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European Journal of Pharmacology 492 (2004) 225–231

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Ouabain increases myofibrillar Ca^{2+} sensitivity but does not influence the Ca^{2+} release in human skinned fibres

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Received 17 March 2004; accepted 30 March 2004

Abstract

The present study investigated the impact of the Na^+ pump inhibitor ouabain (g-strophanthin) on Ca^{2+} sensitivity and Ca^{2+} release in human right auricular trabeculae (coronary bypass) and in skinned muscle fibres from left ventricular myocardium (cardiac transplantation, dilated cardiomyopathy). A time-dependent increase in force of contraction was observed in right auricular trabeculae in response to ouabain (100 nM) before the intracellular Ca^{2+} transient (fura-2) increased ($n=6$). In triton X-skinned fibres (no sarcoplasmic reticulum) of human failing myocardium, ouabain (1–100 nM) concentration-dependently increased tension at a free extracellular Ca^{2+} concentration of 1 μM and the Hill coefficient of the Ca^{2+}-dependent tension development. Ouabain (1–100 nM) did not directly induce a Ca^{2+} release out of the sarcoplasmic reticulum, nor did it alter the caffeine (10 mM) induced sarcoplasmic reticulum Ca^{2+} release in saponin-skinned fibre preparations in which the sarcoplasmic reticulum had been Ca^{2+}-loaded.

In conclusion, ouabain increases myofibrillar Ca^{2+} sensitivity possibly due to an increase in the cooperativity of the thick and thin myofilaments. This mechanism may additionally contribute to the positive inotropic effect of ouabain.

Keywords: Human heart; Skinned fibre; Ouabain; Caffeine; Ryanodine channel

1. Introduction

Cardiac glycosides increase force of contraction and are clinically used in the treatment of symptomatic heart failure. Yet, there is an ongoing discussion on the mechanisms underlying their positive inotropic effect. Cardiac glycosides, like ouabain, cause an increase of intracellular free Ca^{2+} when applied to cardiac muscle (Allen et al., 1984, 1985; Brixius et al., 1997). This is thought to be primarily due to an inhibition of the Na^+/K^+-ATPase, which increases the intracellular Na^+ concentration and thus the reverse mode of the Na^+/Ca^{2+} exchanger, which finally results in an increase of the intracellular Ca^{2+} concentration (Lee and Dagostino 1982; Levi et al., 1994). An enhancement of the transient Ca^{2+} inward current by glycosides has also been reported and may additionally contribute to the positive inotropic effect of cardiac glycosides (Wier and Hess 1984). Yet, the influence on the transient Ca^{2+} inward current may be dependent on the respective glycoside species (Ruch et al., 2003). However, in nanomolar concentrations, ouabain was even found to enhance Na^+/K^+-ATPase activity in isolated guinea pig ventricular myocytes (Gao et al., 2002). Furthermore, strophanthidin (an aglycon of ouabain) has been shown to cause an increase in developed tension prior to an increase in intracellular Na^+ suggesting that inhibition of the Na^+/K^+-ATPase is not the only mechanism of inducing positive inotropy and it may be preceded by an increase in the Ca^{2+} sensitivity of the cardiac myofilaments (Boyett et al., 1986). Further evidence for this hypothesis is obtained from a previous study on cat ventricular myocytes in which a dramatic increase in cell shortening was only accompanied by a modest increase in Ca^{2+}-transient magnitude (Nishio et al., 2002).

Cardiac glycosides are able to permeate the sarcolemma and to accumulate in the sarcoplasmic reticulum (Dutta et al., 1968). Thus, a direct influence on intracellular Ca^{2+} regulation may be an additional mechanism of action. Consistently, an increase in Ca^{2+} sensitivity of the ryano-

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doi:10.1016/j.ejphar.2004.03.064

dine channels has been described after application of cardiac glycosides in isolated sarcoplasmic reticulum vesicles obtained from skeletal and cardiac muscle of the sheep (McGarry and Williams 1993).

The aim of the present study was to investigate putative additional mechanisms of action of cardiac glycosides besides the inhibition of Na^+/K^+-ATPase in human myocardium. In right auricular trabeculae obtained from patients undergoing aortocoronary bypass surgery, simultaneous measurements of force of contraction and the intracellular Ca^{2+} transient were performed. In addition, the influence of ouabain was studied on Ca^{2+}-dependent tension in triton X-skinned fibres and the sarcoplasmic reticulum Ca^{2+} release in saponin-skinned fibres from human failing left ventricular myocardium.

2. Materials and methods

2.1. Myocardial tissue

Failing human myocardium was obtained during cardiac transplantation ($n=10$; mean age: 48 ± 5 years, 3 female, 7 male). The transplanted patients had heart failure due to dilated cardiomyopathy which was classified as New York Heart Association Class IV (NYHA IV) on the basis of clinical symptoms and signs. All patients gave written informed consent before surgery. The medical therapy consisted of angiotensin-converting enzyme inhibitors, nitrates, diuretics, β-adrenoceptor antagonists and spironolactone. Right atrial myocardium was obtained from patients who underwent aortocoronary bypass surgery without clinical signs of heart failure ($n=6$, mean age: 59 ± 2 years, 3 female, 3 male). Immediately after explantation, the myocardial tissue was placed in ice-cold aerated modified Tyrode's solution (see below) and delivered to the laboratory within 10 min. The study was approved by the local ethics committee and conforms with the Declaration of Helsinki.

2.2. Ca^{2+} transient and force measurements

Intracellular Ca^{2+} was measured in isolated muscle strip preparations from human atrial myocardium obtained from patients undergoing aortocoronary bypass surgery using the fluorescence indicator fura-2 (Grynkiewicz et al., 1985). To facilitate cell loading, fura-2 was used as acetoxymethyl (AM) ester as described previously (Brixius et al., 1997).

After fura-2 loading, the muscle strips were rinsed with oxygenated Tyrode solution for 15 min. Afterwards, the muscle strips were fixed between a tweezer and the force transducer. The force transducer was connected by an analog-digital converter to a personal computer. For online data analysis, special software was used (Scientific Instruments, Heidelberg, Germany). Fura-2 fluorescence was measured by using a dual-wavelength fluorometer equipped with an inverted microscope. Experiments were performed as described previously (Brixius et al., 1997). The bathing solution used was a modified Tyrode's solution containing (mM) NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHPO_4$ 0.42, $NaHCO_3$ 22.6, Na-EDTA 0.05 and glucose 5.0. It was continuously gassed (5% CO_2, 95% O_2) and maintained at 37 °C (pH 7.4). Isometric force of contraction was measured with an inductive force transducer attached to a recorder. The preparations were electrically paced at 1 Hz with rectangular pulses of 5-ms duration. The voltage was 20% above threshold.

2.3. Chemically skinned left ventricular fibres

Muscle pieces of about 10 mm in length and 1 mm in diameter were excised from the anterior left ventricular papillary muscle from failing human hearts while being incubated in ice-cold Bretschneider solution. The muscle pieces were incubated in a *preparation* solution containing 50% glycerol and (mM) imidazole 20, NaN_3 10, ATP 5, $MgCl_2$ 5, EGTA 4 and dithioerithrol 2 (pH 7.0 at 4 °C for 1 h) and then were divided with tweezers into small fibre bundles.

Triton X-skinning (1%, 20 h, 4 °C) was performed as previously described (Schwinger et al., 1994; Brixius et al., 2000). For saponin skinning, the small fibre bundles were placed in the *preparation* solution, but this time with the addition of 50 μg/ml saponin for 30 min (pH 7.0 at 4 °C). This treatment disrupts the sarcolemma but does not affect the ability of the sarcoplasmic reticulum to accumulate and release Ca^{2+} (Endo and Iino 1980). The preservation of sarcoplasmic reticulum function by saponin treatment is shown by the ability of caffeine to evoke contractures (D'Agnolo et al., 1992). Finally, the fibres were stored at −20 °C in the preparation solution without triton X or saponin until use.

For contraction measurements, fibre bundles were further dissected under the microscope and then mounted to a force transducer (KG7, Scientific Instruments). The fibre length was adjusted to the point where resting tension was just threshold (slack position). Fibre diameter (125–175 μm) and length (7–8 mm) were the same in all preparations studied. All experiments were performed at 21 °C.

2.4. Influence of ouabain on myofibrillar Ca^{2+} sensitivity

After the initial precontraction and relaxation, fibres were incubated in a solution with free Ca^{2+} concentration of 1 μM in the absence and presence of ouabain (1–100 nM). At the end, a contraction solution (see below) was added.

2.5. Measurements of sarcoplasmic reticulum Ca^{2+} release

Saponin-skinned muscle fibres were relaxed in a solution of the following composition (relaxation solution, in mM): imidazole 20, Na_2ATP 10, NaN_3 5, EGTA 5, $MgCl_2$ 12.5, dithiothreitol 1, creatine phosphate 10, creatine kinase 1 mg/ml, free Ca^{2+}: 10 nM, pH 7.0. Afterwards, they were

maximally stimulated (contraction solution, as above, but free Ca^{2+}: 12.9 μM) and then relaxed again. After complete relaxation, Ca^{2+} was completely depleted from the sarcoplasmic reticulum by incubating the fibres in a relaxation solution with the addition of caffeine (25 mM). The sarcoplasmic reticulum was loaded again using the relaxation solution with the addition of 0.1 μM free Ca^{2+} ions for 30 s. Caffeine (10 mM) was applied [relaxation solution with low EGTA (0.025 mM) and Mg^{2+} concentrations (0.05 mM)] in the absence and presence of ouabain (100 nM) and force development was recorded as a measure of Ca^{2+} release. To assess a direct Ca^{2+} releasing effect, ouabain (1 nM) was applied instead of caffeine at the end of the experiments. The Ca^{2+} release from the sarcoplasmic reticulum was quantified by the ratio of caffeine/ouabain-induced tension over maximal Ca^{2+} activated tension. Free Ca^{2+} concentrations were estimated using the program of Fabiato and Fabiato (1979). Experiments were performed as previously described (D'Agnolo et al., 1992).

2.6. Materials

Ouabain was obtained from Boehringer-Mannheim. All other chemicals were of analytical grade or the best grade commercially available. All compounds were dissolved in twice distilled water. Applied agents did not change the pH of the medium.

2.7. Statistics

All values are expressed as means ± S.E.M. Student's paired *t*-test or one-way ANOVA was used to compare the different parameters among groups. Statistical significance was defined when $P < 0.05$.

3. Results

3.1. Inotropic effects of ouabain

Fig. 1 shows original tracings; Fig. 2 and Table 1 summarize the results for the simultaneous measurements of isometric force of contraction and the intracellular Ca^{2+} transient in right auricular trabeculae. When ouabain was applied to contracting isolated trabeculae, force of contraction was increased after 20 min, while Ca^{2+} transient was not. After 50 min, force of contraction increased further and the intracellular diastolic and systolic Ca^{2+} transient were significantly increased as well (Fig. 1, Table 1). Application of ouabain did not alter the time parameters of the contractile twitch or of the intracellular Ca^{2+} transient (Table 1). No significant alterations in force of contraction and the intracellular Ca^{2+} transient were observed when experiments were performed under similar conditions without application of ouabain (50 min vs. 0 min force of contraction: 102 ± 7% of basal force, systolic fura-2 ratio: 93 ± 5% basal ratio, $n = 4$).

3.2. Influence of ouabain on myofibrillar tension

In triton X-skinned fibres of human failing myocardium, in which the sarcolemma and the sarcoplasmic reticulum is removed, it was investigated whether ouabain may sensitize the myofilaments towards Ca^{2+}. Fig. 3 summarizes the

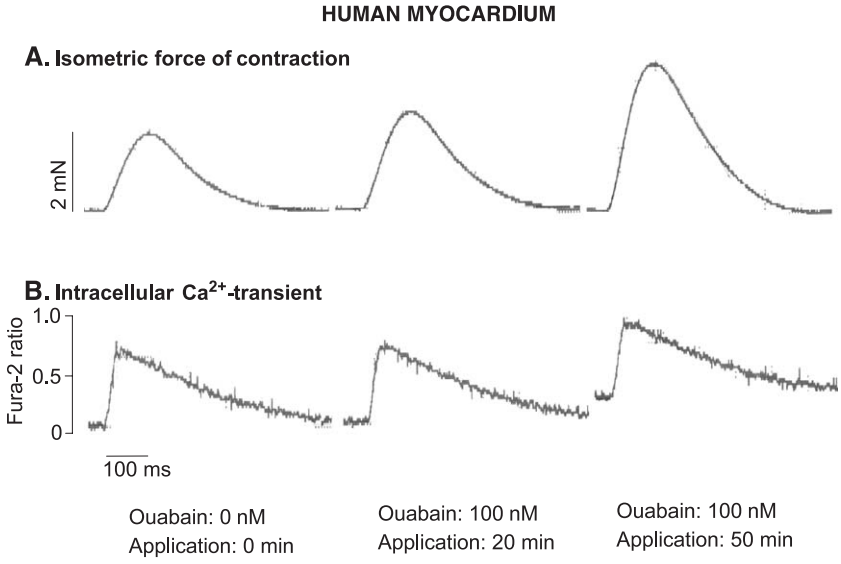

Fig. 1. Original tracings of the intracellular Ca^{2+} transient measured by the fluorescence indicator fura-2 (lower panel) and the isometric force of contraction (upper panel) 0, 20 and 50 min after application of ouabain (0.1 μM). The positive inotropic effect of ouabain was attributed to both an increase in the intracellular Ca^{2+} transient and in force of contraction. Yet, the increase in force of contraction occurred prior compared to the increase in the intracellular Ca^{2+} transient.

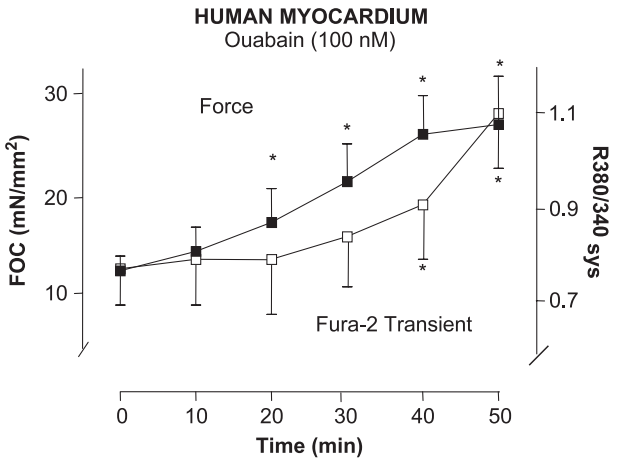

Fig. 2. Time-dependent inotropic effects of ouabain on the intracellular Ca^{2+} transient and force of contraction ($n=6$ from 6 hearts). * $P<0.05$ vs. 0 μM ouabain.

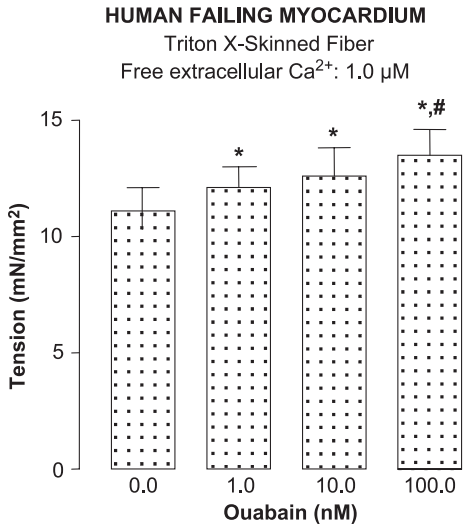

Fig. 3. Measurement of Ca^{2+}-activated tension in left ventricular triton X-skinned fibre preparations of human failing myocardium at increasing concentrations of ouabain. Application of ouabain concentration-dependently increased Ca^{2+}-dependent tension.

results. The fibres were suspended into a solution with a free Ca^{2+} concentration of 1 μM, which corresponds to a free Ca^{2+} concentration at which a half-maximal activation of the Ca^{2+}-dependent tension development is achieved in human failing myocardium (Brixius et al., 2000). After a plateau of tension was reached (11.1 ± 1.0 mN/mm^2), ouabain (1–100 nM) was added to the incubation solution. Ouabain concentration-dependently increased the developed tension (Fig. 3). To further investigate the Ca^{2+}-sensitizing properties of ouabain, Ca^{2+}-dependent tension development was studied in triton X-skinned fibre preparations under control conditions, as well as in the presence of ouabain (1, 10 or 100 nM). Results are summarized in Fig. 4. Application of ouabain (1 nM) significantly shifted the Ca^{2+} concentration response curve to the left, as indicated by an increase of the EC_{50}, i.e. the Ca^{2+} concentration needed to achieve a 50% decrease in myofibrillar tension (control: 1.21 ± 0.08 μM Ca^{2+}, +ouabain (1 nM): 0.97 ± 0.06 μM Ca^{2+}). Higher concentrations of ouabain did not further alter the myofibrillar Ca^{2+} sensitivity since no significant alterations of the EC_{50} Ca^{2+} were observed in the presence of 10 nM (EC_{50} Ca^{2+}: 0.92 ± 0.04 μM Ca^{2+}) or 100 nM ouabain (EC_{50} Ca^{2+}: 0.96 ± 0.03 μM Ca^{2+}). Instead, especially at higher concentrations, ouabain increased the steepness of the Ca^{2+} tension response curves as indicated by a concentration-dependent increase of the Hill coefficient (nHill, control: 2.31 ± 0.11, +ouabain (10 nM: 6.65 ± 0.69)).

Table 1
Influence of ouabain (100 nM) on isometric force of contraction and the fura-2 transient in human myocardium ($n=6$ from 6 hearts)

Parameter	Control	Ouabain (100 nM)
Isometric force		
FOC (mN/mm^2)	12.6 ± 2.5	27.6 ± 5.3[a]
TPT (ms)	121 ± 13	116 ± 9
T1/2T (ms)	121 ± 17	126 ± 15
+T (mN/s)	9.7 ± 3.2	20.2 ± 7.8[a]
−T (mN/s)	5.8 ± 1.7	9.9 ± 2.9[a]
Fura-2 transient		
$R340/380_{sys}$	0.77 ± 0.11	1.11 ± 0.13[a]
$R340/380_{dia}$ (Δ)		$+0.35 \pm 0.11$
TPR (ms)	49 ± 7	44 ± 5
T1/2R (ms)	188 ± 31	185 ± 24

Ouabain data were obtained 50 min after application.
FOC: force of contraction, TPT: time to peak force, T1/2T: time to half peak relaxation, +T: maximal contraction velocity, −T: maximal relaxation velocity, $R340/380_{sys}$: systolic fura-2 ratio, $R340/380_{dia}$: diastolic fura-2 ratio, TPR: time to peak fura-2 transient, T1/2R: time to half peak fura-2 transient decay.
[a] $P<0.05$ vs. control.

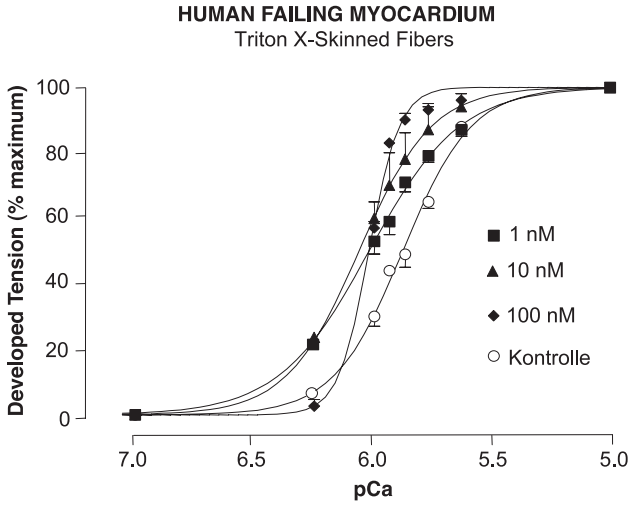

Fig. 4. Influence of ouabain on the Ca^{2+}-tension relationship in triton X-skinned fibre preparations.

3.3. Effect of ouabain on the sarcoplasmic reticulum Ca^{2+} release

Fig. 5A shows an original tracing of the caffeine-induced Ca^{2+} release from a saponin-skinned fibre obtained from human failing left ventricular myocardium. After a precontraction in the presence of free Ca^{2+} (Ca^{2+}: 12.9 μM), a maximal Ca^{2+} activated tension [(DT_{max}): 28.0 ± 2.7 mN/mm^2, $n = 6$] was measured. The caffeine-induced maximal tension increase was 9.7 ± 1.2 mN/mm^2 (34.1 ± 1.8% of DT_{max}). The caffeine-induced tension increase declined, at least partly, after a while which may be attributed to a reloading of the sarcoplasmic reticulum by SERCA 2a. When the experiments were performed in the presence of ouabain (1, 10 or 100 nM) instead of caffeine (10 mM) at the end of the experiment,

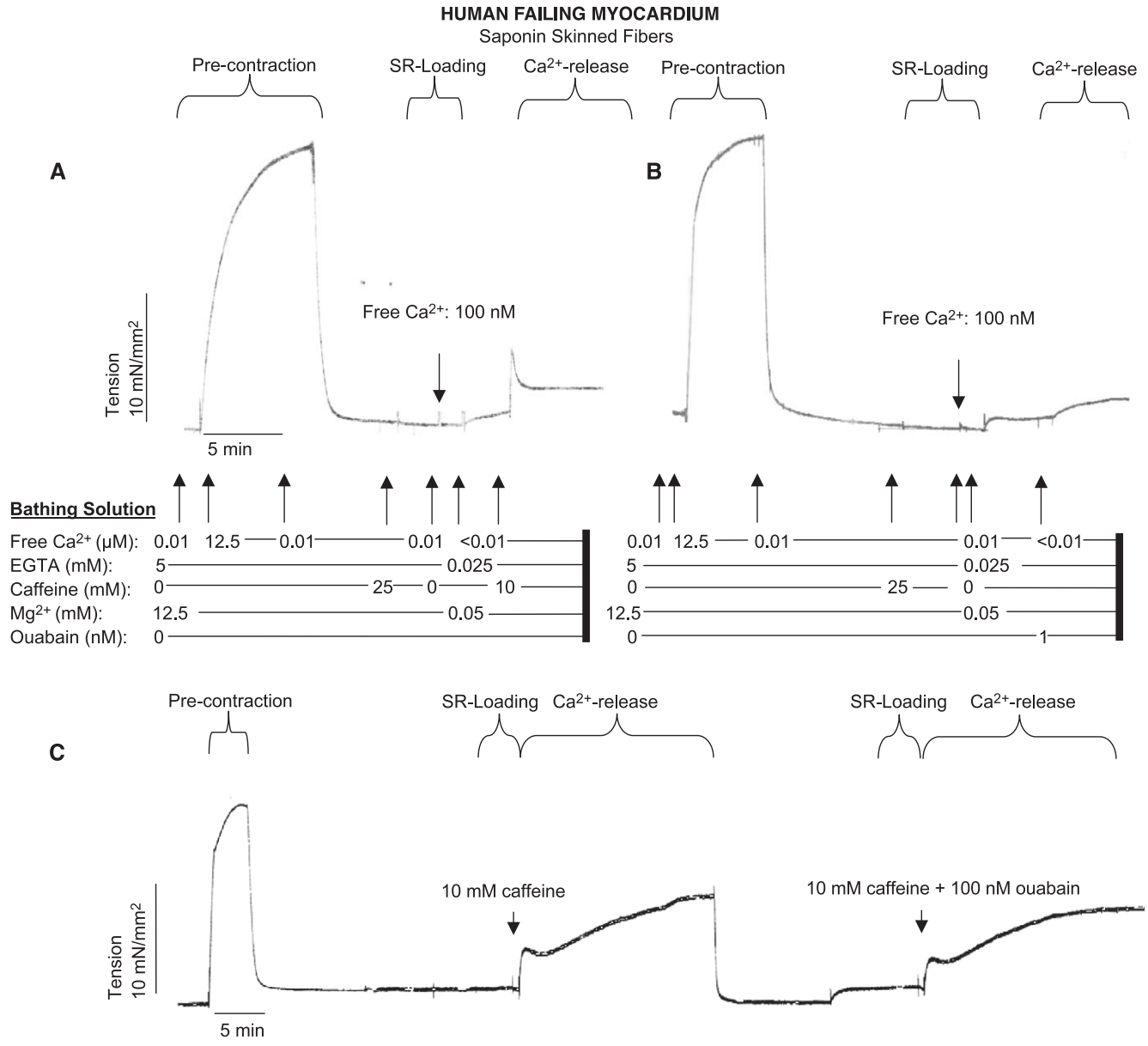

Fig. 5. Original tracings of the measurement of the caffeine-induced Ca^{2+} release from the sarcoplasmic reticulum. (A) In the beginning, all fibres were relaxed (free Ca^{2+}: 10 nM). Afterwards, the fibres were maximally Ca^{2+}-stimulated (free Ca^{2+}: 12.9 μM). Fibres were relaxed again and then incubated in a relaxing solution with the addition of 25 mM caffeine to open the sarcoplasmic reticulum Ca^{2+} channels and to empty the sarcoplasmic reticulum. The sarcoplasmic reticulum was reloaded again with Ca^{2+} under definite conditions (free Ca^{2+}: 0.1 μM, 30 s). Then, fibres were dipped into a solution in which the EGTA (0.025 mM) and Mg^{2+} concentrations (0.05 mM) were diminished to optimize the conditions for the sarcoplasmic reticulum Ca^{2+} release. At the end, fibres were incubated in a solution containing caffeine (10 mM). (B) The same experimental procedure as described in (A). This time, ouabain (1 nM) was added at the end of the experiment instead of caffeine. In contrast to caffeine, no Ca release, i.e. no increase in tension, was observed. (C) Effect of ouabain on the Ca^{2+} release-dependent contraction of saponin-skinned fibres. Ouabain did not influence the caffeine-induced contraction.

no spontaneous increase in tension was observed (Fig. 5B). To investigate whether ouabain may potentiate the sarcoplasmic reticulum Ca^{2+} release, the caffeine-induced sarcoplasmic Ca^{2+} release was measured either in the absence or in the presence of ouabain (100 nM). In the absence of ouabain, the caffeine-induced tension was $25.1 \pm 4.5\%$ of DT_{max} ($n = 8$, Fig. 5C); in the presence of ouabain, caffeine-induced tension was $24.3 \pm 5.3\%$ of DT_{max} (both, $n = 8$, Fig. 5C). Thus, ouabain did not alter the caffeine-induced Ca^{2+} release out of the sarcoplasmic reticulum in saponin-skinned fibre preparations.

4. Discussion

It is widely accepted that the main pharmacodynamic positive inotropic mechanism of cardiac glycosides is the result of binding and inhibition of the sarcolemmal Na^+/K^+-ATPase. Besides this well-established mechanism, there have been numerous reports over the past few years that additional actions of cardiac glycosides might also contribute to their positive inotropic effect. We therefore investigated whether a Ca^{2+} sensitizing effect or a potentiation of the sarcoplasmic reticulum Ca^{2+} release may be mechanisms involved in the positive inotropic effect of ouabain in human myocardium.

4.1. Ouabain—a cardiac Ca^{2+} sensitizer?

From simultaneous measurements of the isometric force of contraction and the intracellular Ca^{2+} transient, evidence is provided by the present study that a Ca^{2+} sensitizing component may be involved in the positive inotropic effect of ouabain since force of contraction was increased before an increase in the intracellular Ca^{2+} transient was observed. Similar results have been described after application of WS-1442, a standardized extract from Crataegus, the inotropic action of which has been also suggested to be due, at least partly, to an inhibition of the sarcolemmal Na^+/K^+-ATPase (Brixius et al., 1998; Schwinger et al., 2000). In addition, our results are in line with experiments showing that strophanthidin, an aglycon of ouabain, may cause an increase in developed tension prior to an increase in intracellular Na^+ in isolated sheep Purkinje fibres (Boyett et al., 1986) as well as with studies in isolated cat ventricular myocytes, in which a dramatic increase in cell shortening was only accompanied by a modest increase in Ca^{2+}-transient magnitude (Nishio et al., 2002).

Unlike the Ca^{2+} sensitizers, EMD 57033 [the (+)enantiomer of the thiadiazinone derivative EMD 53998 (5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one] or CGP 48506 [the (+)enantiomer of the racemic 1,5-benzodiazocine derivative 5-methyl-6-phenyl-1,3,5,6-tetrahydro-3,6-methano-1,5-benzodia-zocine-2,4-dione], which directly interact with the myofilaments (Palmer et al., 1996; Brixius et al., 2000) and therefore impair cardiac relaxation (Brixius et al., 1997; Brixius et al., 2002), no alterations of cardiac relaxation were observed after application of ouabain. Therefore, it may be possible that the Ca^{2+} sensitizing moiety of ouabain may be attributed to alterations in the Ca^{2+} affinity of troponin C similar to levosimendan (Haikala et al., 1995). Direct evidence for a Ca^{2+} sensitizing effect of ouabain is provided by the results in triton X-skinned fibres, in which ouabain concentration-dependently increased Ca^{2+}-activated force. Very interestingly, a concentration-dependent increase in the steepness of the Ca^{2+}/tension relationship was observed after application of ouabain, indicating that ouabain may influence the interaction of the thick and the thin myofilaments.

4.2. Ouabain—a ryanodine channel agonist?

Previously, digoxin in nanomolar concentrations has been shown to increase the open probability of the ryanodine receptor channel by increasing the number of openings without changing open or closed times in sheep cardiomyocytes (McGarry and Williams, 1993). The effect of glycosides may be the result of an increased sensitivity of channel opening in response to cytosolic Ca^{2+}. In addition, the effect of digoxin was unique to cardiac ryanodine receptor channels; channels isolated from skeletal muscle did not respond to glycoside exposure (McGarry and Williams, 1993). In contrast to these findings, we did not observe a direct effect of ouabain on the Ca^{2+} release from the sarcoplasmic reticulum in saponin-skinned heart muscle fibres. Possible reasons that could explain why our results are different from those reported previously could be the use of different experimental models [saponin-skinned fibres (our study) vs. artificial planar bilayer (Sagawa et al., 2002)]. It may be feasible that transporter systems which are normally required to transport the hydrophilic drug ouabain into the intracellular space and even into the sarcoplasmic reticulum may not work after saponin treatment or the condition under which the caffeine-induced sarcoplasmic reticulum Ca^{2+} release is measured, e.g. low extracellular Ca^{2+} concentration and high Mg^{2+} concentrations. In addition, it may be considered that the low lipophilicity of ouabain may account for the nonreactivity in saponin-skinned fibres.

Our results obtained from simultaneous measurements of force of contraction and the intracellular Ca^{2+} transient clearly demonstrate that positive inotropy of ouabain occurs prior to changes in systolic or diastolic Ca^{2+} and may be also dependent on the Ca^{2+} release out of the sarcoplasmic reticulum or other changes in intracellular Ca^{2+} homeostasis. In conclusion, the present study provides first-time evidence that a Ca^{2+} sensitizing effect of ouabain may play a role in positive inotropy in human myocardium.

Acknowledgements

We are indebted to all colleagues of the Department of the Cardiothoracic Surgery of the University of Cologne (Director: Prof. Dr. R.E. de Vivie) for providing us with human myocardial samples. The authors thank Mrs. Esra Koröglu, Kerstin Schenk and Katja Rössler for their excellent technical help. This study was supported by the Deutsche Forschungsgemeinschaft (R.H.G.S.) and the Köln Fortune Program/Faculty of Medicine, University of Cologne (K.B.). This work contains parts of the doctoral thesis of N.H.

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