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Effects of doxorubicinol on excitation–contraction coupling in guinea pig ventricular myocytes

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Abstract

The cardiotoxicity of the anticancer drug doxorubicin may be related to its main metabolite doxorubicinol. In this study, the acute effects of doxorubicinol on excitation–contraction coupling in isolated guinea pig ventricular myocytes were investigated and compared with doxorubicin using the whole-cell patch-clamp-, fura-2 fluorescence- and cell-edge tracking techniques. Both drugs were applied intracellularly by diffusion from the patch electrode for 15–20 min. Doxorubicin (100 μM) prolonged the action potential duration (APD) by 31% and enhanced cell shortening by 26%. Contrary to doxorubicin, doxorubicinol (10 μM) shortened APD by 25% and decreased cell shortening by 31%. APD shortening by doxorubicinol was due to an increase of the delayed rectifier K^+ current. Neither the inward rectifier K^+ current nor the L-type Ca^{2+} current was influenced by doxorubicinol. The decline in cell shortening induced by doxorubicinol was not exclusively due to APD shortening because doxorubicinol reduced the peak Ca^{2+} transient by 23% in cells clamped with an action potential of constant duration. Despite opposite effects on APD and contractility, both doxorubicin and doxorubicinol produced a considerable delay in the activation and inactivation of contraction and Ca^{2+} transient, compatible with an impaired function of the sarcoplasmic reticulum. It is suggested that doxorubicinol-induced APD shortening may amplify the detrimental effects of both doxorubicin and doxorubicinol on sarcoplasmic reticulum Ca^{2+} load and hence on contractile function. The accumulation of doxorubicinol in the cardiac myocytes may play an important role in the time-dependent development of doxorubicin-induced ventricular dysfunction.

Keywords: Action potential duration; Cardiac myocyte; Doxorubicin; Doxorubicinol; Inotropic effect

1. Introduction

The anthracycline derivative doxorubicin is widely used to treat patients with neoplastic diseases. Cumulative dose-related cardiotoxicity, however, remains a significant and dose-limiting clinical problem. Acute manifestations of doxorubicin-induced cardiotoxicity are electrocardiographic abnormalities such as QT_c prolongation, QT_c dispersion and occasionally afterpotentials, followed later by a life-threatening cardiomyopathy (Ferrari et al., 1996; Sarubbi et al., 1997; Nousiainen et al., 1999; Singal and Iliskovic, 1998). Several lines of evidence indicate that an abnormal Ca^{2+} handling of myocardial cells may explain the cardiac dysfunction seen in doxorubicin-induced cardiomyopathy. Doxorubicin has been shown to inhibit the gene transcription of the sarcoplasmic reticulum Ca^{2+}-ATPase (Arai et al., 1998, 2000) and to activate cardiac Ca^{2+} release channels (ryanodine receptors), which are localized to the terminal cisternae of sarcoplasmic reticulum (Nagasaki and Fleischer, 1989; Holmberg and Williams, 1990). A decrease of sarcoplasmic reticulum Ca^{2+} load and hence Ca^{2+}-induced Ca^{2+} release has been observed with doxorubicin in isolated guinea pig ventricular myocytes (Wang and Korth, 1995). This effect was reminiscent of ryanodine, which at submicromolar concentrations locks the Ca^{2+} release channel of the sarcoplasmic reticulum in the open state, facilitating spontaneous sarcoplasmic reticulum Ca^{2+} release (Meissner, 1986; Rousseau et al., 1987). In the same study (Wang and Korth, 1995),

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doxorubicin was found to prolong action potential duration (APD) substantially, an effect which probably accounts for QT_c prolongation and afterpotentials in the clinical setting. The mechanism by which doxorubicin affects Ca^{2+} homeostasis of cardiac myocytes has not been fully defined but may involve an iron-catalyzed direct effect of doxorubicin (Boucek et al., 1993), doxorubicin-induced formation of reactive oxygen intermediates (Arai et al., 2000), and conversion of doxorubicin to a toxic metabolite (Olson and Mushlin, 1990). Doxorubicin has been shown to be slowly converted in cardiac cells to doxorubicinol, which is the major metabolite of doxorubicin (Olson et al., 1988; Forrest et al., 1991; Stewart et al., 1993). Doxorubicinol differs from the parent compound only in that the C-13 carbonyl group in the side chain is reduced to a secondary alcohol (Fig. 1). Doxorubicinol was found to be more potent than doxorubicin at compromising both systolic and diastolic cardiac function (Olson et al., 1988). Similarly, doxorubicinol was much more potent than doxorubicin at inhibiting the sarcoplasmic reticulum calcium pump (Olson et al., 1988; Boucek et al., 1987). In addition, doxorubicinol shares the property of doxorubicin to activate the sarcoplasmic reticulum Ca^{2+} release channel (Nagasaki and Fleischer, 1989). The effects of doxorubicinol on membrane electrical properties, however, are not known.

In the present study, we investigated the acute effects of doxorubicinol on isolated ventricular cardiomyocytes. The whole-cell clamp technique was used to dialyze the anthracyclines directly into the cytosol of the myocardial cell and to record cardiac action potentials and membrane currents. Furthermore, the effects of doxorubicinol on Ca^{2+} transients and the accompanying cell shortening were investigated in current-clamped and action-potential-clamped cells.

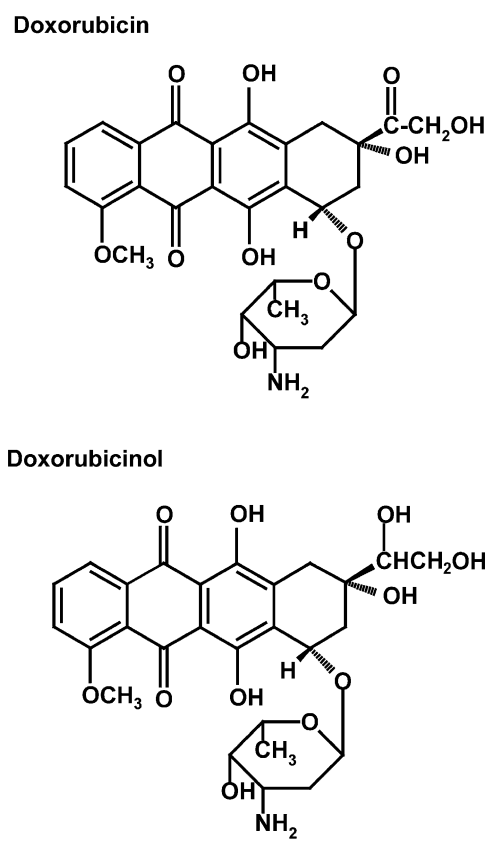

Fig. 1. Chemical structures of doxorubicin and doxorubicinol.

2. Materials and methods

2.1. Chemicals

Doxorubicinol hydrochloride (batch CZ 10826/31) and doxorubicin hydrochloride (batch 5018D47F) were kindly provided by Pharmacia & Upjohn (Erlangen, Germany) and were dissolved in distilled water to give 10- and 50-mM stock solutions, respectively. Appropriate portions of this stock solution were added to the electrode filling solution just before use to achieve final concentrations. Nisoldipine was kindly provided by Bayer AG (Elberfeld, Germany) and was dissolved in dimethyl sulfoxide to give a 10-mM stock solution. Fura-2 acetoxymethyl ester and ionomycin were obtained from Calbiochem (La Jolla, CA).

2.2. Single-cell isolation

Isolated myocytes were prepared from ventricles of adult guinea pigs by enzymatic dissociation according to Powell et al. (1980) with small modifications. Briefly, the heart was retrogradely perfused at 37 °C and at a constant rate of 10 ml/min with the following solutions: 5 min with a nominally Ca^{2+}-free Joklik solution (Joklik-MEM, Biochrom) supplemented with $NaHCO_3$, and then 5–10 min with the same solution to which had been added 50 μM $CaCl_2$, collagenase (Worthington type II, 25 mg/50 ml, Biochrom), protease (type XIV, 10 mg/50 ml, Sigma), and 0.1% bovine serum albumin (fraction V, Sigma). All solutions were gassed with 5% CO_2 in O_2; the pH was 7.4. After perfusion, the heart was minced and incubated for another 5 min in fresh enzyme solution. The cells were then disaggregated by gentle mechanical agitation. After filtration through a nylon mesh, the cells were centrifuged at $37 \times g$ for 3 min and then resuspended in Joklik solution containing 300 μM $CaCl_2$ and 1% bovine serum albumin and kept for use at room temperature under a continuous stream of 5% CO_2 in O_2.

2.3. Whole-cell patch-clamp

A drop of cell suspension was added to the Tyrode solution in the recording chamber (volume, 0.5 ml) mounted on an inverted microscope (Axiovert 10, Carl Zeiss, Germany). After the cells had attached to the bot-

tom, the bath was perfused at a flow rate of 4 ml/min with pre-warmed Tyrode solution continuously gassed with O_2. The temperature in the bath (34 to 35 °C) was continuously monitored. The Tyrode solution contained (mM): NaCl 138, $MgSO_4$ 1.2, $CaCl_2$ 2, KCl 5, Glucose 10, HEPES 5; the pH was 7.4.

Action potential and membrane currents were measured in the whole-cell patch-clamp configuration (Hamill et al., 1981). Patch electrodes were fabricated from borosilicate glass capillaries (World Precision Instruments) and filled with prefiltered solution containing (mM): potassium aspartate 80, KCl 50, KH_2PO_4 10, $MgSO_4$ 3, NaCl 5, HEPES 5, and K_2ATP 5. The resistance of the electrodes ranged from 1.5 to 3 MΩ. The whole-cell clamp was achieved by the use of a patch-clamp amplifier (EPC7, List Medical Electronics, Germany), connected via a 16 bit A/D interface to a pentium IBM clone computer. The cell capacitance (88.3 ± 2.2 pF, $n = 23$) and series resistance (≤ 5 MΩ) were compensated. The data were sampled at 2 kHz. Data acquisition and analysis was performed with an ISO-3 multitasking patch-clamp program (MFK, Niedernhausen, Germany).

Action potentials were elicited at a stimulation frequency of 0.5 Hz. The L-type Ca^{2+} current ($I_{Ca(L)}$) was recorded by applying a test pulse of 300-ms duration every 5 s from a holding potential of −80 mV. To inactivate both fast Na^+ and T-type Ca^{2+} currents, a prepulse to −40 mV of 40-ms duration preceded the test pulses. To eliminate interfering K^+ currents, K^+ in the electrode and bath solutions were substituted by Cs^+. The amplitude of $I_{Ca(L)}$ was measured as peak inward current with respect to the zero current level. Steady state membrane currents were obtained by applying hyperpolarizing and depolarizing clamp steps for 5 s from a holding potential of −40 mV at a rate of 0.1 Hz. The steady state membrane current was measured as the net current at the end of the clamp step with respect to the zero current level. The delayed rectifier K^+ current (I_K) was obtained by measuring the outward tail currents elicited on repolarization to −40 mV at the end of 5-s depolarizing clamp steps. The amplitude of the deactivating I_K tail current was measured as the difference between the peak outward tail current and the steady state current level after decay of the tail current. When measuring K^+ currents, a holding potential of −40 mV was used to inactivate fast Na^+ and T-type Ca^{2+} currents, and the external bath solution contained 0.3 μM nisoldipine in order to block interfering $I_{Ca(L)}$.

2.4. Cell shortening

Myocytes were stimulated at 0.5 Hz under current clamp. Cell length was monitored using a stable light source (Gossen-Konstanter, Erlangen, Germany) to form a bright field image of the cell, which was projected onto a photodiode array (Laser 2000, Weßling, Germany) with a 4-ms scan rate, and changes in cell length during contraction were quantified via edge tracking. The signal was then transmitted to a computer for on-line analysis. Peak shortening, time to peak shortening, and relaxation time (measured at 90% of relaxation) were evaluated.

2.5. Ca^{2+} transients

Myocytes were stimulated at 0.5 Hz by the action potential clamp. Action potentials were recorded from a control cell in the current-clamp mode and stored in a computer. A typical action potential was then chosen to serve as voltage-command to clamp other cells and to elicit Ca^{2+} transients. Ca^{2+} transients were recorded by using the Ca^{2+}-sensitive dye fura-2. Myocytes were loaded with 3 μM fura-2 acetoxymethyl ester (diluted in Tyrode solution) for 15 min at room temperature. The cells were then transferred to the recording chamber mounted on the inverted microscope adapted for epifluorescence measurement. To allow for intracellular dye conversion and to wash away extacellular fura-2 acetoxymethyl ester, cells were superfused with the Tyrode solution for > 30 min. The dye was alternately (200 Hz) excited at 340- and 380-nm wavelength of light generated by a Deltascan illumination system (Photon Technology International, Brunswick, NJ). Emission fluorescence at 510 nm was detected with a photon-counting photomultiplier tube. Intracellular calibration procedure was adapted from a method described previously (Ganitkevich and Isenberg, 1991). The ratio (R) of fluorescence signals recorded at 340 and 380 nm excitation wavelengths was converted to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by the following equation:

$$[Ca^{2+}]_i = K_d \times [(R - R_{min})/(R_{max} - R)] \times Sf_{380}/Sb_{380}$$

where K_d is the dissociation constant of fura-2, which was taken as 224 nM (Grynkiewicz et al., 1985); R_{min} and R_{max} are the fluorescence ratio values under Ca^{2+}-free and Ca^{2+}-saturating conditions, respectively, and Sf_{380} and Sb_{380} are the fluorescence values for Ca^{2+}-free and Ca^{2+}-saturating forms of fura-2 measured at 380-nm excitation wavelength. R_{max} and Sb_{380} were determined by superfusing the cells with bath solution containing 2 mM Ca^{2+} and 10 μM of the Ca^{2+}-ionophore ionomycin, and by voltage-clamping the membrane potential to −200 mV. To obtain the values of R_{min} and Sf_{380}, cells were perfused with an electrode filling solution containing 10 mM EGTA. The values for R_{max} and R_{min} obtained from 10 cells were 2.5301 ± 0.1643 and 0.2142 ± 0.0178, respectively.

2.6. Statistics

Where appropriate, results are presented as means ± S.E. Significance tests were performed using Student's *t*-test for paired observations. Differences between means were regarded statistically significant at $P < 0.05$.

3. Results

3.1. Action potential duration

The effect of doxorubicin and doxorubicinol on action potential duration (APD) was investigated in isolated ventricular myocytes that had been current-clamped in the whole-cell clamp configuration (Fig. 2). Both compounds were applied directly to the interior of the myocytes by diffusion from the patch electrode. The final drug concentrations in the patch pipette were 100 μM for doxorubicin and 10 μM for doxorubicinol. In the absence of drugs, cells stimulated at 0.5 Hz had resting potentials in the range of −77 to −82 mV (mean of 10 cells -79.4 ± 0.5 mV) and displayed action potentials of 377.6 ± 14.3 ms duration ($n = 10$; drug-free in Fig. 2B) when measured at 90% repolarization (APD_{90}). As shown in Fig. 2B, without drug-intervention (drug-free), APD_{90} was not significantly changed during the recording time of 20 min (369.2 ± 19.1 ms after 20 min, $n = 10$). Diffusion of doxorubicin into the myocytes, however, resulted within 20 min in a time-dependent prolongation of APD_{90}, on the average by 31.2%. In nine myocytes, APD_{90} was 388.3 ± 14 ms after the establishment of the current clamp protocol and 509.3 ± 21.4 ms 20 min later ($n = 9$). Contrary to doxorubicin, doxorubicinol produced a shortening of APD_{90} by 24.6%. In 10 cells, APD_{90} decreased within 20 min from 382.4 ± 17.2 to 288.6 ± 26 ms ($n = 10$). As shown by the representative superimposed action potential recordings of Fig. 1A, besides prolonging APD, neither doxorubicin nor doxorubicinol had any influence on other action potential parameters such as membrane resting potential and action potential amplitude.

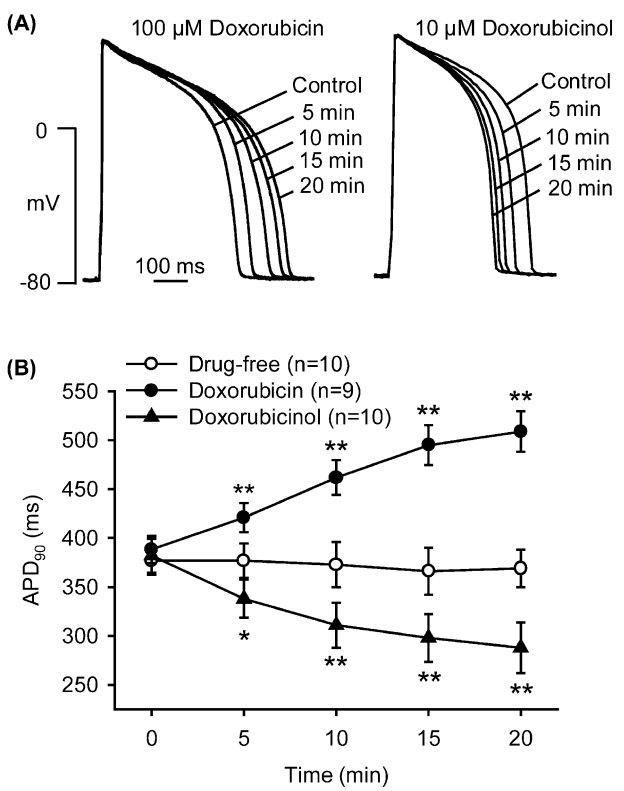

Fig. 2. Opposite effects of doxorubicin and doxorubicinol on action potential duration in guinea pig ventricular myocytes. Action potentials were evoked with current clamp at a frequency of 0.5 Hz. Doxorubicin (100 μM) and doxorubicinol (10 μM) were dialyzed into the cells by diffusion via the patch electrode. (A) Superimposed original recordings showing doxorubicin-induced prolongation and doxorubicinol-induced shortening of action potential duration. (B) Summarized data showing time-dependent changes of action potential duration measured at 90% repolarization (APD_{90}) in the absence (drug-free) or in the presence of doxorubicin or doxorubicinol. $^{*}P < 0.05$; $^{**}P < 0.01$ versus respective control.

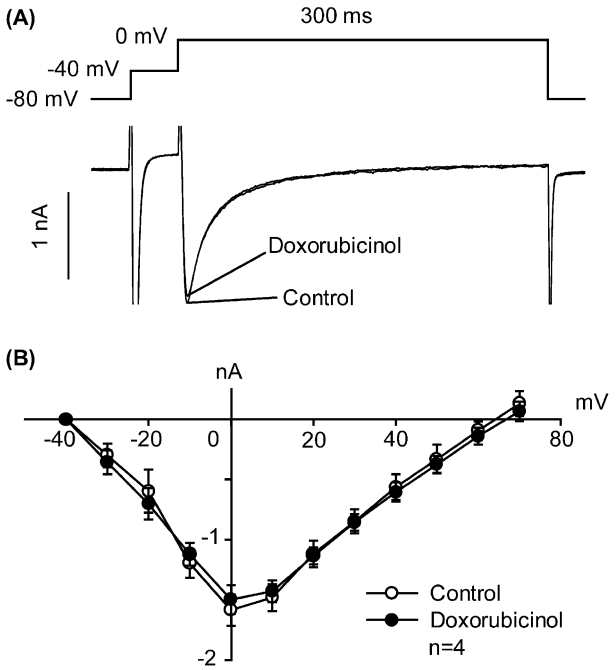

Fig. 3. Failure of doxorubicinol to influence $I_{Ca(L)}$ in guinea pig ventricular myocytes. Doxorubicinol (10 μM) was dialyzed into the cells by diffusion via the patch electrode for 15 min. (A) Superimposed original recordings showing lack of effect of doxorubicinol on $I_{Ca(L)}$. The voltage protocol used to elicit $I_{Ca(L)}$ is depicted above the current traces. (B) Summarized data showing no effects of doxorubicinol on the current–voltage relation of $I_{Ca(L)}$. The $I_{Ca(L)}$ was elicited by depolarizing pulses of 300-ms duration to various test potentials in 10-mV steps preceded by a 40-ms prepulse to −40 mV from a holding potential of −80 mV at 0.2 Hz.

3.2. L-type Ca^{2+} current

Doxorubicinol-dependent shortening of APD could have been due to inhibition of the L-type calcium current $I_{Ca(L)}$. In the experiments shown in Fig. 3A and B, peak $I_{Ca(L)}$ was measured in myocytes immediately after membrane disruption (control) and 15 min after dialysis with 10 μM doxorubicinol. The Na^{+} current was inactivated by using a preceding depolarization step from −80 to −40 mV for 40 ms. The second depolarization to more positive poten-

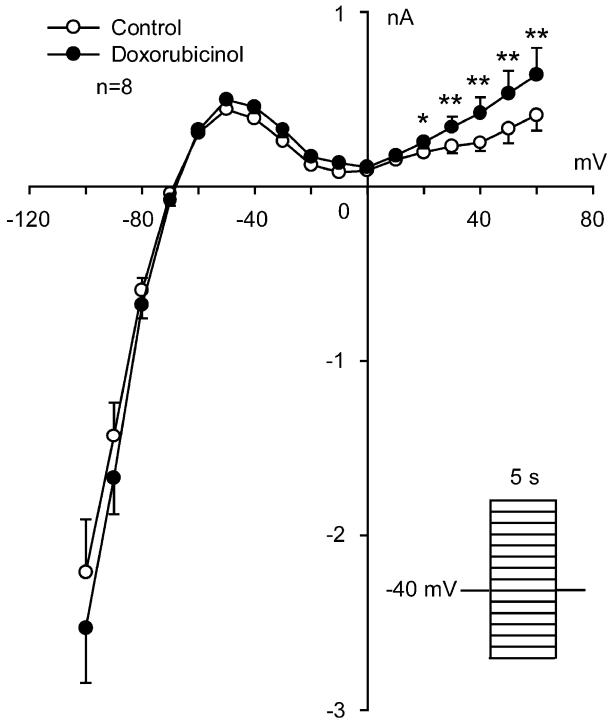

Fig. 4. Effects of doxorubicinol on the current–voltage relation of the steady state K^+ currents in guinea pig ventricular myocytes. The voltage protocol used to elicit the currents is shown as inset. The cells were clamped by 5-s test pulses to various potentials in 10-mV steps from a holding potential of −40 mV at 0.1 Hz. Doxorubicinol (10 μM) was dialyzed into the cells by diffusion via the patch electrode for 15 min. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control.

tials activated $I_{Ca(L)}$. The superimposed $I_{Ca(L)}$ traces of Fig. 3A demonstrate that doxorubicinol produced no significant effect on either the peak current or the current kinetics when the cell was clamped to 0 mV for 300 ms. Peak $I_{Ca(L)}$ was −1.59 nA immediately after membrane disruption (control) and −1.51 nA 15-min later. The current–voltage relations obtained from four cells show that doxorubicinol failed to influence $I_{Ca(L)}$ at all relevant membrane potentials (Fig. 3B).

3.3. Voltage-dependent K^+ currents

To test for a possible interaction of doxorubicinol with K^+ currents, myocytes were clamped from a holding potential of −40 mV to voltages between −100 and +60 mV in 10-mV steps for 5 s. As shown by the current–voltage relations in Fig. 4, treatment of cells by dialysis with 10 μM doxorubicinol for 15 min produced no significant effect on outward currents at voltages between −100 and +10 mV. K^+ currents activated at potentials positive to +10 mV, however, were enhanced by doxorubicinol. At 60 mV, K^+ currents were 410.6 ± 90.0 pA immediately after membrane disruption (control) and 639.3 ± 155.2 pA 15 min later ($n = 8$). Because I_K activates at these positive potentials, the effect of doxorubicinol on I_K was investigated in more detail by measuring the outward tail currents elicited on repolarization to −40 mV after depolarizing step potentials from a holding potential of −40 to +60 mV in 10-mV increments for 5 s. Typical recordings obtained from a myocyte clamped from −40 to +50 mV at the beginning and 15 min after the initiation of dialysis with 10 μM doxorubicinol are shown in Fig. 5A. The peak tail current was 98 pA in the absence (control) and 145 pA in the presence of doxorubicinol; i.e., doxorubicinol increased the current by 48%. In Fig. 5B, tail currents of eight myocytes were plotted as a function of membrane potential, and it can be seen that currents in the presence of doxorubicinol were enhanced at voltages between −10 and +60 mV. Inspection of Fig. 5B reveals that the effect of doxorubicinol is small in the potential range between −10 and +10 mV, but becomes larger at more positive potentials. In Fig. 5C, the same tail-current data as those shown in Fig. 5B were normalized relative to the respective maximal tail current amplitude. The results show that both curves are superimposable, which excludes a voltage

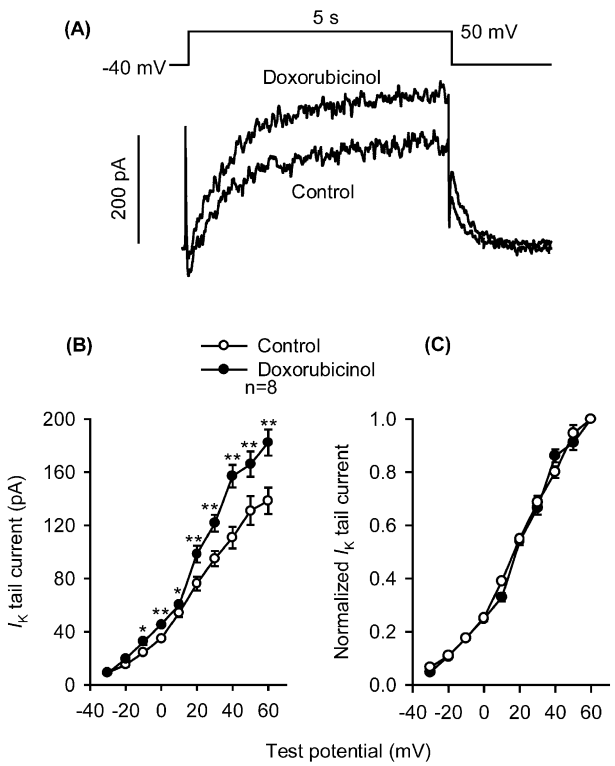

Fig. 5. Augmentation of I_K by doxorubicinol in guinea pig ventricular myocytes. Doxorubicinol (10 μM) was dialyzed into the cells by diffusion via the patch electrode for 15 min. (A) Superimposed original recordings showing the increase of I_K induced by doxorubicinol. The voltage protocol used to elicit I_K is shown above the current traces. (B) Summarized data showing the influence of doxorubicinol on the current–voltage relation of the I_K tail current. The I_K tail currents were elicited on repolarization to −40 mV at the end of 5-s depolarizing pulses to various test potentials in 10-mV steps at 0.1 Hz. (C) Normalized I_K tail currents showing no influence of doxorubicinol on the voltage dependence of I_K activation. Same data as in panel (B). $^{*}P < 0.05$; $^{**}P < 0.01$ versus control.

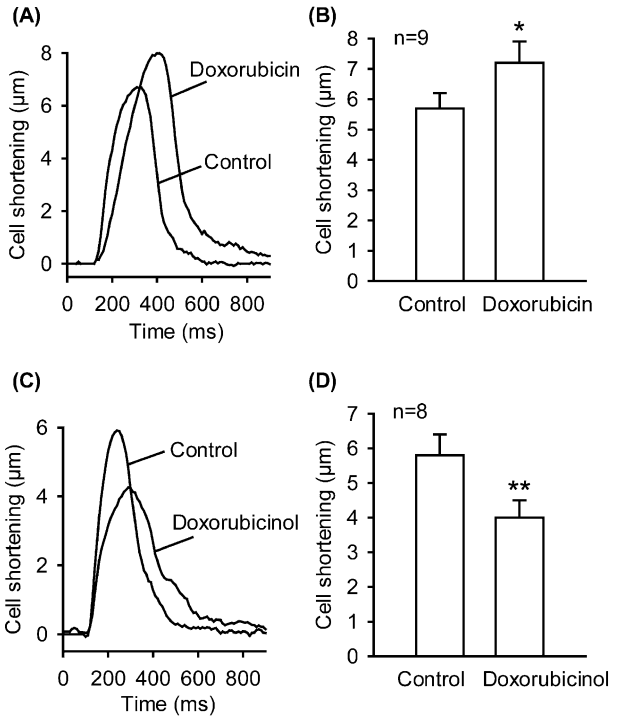

Fig. 6. Opposite effects of doxorubicin and doxorubicinol on cell shortening in guinea pig ventricular myocytes. Cell shortening was induced by current clamp at a frequency of 0.5 Hz. Doxorubicin (100 μM) and doxorubicinol (10 μM) were dialyzed into the cells by diffusion via the patch electrode for 15 min. (A) and (C) Superimposed original recordings as well as (B) and (D) summarized data showing the enhancing effect of doxorubicin and inhibitory effect of doxorubicinol on cell shortening, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$ versus respective control.

dependence of the action of doxorubicinol on I_K activation. In another set of experiments, test pulses of shorter duration (250 ms instead of 5 s) were used to preferentially activate the rapid component of I_K. Dialysis of the cells with 10 μM doxorubicinol for 15 min, however, failed to induce any significant effect on either the steady-state- or the tail currents elicited by test potentials ranging from −40 to 0 mV (data not shown), indicating that doxorubicinol did not affect the rapid component of I_K.

3.4. Cell shortening

Fig. 6A and C show superimposed original recordings of cell shortening from two typical experiments in which ventricular myocytes were current-clamped at 0.5 Hz. It is shown that doxorubicin enhanced cell shortening within 15 min from 6.7 to 8.0 μm, whereas doxorubicinol decreased cell shortening from 5.9 to 4.2 μm. In nine cells dialyzed with 100 μM doxorubicin, cell shortening was significantly enhanced within 15 min by 26.3% (from 5.7 ± 0.5 to 7.2 ± 0.7 μm; Fig. 6B). In eight cells dialyzed with 10 μM doxorubicinol, cell shortening decreased within 15 min by 31.0% (from 5.8 ± 0.6 to 4.0 ± 0.5 μm; Fig. 6D). Despite opposing effects on cell shortening, both anthracyclines prolonged shortening duration (Fig. 6A and C). Doxorubicin prolonged the time to peak shortening and the relaxation time in nine cells by 41.7% from 129.6 ± 12.8 to 183.7 ± 17.9 ms and by 35.4% from 163.4 ± 10.8 to 221.3 ± 12.4 ms, respectively. Doxorubicinol prolonged the two parameters by 14.5% from 131.4 ± 10.2 to 150.4 ± 11.7 ms and by 51.1% from 188.3 ± 7.8 to 284.5 ± 26.1 ms ($n = 8$), respectively.

3.5. APD and Ca^{2+} transients

To investigate the influence of an APD-independent effect of doxorubicinol, intracellular Ca^{2+} transients were elicited in myocytes that were clamped with an action potential of constant duration. In the experiments shown in Fig. 7, action potential recorded from a control cell was

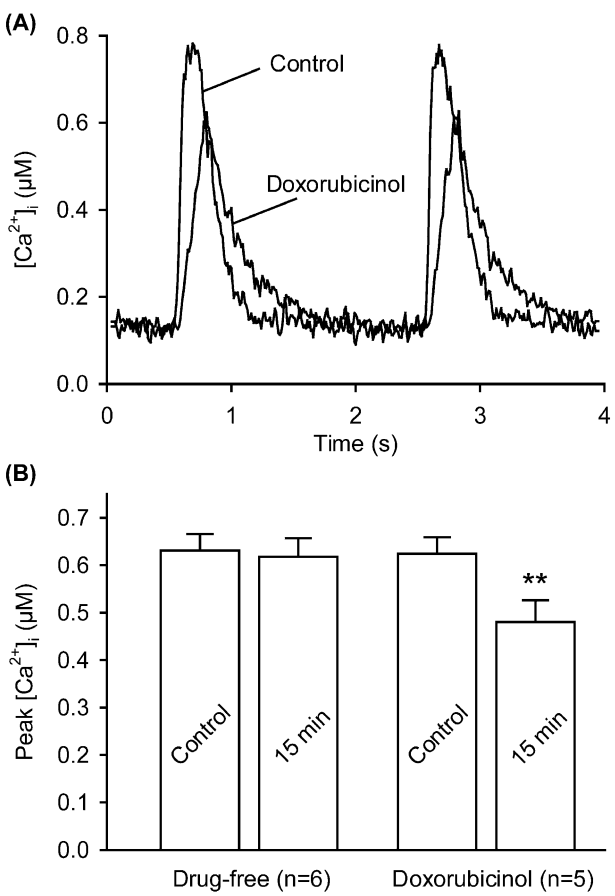

Fig. 7. Decrease of intracellular Ca^{2+} transient by doxorubicinol in guinea pig ventricular myocytes. Myocytes were clamped at 0.5 Hz with an action potential of constant duration ($APD_{90} = 360$ ms). The action potential was recorded before from a control cell. This action potential was then used as a voltage command for other cells to elicit Ca^{2+} transients. The Ca^{2+} transients were measured by loading the cells with 3 μM fura-2 acetoxymethyl ester for 15 min. Doxorubicinol (10 μM) was dialyzed into the cells by diffusion via the patch electrode for 15 min. (A) Superimposed original recordings showing inhibition of the Ca^{2+} transients by doxorubicinol. (B) Summary of changes of the peak Ca^{2+} transient in the absence (drug-free) and in the presence of doxorubicinol. $^{**}P < 0.01$.

used as voltage command to clamp other cells to elicit Ca^{2+} transients. In Fig. 7A, a cell was clamped at 0.5 Hz with an APD_{90} of 360 ms. Immediately after membrane disruption, transients with a peak $[Ca^{2+}]_i$ of 0.775 μM were recorded. Diffusion of 10 μM doxorubicinol into the cell induced within 15 min a decline of peak $[Ca^{2+}]_i$ to 0.623 μM. The combined data from five cells showed a significant decrease in Ca^{2+} transients by 23% from 0.624 ± 0.035 to 0.48 ± 0.046 μM (Fig. 7B). The mean diastolic $[Ca^{2+}]_i$ in all five cells was 0.140 ± 0.013 μM. In control cells that were not treated with doxorubicinol, no significant decline of the peak $[Ca^{2+}]_i$ was observed after 15 min (0.631 ± 0.035 μM at the beginning of the experiment and 0.618 ± 0.039 μM 15 min later; Fig. 7B). Furthermore, inspection of Fig. 7A discloses a marked doxorubicinol-dependent prolongation of both the time to peak $[Ca^{2+}]_i$ and the decaying time of the Ca^{2+} transients. In five cells, doxorubicinol prolonged within 15 min the time to peak $[Ca^{2+}]_i$ by 41.5% from 202 ± 17 to 286 ± 12 ms and slowed the time at which the transient had decayed to 90% of its peak by 52.1% from 514 ± 70 to 782 ± 87 ms.

4. Discussion

This study provides new insight into the toxicity of doxorubicin. We find that doxorubicinol, the major metabolite of doxorubicin, impairs cardiac contractility by both shortening of APD due to activation of I_K and partial depletion of the sarcoplasmic reticulum Ca^{2+} content. Both effects reduce the amount of Ca^{2+} that is available for activation of myofilaments during depolarization.

In the present study, we applied the anthracyclines doxorubicin and doxorubicinol directly into the cytosol of patch-clamped cardiomyocytes via diffusion from the micropipette. The concentration of doxorubicinol in the patch pipette was 10-times lower (10 μM) than that of doxorubicin (100 μM). We used this ratio between both compounds because doxorubicinol was reported to be 10 times more potent than doxorubicin in impairing contractile function of right ventricular papillary muscles from the rabbit (Olson et al., 1988). Furthermore, the IC_{50} of doxorubicinol in inhibiting the Ca^{2+}-stimulated ATPase activity of cardiac sarcoplasmic reticulum was approximately 10 μM (Boucek et al., 1987). Doxorubicin was at least 200 times less potent than doxorubicinol in inhibiting the sarcoplasmic reticulum Ca^{2+} pump.

Contrary to what one would expect from a compound that induces cardiomyopathy and may finally lead to congestive heart failure, doxorubicin enhanced single-cell contractility by significantly increasing cell shortening. This is consistent with earlier findings showing that doxorubicin produced a time- and concentration-dependent positive inotropic effect in isolated papillary muscles from different species (Wang and Korth, 1995; Van Boxtel et al., 1978; Kim et al., 1980; Temma et al., 1993). The clinical relevance of this effect is underscored by studies demonstrating an increase in left ventricular systolic and diastolic function 4 and 24 h after administration of doxorubicin to patients with neoplastic diseases and normal ventricular function (Unverferth et al., 1981; Brown et al., 1989). The positive inotropic effect of doxorubicin was probably due to its prolonging effect on APD. It is well known that an increase in APD, particularly at the plateau level, is associated with an increase in contraction force (Morad and Trautwein, 1968; Terrar and White, 1989; Wang et al., 2000). The increased inotropic response to action potential prolongation may be the result of a reduced driving force for Ca^{2+} extrusion via the Na^+/Ca^{2+} exchange and/or due to a prolonged Ca^{2+} influx via noninactivated Ca^{2+} channels. Other mechanisms such as an increase of Ca^{2+} inward current or inhibition of Na^+/K^+ pump have been ruled out as mechanisms contributing to the positive inotropic effect of doxorubicin (Wang and Korth, 1995; Boucek et al., 1987; Kim et al., 1980). The finding that doxorubicin increased the peak of Ca^{2+} transients in fura-2 loaded cardiomyocytes (Wang and Korth, 1995) suggests that the positive inotropic effect of doxorubicin cannot be exclusively explained by a direct activation of the force generating myofilaments by doxorubicin as has been demonstrated before in skinned trabeculae of the rat heart (Bottone et al., 1997). APD prolongation finds its clinical correlate in a prolonged QT_c interval, QT_c dispersion and in afterpotentials (Ferrari et al., 1996; Sarubbi et al., 1997; Nousiainen et al., 1999). QT_c dispersion and afterpotentials are both known to be associated with an increased risk of serious ventricular dysrhythmias and sudden death.

Contrary to doxorubicin, doxorubicinol produced a significant shortening of APD. Both Ca^{2+} inward current ($I_{Ca(L)}$) and K^+ outward currents conducted through delayed rectifier K^+ channels (I_K) play a key role in regulating cardiac APD. Shortening of APD was not due to inhibition of Ca^{2+} inward current, because doxorubicinol had no influence on the amplitude or on the kinetics of $I_{Ca(L)}$ activated by depolarizing steps to various potentials. Analysis of activation of I_K is difficult because of additional currents that may be simultaneously activated by depolarizing pulses. Upon repolarization from activating voltage steps, an outward tail current is observed which is thought to represent the slow deactivation of I_K and serves as a more reliable index of the I_K current (Heath and Terrar, 1996). Compatible with APD shortening, the experiments showed that doxorubicinol caused a substantial stimulation of I_K tail currents. This effect was opposite of what was found earlier under otherwise identical experimental conditions with doxorubicin which induced inhibition of I_K and consequently prolonged APD (Wang and Korth, 1995). Because doxorubicinol differs from doxorubicin only by a hydroxyl group instead of a carbonyl moiety in the C-13 position, this single modification in chemical structure seems to play a pivotal role in modifying the property of the cardiac delayed rectifier K^+ chan-

nel. As expected from a shortening of APD, doxorubicinol produced a decrease of cell shortening which corresponds to the negative inotropic effect observed earlier in multicellular heart preparations (Olson et al., 1988; Mushlin et al., 1993). APD shortening, however, cannot completely account for the decline in cell shortening because doxorubicinol produced still a decrease of the peak Ca^{2+} transient in cardiomyocytes that were clamped with an action potential of a constant APD. Doxorubicinol has been shown to stimulate sarcoplasmic reticulum Ca^{2+} release channels by increasing their open probability (Nagasaki and Fleischer, 1989; Mushlin et al., 1993) and to inhibit sarcoplasmic reticulum Ca^{2+}-ATPase which leads to an impaired Ca^{2+} sequestration (Boucek et al., 1987; Olson et al., 1988). Both effects cause the sarcoplasmic reticulum Ca^{2+} load to decline and are compatible with an APD-independent decrease of cell shortening and peak Ca^{2+} transient by doxorubicinol. Previous work on rat and guinea pig cardiomyocytes has found that both ryanodine, which enhances Ca^{2+} release from sarcoplasmic reticulum without inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase (Feher and Lipford, 1985), and thapsigargin, which specifically inhibits sarcoplasmic reticulum Ca^{2+} ATPase (Kijima et al., 1991; Kirby et al., 1992), slow the rate of rise and the decay rate of the Ca^{2+} transient and of cell shortening (Negretti et al., 1993; Lewartowski et al., 1994). Simultaneously, the time to the peak of the respective signal was significantly prolonged. By using a pump inhibition (thapsigargin) and leak (ryanodine) model, it was proposed that in the presence of either drug, the sarcoplasmic reticulum will loose its role in Ca^{2+} buffering, so that Ca^{2+} removal from the cytosol will be controlled predominantly by the sarcolemmal Na^{+}/Ca^{2+} exchange and the recovery will be slowed (Negretti et al., 1993). Moreover, a reduction of Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum slowed the velocity of cell shortening and the rising phase of the Ca^{2+} transient. From this, it is evident that sarcoplasmic reticulum leak and pump inhibition will produce qualitatively identical effects on the contraction and Ca^{2+} transient. The present study demonstrates that both doxorubicin and doxorubicinol, although producing opposite effects on APD and contractility, modify cell shortening and Ca^{2+} transient as predicted by the leak and pump inhibition model. Whereas both sarcoplasmic reticulum Ca^{2+} release and pump inhibition probably account for the APD-independent decrease of contractility by doxorubicinol, only Ca^{2+} leak seems to be the mechanism by which doxorubicin slows the rising and decay phase of cell shortening and Ca^{2+} transient (Nagasaki and Fleischer, 1989; Mushlin et al., 1993). In order to explain the positive inotropic effect of doxorubicin, one must assume that APD prolongation provided more activator Ca^{2+} for contraction than had been lost by the sarcoplasmic reticulum Ca^{2+} leak. It has been shown that doxorubicin inhibits sarcoplasmic reticulum Ca^{2+}-ATPase gene transcription in cardiomyocytes cultured for 24 h (Arai et al., 1998, 2000). Such a genomic effect, however, should not play a role in the present study describing the acute effects of doxorubicin, but may become important in delayed cardiotoxicity of doxorubicin. Taken together, although both anthraquinones impair the Ca^{2+} storage function of sarcoplasmic reticulum, doxorubicinol and doxorubicin differ from each other in their effects on myocardial contraction due to the opposite action on APD. It should be kept in mind that in a situation in which sarcoplasmic reticulum function is severely impaired, APD plays the predominant role in determining how much Ca^{2+} will be available for contraction. As cardiac levels of doxorubicinol increase during prolonged exposure to doxorubicin (Olson et al., 1988), APD shortening may progressively amplify the detrimental effects of both anthraquinones on sarcoplasmic reticulum Ca^{2+} load and hence on contractile function. Thus, owing to its APD-shortening action, doxorubicinol may play an important role in the time-dependent development of doxorubicin-induced ventricular dysfunction. On the other hand, the early arrhythmogenic effect of doxorubicin as a result of QT_c prolongation may gradually disappear as doxorubicinol accumulates within the cardiac cell.

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