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Effects of cantharidin on force of contraction and phosphatase activity in nonfailing and failing human hearts

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1 The effect of the phosphatase inhibitor, cantharidin (3-300 μM) on force of contraction was studied in isolated electrically driven right ventricular trabeculae carneae from human myocardium.

2 The positive inotropic effect of cantharidin started at a concentration of 100 μM with a positive inotropic effect to 199% and to 276% of the predrug value in nonfailing and failing human hearts, respectively.

3 Under basal conditions the contraction time parameters were prolonged in human heart failure vs. nonfailing preparations. However, the positive inotropic effect of cantharidin did not affect contraction time parameters. Thus, time to peak tension, time of relaxation and total contraction time were not shortened by cantharidin in nonfailing and failing preparations.

4 The phosphatase activity was unchanged in preparations from failing hearts compared to nonfailing hearts.

5 Cantharidin inhibited phosphatase activity in a concentration-dependent manner. The IC_{50} value of cantharidin was about 3 μM in both nonfailing and failing human myocardium.

6 The positive inotropic effect of cantharidin was similar in nonfailing and failing human hearts, accompanied by a similar inhibitory effect of cantharidin on the phosphatase activity. The positive inotropic effect of cantharidin in failing hearts was as strong as the effect of isoprenaline in nonfailing hearts.

7 It is concluded that the treatment with a phosphatase inhibitor may offer a new positive inotropic modality for the treatment of human heart failure.

Keywords: Cantharidin; contractility; phosphatase activity; human myocardium

Introduction

In human heart failure the β-adrenoceptor/adenylyl cyclase signalling pathway is desensitized with a consequently diminished inotropic effect to β-adrenoceptor agonists (Bristow *et al.*, 1982). Furthermore, the positive inotropic effect of phosphodiesterase inhibitors (unspecific and type III inhibitors), which act independently of the β-adrenoceptor signalling pathway, is attenuated (Feldman *et al.*, 1987; Steinfath *et al.*, 1992). Reductions in the number of β-adrenoceptors and an increase of inhibitory GTP-binding proteins were suggested to be involved (Bristow *et al.*, 1982; Feldman *et al.*, 1988; Neumann *et al.*, 1988). Fittingly, the cyclic AMP formation after β-adrenoceptor stimulation is diminished in failing myocardium (Danielsen *et al.*, 1989).

Beyond the β-adrenoceptor/adenylyl cyclase signalling pathway the contractility of the heart is controlled by the phosphorylation state of regulatory proteins. The phosphorylation state reflects the activity of both, protein kinases and phosphatases. Protein kinase A is activated by cyclic AMP and mediates the β-adrenoceptor effects. Protein kinases phosphorylate regulatory proteins in the heart and thereby induce a positive inotropic effect. Phosphatases induce via dephosphorylation a reduction in the phosphorylation state of regulatory proteins and influence the contractility of the heart.

Only scant information is available about the phosphatase system in human hearts. However, phosphatases are theoretically as important as protein kinases in regulating the activity of regulatory proteins in the heart. Two major classes of phosphatases have been identified, namely phosphatases type 1 and type 2, but their functional role in the heart is not well characterized (Shenolikar *et al.*, 1991). Because of the attenuated positive inotropic effect of β-adrenoceptor agonists, it is conceivable that phosphatase inhibitors could act as a new therapeutic agent in the treatment of heart failure. Thus, we studied cantharidin, a phosphatase inhibitor which inhibits phosphatases type 1 and type 2A (Honkanen, 1993). Both types of phosphatases are present and functionally relevant in the heart (Shenolikar *et al.*, 1991) and may contribute to the diminished responsiveness to positive inotropic agents.

Thus, we have studied the effect of cantharidin, a phosphatase inhibitor, on force of contraction and contraction time parameters in preparations from nonfailing and failing human hearts to investigate whether phosphatase inhibitors might be possible positive inotropic agents in human heart failure. Since the inotropic effect of cantharidin may depend on the level of phosphatase activity in the heart, the phosphatase activity in failing and nonfailing human hearts was compared.

Methods

Heart tissue

Procedures for obtaining human tissue complied with the Helsinki Declaration. Permission for these experiments was obtained from the local Ethics Committee. Nonfailing hearts were obtained from prospective organ donors whose hearts could not be used because of surgical reasons or blood group incompatibility ($n=5$). On inspection these hearts appeared to have normal ventricles. Failing hearts were obtained from patients undergoing orthotopic heart transplantation due to end-stage heart failure resulting from idiopathic dilated cardiomyopathy ($n=5$). All of the patients were classified as New York Heart Association (NYHA) class IV with markedly

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abnormal pretransplant haemodynamics. Aortic and pulmonary valves were excised from nonfailing hearts and later used for valve replacement. Medical treatment for the patients with heart failure consisted of nitrates, cardiac glycosides, diuretics and angiotensin-converting enzyme inhibitors. Cardiac surgery was performed with neuroleptic narcotic combination (haloperidol, fentanyl, nitrous oxide). Right ventricular tissue was quickly transferred in gassed bathing solution at 4°C from the theatre to the laboratory and used for functional studies. In addition, pieces of right ventricular myocardium were frozen in liquid nitrogen immediately after cardiectomy and used for biochemical assays.

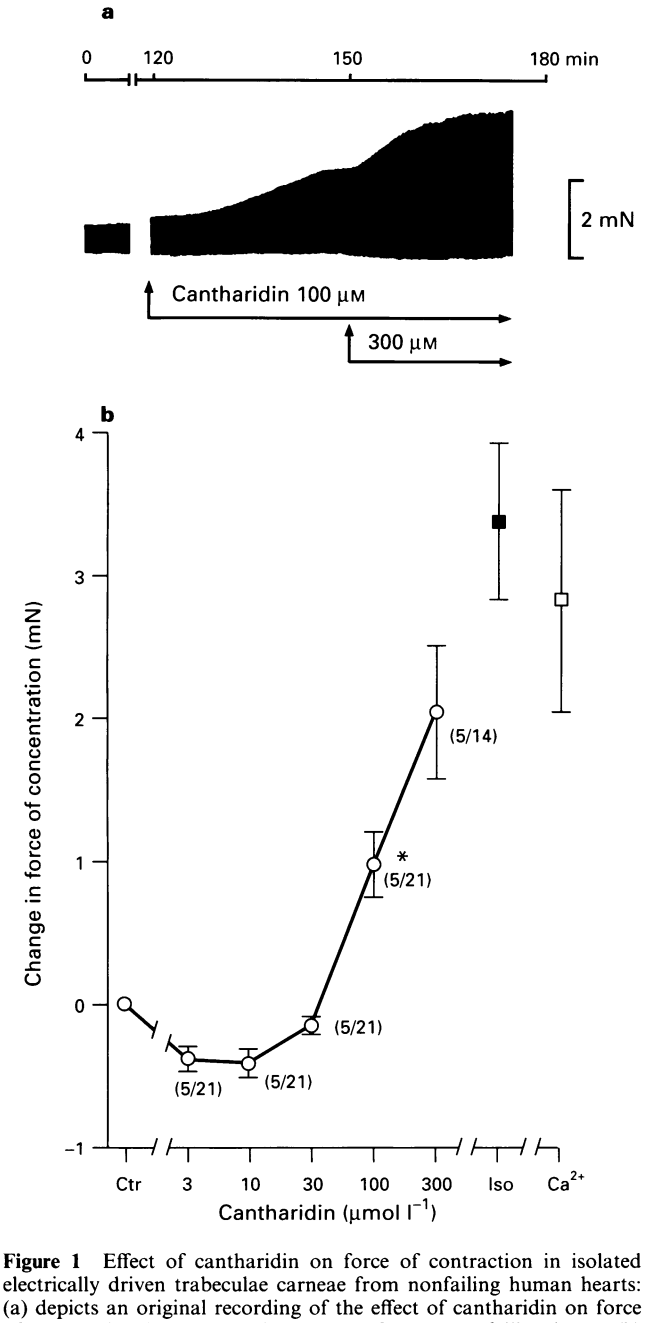

Figure 1 Effect of cantharidin on force of contraction in isolated electrically driven trabeculae carneae from nonfailing human hearts: (a) depicts an original recording of the effect of cantharidin on force of contraction in a trabeculae carneae from a nonfailing heart; (b) represents the effect of cantharidin (○). Ctr = predrug value. Abscissa scale: concentration of cantharidin. Ordinate scale: change in force of contraction in milliNewtons (mN). Numbers are indicated in parentheses. The first number represents the hearts and the second number the corresponding trabeculae carneae. The effects of 100 μM isoprenaline (Iso ■) and the effect of 16.2 mM Ca^{2+} (□) are indicated. Significant differences (*$P<0.05$) vs. control (Ctr).

Contraction experiments

Contraction experiments were performed as described previously (Danielsen *et al.*, 1989). In brief, trabeculae carneae were isolated from right ventricles (diameter less than 1 mm, length 5–8 mm) which were dissected in gassed bathing solution at 4°C. The bathing solution contained (in mM): NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2EDTA (ethylenediaminetetraacetic acid) 0.05, ascorbic acid 0.28, glucose 5.05, continuously gassed with 95% O_2 and 5% CO_2 and maintained at 35°C (pH 7.4). Preparations were attached to a bipolar stimulating electrode and suspended individually in 10 ml glass tissue chambers for recording isometric contractions. Each muscle was stretched to the length of maximal force of contraction. Resting force (about 5 mN) was kept constant throughout the experiments. Trabeculae carneae were electrically stimulated by 0.5 Hz with rectangular pulses of 5 ms duration, the voltage was about 10–20% greater than threshold. All preparations were equilibrated in bathing solution until complete stabilization. During this period the bathing solution was changed every 15 min. Concentration-response curves were obtained cumulatively. Trabeculae were exposed to increasing concentrations of cantharidin (30 min each). After complete washout Ca^{2+} was added in a concentration of 12.6 mmol l^{-1} for 10 min. In parallel trabeculae were exposed to increasing concentrations of isoprenaline (5 min each). All experiments were performed in the presence of adenosine deaminase (1 μg ml^{-1}) in order to prevent interference from endogenous adenosine.

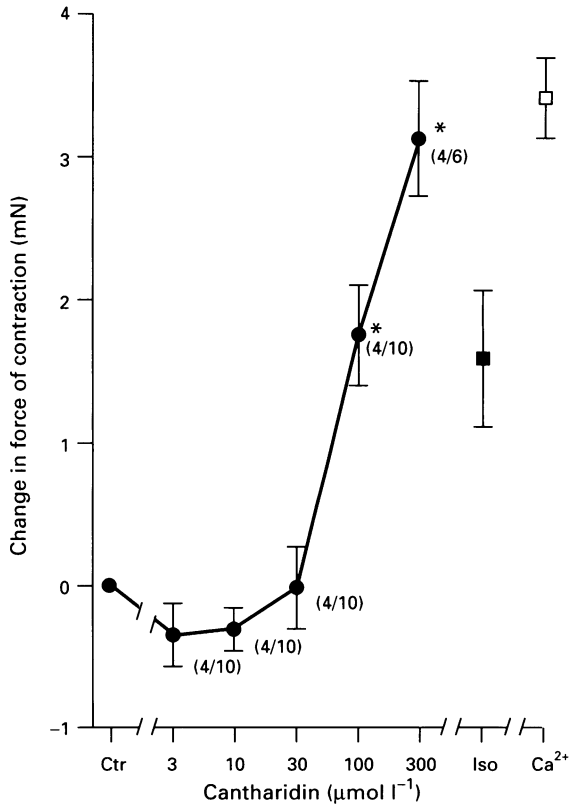

Figure 2 Effect of cantharidin on force of contraction in isolated electrically driven trabeculae carneae from failing human hearts (●). Ctr = predrug value. Abscissa scale: concentration of cantharidin. Ordinate scale: change in force of contraction in milliNewtons (mN). Numbers are indicated in parentheses. The first number represents the hearts and the second number the corresponding trabeculae carneae. The effect of 100 μM isoprenaline (Iso) (■) and the effect of 16.2 mM Ca^{2+} (□) are indicated. Significant difference (*$P<0.05$) vs. control (Ctr).

Preparation of homogenates

Homogenates were prepared as described previously (Neumann *et al.*, 1993). Frozen tissue was homogenized in 10 ml of a medium containing (in mM) EDTA 4.0, $Na_4H_2PO_7$ 1.0, NaF 10.0 and 0.1% (v/v) β-mercaptoethanol. The tissue was homogenized three times for 30 s each with a Polytron PT-10 (Kinematica, Lucern, Switzerland). The sample was sedimented for 20 min at 14000 *g*. The supernatant (subsequently referred as homogenate) was immediately frozen at −80°C.

Phosphatase assay

Assays for phosphatase activity were performed as described by Ahmad *et al.* (1989). Phosphatase activity was measured at 30°C using ^{32}P-phosphorylase a, as substrate. The 50 μl incubation mixture contained (in mM): Tris HCl 20.0 (pH 7.0) caffeine 5.0, EDTA 0.1, 0.1% (v/v) β-mercaptoethanol. The reaction was started by adding homogenate and terminated after 10 min by the addition of 10 μl 50% trichloroacetic acid. After 10 min on ice, precipitated protein was collected by centrifugation. An aliquot of the supernatant was counted in a liquid-scintillation counter.

Protein was measured according to the method of Bradford (1976).

Materials

^{32}P-phosphorylase a was prepared as described by Neumann *et al.* (1991). Compounds used were adenosine deaminase (Boehringer Mannheim, Mannheim, Germany) 1 gamma ^{32}P-ATP (NEN Du Pont, Bad Homburg; Germany), cantharidin (Sigma, St. Louis, U.S.A.), dimethyl sulphoxide (Sigma, St. Louis, U.S.A.), (±)-isoprenaline hydrochloride (Boehringer Ingelheim, Ingelheim, Germany). All other chemicals were of analytical or best commercial grade available. Deionized and twice distilled water was used throughout.

Analysis of data

Data shown are means ± s.e.mean. IC_{50} values indicating the concentration for half maximal inhibition were determined graphically. Statistical analysis was performed by means of one-way analysis of variance followed by Bonferroni's *t* test. A *P* value less than 0.05 was considered significant.

Results

In isolated electrically driven trabeculae carneae from nonfailing and failing human myocardium isoprenaline exerted a concentration-dependent positive inotropic effect. The positive inotropic effect of 10 μM isoprenaline was lower in failing than in nonfailing human hearts as reported before (Bristow *et al.*, 1982). This was additional functional evidence that the human hearts used for subsequent functional and biochemical studies were from diseased or nondiseased ventricles, respectively. Cantharidin increased force of contraction in a concentration-dependent manner in nonfailing (Figure 1) and failing (Figure 2) human hearts. The positive inotropic effect started at 100 μM; 300 μM cantharidin further increased force of contraction. A concentration of 300 μM cantharidin led to contracture in some trabeculae carneae which were therefore excluded from further analysis. Higher concentrations were not tested. Figure 1a depicts an original recording of the effect of cantharidin on force of contraction in electrically driven trabeculae carneae from a nonfailing heart. In nonfailing hearts (Figure 1b) force of contraction increased by 0.99 ± 0.23 mN (=99%) in 21 trabeculae carneae from 5 hearts ($n = 5/21$) after stimulation with 100 μM cantharidin and

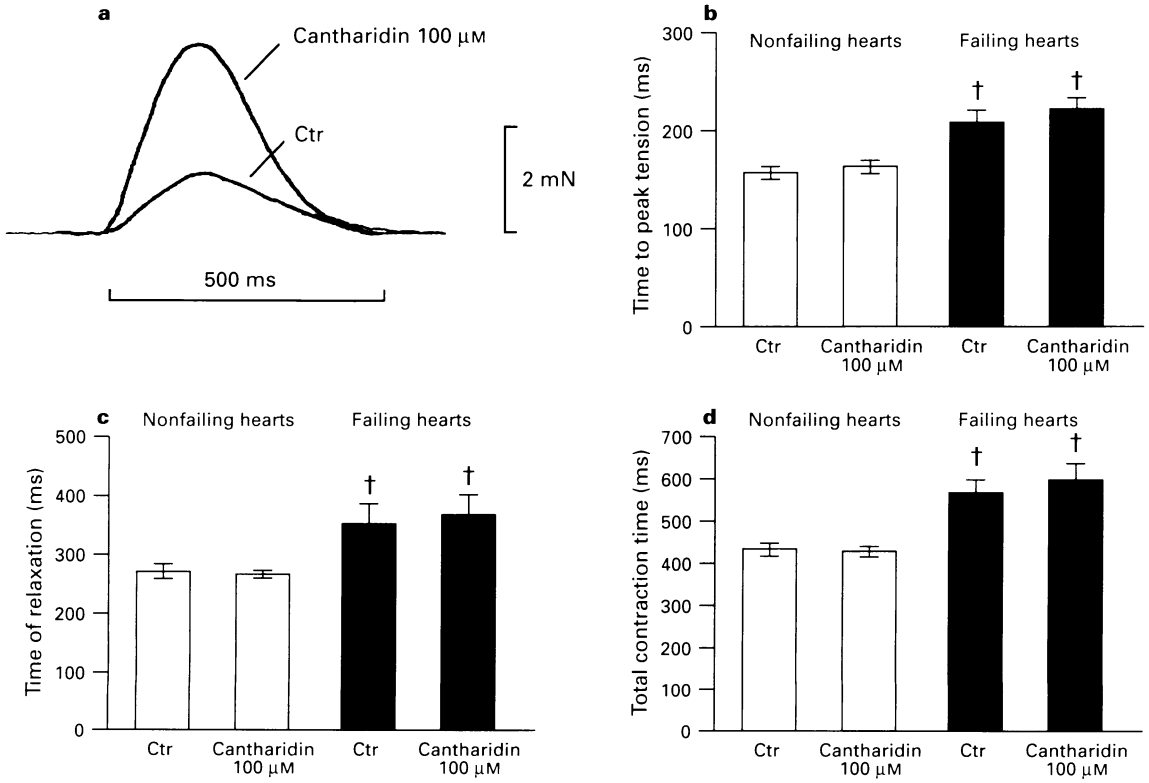

Figure 3 Effect of cantharidin on time parameters in isolated electrically driven trabeculae carneae: (a) shows an original recording of the effect of cantharidin on contractile parameters; (b) shows time to peak tension before (Ctr) and after stimulation with 100 μM cantharidin in nonfailing (open columns) and failing (solid columns) hearts; (c) depicts time of relaxation and (d) total contraction time before (Ctr) and after stimulation with 100 μM cantharidin. Ordinates represent the time parameter in milliseconds (ms). †Significant difference vs. control (Ctr).

by 2.05±0.46 mN (=205%) in 14 trabeculae from 5 hearts (n=5/14) after stimulation with 300 μM cantharidin. In comparison to failing hearts the force of contraction before stimulation with cantharidin was significantly lower in nonfailing than in failing hearts. In failing hearts (Figure 2) stimulation with 100 μM cantharidin increased force of contraction by 1.76±0.35 mN (=176%) (n=4/10) and 300 μM cantharidin further increased force of contraction by 3.13±0.41 mN (=310%) (n=4/6).

Figure 3a depicts an original recording of the effect of 100 μM cantharidin on contractile parameters. In trabeculae carneae from failing hearts time to peak tension (Figure 3b), time of relaxation (Figure 3c) and total contraction time (Figure 3d) were increased under control conditions in comparison to nonfailing hearts. Time to peak tension was 158±8 ms (n=5/21) and 211±13 ms (n=4/10), respectively.

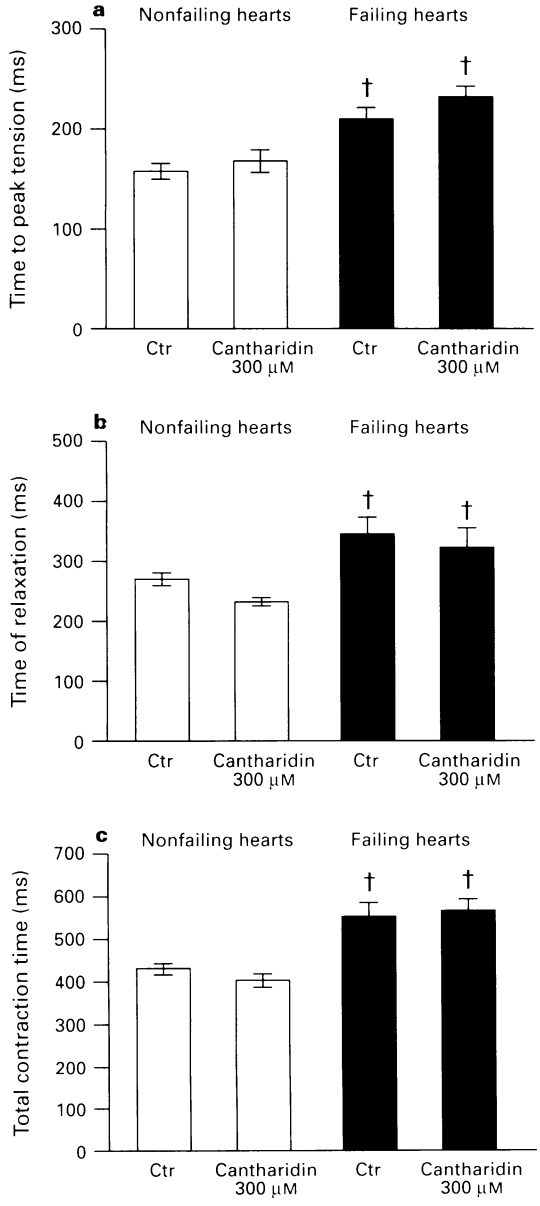

Figure 4 Effect of cantharidin on time parameter in isolated electrically driven trabeculae carneae from nonfailing (open columns) and failing (solid columns) hearts; (a) depicts time to peak tension before (Ctr) and after stimulation with 300 μM cantharidin; (b) depicts time of relaxation and (c) total contraction time before (Ctr) and after stimulation with 300 μM cantharidin. Ordinates represent the time parameter in milliseconds (ms). †Significant difference vs. control (Ctr).

Time of relaxation was increased from 272±11 ms (n=5/21) to 350±28 ms (n=4/10) and total contraction time was increased from 431±15 ms (n=5/21) to 561±32 ms (n=4/10) in failing vs. nonfailing hearts. Stimulation with 100 μM cantharidin did not alter contraction time parameters either in nonfailing or in failing human hearts (Figure 3b–d). Likewise, stimulation with 300 μM cantharidin did not affect time parameters (Figure 4a–c).

Phosphatase activity, more precisely using phosphorylase a as a substrate, measured in ventricular homogenates from nonfailing and failing hearts was not significantly different (Figure 5). Cantharidin concentration-dependently inhibited phosphatase activity in samples from nonfailing and failing hearts (Figure 6). The inhibitory effect started at 0.1 μM cantharidin in nonfailing and failing hearts. The IC_{50} was approximately 3 μM in homogenates from failing as well as nonfailing hearts. Thus, the inhibitory potency of cantharidin on phosphatase activity was identical in preparations from nonfailing and failing hearts. Moreover, phosphatase activity could be blocked completely by high concentrations of cantharidin in preparations from both failing and nonfailing human hearts.

Discussion

Cantharidin elicited a positive inotropic effect without affecting contraction time parameters in trabeculae carneae from nonfailing and failing human hearts. In contrast, β-adrenoceptor stimulation reduced time to peak tension and time of

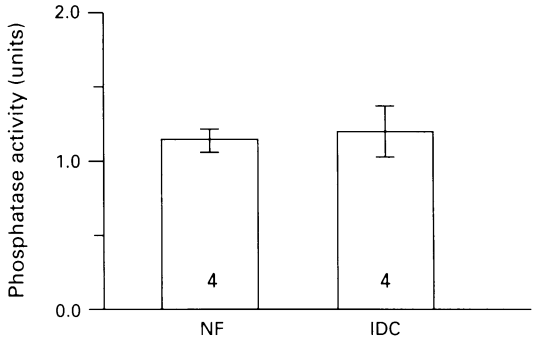

Figure 5 Phosphatase activity in preparations from nonfailing (NF) and failing human hearts (idiopathic dilated cardiomyopathy, IDC). Ordinate scale depicts the phosphatase activity in units. Numbers in columns indicate number of hearts studied.

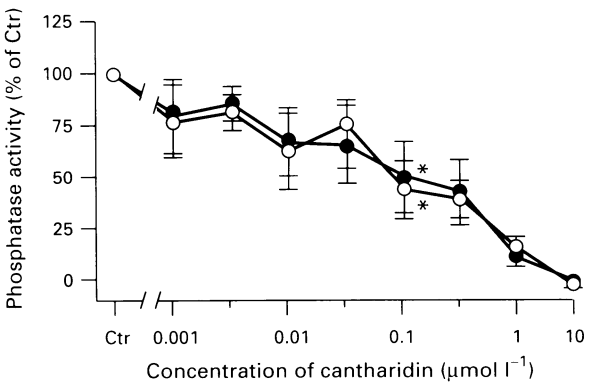

Figure 6 Inhibition of phosphatase activity by cantharidin in homogenates from nonfailing (○) and failing (●) human hearts. Abscissa scale: concentration of cantharidin. Ordinate scale: phosphatase activity as % of control (=DMSO). *The first concentration showing significant difference vs. Ctr.

relaxation in parallel experiments. The positive inotropic effect reached significant levels at 100 μM cantharidin. Interestingly, there was no difference in the apparent efficacy of cantharidin between nonfailing and failing human hearts. In preparations from the same patients, isoprenaline induced a concentration-dependent positive inotropic effect. The positive inotropic effect of isoprenaline was diminished in failing compared to nonfailing human hearts (Figure 2) as reported by Bristow *et al.* (1982). However, isoprenaline induced shortening of contraction time parameter (data not shown). Contraction time parameters (time to peak tension, time of relaxation and total contraction time) under basal conditions (that is absence of any inotropic drugs) were increased in preparations from failing vs. nonfailing human myocardium which is in agreement with previous data (Gwathmey *et al.*, 1987; Feldman *et al.*, 1987).

In end-stage human heart failure the β-adrenoceptor/adenylyl cyclase signalling pathway is desensitized, characterized by a reduction of β-adrenoceptor density and an increase in inhibitory GTP-binding proteins (Bristow *et al.*, 1982; Feldman *et al.*, 1988; Neumann *et al.*, 1988). The positive inotropic effect of β-adrenoceptor agonists and of phosphodiesterase inhibitors, which act via cyclic AMP are diminished in human heart failure (Bristow *et al.*, 1982; Feldman *et al.*, 1987). Cyclic AMP via cyclic AMP-dependent protein kinase leads to phosphorylation of cardiac regulatory proteins (e.g. L-type Ca^{2+} channels; phospholamban) and thus eventually to inotropy. The phosphorylation state of these proteins can also be increased by reduced activities of phosphatases. Therefore, we hypothesized that the phosphatase system is altered in human heart failure and may contribute to the impaired inotropy and relaxation. Phosphatases can be classified into type 1, 2A, 2B and 2C (Shenolikar *et al.*, 1991). Under our assay conditions we determined type 1 and type 2A phosphatases using phosphorylase a as a substrate. Moreover, 90% of cardiac phosphatases belongs to type 1 or type 2A (Shenolikar *et al.*, 1991). Furthermore, type 1 and type 2A phosphatases are of functional relevance because of the ability of type 1 and type 2A phosphatases to dephosphorylate important cardiac proteins like phospholamban and myosin light chains (Ishihara *et al.*, 1989; MacDougall *et al.*, 1991). Moreover, *in vitro* experiments demonstrated a dephosphorylation of troponin I by type 2A phosphatase (Mumby *et al.*, 1987). The phosphorylation of cardiac regulatory proteins influences the contractility of the heart. For instance, phospholamban regulates the function of the SR-Ca^{2+}-ATPase, which has been reported to be important for both relaxation and contraction (Lindemann *et al.*, 1983; Luo *et al.*, 1994). Phosphorylation of phospholamban is associated with activation of the SR-Ca^{2+}-ATPase and dephosphorylation reverses activation. Hence, the phosphorylation state of proteins indeed influences contractile parameters and may be involved in the impaired relaxation and diminished contractile response to positive inotropic agents in human heart failure.

The phosphorylation state and thus the function of regulatory proteins is tightly controlled by the activities of protein kinases and protein phosphatases. However, the activity of cyclic AMP-dependent protein kinase is unaltered in human heart failure (Böhm *et al.*, 1994). Therefore, the phosphatase system could be altered and responsible for the reduced contractility in human end-stage heart failure. However, the phosphatase activity was unchanged in homogenates from nonfailing and failing myocardium. It is practically impossible to label human heart preparation with ^{32}P-orthophosphate to study the effects of phosphatase inhibitors on the phosphorylation state of cardiac proteins in contracting samples from nonfailing and failing hearts. However, previous data using ^{32}P-orthophosphate labelled guinea-pig ventricular cardiomyocytes reported an increased phosphorylation of phospholamban, troponin I and myosin light chain after stimulation with phosphatase inhibitors like okadaic acid, calyculin A and cantharidin (Neumann *et al.*, 1993; 1994; 1995), indicate that indeed phosphatases and their inhibition influence the phosphorylation state of regulatory phosphoproteins. Moreover, we have reported a reduced phosphorylation of phospholamban under basal conditions in failing hearts (Bartel *et al.*, 1996). Therefore, it is still conceivable that an altered activity of a special phospholamban phosphatase or of a phosphatase in a specialized subcellular compartment (e.g. sarcoplasmic reticulum) of the heart may be detectable in future work.

Next, we studied whether or not phosphatase inhibitors are active in human cardiac preparations and whether the increased activity of phosphatases in heart failure alters the potency and efficacy of phosphatase inhibitors. Cantharidin inhibits phosphatase activity of phosphatase type 1 and type 2A (Honkanen, 1993). Although, it has been reported that the inhibition of phosphatase type 2A by cantharidin is more potent than inhibition of type 1 phosphatases the selectivity for inhibition of 2A phosphatases is not high enough to attribute the effects of cantharidin solely to an inhibition of type 2A phosphatase (Neumann *et al.*, 1995). It has been reported that cantharidin increased the phosphorylation of phospholamban, troponin I, myosin light chains 2 and probably C-protein. Furthermore, cantharidin is capable of increasing ion current through L-type calcium channels. This was accompanied by an increased positive inotropic effect of cantharidin in isolated electrically stimulated guinea-pig papillary muscles and furthermore, accompanied by reduced contraction time parameter in guinea-pig papillary muscles (Neumann *et al.*, 1995).

This is the first account of phosphatase inhibitors exerting a positive inotropic effect in the human heart. The efficacy in nonfailing and failing human hearts was not different. However, due to toxic effects (contracture) a clear plateau of the concentration-response curve cannot be reached. In contrast a clear plateau was observed in guinea-pig papillary muscle. The positive inotropic effect even seemed to decrease at higher concentrations of cantharidin (30 μM). However, this apparent decline in force of contraction was not statistically significant. In contrast to human preparations, in guinea-pig papillary muscles we never observed contractures. Clearly one cannot rule out that mechanisms apart from inhibition of phosphatases contribute to the positive inotropic effect of cantharidin. Evidence for this is lacking at present. There is no evidence that cantharidin can increase cyclic AMP, or inhibit phosphodiesterase activity. Conceivably even contractures might be due to phosphatase inhibition. One can speculate that extensive phosphorylation of unidentified substrates in the human sarcoplasmic reticulum and an imbalance between release and uptake of calcium and finally the elevated diastolic calcium may lead to contracture.

In human in contrast to guinea-pig preparations (Neumann *et al.*, 1995) cantharidin did not affect time parameters of contraction. The positive inotropic effect after stimulation with cantharidin did not alter contraction time parameters in either nonfailing or failing human myocardium, respectively. It could be argued that the preparations were mechanically not able to exhibit reductions in time parameters or that the positive inotropic effect was too weak to shorten relaxation. However, β-adrenoceptor agonists reduced contraction time in the same preparations. Moreover, at equieffective concentrations compared to 300 μM cantharidin in our study in guinea-pig papillary muscles, cantharidin reduced relaxation time. Furthermore, the threshold for the positive inotropic effect of cantharidin was much higher in human (100 μM) than in guinea-pig ventricular preparations (1 μM). This indicates that species differences clearly exist. The phosphatase activity in homogenates of nonfailing and failing myocardium could be inhibited by incubation with cantharidin. The IC_{50} of phosphatase inhibition was about 3 μM in nonfailing human preparations and was in the same range as in guinea-pig myocardium ($IC_{50} = 0.6$ μM). The IC_{50} was about the same in preparations from failing human hearts. There is an apparent discrepancy in the IC_{50} (3 μM cantharidin) for phosphatase inhibition and the beginning of the positive inotropic effect

(100 μM cantharidin). However, the same was reported with other phosphatase inhibitors (Neumann *et al.*, 1993; 1994). This could be caused by an impaired diffusion of cantharidin into papillary muscle in comparison to homogenate in the phosphatase assay. Additional mechanisms must come into play because it is not easy to explain why diffusion should be much more impaired in human traceculae than in guinea-pig papillary muscles. For instance, the phosphatase assay employed reflects only the phosphatase activity whereas the *in vivo* contraction experiments are also influenced by the activity of protein kinases which might be different in guinea-pig and human hearts.

The EC_{50} for isoprenaline in guinea-pig papillary muscles and trabeculae from nonfailing and failing human hearts is identical (about 0.02 μM in both cases, e.g. Steinfath *et al.*, 1992). Thus, the much higher concentrations of cantharidin necessary for a positive inotropic effect in failing and nonfailing human preparations cannot be explained by a generally higher sensitivity of guinea-pig preparation to positive inotropic drugs.

This paper clearly demonstrates that phosphatase inhibition is a viable positive inotropic mechanism even in failing human hearts. Future work should address the question of the relative importance of type 1 and type 2 phosphatase. To that end contraction experiments with okadaic acid would be useful. This would further strengthen the causal link between phosphatase inhibition and positive inotropy. Obviously, cantharidin is a toxic compound that cannot be applied in patients. However, its simple chemical structure might lead to usable derivatives. In summary, phosphatases inhibitors might be a new positive inotropic modality in the treatment of human end-stage heart failure.

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