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Ouabain treatment is associated with upregulation of phosphatase inhibitor-1 and Na^+/Ca^{2+}-exchanger and β-adrenergic sensitization in rat hearts

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Abstract

Cardiac glycosides are widely used in the treatment of congestive heart failure. While the mechanism of the positive inotropic effect after acute application of cardiac glycosides is explained by blockade of the Na^+/K^+-pump, little is known about consequences of a prolonged therapy. Here male Wistar rats were treated for 4 days with continuous infusions of ouabain (6.5 mg/kg/day) or 0.9% NaCl (control) via osmotic minipumps. Electrically driven (1 Hz, 35 °C) papillary muscles from ouabain-treated rats exhibited shorter relaxation time (−15%) and a twofold increase in the sensitivity for the positive inotropic effect of isoprenaline. The density and affinity of β_1- and β_2-adrenoceptors as well as mRNA and protein levels of stimulatory ($G_{s\alpha}$) and inhibitory ($G_{i\alpha\text{-}2}$, $G_{i\alpha\text{-}3}$) G-proteins were unaffected by ouabain. Similarly, SR-Ca^{2+}-ATPase 2A, phospholamban, ryanodine-receptor expression as well as the oxalate-stimulated ^{45}Ca-uptake of membrane vesicles remained unchanged. However, mRNA abundance of the protein phosphatase inhibitor-1 (I-1) and the Na^+/Ca^{2+}-exchanger (NCX) were increased by 52% and 26%, respectively. I-1 plays an amplifier role in cardiac signaling. Downregulation of I-1 in human heart failure is associated with desensitization of the β-adrenergic signaling pathway. The present data suggest that the ouabain-induced increase in I-1 expression might be at least partly responsible for the increased isoprenaline sensitivity and increased expression of NCX for the accelerated relaxation after chronic ouabain in this model.

Keywords: Cardiac glycosides; Rat; Gene expression; Protein phosphatase inhibitor-1; Na^+/Ca^{2+}-exchanger; β-Adrenergic signaling

Cardiac glycosides are widely used in the treatment of congestive heart failure. The positive inotropic effect of cardiac glycosides on the myocardium is explained by partial inhibition of the cardiac Na^+/K^+-pump, causing an increase in intracellular Na^+, which in turn affects the sarcolemmal Na^+/Ca^{2+}-exchanger, leading to increases in intracellular Ca^{2+} and in the force of contraction [1,2]. Whether this acute effect on cardiac contractility accounts for the therapeutic benefit of these drugs in the treatment of congestive heart failure or rather a reduction in sympathetic nervous tone seen already at low plasma levels is currently disputed [3,4]. Little is known about molecular and functional changes in the heart caused by chronic application of cardiac glycosides. The present study aimed: (1) to investigate the influence of chronic ouabain on cardiac contraction parameters and their sensitivity to isoprenaline; (2) to search for changes in gene expression of various components of the β-adrenergic signal transduction pathway that may affect the sensitivity of ventricular heart muscle to catecholamines [candidates were β_1-, β_2-, α_1-adrenoceptors, G-proteins of the adenylyl cyclase pathway (G_s, $G_{i\alpha\text{-}2}$, $G_{i\alpha\text{-}3}$), and the newly recognized protein phosphatase inhibitor-1 (I-1)];

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and (3) to test the influence of chronic ouabain on Ca^{2+}-uptake into membrane vesicles and on gene expression of proteins involved in myocardial calcium homeostasis [candidates were the SR-Ca^{2+}-ATPase 2a (SERCA), phospholamban (PLB), calsequestrin (CSQ), the ryanodine-receptor (RyR), and the Na^+/Ca^{2+}-exchanger (NCX)].

Methods

Animal model

Male Wistar rats (240–300 g) were treated with subcutaneous infusions by osmotic minipumps (Alzet ML23, AIza Corporation, Palo Alto, CA, USA) as described before [5,6]. Rats were treated for 4 days with either 0.9% NaCl as control or ouabain (6.5 mg/kg/day; Sigma, Deisenhofen, Germany). Heart rate and PQ-time were measured daily by recording electrocardiogram in conscious rats 2 days prior and during treatment. The rats were killed by a blow to the neck and bleeding from the carotid arteries. Hearts were removed rapidly and blood was removed in ice-cold, gassed bathing solution [6]. Contraction experiments were begun immediately thereafter whereas the other experiments were carried out on ventricular tissue samples frozen in liquid nitrogen and stored at −80 °C until use.

Contraction experiments

The experiments were performed on electrically driven (1 Hz) left papillary muscles as described previously [5,6]. The mean diameter of papillary muscles was 1.08 ± 0.28 mm in the ouabain-treated group and 1.18 ± 0.27 mm in controls (NaCl).

Radioligand binding

β-Adrenoceptors. Experiments were performed on crude ventricular membranes as previously reported [5,6] using six different concentrations of [(−)-^{125}I]iodocyanopindolol (ICYP). The relative amounts of β_1- and β_2-adrenoceptors were determined by binding of ICYP (50 pmol/L) in the presence and absence of 300 nmol/L of the highly selective β_1-adrenoceptor antagonist (±)-CGP 20712A.

α_1-Adrenoceptors. Binding experiments were performed as described [7] using nine different concentrations (0.01–1.0 nmol/L) of the radioligand [^{3}H]prazosin (New England Nuclear, Dreieich, Germany).

Pertussis-toxin-catalyzed ADP-ribosylation

Homogenization of ventricular tissue, pertussis-toxin (PTX)-catalyzed ADP-ribosylation, SDS–polyacrylamide gel electrophoresis, and quantification of autoradiographic signals were performed as described in detail previously [5].

Western-blotting

Western-blots of SDS extracts were performed as previously published [8]. After blotting on nitrocellulose, sheets were incubated with monoclonal antibody AI raised against PLB [9], monoclonal antibody AB 465 against SERCA [10], and polyclonal antibody Ca1 against CSQ [11]. Antibodies were kindly provided by Dr. L.R. Jones, Indianapolis.

Measurement of oxalate calcium-uptake

Oxalate-stimulated calcium-uptake was determined on crude frozen homogenates prepared from frozen tissue essentially as described by [12] in the presence of 30 mM of the phosphatase inhibitor NaF.

Total RNA preparation

Total RNA was isolated from ventricular tissue (frozen immediately after sacrifice) according to the protocol of Chomczynski and Sacchi [13].

cDNA probes

cDNAs for the rat RyR and the rat NCX were cloned from a rat λ-ZAP library as previously described [14] using as probe human cDNA generously furnished by P. Allen and K.D. Philipson (NCX), respectively. The identity of the clones was confirmed by double-strand sequencing. The cDNAs of GAPDH and I-1 were cloned by reverse transcriptase PCR using the following primers: I-1 fw 5′-GCGGCC ATGGAGCCCGACAAC-3′ and rv 5′-CATCCAGTGTCCATGA ACTTCC-3′; GAPDH fw 5′-CTTCACCACCATGGAGAAGG-3′ and rv 5′-ATTGAGAGCAATGCCAGCC-3′. cDNAs of PLB and SERCA 2a were used as described before [14,15]. cDNAs of $G_{i\alpha\text{-}2}$, $G_{i\alpha\text{-}3}$, and G_s were kind gifts of Dr. R.R. Reed and used as previously described [6]. The cDNA probes were labeled (Mega prime kit, Amersham Buchler, Braunschweig, Germany) with [^{32}P]dCTP (3.000 Ci/mmol, New England Dupont, Bad Homburg, Germany) to a specific activity of greater 1×10^9 dpm/μg.

cRNA standard

For an accurate quantification of mRNAs sense cRNA standards of each cDNA were obtained by in vitro transcription of unilaterally linearized plasmids using T3 and T7 RNA polymerase (Boehringer–Mannheim, Mannheim, FRG). Six to eight different cRNA concentrations adjusted to 6 μg of total RNA by addition of tRNA were blotted in duplicate on each slot-blot membrane as standard [16].

Hybridization procedure

Hybond N nylon membranes were hybridized as described before [8]. The membranes were washed with increasing stringency to a final wash in 0.2× SSC, 0.1% SDS at 50–65 °C and exposed to X-ray films (X-OMAT AR; Kodak) for 2–5 days. The intensity of the autoradiographic signal was measured by two-dimensional densitometry (TLC II, CAMAG, Berlin, Germany). Each blot was hybridized with the GAPDH-cDNA probe, an end-labeled poly(A)$^+$ oligo of 16–21 bp, and a 18s rRNA oligo 5′-ACGGTATCTGATCGTCTTCGAACC-3′ in order to normalize the results and to rule out uneven loading.

RNAse protection assay

As Northern-blot signals of RyR, NCX, and I-1 were just seen with 20 μg poly(A)$^+$-RNA, the more sensitive RNAse protection assay was used. The experiments were accomplished using the RPA II Ribonuclease Protection Assay Kit (Ambion, Austin, TX, USA). Experiments were performed as a multiplex assay for quantification of up to 4 mRNAs per tube. The following cRNA probes were used: a 520 bp I-1-probe of 6.61×10^8 dpm/μg, a 330 bp NCX-probe of 1.35×10^9 dpm/μg, a 240 bp RyR-probe of 1.35×10^9 dpm/μg, and a 110 bp $G_{s\alpha}$-probe of 1.35×10^8 dpm/μg. Quantification of $G_{s\alpha}$-mRNA, which was unaffected by ouabain application, was performed as internal standard to control for even loading and for loss of RNA during the procedure. Protected fragments were separated by denaturing polyacrylamide gel electrophoresis using Long-Ranger solution (AT Biochem, Malvern, PA, USA).

Statistics

Values presented are arithmetic means ± SEM. Statistical significance was determined by Student's t test for paired and unpaired observations; $p < 0.05$ was considered significant.

Results

Animal model

Body weight (248–272 g), heart rate (402–473 beats/min), and PQ-time (41–52 ms at a heart rate of 450 beats/min) did not differ between ouabain- and NaCl-treated rats before and during the experiment (NaCl $n = 13$ and ouabain $n = 8$). Ouabain did not affect the weight of ventricles (753.6 ± 15.8 vs. 765.3 ± 10.9 mg in NaCl), the weight of right atrium (33.2 ± 2.6 vs. 36.5 ± 2.8 mg), the weight of left atrium (46.6 ± 6.1 vs. 43.3 ± 3.5 mg), the yield of total RNA per milligram of cardiac tissue (1.27 ± 0.07 vs. 1.28 ± 0.04 μg), the protein concentration per cardiac tissue (148 ± 16 vs. 153 ± 22 mg/g) or the vesicular protein concentration (10.8 ± 1.1 vs. 11.1 ± 1.3 mg/g).

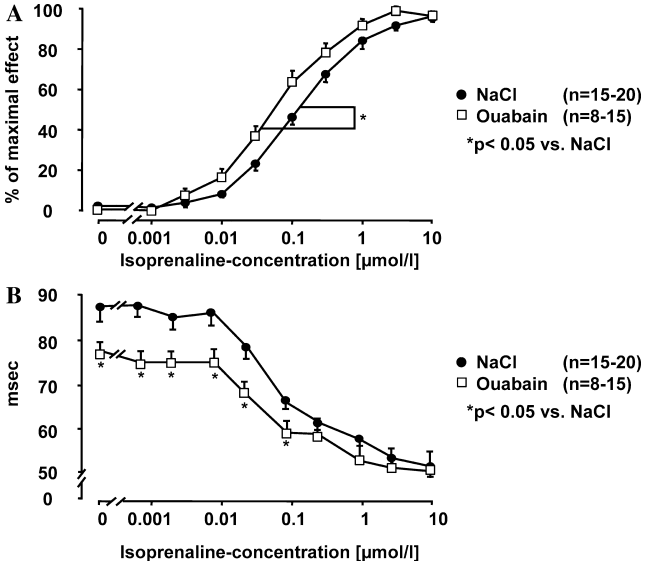

Fig. 1. Effect of isoprenaline. Influence of a 4-day infusion of 0.9% NaCl and 6.5 mg/kg/day ouabain on the effect of isoprenaline on force of contraction (A) and time-to-90% relaxation (B) of electrically driven left ventricular papillary muscles of rats at different isoprenaline concentrations. Ordinate in (A) indicates change in force of contraction in percent of the maximal effect.

Contractile function of isolated papillary muscles

Under basal conditions (35 °C, 1 Hz, 1.8 mmol/L calcium) papillary muscles from ouabain-treated rats contracted with the same force of contraction [twitch amplitude: 3.9 ± 0.7 vs. 3.8 ± 0.7 mN (NaCl)] and time-to-peak tension [58.2 ± 1.5 vs. 56.5 ± 1.9 ms (NaCl)] as control muscles. The positive inotropic response to increased extracellular calcium (1.8–5.4 mmol/L) was similar in both groups [increase from 3.9 ± 0.7 to 8.0 ± 0.8 (ouabain) and from 3.8 ± 0.7 to 8.6 ± 1.3 mN (NaCl)]. After five washes with fresh Tyrode's solution an isoprenaline concentration–response curve (0.001–10 μmol/L) was performed. The maximal positive inotropic effect was similar in both groups [increase from 2.7 ± 0.4 to 7.4 ± 1.0 mN (ouabain) and from 2.5 ± 0.3 to 7.9 ± 1.2 mN (NaCl)]. However, the concentration–response curve of isoprenaline was shifted to the left by a factor of 2 in ouabain-treated muscles (EC_{50} 76.8 ± 16.0 vs. 170.9 ± 28.7 nM; Fig. 1A). In addition, the time of 90% relaxation was significantly abbreviated by 10–15 ms or 11–17% (Fig. 1B). It is noteworthy that 6 out of 14 papillary muscles from the ouabain-treated rats became arrhythmic during stimulation with isoproterenol compared to 5 out of 20 preparations from control animals, although this effect did not reach statistical significance.

β/α-Adrenoceptor signal transduction

The density of β_1- and β_2-adrenoceptors and the affinity of [^{125}I]ICYP were unchanged after ouabain application (Table 1). Likewise, the density of α_1-adrenoceptors and the affinity of [^{3}H]prazosin binding were unaffected by ouabain pretreatment (Table 1).

Table 1
Influence of a 4-day infusion of 0.9% NaCl or 6.5 mg/kg/day ouabain on mRNA and protein levels of the GTP-binding proteins $G_{i\alpha\text{-}2}$, $G_{i\alpha\text{-}3}$, and $G_{s\alpha}$, and on the density and affinity of α- and β-adrenoceptors

	Method	NaCl	*n*	Ouabain	*n*	Unit
$G_{i\alpha\text{-}2}$-mRNA	Slot-blot	72 ± 2	9	73 ± 2	9	pg/μg total RNA
$G_{i\alpha\text{-}3}$-mRNA	Slot-blot	19 ± 2	9	18 ± 2	9	pg/μg total RNA
$G_{i\alpha}$-protein	PTX ADP-ribosylation	1183 ± 62	10	991 ± 51	10	U/μg protein
$G_{s\alpha}$-mRNA	Slot-blot	230 ± 19	9	260 ± 23	9	pg/μg total RNA
α_1-Adrenoceptor-densitiy	[^{3}H]prazosin-binding	42.5 ± 2.3	8	41.3 ± 2.4	8	fmol/mg protein
[^{3}H]prazosin-affinity	[^{3}H]prazosin-binding	0.057 ± 0.0007	8	0.0052 ± 0.0008	8	nmol/L
β-Adrenoceptor-density	[^{125}I]ICYP-binding	61 ± 3	6	62 ± 6	6	fmol/mg protein
[^{125}I]ICYP-affinity	[^{125}I]ICYP-binding	0.021 ± 0.005	6	0.016 ± 0.003	6	nmol/L
β_1-Adrenoceptor-density	[^{125}I]ICYP-binding	38.6 ± 2.3	6	43.4 ± 2.5	6	fmol/mg protein
β_2-Adrenoceptor-density	[^{125}I]ICYP-binding	23.1 ± 1.2	6	19.8 ± 1.1	6	fmol/mg protein

Data are presented as means ± SEM.

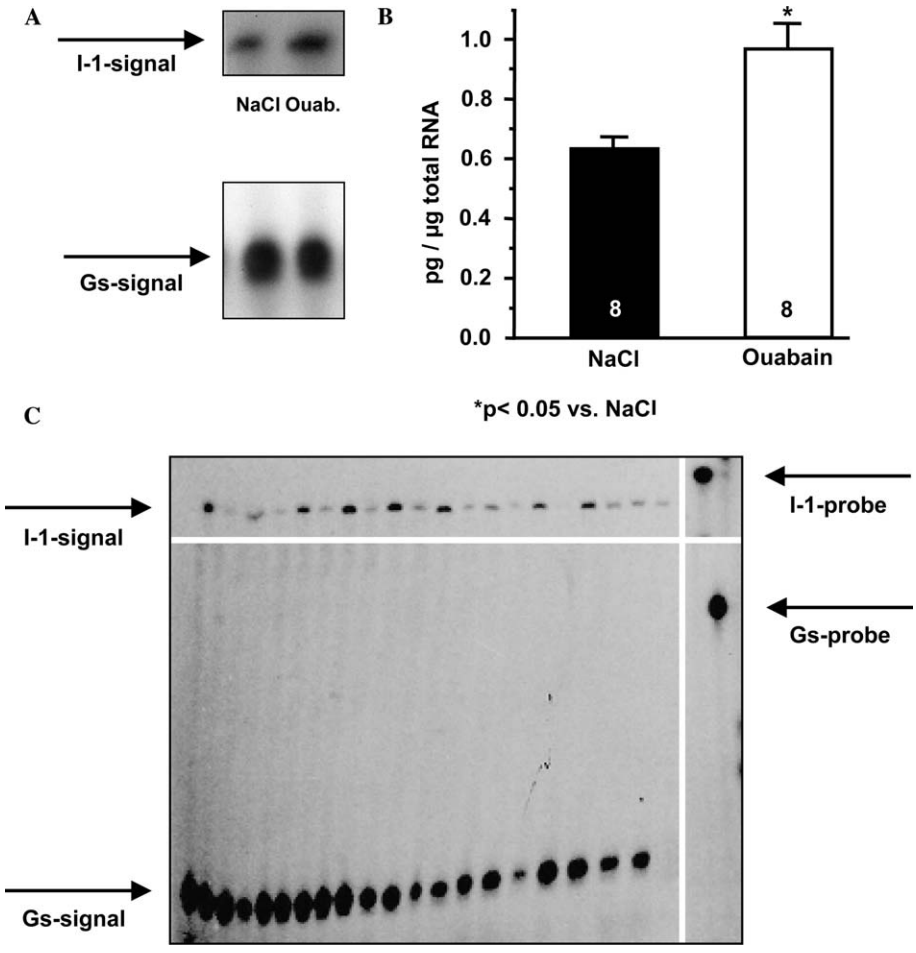

Fig. 2. Protein phosphatase inhibitor-1 mRNA. Influence of a 4-day infusion of 0.9% NaCl and 6.5 mg/kg/day ouabain on the mRNA level of the protein phosphatase inhibitor-1 (I-1). (A) Representative multiplex RNase protection assay of I-1 and $G_{s\alpha}$. (B) Statistical analysis of I-1 normalized to expression of $G_{s\alpha}$, which was unchanged in this model (Table 1). Number in columns = number of hearts studied. (C) Representative multiplex RNase protection assay for quantification of I-1 and $G_{s\alpha}$-mRNA after treatment with different pharmacological substances. The difference in size between cRNA probe and protected fragment results from additional sequences of the plasmid vector generated by in vitro transcription. Original size: 40 × 50 cm.

mRNA abundance of the most prevalent cardiac inhibitory G-proteins $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$ and of $G_{s\alpha}$ [6] was unchanged as was the amount of $G_{i\alpha}$-proteins as determined by PTX- catalyzed ADP-ribosylation (Table 1). However, transcript levels of I-1 were increased by 52% after 4 days of ouabain application (Fig. 2).

Table 2
Influence of a 4-day infusion of 0.9% NaCl or 6.5 mg/kg/day ouabain on mRNA and protein levels of SERCA, PLB, and calsequestrin and on the mRNA level of the RyR

	Method	NaCl	*n*	Ouabain	*n*	Unit
SERCA-mRNA	Slot-blot	176 ± 11	13	162 ± 16	8	pg/µg total RNA
SERCA-protein	Western-blot	4.92 ± 0.47	8	5.03 ± 0.70	5	Mio. dpm/µg protein
PLB-mRNA	Slot-blot	70 ± 2.22	13	59 ± 4.18*	8	pg/µg total RNA
PLB-protein	Western-blot	7.23 ± 0.85	8	7.26 ± 0.32	5	Mio. dpm/µg protein
PLB/SERCA ratio mRNA		0.38 ± 0.11	13	0.42 ± 0.09	8	
PLB/SERCA ratio protein		1.53 ± 0.23	8	1.51 ± 0.16	5	
Calsequestrin-mRNA	Slot-blot	4.1 ± 0.2	13	3.8 ± 0.2	8	pg/µg total RNA
Calsequestrin-protein	Western-blot	7.15 ± 0.66	8	6.72 ± 0.23	5	Mio. dpm/µg protein
Ryanodine-rec.-mRNA	Protection-assay	7.5 ± 0.5	13	6.8+0.5	8	pg/µg total RNA

SERCA, PLB, and calsequestrin mRNA levels were determined by slot-blotting. The same results were obtained after normalization to expression of GAPDH-mRNA, 18s rRNA, and poly(A)$^+$-RNA (data not shown). RyR mRNA was quantified by RNAse protection assay. The raw data were normalized to $G_{s\alpha}$ mRNA levels that were unaffected by ouabain (Table 1). Data are presented as means ± SEM.

*$p < 0.05$ vs. NaCl.

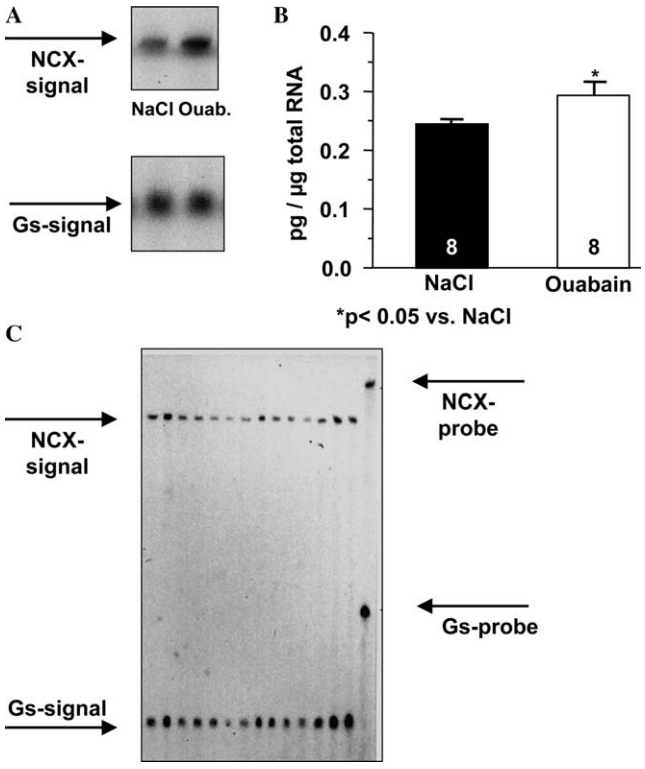

Fig. 3. Na^+/Ca^{2+}-exchanger mRNA. Influence of a 4-day infusion of 0.9% NaCl and 6.5 mg/kg/day ouabain on the mRNA level of Na^+/Ca^{2+}-exchanger (NCX). (A) Representative multiplex RNase protection assay of NCX and $G_{s\alpha}$. (B) Statistical analysis of NCX normalized to expression of $G_{s\alpha}$. Number in columns = number of hearts studied. (C) Representative multiplex RNase protection assay for quantification of NCX and $G_{s\alpha}$-mRNA after treatment with different pharmacological substances.

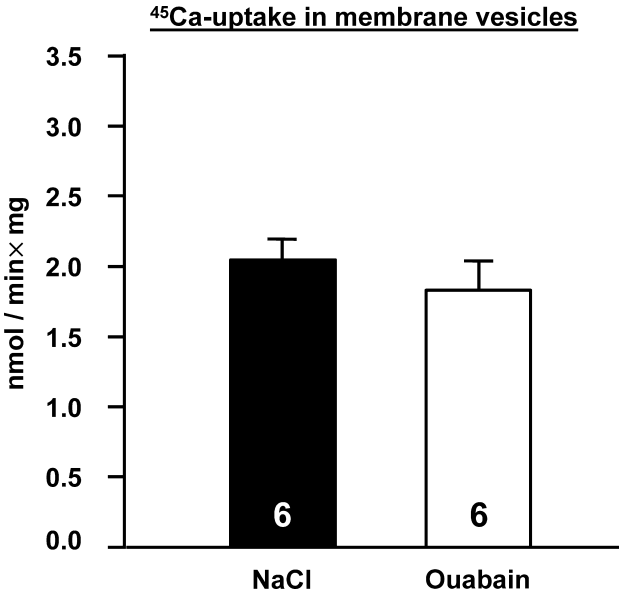

Fig. 4. ^{45}Ca-uptake. Influence of a 4-day infusion of 0.9% NaCl and 6.5 mg/kg/day ouabain on rate of oxalate-stimulated ^{45}Ca-uptake rate in cardiac membrane vesicles after 4 min of incubation. Number in columns = number of hearts studied.

Proteins involved in myocardial Ca^{2+} homeostasis

SERCA 2a mRNA and protein levels were unaffected by ouabain pretreatment. The mRNA of PLB was slightly decreased by 15% but PLB protein abundance was unchanged (Table 2). Ouabain did not alter the PLB/SERCA ratio either at the protein or mRNA level (Table 2). Likewise, neither CSQ mRNA and protein nor RyR mRNA abundance differed between ouabain-treated animals and controls (Table 2). However, ouabain treatment increased the amount of NCX by 26% (Fig. 3).

^{45}Ca-uptake in membrane vesicles

In control experiments calcium-uptake was dependent upon the addition of ATP and could be completely inhibited by thapsigargin, a specific antagonist of the SERCA. The ^{45}Ca-uptake was constant over a period of >6 min (not shown). Measurements were performed for 4 min incubation at 30 °C. No significant differences in rates of ^{45}Ca-uptake could be demonstrated between ventricles obtained after 4 days of ouabain treatment compared to NaCl controls (2.04 ± 0.09 nmol/min/mg NaCl vs. 1.91 ± 0.13 nmol/min/mg ouabain; Fig. 4).

Discussion

Despite their widespread use, the functional and molecular consequences of chronic treatment with cardiac glycosides in the heart are poorly documented. This is remarkable since chronic hemodynamic and cardiac responses to cardiac glycosides differ considerably from acute effects [2,17]. The present study demonstrates to our knowledge for the first time hastened cardiac relaxation of isolated heart muscle preparations and increased sensitivity to positive inotropic and lusitropic effects of isoprenaline after long-term ouabain treatment. These effects were accompanied by an increased expression of I-1 and NCX, whereas all other components of the β-adrenergic signal transduction pathway as well as the Ca^{2+} handling proteins investigated were unaffected.

The dose of ouabain of 6.5 mg/kg/day used in this study has been shown to be effective in rats without heart failure [18]. Given that acute ouabain application did not affect the time parameters of contraction in rat cardiac myocytes [19,20] the observed alterations in function cannot be ascribed to the presence of residual ouabain in the muscle tissue. In addition, in Langendorff-perfused rat hearts ouabain was completely washed out after 30 min [21]. We allowed equilibration with >2 changes in Tyrode's solution for at least

60 min. Hence, the functional changes are likely secondary to long-term changes in cardiac gene expression.

Increased sensitivity to isoprenaline

Long-term ouabain treatment resulted in a moderate, but significant, increase in the sensitivity of isolated papillary muscles to isoprenaline with regard to force of contraction, maximal contraction- and relaxation velocity, and relaxation time. In principle, the increased β-adrenergic sensitivity could be due to alterations in all components of the β-adrenergic signaling cascade. However, the density and affinity of β_1-, β_2-, and α_1-adrenoceptors as well as mRNA and protein levels of $G_{s\alpha}$, $G_{i\alpha\text{-}2}$, and $G_{i\alpha\text{-}3}$ remained unchanged. Protein levels of SERCA, PLB, and CSQ as well as ^{45}Ca-uptake rates were unaffected too. In contrast, expression level of the cardiac regulatory protein I-1 was markedly increased by >50%. I-1 protein levels are difficult to determine since expression levels are below the detection limit of standard Western-blots [22]. However, we found recently that cardiac I-1 protein and transcript levels are affected similarly in human heart failure, both qualitatively and quantitatively [23]. This suggests that expression of I-1 is predominantly regulated on the mRNA level and that ouabain infusion also increased I-1 protein levels.

I-1 is a highly specific and potent inhibitor (IC_{50} 1 nM) [24] of type 1 phosphatases (PP1). It is active only in its PKA-phosphorylated form [25–27]. Stimulation of the β-adrenergic signaling pathway in cardiac myocytes results in activation of cAMP-dependent protein kinase A (PKA) and phosphorylation of several PKA substrates, e.g., the sarcolemmal L-type Ca^{2+} channel, the ryanodine-receptor (RyR), and phospholamban (PLB) [28]. Phosphorylation of these substrates acts in concert to enhance contractility and accelerate relaxation in response to β-adrenergic stimulation. Isoprenaline also increases I-1 phosphorylation via cAMP-dependent PKA, which results in the inhibition of PP1 activity in the myocardium [29–31]. This in turn prevents dephosphorylation of the regulator proteins. The role of I-1 in the overall regulation of heart muscle function remained obscure for long. Recently, Carr et al. [32] showed that targeted ablation of I-1 increased PP1 activity, reduced isoprenaline-stimulated PLB-phosphorylation and impaired β-adrenergic contractile responses in the mouse heart. Our group provided direct evidence that I-1 plays an amplifier role in β-adrenergic signaling of cardiac myocytes. Adenoviral overexpression of I-1 significantly sensitized three-dimensional engineered heart tissue and freshly isolated adult rat cardiac myocytes to the positive inotropic effect of isoprenaline [22]. In the light of these data, the increase in I-1 after chronic ouabain offers an obvious explanation for the observed increase in sensitivity to β-adrenergic stimulation. One may argue that increased PLB-phosphorylation should accelerate ^{45}Ca-uptake in SR vesicles, but our assay was performed in SR preparations from unstimulated hearts in the absence of β-adrenergic stimulation where such influence is unlikely to be seen.

Hastened relaxation

Papillary muscles from ouabain-treated rats also exhibited hastened relaxation. Since major SR-Ca^{2+} handling proteins (i.e., SERCA, PLB or CSQ protein levels) as well as Ca^{2+}-uptake rates were unaffected by ouabain, alterations in SR function are unlikely to account for hastened relaxation in our study. In contrast, the 26% increase in NCX expression could theoretically well contribute to faster relaxation. Previously, an increased activity of the NCX was observed in neonatal rat cardiac myocytes exposed to ouabain over a 24 h period [33]. Furthermore, it was demonstrated in accordance with our findings that chronic ouabain (30 μmol/L, 48 h) treatment increases NCX abundance in isolated adult rat cardiomyocytes [34] while all other investigated proteins (SERCA 2a, PLB, and Na^+/K^+-ATPase α_1 and β_1) remained unchanged. The NCX has originally been considered as an ion transporting system that extrudes Ca^{2+} from the cytoplasm during diastole, driven by membrane potential and the Na^+ gradient ("forward mode"). However, it became clear that, at depolarized membrane potentials, i.e., the early phase of the action potential, and relatively high intracellular Na^+ concentrations, the NCX passes Na^+ in the outward and Ca^{2+} in the inward directions ("reverse mode" [35]). Under conditions of increased $[Na^+]_i$ this reverse mode predominates and gives rise to a net outward current (3 Na^+ vs. 1 Ca^{2+}) and thereby to action potential shortening [36–38]. NCX-mediated shortening of action potential may lead to shortening of relaxation. Several evidences argue for this assumption. (1) Transgenic overexpression of NCX resulted in faster relaxation under basal conditions [39,40]. (2) An increased abundance of the NCX is indicative of preserved diastolic function in human heart failure [41]. In the absence of cardiac glycosides (organ baths experiments) the increased NCX would favor Ca^{2+} efflux (forward mode) and thereby faster relaxation, in the presence of cardiac glycosides (in vivo, high $[Na^+]_i$) the increase in NCX would favor Ca^{2+} influx (reverse mode), but also repolarization and thereby shortening of the action potential. Therefore, the increase in NCX expression after long-term ouabain application could contribute to the observed hastened relaxation in our model. On the other hand, increased NCX is assumed to cause less Ca^{2+} filling of the SR and, due to its electrogenic current, an increased risk of arrhythmia [41], the most relevant side effect of cardiac glycosides. This has also been observed

in our studies as 6 out of 14 papillary muscles became arrhythmic after stimulation with isoproterenol compared to just 5 out of 20 preparations from control animals.

Taken together, chronic ouabain treatment was associated with increased expression of I-1 and NCX, increased β-adrenergic sensitivity, and accelerated relaxation of isolated papillary muscles. The molecular alterations are plausible candidates to explain the observed functional changes. Though the effects were of moderate size, hastened relaxation and β-adrenergic sensitization could participate in the beneficial effects of cardiac glycosides in the therapy of human heart failure, but could also contribute to their adverse effects.

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