Deficient production of cyclic AMP: pharmacologic evidence of an important cause of contractile dysfunction in patients with end-stage heart failure

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ABSTRACT We studied the effects of different classes of inotropic drugs belonging to the second general class bypass cyclic AMP as a mediator and act by other mechanisms to increase the free ionized calcium (Ca++) available for activation of the contractile apparatus. The major examples of clinically useful agents belonging to this second class are digitalis and systemic infusions of Ca++, although other potentially useful drugs such as the calcium-channel blockers remain under development.

Over the past decade, many new inotropic agents have been developed, some of which, like the phosphodiesterase inhibitors amrinone and milrinone and drugs with β-agonist properties like dobutamine and prenalterol, have reached the stage of active clinical testing. The predominant action of each of these agents is to stimulate production of intracellular cyclic AMP. Although some of these drugs have shown promise as therapeutic agents, their relative effectiveness in patients with the most severe degrees of heart failure and markedly diminished cardiac reserve remains to be determined. The purpose of this study was to deter-
mine the relative effectiveness of different classes of positive inotropic agents on working myocardium isolated from patients with end-stage heart failure and to further define the functional abnormalities of failing heart muscle.

Methods

Hearts were obtained from seven patients with end-stage heart failure undergoing cardiac transplantation at the Brigham and Women’s Hospital. Pertinent clinicopathologic details are summarized in table 1. Diagnoses included idiopathic dilated cardiomyopathy (patients 1 to 3), “ischemic cardiomyopathy,” i.e., heart failure resulting from coronary artery disease (patients 4 to 6), and heart failure secondary to mitral valve disease (patient 7). In brief, gross examination of all hearts (the specimens included predominantly right and left ventricles with only a rim of atria) revealed biventricular hypertrophy and dilatation. Each of the patients with coronary artery disease had severe chronic coronary occlusions with remote, large transmural myocardial infarcts.

Histologically, patients with coronary artery disease had essentially normal but hypertrophied myocardium in areas distant from discrete infarcts; patients with idiopathic dilated cardiomyopathy and myocardial failure following valvular disease had generalized hypertrophy and multifocal perivascular replacement and interstitial fibrosis diffusely throughout the ventricular myocardium. Medications being administered at the time of transplantation included furosemide (n = 7), spironolactone (n = 3), captopril (n = 4), hydralazine (n = 2), digoxin (n = 7), coumadin (n = 6), milrinone (n = 1), intravenous amrinone (n = 1), intravenous dopamine (n = 1), and intravenous propranolol (n = 1). No patients were receiving oral β-blockade. Control hearts were obtained from five donors without heart failure who died from noncardiac causes. Three of the patients were trauma victims who suffered brain death, one was a patient who died secondary to rupture of a cerebral aneurysm, and the fifth was a patient who died from respiratory causes in the intensive care unit and subsequently underwent rapid autopsy. On gross inspection, all five of these hearts showed normally structured left and right ventricles. Four of these patients were men and one was a woman; their ages ranged from 15 to 58 years. Informed consent was obtained from all recipients and from the families of all prospective brain-dead donors before transplantation or excision.

After removal from the thoracic cavity, the left and right ventricles were opened surgically and experimental tissue was removed and placed into a container of oxygenated physiologic salt solution (see composition below) at room temperature. Thin trabeculae carneae were selected for use in the experiments; the mean fiber diameter and length of the trabeculae carneae were, respectively, 1.1 ± 0.15 and 7.3 ± 1.9 mm in the control hearts (n = 20) and 1.0 ± 0.3 and 8.2 ± 2.4 mm in the heart failure group (n = 25). Five of 20 muscles in the control group and seven of 25 muscles in the heart failure group were obtained from the left ventricle; the remainder were obtained from the right ventricle. Due to the relatively small total number of fibers studied and the similarity of results, data from left and right ventricular trabeculae carneae were analyzed together for both the control and failure groups. The muscles were placed in an organ bath at 30°C and connected to a Statham force transducer (UC-2) for recording of isometric tension development. The preparations were stimulated to contract at 3 sec intervals through a punctate electrode with the use of voltage that was 10% above threshold and pulse durations of 5 msec, and were allowed to equilibrate for 1 to 1 ½ hr, during which time they were stretched to the length at which maximal isometric tension

### TABLE 1

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/sex</th>
<th>Specimen wt. (g)</th>
<th>Gross appearance</th>
<th>Coronary artery obstructions</th>
<th>Microscopic findings</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35/M</td>
<td>420</td>
<td>Moderate generalized dilatation</td>
<td>Focal 50% LAD</td>
<td>Moderate</td>
<td>Focal</td>
</tr>
<tr>
<td>2</td>
<td>48/M</td>
<td>490</td>
<td>Moderate generalized dilatation</td>
<td>Focal 40% LAD</td>
<td>Moderate</td>
<td>Focal</td>
</tr>
<tr>
<td>3</td>
<td>35/M</td>
<td>460</td>
<td>Mild generalized dilatation</td>
<td>Mild</td>
<td>Mild</td>
<td>Mild</td>
</tr>
<tr>
<td>4</td>
<td>44/M</td>
<td>620</td>
<td>Moderate dilatation; remote transmural anteroposteri- apical MI</td>
<td>100% LAD; 100% RCA</td>
<td>Moderate</td>
<td>Focal</td>
</tr>
<tr>
<td>5</td>
<td>40/M</td>
<td>510</td>
<td>Moderate dilatation; remote anteroseptal MI with aneurysm and mural thrombus</td>
<td>100% LAD; 90% LCX</td>
<td>Moderate</td>
<td>Focal</td>
</tr>
<tr>
<td>6</td>
<td>33/M</td>
<td>387</td>
<td>Moderate dilatation; remote anterior MI with aneurysm</td>
<td>90% LAD</td>
<td>Moderate</td>
<td>Minimal</td>
</tr>
<tr>
<td>7</td>
<td>55/M</td>
<td>477</td>
<td>Moderate generalized dilatation</td>
<td>Focal 50% LAD</td>
<td>Severe</td>
<td>Focal</td>
</tr>
</tbody>
</table>

LAD = left anterior descending artery; LCX = left circumflex artery; RCA = right coronary artery; MV = mitral valve; LV = left ventricular; MI = myocardial infarction.

*Areas distant from MIs.
developed. Isometric tension development was recorded on a Gould strip-chart recorder at a paper speed of 0.25 mm/sec. To record time course changes, paper speed was increased to 100 mm/sec for several twitches. The data were analyzed with regard to peak isometric tension development, time to peak tension measured from the stimulus artifact, and time to 50% decline from peak tension (RT 1/2). The composition of the physiologic salt solution used in these experiments was (in mM): NaCl, 120; KCl, 5.9; glucose, 11.5; NaHCO3, 25; NaH2PO4·H2O, 1.2; MgCl2·6H2O, 1.2; CaCl2, 2.5. The solution was bubbled with a gaseous mixture containing 95% O2, 5% CO2, to pH 7.4.

Cumulative dose-response curves were obtained for a variety of inotropic drugs and were expressed as a percentage of the maximal response to Ca++ obtained in each muscle or as the percent change from control tension. With the exceptions of milrinone and forskolin, each of these agents was dissolved in distilled water before being added to the bath. To obtain the calcium dose-response curves, a phosphate-free salt solution was placed in the bath and calcium was added in incremental doses. This process avoids calcium precipitation from the standard salt solution. Milrinone was dissolved in hydrochloric acid and the appropriate amount of this acidic stock solution was added to the bathing medium to produce the desired final concentration of drug. Before the solution was allowed to come into contact with the muscle, the pH was adjusted to 7.4 by the addition of small amounts of NaOH. The pH was again checked at the end of each response to make certain it did not change. No visible precipitate formed when the drug solutions were prepared in this manner. Similar amounts of acidic solution without milrinone were neutralized with NaOH; these solutions had no inotropic or time course effect on the muscle. Stock solutions of forskolin were prepared by dissolving the drug in an aqueous solution containing 40% ethanol; in the amounts used in these experiments, the diluent had no effect on force development. The following drugs and chemicals were used: milrinone (Sterling-Winthrop, Inc.), l-isoproterenol bitartrate (Sigma Chemical Co.), caffeine (Sigma Chemical Co.), forskolin (Calbiochem-Behring, Division of American Hoechst Corp.), CaCl2 (BDH Chemicals LTD), 3-isobutylmethylxanthine (Sigma Chemical Co.), and acetylthrophanthidin (Sigma Chemical Co.). The concentration of each drug or chemical is expressed as the final bath concentration.

Analysis of variance was used for statistical analysis; p values < .05 were considered indicative of a significant difference.

**Results**

Figure 1 shows the dose-response relationship for varying concentrations of extracellular calcium ([Ca++]). In a ventricular trabecula from a control heart and from a patient with end-stage biventricular failure. Figure 2 shows the mean responses for all of the muscles in both groups. Of interest, muscles from both the control and failing hearts generated similar mean tensions in response to [Ca++]. The calcium dose-response curves for both groups were parallel and essentially superimposable by statistical criteria (p > .1). These results indicate that the efficacy and potency of [Ca++], as an agonist was similar in muscles from both the control and heart failure groups.

Figure 3 shows the relative time course of isometric contraction and relaxation in the control and heart failure groups. Note that at all concentrations of [Ca++], studied, the time to peak tension and the RT 1/2 were significantly longer in the heart failure group than in the control muscle tissue. Moreover, changes in [Ca++], did not appear to alter the relative duration of the contraction in the muscles from the control or heart failure groups except at the highest concentrations, at which slight prolongation appeared to occur. The prolonged time course of the twitch in the heart failure group can be seen also in figure 1.

Figure 4 shows the effects of three different inotropic agonists on peak isometric tension development. In control muscles, isoproterenol (panel A) produced 85% of the peak isometric contractile response to
Ca++, in contrast, in the muscles from the failing hearts, isoproterenol was able to produce only 60% of peak isometric tension development, indicating that its efficacy is reduced in heart failure. In contrast, forskolin (figure 4, B), which activates adenylate cyclase, thereby increasing intracellular cyclic AMP concentrations, produced similar degrees of activation of muscles in the control and heart failure groups. Note that the dosage of forskolin that could be used was limited by the relative insolubility of this drug in physiologic solution. Figure 4, C, shows the effects of acetylstrophanthidin, a cardiotonic steroid that produces a positive inotropic effect by mechanisms unrelated to generation of cyclic AMP. Note that acetylstrophanthidin produced similar peak responses in both the control and failing muscle. The dosage of acetylstrophanthidin was limited by the occurrence of toxicity (i.e., an increase in diastolic tone or development of arrhythmias); the doses illustrated in this figure are those that produced an inotropic effect without evidence of toxicity.

Figure 5 shows the effects of three different agents known to increase intracellular cyclic AMP concentrations through inhibition of phosphodiesterase: milrinone (panel A), caffeine (panel B), and isobutylmethylxanthine (panel C). Note that, in contrast to the control cardiac muscle fibers, the fibers from the heart failure group showed little response to any of these agents. In contrast, in the presence of a minimally effective dose of forskolin (i.e., a dose that produced a barely detectable increase in isometric tension development), all phosphodiesterase inhibitors were capable of producing a marked positive inotropic response in the heart failure group.

**FIGURE 3.** Effects of changing the calcium concentration in the bathing medium on time to peak isometric tension development (A) and RT \( \frac{1}{2} \) (B). Open circles represent control values, and open triangles values from the heart failure group. Time is expressed in milliseconds.

**FIGURE 4.** Dose-response curves for isoproterenol (A), forskolin (B), and acetylstrophanthidin (C) expressed as percents of maximum isometric tension development in response to calcium. Open circles represent control values, and open triangles values from the heart failure group.
Discussion

Cyclic AMP is a major mediator of the amplitude and time course of cardiac contraction.\(^1, 2, 9\) This effect occurs through activation of a variety of cyclic AMP-dependent protein kinases that are capable of phosphorylating a series of proteins that can alter the handling of calcium in the sarcoplasm. Three major sites of phosphorylation include the regulatory proteins of the contractile elements, specifically troponin I; the sarcoplasmic reticulum, specifically phospholamban; and structures in the sarcolemma involved with regulation of the calcium channels (figure 6).\(^10, 11\) Cyclic AMP levels in lymphocytes from patients with congestive heart failure have been analyzed after the lymphocytes were incubated with isoproterenol and a threefold decrease in the amount of isoproterenol liberated into the reaction medium was found compared with normal.\(^12\) The Stanford group extended this observation by exposing cardiac muscle from transplant recipient and donor hearts to isoproterenol and determining the adenylate cyclase activity, normalizing their data for the adenylate cyclase activity that could be generated with sodium fluoride, an agent that stimulates adenylate cyclase directly.\(^13, 14, 15\) Their studies demonstrated a significant decrease in the amount of adenylate cyclase activity that could be generated in failing hearts, suggesting that basal cyclic AMP concentrations might be reduced in the presence of heart failure; these results were consistent with earlier reports of animal experiments.\(^16\)

Our results show that the response of failing human myocardium to inotropic drugs that act by mechanisms independent of cyclic AMP (i.e., increases in \([Ca^{++}]_o\), acetylcholine) is the same as that of control muscle, indicating that, under the appropriate conditions, working myocardium from failing human hearts can generate a normal increase in contractile force development (figures 2 and 4, C). On the other hand, drugs that act via sarcolemmal receptors to increase intracellular cyclic AMP concentrations, and drugs that act to prevent the breakdown of cyclic AMP, show markedly diminished effectiveness in muscle from the failing hearts compared with their effectiveness in control muscle (figures 4, A, and 5). In this regard, it is important to note that the bath concentrations of drugs used in this study exceeded the serum concentrations in patients receiving therapeutic doses of these agents. For example, therapeutic serum concentrations of milrinone\(^17\) are around 500 ng/ml, which corresponds to a bath concentration of \(2 \times 10^{-6} \text{M}\); however, bath concentrations of milrinone up to \(3 \times 10^{-4}\text{M}\) were

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**Figure 5.** Dose-response curves for milrinone (A), caffeine (B), and isobutylmethylxanthine (C) expressed as percent changes in isometric tension from baseline. Open circles represent control values, open triangles the values from the heart failure group, and closed triangles values from the heart failure group in the presence of a minimally effective dose of forskolin.
achieved in these experiments. Acetylstrophanthidin, at a total intravenous dose of 1.5 mg, has been shown by radionuclide scanning to significantly increase ventricular ejection fraction. A similar dose produced a serum level of 10 ng/ml, which corresponds to an organ bath concentration of $2 \times 10^{-8}$M. In our experiments, dose-response curves with isolated human muscle exceeded this level, reaching $4 \times 10^{-7}$M.

The responsiveness of the trabecular strips from patients with heart failure to phosphodiesterase inhibitors was restored in the presence of a low dose of forskolin, which acts to increase intracellular cyclic AMP levels through direct activation of adenylate cyclase. This suggests that generation of cyclic AMP can proceed normally under the appropriate stimulus and, moreover, that this stimulus may be lacking in the failing heart. Of interest, the dose-response curves for acetylstrophanthidin in the muscles from the heart failure group (figure 4, C) were located to the left of those in the control muscles, suggesting an increased potency of the cardiotonic steroids in muscles from patients with heart failure. This shift may also be related to decreased intracellular concentrations of cyclic AMP in the presence of heart failure, since cyclic AMP-mediated phosphorylation of troponin I has been reported to decrease the sensitivity of the contractile apparatus to $Ca^{++}$. 

We were surprised to find that muscles from the failing hearts were able to generate levels of isometric tension similar to those in the control muscle (figure 2). This has been reported by other investigators using human tissue and may reflect the selection for study of relatively viable trabeculae carneae from the failing hearts, since areas of dense fibrosis or necrosis were avoided. This raises the possibility that the peak isometric tension generated by our muscles in vitro may not reflect the contractile reserve of the heart as a whole. Moreover, the trabecular strips obtained from patients with heart failure showed varying degrees of compensatory hypertrophy; in animal experiments the development of hypertrophy alone without failure has been reported to affect the response of the heart to drugs. However, even if the validity of these concerns is proven by additional experiments, we emphasize that our experiments were designed to look at the "best case" response to inotropic interventions; pharmacologic effectiveness would be expected to be diminished even further in less viable muscle.

A more relevant question that needs to be addressed is the extent to which the remaining viable myocytes can contribute to the overall function of the heart in vivo even under the influence of optimal inotropic stimulation. The mean force per cross-sectional area generated by our control trabeculae carneae was somewhat lower than that reported for papillary muscles from other mammals studied under similar experimental conditions. This raises the possibility of damage to some of the experimental preparations during acquisition.

FIGURE 6. Diagram of excitation-contraction coupling in mammalian working myocardium (see text for details).
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sition. However, the standard level of force generation by human ventricular trabeculae carneae has not yet been determined, and it is important to note the levels of force generated by our muscles appear to be significantly higher than those reported by other investigators using isolated human working myocardium.\textsuperscript{13, 14, 30}

In contrast to peak tension development, the time course of isometric contraction and relaxation in muscle from the patients with heart failure was significantly longer than that in the control muscles (figures 1 and 3). We and others have previously reported that drugs and interventions that inhibit the uptake (i.e., caffeine) or release (i.e., ryanodine) of Ca\textsuperscript{2+} by the sarcoplasmic reticulum prolong the duration of the contraction and those that increase the rate of uptake (i.e., norepinephrine) abbreviate the contraction.\textsuperscript{11, 29} Our results suggest that heart failure may be associated with a decrease in the rate of Ca\textsuperscript{2+} release and/or uptake by intracellular stores, thereby prolonging the time course of tension development and impairing relaxation. This interpretation is consistent with reports of studies performed on isolated preparations of sarcoplasmic reticulum that indicate that failure is associated with depression of function.\textsuperscript{1, 31} However, prolongation of the time course of contraction has been reported to occur in cardiac hypertrophy without failure,\textsuperscript{32, 33} and the extent to which hypertrophy alone contributes to our findings remains to be determined. Attempts have been made to relate changes in the rate of Ca\textsuperscript{2+} uptake to alterations in the activity of Ca\textsuperscript{2+}-ATPase isolated from sarcoplasmic reticulum vesicles,\textsuperscript{34} but the results are not yet conclusive.

We have measured a prolonged time course of the calcium transient using aequorin in isolated ventricular trabecular muscles from patients with heart failure; in these muscles, the prolonged Ca\textsuperscript{2+} transient correlates well with prolonged relaxation of tension.\textsuperscript{35} These changes could also be due to a relative decrease in intracellular concentrations of cyclic AMP, which might be expected to result in decreased phosphorylation of phopholamban and a diminished rate of Ca\textsuperscript{2+} uptake by the sarcoplasmic reticulum. In support of this point, we found in the present study that maximally effective doses of forskolin produced a relatively greater abbreviation of RT \(\sqrt{2}\) in the heart failure group (35% reduction) than in the control muscle (19% reduction). It is difficult to attribute our findings to prior pharmacologic treatment since, with the exception of digoxin, the drug regimens that patients were receiving varied quite a bit (see Methods).

The \(\beta\)-receptor down-regulation that occurs in end-stage heart failure\textsuperscript{13-15, 36} would be expected to reduce basal concentrations of cyclic AMP within the cardiac cells, and provides a plausible explanation for our observations. Regardless of the underlying mechanism, these data indicate that agents that inhibit the degradation of cyclic AMP are not effective as single inotropic agents in myocardium from patients with heart failure. However, when myocardium from patients with heart failure is pretreated with an agent that increases the basal levels of cyclic AMP, phosphodiesterase inhibition becomes an effective inotropic intervention. These findings, if extrapolated to the failing heart in vivo, make a strong argument for some form of combined therapy (figure 5). Unfortunately, no cardioselective adenylyl cyclase activator is currently available and the generalized systemic actions of forskolin limit its usefulness as a therapeutic agent.\textsuperscript{5, 6} However, it is possible that another drug that acts to stimulate cyclic AMP production (for example, an orally active \(\beta\)-adrenergic agonist) in combination with a phosphodiesterase inhibitor may be effective inotropic therapy. Preliminary reports have appeared in the clinical literature that support this hypothesis.\textsuperscript{37, 38} The cardiac effects of most of the clinically available positive inotropic agents, including dopamine, dobutamine, isoproterenol, norepinephrine, epinephrine, and amrinone, occur by mechanisms that are dependent on the production of cyclic AMP within the cell, and, based on our data, each of these agents would be expected to lose its effectiveness with advancing degrees of heart failure.\textsuperscript{26} Several clinical reports have described a positive inotropic action of phosphodiesterase inhibitors in patients with heart failure.\textsuperscript{17, 20-23} Our data are not incompatible with these clinical observations since, although the cardiac \(\beta\)-adrenergic receptors may be down-regulated in heart failure, phosphodiesterase inhibition may be potentiated by the \(\beta\)-adrenergic effects of the high circulating levels of catecholamines that characterize this condition.\textsuperscript{39} As seen in figure 4, \(\beta\)-receptor stimulation did retain significant positive inotropic efficacy in failing muscle. Moreover, we emphasize that our studies examined only the direct positive inotropic actions of these agents in vitro. In vivo, drugs like amrinone and milrinone possess peripheral vascular actions that lead to vasodilation and significant afterload reduction, which may contribute to a favorable therapeutic response.\textsuperscript{26, 40, 41} The preservation of a positive inotropic response to acetylstrophanthidin in myocardium from patients with heart failure was striking in contrast to the relative diminution of the effects of the other standard agents; similar results have been reported by Wilmshurst et
These data suggest that, despite its low therapeutic-to-toxic ratio, digitalis may be relatively more effective as a single agent than the newer inotropic drugs with phosphodiesterase-agonist or β-adrenergic-agonist properties, at least in patients with more severe degrees of heart failure. However, we caution that in man drug toxicity may ensue before achievement of concentrations necessary to duplicate our results in vitro.

Our results suggest that future efforts at development of new inotropic drugs could be fruitfully directed toward introducing agents that act by additional mechanisms to increase calcium availability at the level of the myofilaments. Such drugs might include selective calcium-channel agonists or agents that selectively activate cardiac adenylate cyclase. In cases of predominant systolic failure, a drug that enhances the calcium sensitivity of the myofilaments (a possible action of higher doses of the experimental agent salmazole) might be useful, as would an agent that could increase the maximal Ca**+-activated force that can be generated by the heart.

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