Mechanism of preserved positive lusitropy by cAMP-dependent drugs in heart failure

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In heart failure, the contractile response to β-adrenergic stimulation is attenuated through the mechanism by which the basal intracellular cAMP level is decreased, i.e., downregulation of myocardial β-adrenergic receptor and increase in inhibitory guanine-nucleotide binding proteins (Gᵢ) (4, 5, 29). Both β-adrenergic agonists and phosphodiesterase (PDE) inhibitors have the capability to increase cAMP levels, leading to an enhancement of cardiac contractility (22). Although the clinical benefits of positive inotropism by these drugs have been well established in heart failure, the beneficial role of these cAMP-dependent inotropic agents on left ventricular (LV) relaxation in heart failure remained to be elucidated. In this regard, diastolic dysfunction is a major clinical problem in cardiac hypertrophy and/or failure as well as systolic dysfunction, and sometimes cardiac failure can be induced only by diastolic dysfunction even though systolic function is well preserved (3, 9, 10). Therefore, for the clinical use of these cAMP-dependent drugs, it is important to clarify the difference in positive lusitropic effects between the drugs.

Abnormal regulation of intracellular Ca²⁺ by the sarcoplasmic reticulum (SR) has been shown to be involved in the mechanism of contractile and relaxation dysfunction in heart failure (30, 32). Several investigators demonstrated that Ca²⁺ uptake by SR is decreased in association with the increased density of Ca²⁺-ATPase in cardiac hypertrophy and/or failure (8, 11, 18, 21, 23, 25, 28). In a previous report (42), we demonstrated that a low dose of milrinone substantially improved LV relaxation in normal dogs and that this positive lusitropic effect of milrinone was coupled with a direct acceleration of Ca²⁺ uptake by SR, probably caused by an inhibition of membrane-bound PDE III in SR and hence local elevation of cAMP.

The goal of this study was to evaluate the effects of two different cAMP-dependent drugs, milrinone and dobutamine, on LV relaxation in parallel with the assessment of SR Ca²⁺-ATPase activity in tachycardia-induced heart failure. Tachycardia induced by chronic pacing causes well-defined, predictable, and progressive LV dilatation, contractile dysfunction, and neurohormonal activation (2, 7, 27, 33, 41), and hence this model may more clearly resemble cardiac failure in humans than do previous studies of small-animal models of cardiac hypertrophy and/or failure.

MATERIALS AND METHODS

Heart failure was induced in beagle dogs of either sex by 3 wk of rapid ventricular pacing at a rate of 250 beats/min using an externally programmable miniature pacemaker (Medtronic, Minneapolis, MN). The specific details of the chronic instrumentation were as follows. Beagle dogs (n = 7 for control; n = 7 for rapid ventricular pacing) were sedated with morphine sulfate (15 mg sc) and thiopental sodium (150 mg iv). They were then anesthetized with isoflurane (2%, 1.5 l/min) and a mixture of nitrous oxide and oxygen (2:1), intubated with auffed endotracheal tube, and ventilated at a tidal volume of 22 ml/kg and a respiratory rate of 15 breaths/min. A bipolar pacing lead was fixed to the endocardial rapid
ventricular pacing surface and the distal lead was tunneled to a subcutaneous pocket constructed on the animal’s back, and they were connected to a pacemaker (Medtronic). Cefazolin (1 g iv) was administered before and after surgery. The control dogs underwent only a sham operation without pacing.

After 1 wk was allowed for animal recovery, the pacemaker was programmed to 250 beats/min. Dogs were monitored daily for clinical signs and symptoms of heart failure. With the pacing off after 3 wk of rapid ventricular pacing, the dogs were anesthetized after sedation as described above. LV pressure was measured by a 7-Fr micromanometer (Millar) inserted percutaneously via the carotid artery, and two-dimensional short-axis echocardiograms were obtained at the level of the head of the papillary muscle. Before insertion, the catheter was calibrated at 37°C with a mercury manometer. Zero shift of the pressure transducer was checked by a catheter was calibrated at 37°C with a mercury manometer. Dimensional short-axis echocardiograms were obtained at the pressure was measured by a 7-Fr micromanometer (Millar) inserted percutaneously via the carotid artery, and two-dimensional short-axis echocardiograms were obtained at the level of the head of the papillary muscle. Before insertion, the catheter was calibrated at 37°C with a mercury manometer. Zero shift of the pressure transducer was checked by a catheter was calibrated at 37°C with a mercury manometer. Dimensional short-axis echocardiograms were obtained at the level of the head of the papillary muscle. Before insertion, the catheter was calibrated at 37°C with a mercury manometer. Zero shift of the pressure transducer was checked by a catheter was calibrated at 37°C with a mercury manometer.

Experimental protocol. After control recording, a stepwise intravenous infusion of dobutamine (2–10 µg·kg⁻²·min⁻¹) was started. Five to ten minutes were allowed to obtain a steady state at each dose, and hemodynamic measurements were made at the end of each infusion rate. After the dobutamine infusion, pre-milrinone baseline hemodynamic values were established by waiting at least 3 h, by which time all measurements returned almost to the initial baseline values. Milrinone was then intravenously administered by a stepwise cumulative infusion of 4–20 µg/kg with repeat hemodynamic measurements. The order of drug administration was not randomized because of the long duration of milrinone’s hemodynamic effects.

All data were recorded at the end of an expiration on a multichannel recorder (Electronics for Medicine VR12) digitized at intervals of 2 ms with an on-line analog-to-digital converter. To obtain data for analysis, we used the average of 10 consecutive cardiac cycles. End diastole was defined by the peak of the R wave on the electrocardiogram. The time of the peak value of the negative first derivative of LV pressure (−dP/dt), obtained from the digital data of the dP/dt signal, was used to estimate end systole. The time constant of LV pressure decay (τ) was calculated as the negative inverse slope of the natural log of pressure vs. time relationship, with the assumption of a pressure asymptote of 0 mmHg and with use of data from peak −dP/dt to 10 mmHg above the end-diastolic pressure (40).

The care of the animals and the protocols used were in accordance with guidelines laid down by the Animal Ethics Committee of Yamaguchi University School of Medicine.

Preparation of LV crude homogenate. The homogenate was prepared as described previously (12). LV were homogenized in a solution containing 30 mM Tris-maleate, 0.3 M sucrose, 5 mg/l leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride, at pH 7.0. The homogenate was centrifuged at 5,500 g for 10 min, and the resultant supernatant was filtered through four layers of cheesecloth.

Ca²⁺-ATPase activity and cAMP assays. Ca²⁺-ATPase activity in LV crude homogenate (control, n = 6 preparations; heart failure, n = 6 preparations) was obtained by measuring the amount of Pi released during the reaction after ATP was added. The assay mixture in a total assay volume of 500 µl contained 150 mM KCl, 20 mM MES (pH 6.8), 0.3 mM MgCl₂, 10 mM Na₃HPO₄, 10 mM NaF, 6 µM ionophore A-23187, 0.32 mM CaCl₂, 0.5 mM EGTA (free [Ca²⁺] = 1 µM), and crude homogenate (0.125 mg). To start the reaction, 1.0 mM ATP was added to the above priming solution in the presence or absence of dobutamine (0.1–10 µM) or milrinone (0.1–10 µM). The amount of P_i reacted was calculated by converting nanometers (absorbance of 0.1% malachite green) to nanomoles by means of a standard linear line (13, 24).

The cAMP content in LV crude homogenate (control, n = 6 preparations; heart failure, n = 6 preparations) was determined with an enzyme immunoassay kit (Biotrak, cAMP Enzyme immunoassay system, Amersham International) according to the kit instructions.

Statistics. Unpaired t-test was used to compare the hemodynamic data between control and heart failure. Changes within the same group were analyzed by one-way ANOVA for repeated measures and subsequent Fisher’s protected least significant difference (PLSD). Differences between two groups were analyzed by two-way ANOVA and subsequent Fisher’s PLSD. Data are presented as means ± SE. Statistical significance was defined by P < 0.05.

RESULTS

Hemodynamics in the basal condition. Hemodynamics are summarized in Table 1. After chronic rapid ventricular pacing, heart rate, LV end-diastolic pressure, and LV internal diameters were all increased, compared with control. As for the parameters of LV systolic function, the peak +dP/dt of LV pressure, cardiac output, and fractional shortening were significantly decreased. As for the parameters of LV diastolic function, the time constant of LV pressure decay during isovolumic relaxation period (τ) was prolonged after rapid right ventricular pacing.

Hemodynamic changes after administration of milrinone or dobutamine. Hemodynamics before and after infusion of milrinone or dobutamine are summarized in Table 2. After the administration of dobutamine (2–10 µg·kg⁻¹·min⁻¹), LV peak pressure was slightly increased in control conditions and unchanged in heart failure. LV end-diastolic pressure tended to increase in control and was unchanged in heart failure. Peak +dP/dt increased and τ was shortened in both groups. As shown in Fig. 1, peak +dP/dt increased to a lesser extent in heart failure than in control, whereas τ was shortened to a similar extent as in control.

Table 1. Hemodynamics and echocardiographic data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 7)</th>
<th>Heart Failure (n = 7)</th>
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<tbody>
<tr>
<td>HR, beats/min</td>
<td>87 ± 2</td>
<td>107 ± 4*</td>
</tr>
<tr>
<td>LVPSP, mmHg</td>
<td>101 ± 6</td>
<td>105 ± 6</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4.4 ± 0.7</td>
<td>20.0 ± 1.3*</td>
</tr>
<tr>
<td>Peak +dP/dt, mmHg/s</td>
<td>2,124 ± 189</td>
<td>1,398 ± 88*</td>
</tr>
<tr>
<td>τ, ms</td>
<td>29 ± 1.2</td>
<td>42 ± 1.5*</td>
</tr>
<tr>
<td>CO, l/min</td>
<td>1.7 ± 0.1</td>
<td>1.1 ± 0.2*</td>
</tr>
<tr>
<td>SV, ml</td>
<td>19.8 ± 1.0</td>
<td>10.1 ± 1.0*</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>27.8 ± 1.4</td>
<td>31.8 ± 1.2*</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>18.8 ± 0.9</td>
<td>29.0 ± 1.0*</td>
</tr>
<tr>
<td>%FS</td>
<td>32.4 ± 1.0</td>
<td>9.7 ± 1.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of dogs. HR, heart rate; LVPSP, left ventricular (LV) peak systolic pressure; LVEDP, LV end-diastolic pressure; peak +dP/dt, peak value of first derivative of LV pressure; τ, time constant of LV pressure decay during isovolumic relaxation period; CO, cardiac output; SV, stroke volume; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; %FS, % fractional shortening [(LVEDD – LVESD)/LVEDD × 100]. *P < 0.05 vs. control.
After the administration of milrinone, heart rate was slightly increased in control and unchanged in heart failure. LV peak pressure was unchanged in either control or heart failure. LV end-diastolic pressure decreased in both groups. Peak +dp/dt increased and \( \tau \) was shortened in both groups. As shown in Fig. 2, peak +dp/dt increased to a lesser extent in heart failure than in control, whereas \( \tau \) was shortened much more than in control by low doses of milrinone (4–12 \( \mu \)g/kg).

\( \text{Ca}^{2+}\)-ATPase activity and cAMP level in presence of milrinone or dobutamine. As summarized in Table 3, both cAMP and \( \text{Ca}^{2+}\)-ATPase activity in crude homogenate were significantly decreased in heart failure compared with control. After the addition of dobutamine, the cAMP increased in a dose-dependent manner to a lesser extent in heart failure than in control (Fig. 3A). \( \text{Ca}^{2+}\)-ATPase activity was increased in a dose-dependent manner in both groups (Fig. 3B). There was no significant difference in the percent increase of \( \text{Ca}^{2+}\)-ATPase activity from baseline between control and heart failure. On the other hand, after milrinone was added, cAMP increased similarly in both groups (Fig. 4A), whereas \( \text{Ca}^{2+}\)-ATPase activity was increased to a greater extent in heart failure than in control at low doses of milrinone (Fig. 4B).

Figure 5 shows the relationship between the percent change of \( \text{Ca}^{2+}\)-ATPase activity and the percent change of cAMP in the presence of dobutamine (Fig. 5A) or milrinone (Fig. 5B). In the presence of either dobutamine or milrinone, \( \text{Ca}^{2+}\)-ATPase activity (%)-cAMP (%) relationship curves were shifted to the left in heart failure compared with control, indicating higher sensitivity of \( \text{Ca}^{2+}\)-ATPase activity to cAMP in heart failure. Compared with dobutamine, low doses of milrinone exerted a substantial increase in \( \text{Ca}^{2+}\)-ATPase activity in heart failure, at a given increase in cAMP.

In the presence of 1 \( \mu \)M thapsigargin (SR \( \text{Ca}^{2+}\)-ATPase inhibitor), \( \text{Ca}^{2+}\)-ATPase activity was decreased by 17.7 ± 3.1% in normal homogenate and by 19.6 ± 1.8% in heart failure homogenate. There was no significant difference in the percentage of the thapsigargin-sensitive portion of \( \text{Ca}^{2+}\)-ATPase activity to cAMP in heart failure. Compared with dobutamine, low doses of milrinone exerted a substantial increase in \( \text{Ca}^{2+}\)-ATPase activity in heart failure, at a given increase in cAMP.
The major findings of this study are as follows. First, in heart failure, the positive lusitropic effect of either milrinone or dobutamine was well preserved in association with the increased sensitivity of SR Ca\textsuperscript{2+}-ATPase activity to cAMP. Second, in particular, the positive lusitropic effect of low doses of milrinone was more prominent in heart failure than in normal conditions, associated with a marked stimulation of SR Ca\textsuperscript{2+}-ATPase activity.

Preservation of positive lusitropy in heart failure. Much evidence has accumulated that in heart failure, the positive inotropic response to catecholamine is significantly decreased, whereas positive lusitropy is well preserved (26, 31). The reduction of the positive inotropic action of dobutamine after heart failure might be caused by high production of NO (17). Keaney et al. (16) found that intracoronary infusion of the nitric oxide synthase inhibitor \(N^G\)-nitro-L-arginine methyl ester increased peak \(\pm dP/dt\) in response to intracoronary infusions of either dobutamine or isoproterenol in the in situ canine heart. Consistent with these previous reports, we found that both dobutamine and milrinone caused less increase in LV contractility in heart failure than under normal conditions, whereas lusitropic responses to both drugs were well preserved. Although the underlying mechanism is still unclear, in heart failure the lusitropic response might be coupled more efficiently to cAMP than the inotropic responses on stimulation of either dobutamine or milrinone. Sensitivity of the lusitropic cascade (i.e., phosphorylation of phospholamban, interaction of phospholamban to Ca\textsuperscript{2+}-ATPase, etc.) to cAMP may be increased in heart failure. As a matter of fact, we found that Ca\textsuperscript{2+}-ATPase activity and cAMP level

<table>
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<tr>
<th>Table 3. Ca\textsuperscript{2+}-ATPase activity and cAMP level</th>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}-ATPase activity, µM</td>
</tr>
<tr>
<td>cAMP, pmol/mg protein</td>
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Values are means ± SE; n = 6 dogs. *P < 0.05 vs control.
activity in heart failure was more enhanced at a given increase in cAMP either by dobutamine or by milrinone than under normal conditions (Fig. 5). As another possibility, the recently proposed functional compartmentation of cAMP (1, 6) might partly explain the discordance in inotropic or lusitropic action to cAMP-dependent drugs in heart failure. In heart failure, the cAMP produced either by dobutamine or by milrinone may be better compartmentalized for the elevation of local cAMP in lusitropic response than in inotropic response.

Mechanisms by which milrinone exerts predominant acceleration of LV relaxation in heart failure. Using the model of pacing-induced heart failure, two recent studies have shown that 1) gene expression and activity of PDE III are reduced in SR (37) and 2) cAMP and PDE levels are preferentially reduced in the subendocardium (34). These findings may account in part for the reduced positive inotropic effect of milrinone and also the predominant positive lusitropy and activation of Ca$^{2+}$-ATPase by low doses of milrinone.

Recently, a particulate, high-affinity type IV cAMP-PDE activity was shown to exist in cardiac SR (14). In this regard, much evidence has accumulated that certain cardiotonic agents (milrinone, imazodan, and amrinone) inhibit this SR membrane-bound "low K_m" or "cGMP-inhibited" PDE type IV isozyme (35, 38, 39) and exert their contractile effects through subtle alterations in the metabolism of cAMP (15, 19). With regard to this, functional compartmentation of cAMP and protein kinases was previously proposed for cardiac muscle (1, 6), and intracellular Ca$^{2+}$ mobilization might be affected by cAMP located in the particulate compartment of canine cardiac myocytes (6). Because milrinone, at submicromolar concentrations, inhibits specifically SR membrane-bound PDE III activity (19), the acceleration of SR Ca$^{2+}$-ATPase activity by low doses of milrinone might be caused by an inhibition of membrane-bound PDE III in SR, followed by a local elevation of cAMP, not the global cytosolic elevation of cAMP. Indeed, we previously demonstrated (42) that a low dose of milrinone significantly enhanced LV relaxation.
in association with the substantial increase in the rate of Ca\(^{2+}\) uptake by cardiac SR. This effect of milrinone might also explain why, in this study using LV homogenerate, milrinone exerted more increase in the Ca\(^{2+}\)-ATPase activity in heart failure than dobutamine at a given increase in cAMP (Fig. 5).

Limitations. Milrinone exerts a vasodilating effect as well as positive inotropic and lusitropic effects. Therefore, afterload reduction by this drug may possibly induce acceleration of LV relaxation. With regard to this, when LV pressure was increased by ~25% (mean 30 mmHg) by addition of phenylephrine together with milrinone, \(\tau\) was not significantly influenced in normal dogs (unpublished data). Furthermore, in the present study, the low dose of milrinone (4 \(\mu\)g/ml) did not change peak LV pressure and LV end-diastolic pressure, whereas \(\tau\) was shortened by 16% in heart failure. Therefore, the PDE-inhibitory effect of milrinone might be predominantly involved in the positive lusitropic effect, particularly at a low dose. At higher doses of milrinone, the mixed effects of PDE III inhibition and vasodilatation may play important roles in the improvement of LV relaxation.

In the present study, we measured whole Ca\(^{2+}\)-ATPase activities in myocardium. However, the SR Ca\(^{2+}\)-ATPase activity alone comprises ~25% of the total muscle homogenate activity, and ~75% of total Ca\(^{2+}\)-ATPase activity in muscle homogenate is provided by intracellular organs other than SR, i.e., Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase of the plasmalemma and myofibrils (36). Therefore, we should address the reaction of these other Ca\(^{2+}\)-ATPase activities to milrinone or dobutamine in this study. As shown in Fig. 6, thapsigargin-insensitive Ca\(^{2+}\)-ATPase activity, which comprises ~80% of total Ca\(^{2+}\)-ATPase activity, was influenced by neither dobutamine nor milrinone. Only the thapsigargin-sensitive portion of Ca\(^{2+}\)-ATPase activity was changed by these drugs, indicating that these positive inotropic agents indeed affect SR Ca\(^{2+}\)-ATPase activity.

In the present study, the degree of heart failure appears moderate (heart rate ~100 beats/min, no change in LV systolic pressure, LV end-diastolic pressure <25 mmHg) compared with the hemodynamic values reported in the literature (20, 26, 37). In severe heart failure, the cytosolic level of cAMP and the protein expression of Ca\(^{2+}\)-ATPase might substantially
...and decrease, and hence the positive lusitropic responses to cAMP-dependent drugs may deteriorate no matter how SR Ca\(^{2+}\)-ATPase activity is hypersensitized to cAMP. Also, it is likely that the sensitivity of SR Ca\(^{2+}\)-ATPase activity to cAMP may change depending on the severity of heart failure. Clearly, more work is needed.

In conclusion, 1) positive lusitropic effects by cAMP-dependent drugs were well preserved, probably because of the higher sensitivity of SR Ca\(^{2+}\)-ATPase activity to cAMP in heart failure, and 2) a low dose of milrinone substantially improved LV relaxation in association with stimulation of SR Ca\(^{2+}\)-ATPase activity in heart failure much more than under normal conditions.

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