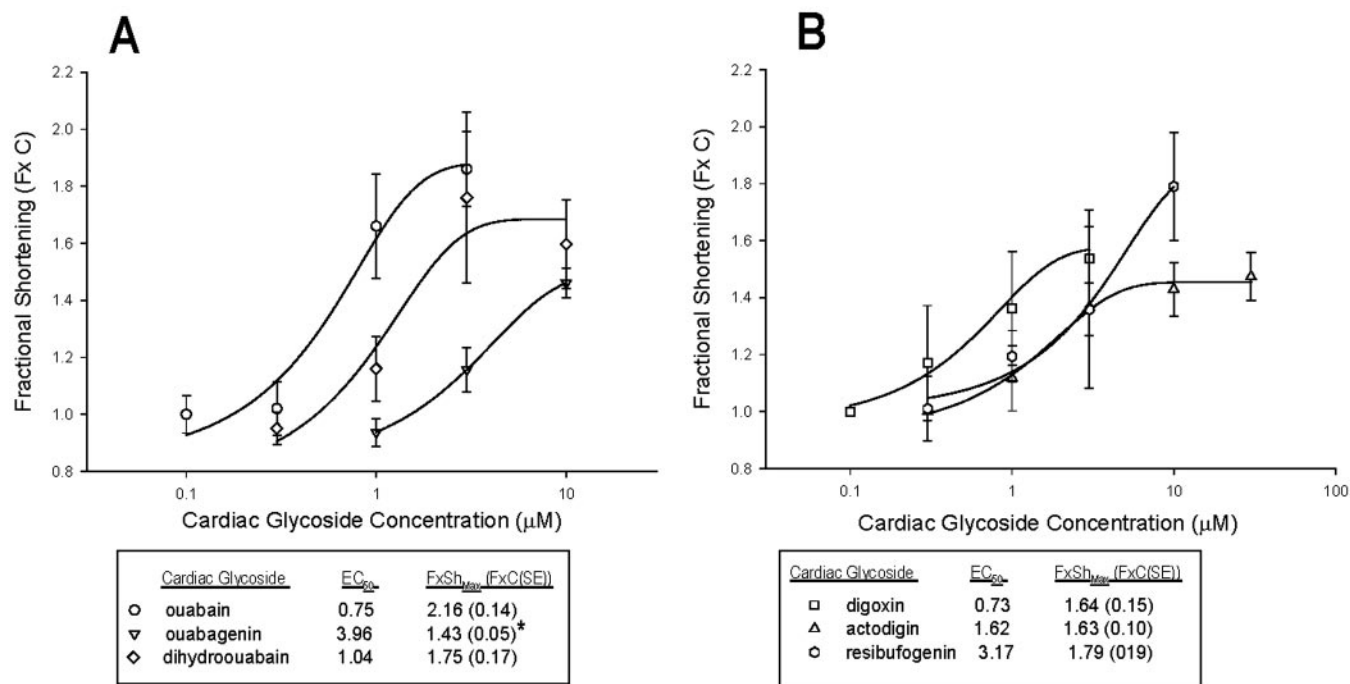


Fig. 4. Concentration-inotropic response curves for six cardiac glycosides. Inotropic response to increasing cardiac glycoside concentrations, expressed as a fraction of control shortening are presented for ouabain, ouabagenin, and DHO in panel A. Digoxin, actodigin, and resibufogenin are presented in panel B. The majority of preparations became toxic at the highest cardiac glycoside concentration indicated in each curve. Tables below each graph denote EC₅₀ values and maximal inotropic response (FxSh_{Max}; expressed as a fraction of control FxSh) for each agent. EC₅₀ values were derived from a sigmoidal fit of each concentration-response curve. In contrast, FxSh_{Max} values were calculated from the maximal inotropic response of each preparation (independent of the drug concentration) where FxSh_{Max} was observed, and only from preparations that subsequently demonstrated toxicity (spontaneous contractions and/or decreased developed force) thereby ensuring that the maximum inotropic response had been reached. For this reason, the FxSh_{Max} values presented in the table closely resemble the apparent maximum of the curves but do not correlate exactly. Of note, ouabagenin produces significantly less maximal positive inotropic response than ouabain ($p < 0.05$).

TABLE 1

Control action potential parameters of the experimental groups

All values are presented as mean (S.E.M.). No statistical differences between groups for the parameters shown.

Cardiac Glycoside	Control RMP	Control APD ₅₀	Control APD ₉₀	Control AP Amplitude
Ouabain	-72.3 (1.4)	254 (12)	312 (13)	114 (2.3)
Dihydroouabain	-71.4 (1.5)	229 (16)	288 (13)	116 (4.0)
Ouabagenin	-72.8 (2.1)	269 (23)	311 (25)	120 (6.2)
Actodigin	-74.7 (1.0)	272 (19)	320 (19)	116 (2.7)
Digoxin	-72.3 (2.3)	245 (29)	307 (28)	109 (8.6)
Resibufogenin	-73.0 (2.7)	273 (24)	343 (21)	111 (3.6)

of reverse mode Na⁺-Ca²⁺ exchange (NCX), markedly attenuated the increase in this current (Fig. 7B), suggesting that the observed current is the result of increased outward (reverse mode) NCX. Composite data show that this cardiac glycoside-sensitive current (Fig. 8; represented as a difference current between control and cardiac glycoside treated conditions) is increased during exposure to maximal inotropic concentrations of both ouabain and DHO but is suppressed by pretreatment with 10 μM KB-R7943. The fact that there is an apparent voltage dependence to this outward current, which increases with test potential, is also consistent with the notion that the electrogenic NCX current may be involved. The finding that both ouabain and DHO are capable of increasing outward NCX current, however, does not explain the divergent effects of ouabain and DHO on the AP.

Effect of Ouabain and DHO on APD with Increased Intracellular Ca²⁺ Buffering. Consideration of the possibility that the observed differences between ouabain and

DHO on APD were secondary to changes in intracellular Ca²⁺ prompted a series of experiments that minimized the cardiac glycoside-induced rise in internal Ca²⁺. First, a series of experiments was performed with patch electrodes using an internal [Na⁺] of 10 mM and near physiological Ca²⁺ buffering (0.056 mM EGTA, measured [Ca²⁺]_i = 82 nM; Vites and Wasserstrom, 1996). Under these conditions (which permitted dialysis and equilibration of the intracellular space with the patch pipette internal solution), APD was observed to shorten upon exposure to 3 μM ouabain (Fig. 9A), but not 10 μM DHO (Fig. 9B), just as we had observed previously using high-resistance electrodes (Fig. 2). The experiment was then repeated with markedly increased internal Ca²⁺ buffering (1 mM EGTA). As shown in Fig. 9, C and D, separate cells were allowed to equilibrate for 20 min with internal solution containing the high EGTA concentration before addition of the cardiac glycoside. During this equilibration time the APD shortened and then stabilized. Addition of a maximal inotropic concentration of both ouabain

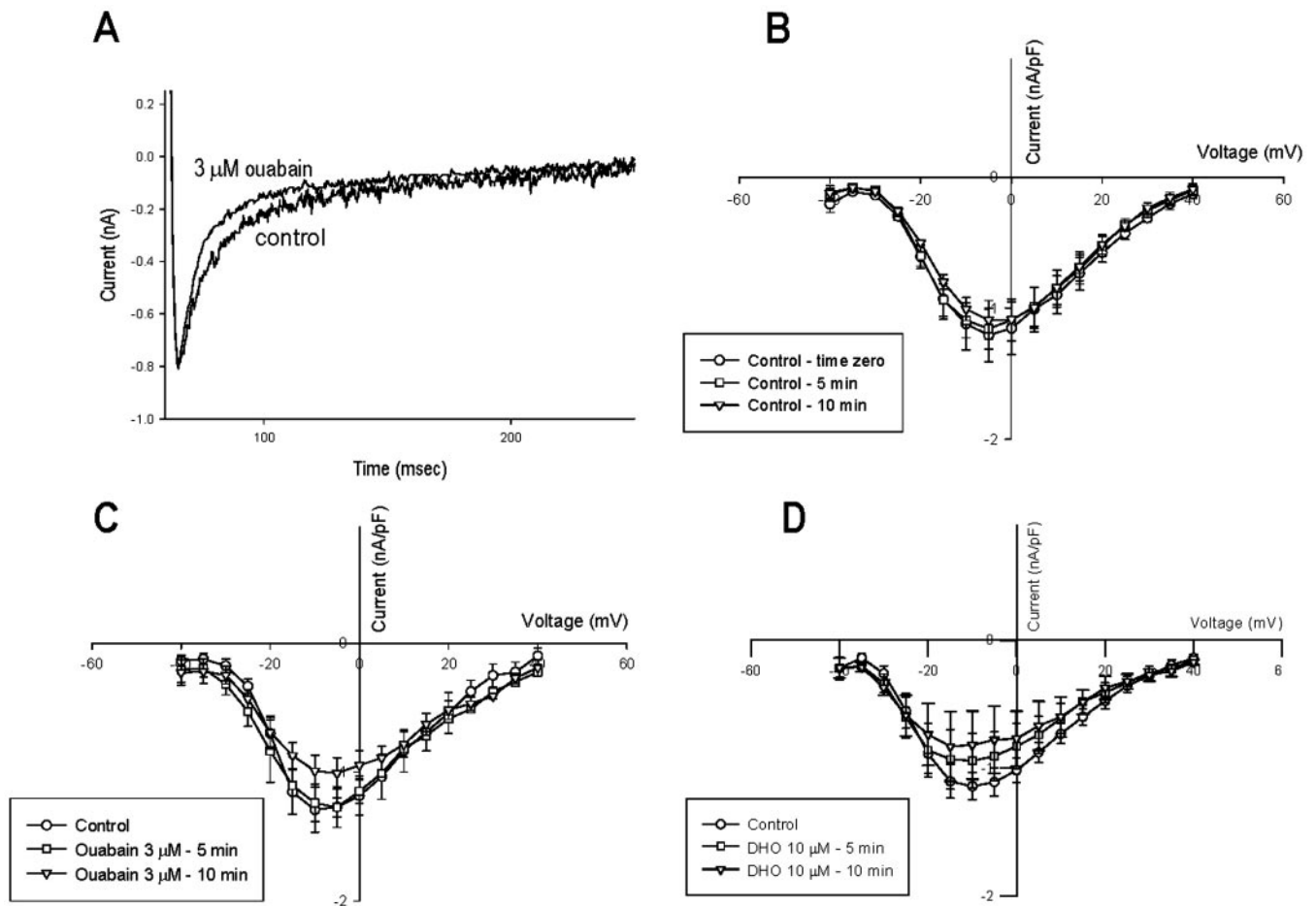


Fig. 5. Effect of ouabain on calcium current (I_{Ca}). Panel A, representative current traces measured with high-resistance microelectrodes are presented. I_{Ca} elicited by voltage steps from -40 to 0 mV is shown for control condition and following a 5-min exposure to $3 \mu\text{M}$ ouabain. As noted, there is no effect of the cardiac glycoside on current magnitude. Panel C, $3 \mu\text{M}$ ouabain has minimal effect on I_{Ca} for the first 5 min of exposure, following which there is a small decrease in maximal current amplitude ($p > 0.05$, NS). Panel D, similarly, exposure to $10 \mu\text{M}$ DHO results in a small, time-dependent decrease in I_{Ca} , which fails to reach statistical significance. Panel B, myocytes not exposed to cardiac glycosides maintain control current levels 10 min or longer without decrement, indicating that high-resistance electrode measurement of I_{Ca} is stable and relatively free of run-down.

(Fig. 9C) and DHO (Fig. 9D) was now able to cause further shortening of the AP. Under these conditions, the decreases in AP_{50} and AP_{90} observed upon exposure to both $3 \mu\text{M}$ ouabain or $10 \mu\text{M}$ DHO were statistically significant, as indicated in Fig. 9E.

Inotropic Action of Ouabain, DHO, and Ouabagenin under AP Clamp. To further characterize the observed diminished inotropic action of ouabagenin compared with ouabain under conditions independent of changes in resting and action potentials, myocytes were voltage-clamped using a protocol that approximated normal RMP (-78 mV) and AP configuration. High-resistance microelectrodes were used to maintain contractility during the experiment. Under these conditions, ouabain, ouabagenin, and DHO did not differ from each other significantly in the extent of their maximal inotropic action; all three cardiac glycosides were able to achieve similar inotropic effects; ouabain increased cell shortening from $4.1 \pm 0.77\%$ to $10.4 \pm 0.76\%$ of resting cell length ($n = 9$); ouabagenin increased shortening from $4.9 \pm 0.87\%$ to $11.4 \pm 0.27\%$ ($n = 5$); DHO increased shortening from $4.7 \pm 0.27\%$ to $12.1 \pm 0.61\%$ ($n = 4$). Unlike the results obtained with the free-running action potential stimulation of contraction, there were no significant differences in the magnitude of the positive inotropic effects between these

three agents. In addition, the magnitude of the current during the plateau phase of the AP clamp increased with exposure to all three of the cardiac glycosides but did not differ significantly between agents (data not shown) as would be expected for a general increase in NCX current. This result suggests that inotropic differences between agents, particularly ouabagenin and ouabain, are minimized under conditions that control resting and excitation potentials.

Discussion

Our investigation of the actions of six cardiac glycoside analogs on action potential configuration and contractility in single cat ventricular myocytes revealed the following. 1) Of all agents tested, only ouabain and actodigin induce AP shortening at high inotropic concentrations. 2) Ouabain-induced AP shortening is associated temporally with the appearance of I_{SS} (reverse mode NCX current, blocked by KB-R7943). 3) DHO is capable of producing AP shortening only if Ca^{2+} overload is prevented or delayed by increased Ca^{2+}_i buffering, suggesting that cardiac glycoside-induced cardiotoxicity is mediated by more than one mechanism—sodium pump inhibition with resulting reverse mode NCX and a second mechanism separate from sodium pump inhibition. 4)

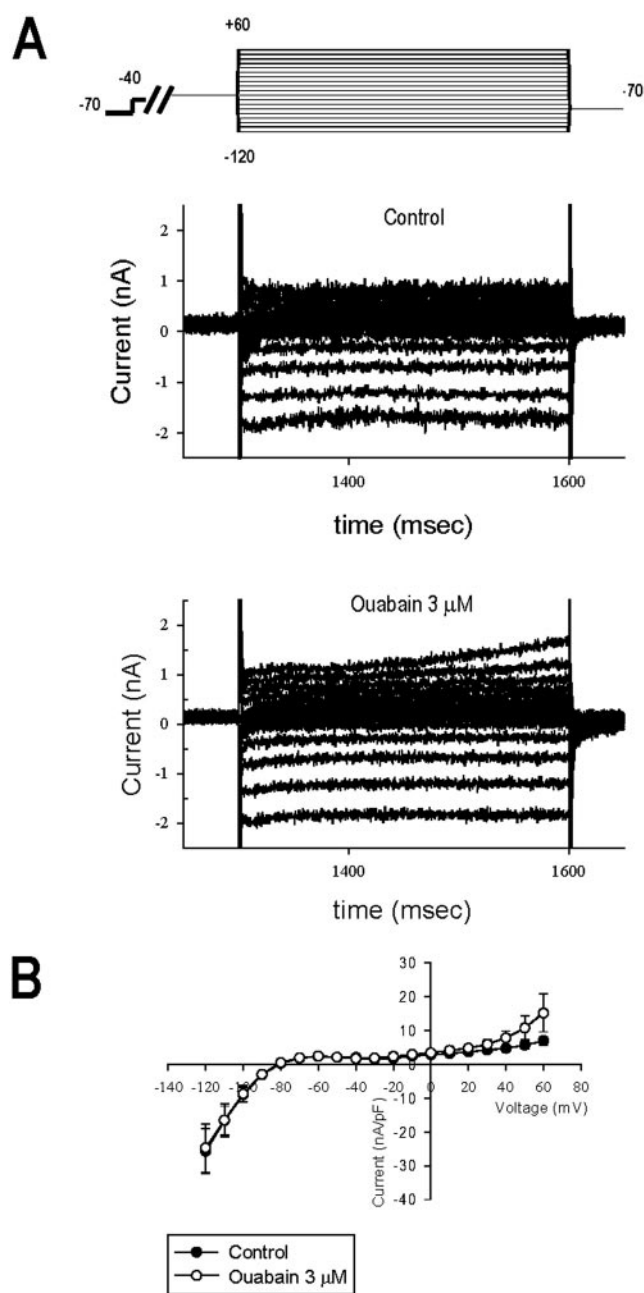


Fig. 6. Effect of ouabain and DHO on I_{K1} . A, raw current traces showing I_{K1} in control condition and following the addition of $3 \mu\text{M}$ ouabain. The increase in outward current noted at positive voltage steps develops over 5 to 10 min after addition of the cardiac glycoside. Inset shows the voltage-clamp protocol used for I_{K1} experiments. B, I_{K1} data averaged from several experiments showing the effect of $3 \mu\text{M}$ ouabain (error bars = S.E.M.). The trend toward increased outward current at positive voltages following addition of ouabain was not statistically significant.

Ouabagenin and certain other cardiac glycosides produce less maximal contractile response before the onset of Ca^{2+} overload toxicity than ouabain but only when the native action potentials trigger excitation-contraction coupling. 5) The apparent difference in inotropic action between cardiac glycosides is significantly reduced under AP voltage clamp, suggesting that a putative second mechanism underlying cardiac glycoside-induced cardiotoxicity is at least partially voltage-dependent.

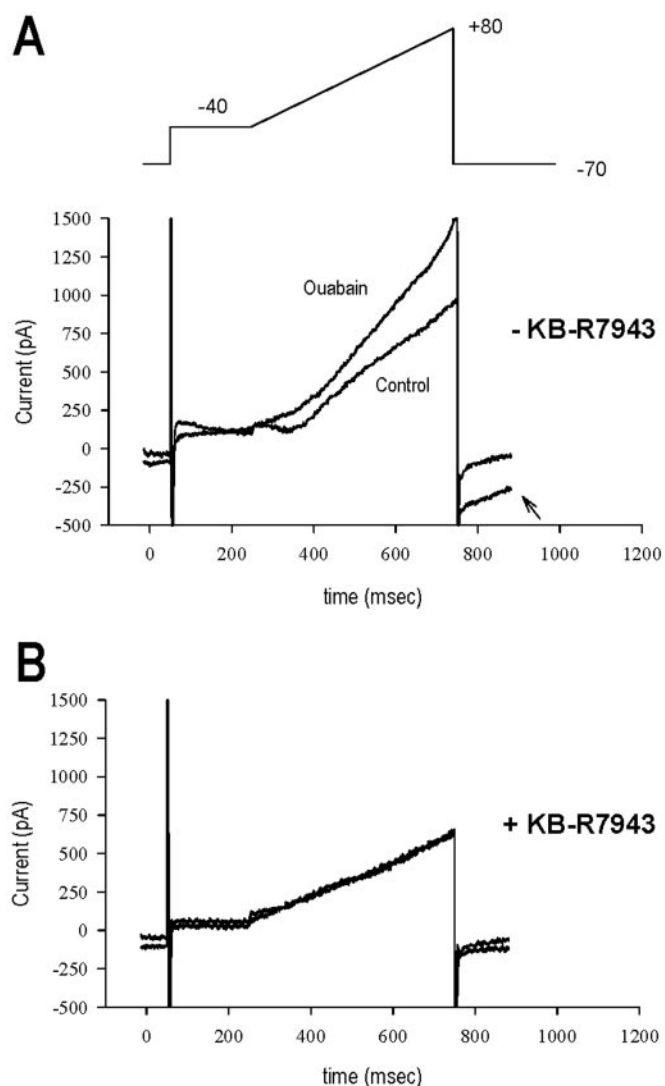


Fig. 7. Ramp protocol experiments: steady-state outward current. Representative current traces elicited by a voltage-clamp ramp protocol (inset). As indicated, the protocol consisted of a step to -40 mV for 200 ms, then a ramp up to $+80 \text{ mV}$ over 500 ms before returning to the holding potential of -70 mV . Cd^{2+} and Ba^{2+} were added to the external solution to diminish I_{Ca} and K^+ currents. Panel A, 10-min exposure to $3 \mu\text{M}$ ouabain produced a dramatic increase in steady-state outward current. An increase in inward current upon return to the holding potential was also observed following exposure to ouabain (noted by arrow). Panel B, pretreatment with KB-R7943 effectively eliminated the development of this cardiac glycoside-sensitive steady-state current.

Cardiac Glycoside Effects on Action Potentials, Inotropy, and Toxicity. Differences in activity among cardiac glycosides have been described both in intact animals and isolated cardiac preparations. However, attempts to explain these differences in action based on specific features of cardiac glycoside molecular structure have produced limited results. Part of this limitation stems from the fact that the majority of cardiac glycoside structure-activity studies are focused on Na^+/K^+ -ATPase binding (De Pover and Godfraind, 1982; Brown and Erdmann, 1984), and few address physiological actions in intact systems. Notable exceptions include studies in guinea pig atria showing stimulation of the sodium pump at low concentrations of ouabain and ouabagenin, but not DHO (Ghysel-Burton and Godfraind, 1979), and the demonstration of differences in toxic to therapeutic ratios

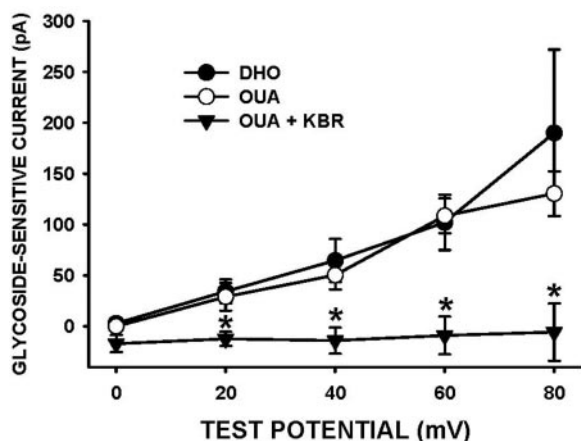


Fig. 8. Effect of DHO and ouabain on cardiac glycoside-sensitive steady-state outward current. Averaged data from ramp protocol experiments is presented. Glycoside-sensitive current is defined as a subtraction (experimental minus control) current. The data summarize the effect of ouabain (3 μ M, empty circles) and DHO (10 μ M, filled circles) on cardiac glycoside-sensitive current; 10 min of exposure demonstrates the time-dependent development of the current. Pretreatment with 10 μ M KB-R7943 eliminates the effect of ouabain on development of outward steady-state current (triangles) producing significantly less current at all test voltages (*, $p < 0.05$ compared with ouabain alone).

of cardiac glycosides in intact dogs, dependent on the position of the attachment of the lactone ring (Mendez et al., 1974). However, none of these studies (individually or collectively) provides a comprehensive foundation for understanding cardiac glycoside structure-activity relationships, and it is likely that multiple factors in structure determine the specific response elicited by a given agent. Information provided by our investigation indicates that subtle differences in structure (including a simple saturation of the lactone ring) cause significant differences in physiological activity. If intracellular sites are indeed important in determining differences in action between cardiac glycoside agents (Fujino and Fujino, 1982; Isenberg, 1984; Sagawa et al., 2002), including the threshold for Ca^{2+} overload, one could speculate that the relative ability of a given glycoside to cross the sarcolemma (either passively or actively; Nunez-Duran et al., 1988) and affinity for specific intracellular sites would be important additional determinants of action. In addition, if the ryanodine receptor is an important determinant of this intracellular mechanism as postulated by some investigators (Isenberg, 1984; Rardon and Wasserstrom, 1990; McGarry and Williams, 1993; Sagawa et al., 2002), then differences in binding affinity to this receptor between cardiac glycosides might contribute to the observed differences in action.

Mechanisms Underlying Shortening of APD. Many studies evaluating the effects of cardiac glycosides on isolated myocardial preparations or single cells have shown a shortening of APD (McDonald et al., 1975; Levi, 1993). Explanations for this finding are reflected in contrasting theories, which attempt to characterize a cardiac glycoside-induced outward current. The first theory (Luk and Carmeliet, 1990) invokes an outward current that is increased by 10 to 100 μ M ouabain in guinea pig cardiac myocytes with similar properties to a single channel current found in inside-out patches. This current is proposed to be a Na^+ -activated K^+ current by virtue of the fact that it is dependent on the presence of Na^+ , displays rectification that is dependent on the K^+ gradient,

and has a reversal potential near the K^+ equilibrium potential. The second theory (Levi, 1993) suggests the involvement of an outward current elicited by 50 μ M strophanthidin also in guinea pig cardiac cells with reversal potential of -54 mV. This current was shown to be consistent with reverse mode NCX by being more pronounced at positive voltages, abolished by removing external Ca^{2+} or addition of Ni^{2+} , and unaffected by increasing intracellular Ca^{2+} buffering (BAPTA) or addition of K^+ current blockers (Ba^{2+} , TEA, and 4-AP). Data from our experiments indicate the cardiac glycoside-induced outward current is most likely due to increased reverse mode NCX because it does not develop in the presence of KB-R7943, a reverse mode NCX blocker (Kimura et al., 1999). In addition, AP shortening appears to be an action of some but not all cardiac glycosides. This could be a result of other drug actions, including the possibility that certain glycosides might also block I_{Ks} , the slowly activating component of the delayed rectifier current (Rocchetti et al., 2003), which could explain why DHO (and not ouabain or actodigin) caused AP prolongation. The balance of opposing direct and indirect actions on net membrane current could then be responsible for the variety of glycoside effects on AP duration, especially in different species with varying dependencies of repolarization on I_{Ks} .

Role of Ca^{2+} Overload in Development of APD Shortening and Inotropy. Data from this study suggest that Ca^{2+} overload is a primary determinant both for maximal inotropic response and alterations in AP configuration and could account for differences in response between cardiac glycoside analogs. This is not a new concept, particularly with regard to inotropic action as addressed by other investigators (Capogrossi et al., 1988). The basic theory, supported by experimental data, contends that the myocardium is capable of increasing inotropy by Ca^{2+} -dependent mechanisms until toxicity develops as indicated by spontaneous sarcoplasmic reticulum Ca^{2+} release and spontaneous contractions. This spontaneous, uncoordinated release of Ca^{2+} depletes the sarcoplasmic reticulum of Ca^{2+} for the next contraction, thereby reducing the inotropic state as well as any contributions of Ca^{2+} -dependent conductances (including NCX current) to AP duration.

We found that Ca^{2+} overload in fact does influence whether or not cardiac glycosides induced AP abbreviation. Although direct evidence for sodium pump inhibition was not evaluated, the presence of a robust increase in reverse mode NCX current within the inotropic range of both ouabain and DHO would suggest that sodium pump inhibition is an important mechanism in the inotropic response of cardiac glycosides in vitro. In addition, the data suggest that a second cardiac glycoside mechanism (independent of sodium pump inhibition) influences the threshold for Ca^{2+} overload and thereby determines the maximal inotropic response observed. For example, the weaker inotropic response and lack of AP shortening observed with ouabagenin compared with ouabain is likely the result of earlier spontaneous SR Ca^{2+} release (lower toxicity threshold) elicited by ouabagenin prior to accumulation of equal levels of intracellular calcium.

How voltage alters spontaneous release from the SR and thereby alters the maximal inotropic response of ouabagenin (as demonstrated in the AP clamp experiments) can only be speculated. The most likely reason is that the positive feedback between SR Ca^{2+} release and membrane depolarization

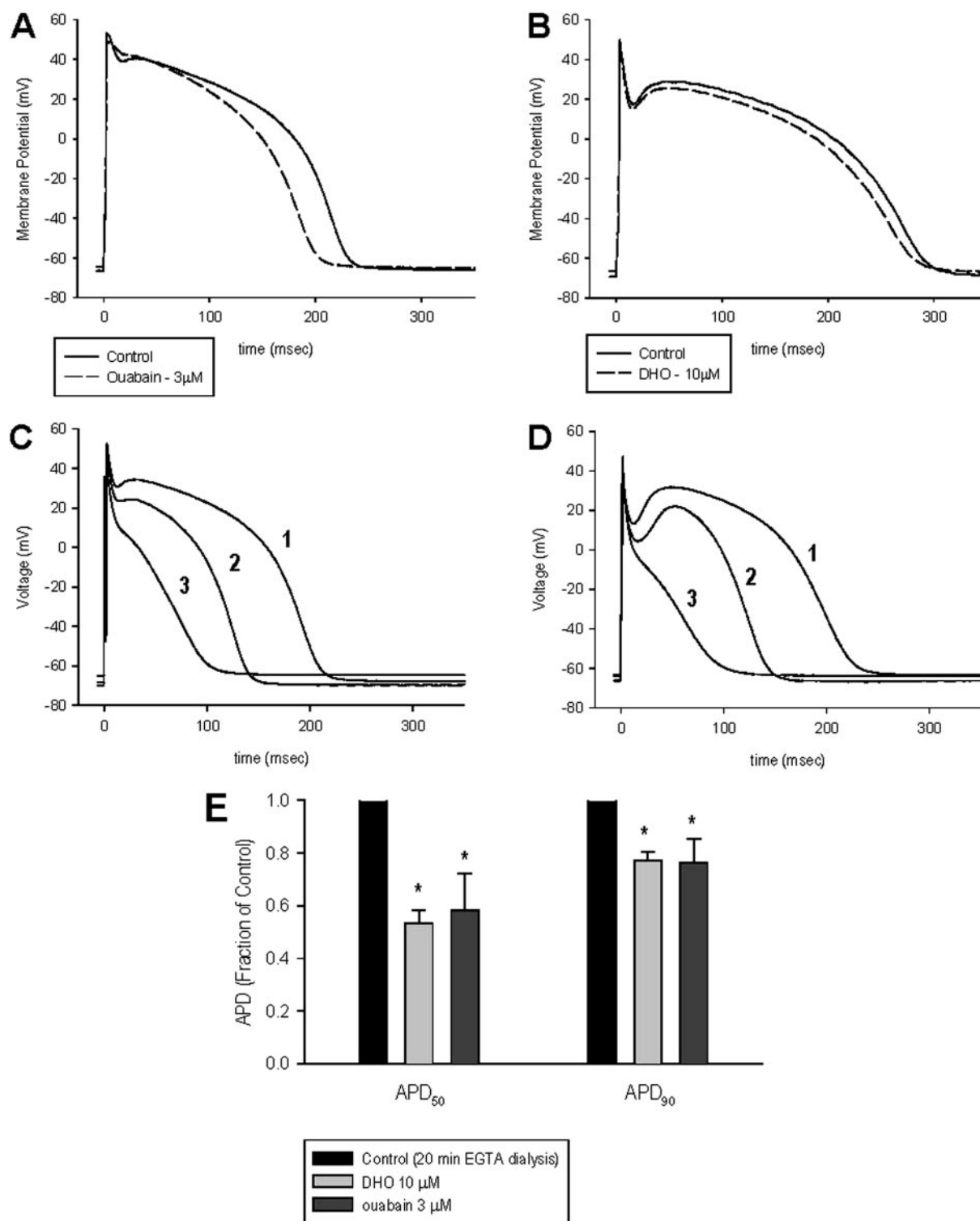


Fig. 9. Effect of ouabain and DHO on action potential configuration in myocytes dialyzed with 1 mM EGTA. Panels A and B, effects of ouabain and DHO on action potentials measured in cat myocytes using patch electrodes. Panel C, equilibration of the myocyte with pipette solution containing increased Ca^{2+} buffer results in dramatic shortening of the AP (from 1 to 2). Addition of 3 μM ouabain results in further shortening of the AP over a period of 5 to 7 min (from 2 to 3). Panel D, a similar experiment using 10 μM DHO produces almost identical AP shortening; under conditions preventing Ca^{2+} overload, both ouabain and DHO are able to induce AP shortening. Panel E, averaged data from several experiments demonstrating that both 3 μM ouabain and 10 μM DHO produce AP shortening (indicated by a decrease in both APD_{50} and APD_{90}) under conditions of increased intracellular Ca^{2+} buffering. *, $p < 0.05$ between experimental and control values. Control APD values were obtained after 20 min of EGTA dialysis.

is prevented under voltage clamp. Thus, differences between drugs displaying maximal inotropic effects are likely to be blunted simply because steady-state drug effects are more

closely approximated. However, it is also possible that this observation may be related to the idea linking membrane potential with SR release (Ferrier and Howlett, 1995). Be-

cause the threshold for spontaneous calcium release from the SR during diastole is a primary determinant of cardiac glycoside inotropy, it may be that voltage clamp prevents or delays diastolic calcium release. This implies that small voltage perturbations during diastole may contribute to spontaneous release or that spontaneous release during diastole is at least partially dependent on membrane potential.

Effects of Low Internal Free Mg^{2+} Concentration on Experimental Results. It is possible that certain of our experimental conditions might influence the results found in this study. One such issue is the low free $[Mg^{2+}]$ concentration in the internal solution (about 10^{-5} M). This is likely to have important effects on Mg^{2+} -dependent process as in the cell. However, it should be noted that even with this low $[Mg^{2+}]$, there are still pronounced differences between different cardiac glycosides just as expected from data obtained using high-resistance microelectrodes in which the intracellular environment is closer to physiological. In addition, the rectifying characteristics of I_{K1} are largely unaffected by the buffering of internal Mg^{2+} (data not shown), suggesting that effective concentrations in critical regions of the cytoplasm remain at normal regulatory levels despite calculated changes in bulk concentration. This fact suggests that it is difficult to extrapolate bulk calculated $[Mg^{2+}]$ to true free concentration at regulatory sites.

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