Decrease in Myocardial Ryanodine Receptors and Altered Excitation-Contraction Coupling Early in the Development of Heart Failure

Dorothy E. Vatner, MD; Naoki Sato, MD; Kaname Kiuchi, MD; Richard P. Shannon, MD; Stephen F. Vatner, MD

Background Rapid ventricular pacing for 1 day reduced myocardial contractile function without inducing heart failure in conscious, chronically instrumented dogs. After 4 to 7 weeks of pacing, myocardial contractility was depressed further and overt signs of congestive heart failure, eg, ascites, dyspnea, and edema, were evident.

Methods and Results The mechanical restitution response, a physiological index of calcium release, was depressed at 1 day of rapid ventricular pacing. Postextrasystolic potentiation was also depressed by a similar amount, 14±3%, at 1 day after pacing. The response to isoproterenol 0.2 μg/kg per minute was depressed by a significantly greater amount (P<.05), 52±7%, at 1 day after pacing. 3H-ryanodine receptor binding fell from 1013±25 to 808±42 fmol/mg after 1 day of pacing and remained depressed at similar levels (782±61 fmol/mg) at 4 to 7 weeks when heart failure was manifest. Ryanodine receptor affinity was unchanged from control values. Neither dihydropyridine binding nor affinity for 3H-PN200-110 was changed from control levels. Within 5 days after recovery from 1 day of pacing, physiological responses to isoproterenol, postextrasystolic potentiation, and mechanical restitution recovered, as did 3H-ryanodine binding density.

Conclusions These findings suggest that the changes in excitation-contraction coupling and potentially the sarcoplasmic reticulum calcium release channel occur early in the development of heart failure and therefore may be important in the pathogenesis of the contractile abnormalities in this disease state. (Circulation. 1994;90:1423-1430.)

Key Words • receptors • calcium channels • pacing • heart failure

Most of the current knowledge regarding the pathogenesis of heart failure is derived from studies on chronic or end stages of the disease process. However, it could be argued that greater insights into mechanisms responsible for early alterations in contractile function before the onset of severe congestive heart failure and its secondary complications, eg, fibrosis, might be more important, particularly for designing therapeutic strategies to reverse the process. The canine model of pacing-induced heart failure is ideal for such investigations because there is a progressive decline in cardiac function resulting in dilated cardiomyopathy and severe congestive heart failure before the development of significant fibrosis or hypertrophy.1,2

This model is characterized by reduced responsiveness to β-adrenergic receptor stimulation, as is observed in chronic human heart failure.1,3,4 We recently observed that even after 1 day of pacing, before heart failure was manifest, there was a decrease in isoproterenol-stimulated adenyl cyclase,1 but the physiological response to isoproterenol stimulation was depressed by a much greater amount.4 Taken together, these data suggest that distal mechanisms, eg, alteration in excitation-contraction coupling, also may be deranged very early in the development of heart failure.

Recently, considerable attention has been directed to alterations in excitation-contraction coupling in the advanced stages of heart failure.2,5-8 At the cellular and molecular levels, however, most of the studies have focused on changes in Ca2+-ATPase9-13 and dihydropyridine-sensitive calcium channels,3,13-17 paying less attention to the sarcoplasmic reticulum (SR) junctional foot process ryanodine receptors.5,18 Even less is known regarding alterations in excitation-contraction coupling during the initial phase in the cardiac decompensation process.

The goal of the present study was to use the pacing-induced heart failure model to examine ryanodine and dihydropyridine receptors early in the development of the disease, that is, at 1 day after initiating pacing and 4 to 7 weeks later, when severe heart failure is manifest. One major deficiency in many prior in vitro studies derived from failing heart tissue is the absence of a physiological correlate for the altered biochemistry. Accordingly, the present investigation not only includes data on left ventricular (LV) function in chronically instrumented animals as contractile dysfunction was induced, but also the mechanical restitution and postextrasystolic potentiation responses, that is, physiological indices of excitation-contraction coupling,19-23 were examined along with the inotropic responses to β-adrenergic receptor stimulation with isoproterenol. Finally, the extent to which these incipient changes in the development of heart failure 1 day after pacing are reversible upon cessation of pacing also was investigated.
Methods

Surgical Instrumentation

Using halothane anesthesia (0.5 to 1.5 vol/100 mL in oxygen) and through a left thoracotomy approach, Tygon catheters were placed in the descending thoracic aorta and left atrium, a solid-state miniature pressure transducer was implanted in the apex of the left ventricle, and stainless steel pacing wires (model 4312, CPI) were placed on the right ventricle. In subgroups of animals, LV diameter and wall thickness crystals were implanted. At 2 to 3 weeks after recovery from surgery, hemodynamic measurements were made in the control state with the dogs fully awake, lying quietly on their right side before pacing (n=27), and at either 1 day (n=17) or 4 to 7 weeks (n=10) after rapid ventricular pacing at a rate of 240 beats per minute using a programmable pacemaker (Pace Medical, Inc, model EV 4543), which was worn externally in a vest. Nine animals were instrumented and studied similarly but did not undergo rapid ventricular pacing and served as sham-operated controls for the purpose of biochemical studies. Animals used in this study were maintained in accordance with guidelines of the Committee on Animals of the Harvard Medical School and the Guide for Care and Use of Laboratory Animals (Department of Health and Human Services publication No. [National Institutes of Health] 83-23, revised 1985).

Measurements

LV pressure was measured using a solid-state miniature pressure transducer calibrated in vitro with a mercury manometer and in vivo using left atrial and aortic catheters and Statham strain gauge manometers (P23 ID, Statham Instruments). LV diameter and wall thickness were measured using the ultrasonic transit-time method.

Protocols

Physiological measurements were made after a 30-minute stabilization period after deactivation of pacing. The mechanical restitution response\textsuperscript{19-22} was determined before and after 1-day pacing and after 4 to 7 weeks of pacing. To study this, the heart was paced at a constant rate (150 beats per minute) with left atrial pacing until the priming frequency had reached a steady state (up to 3 minutes). Two extrastimuli then were introduced after cessation of steady-state pacing. The interval between the end of steady-state pacing and the first extrastimulus was increased in 50-millisecond increments from 200 milliseconds to 900 milliseconds for the determination of mechanical restitution, while the interval between the first and the second extrastimulus was fixed at 600 milliseconds for the determination of postextrasystolic potentiation. Mechanical restitution and postextrasystolic potentiation were plotted as the response of LV dP/dt to the extrastimuli. All LV dP/dt values were normalized to the steady-state LV dP/dt of the priming frequency being investigated.

Mechanical restitution curves were obtained from a monoeponential function\textsuperscript{23}:

\[
(dP/dt (ES))/(dP/dt(SS)) = CR_{max}[1-e^{-T_{SI}/T_{e}}]
\]

where dP/dt (ES) is the maximum dP/dt of the extrastysolic beat and dP/dt (SS) is the maximum dP/dt of the steady state (basic cycle length, 400 milliseconds). TSI\textsubscript{e} is the longest stimulus duration at which no mechanical force develops, T\textsubscript{e} is the time constant, and CR\textsubscript{max} is the plateau value.

Postextrasystolic potentiation curves were also fit to a monoeponential function\textsuperscript{23}:

\[
(dP/dt (PES))/(dP/dt(SS)) = A e^{-PES/T_{e}} + B
\]

where dP/dt (PES) is the maximum dP/dt of postextrasystolic beats, PES is the postextrasystolic interval, and B is the plateau value.

In 4 control dogs, LV dP/dt was depressed by a combination of ganglionic blockade and diltiazem (5 to 10 \(\mu\)g/kg per minute) to match the decreased level of contractility that occurred after 1 day of ventricular pacing. In these 4 dogs, mechanical restitution was examined at this reduced level of LV dP/dt. In 3 control dogs, the sensitivity of the mechanical restitution response to changes in preload and afterload was assessed by infusing phenylephrine and nitroglycerin with heart rate constant.

In 6 dogs, the effects of mechanical restitution and of isoproterenol 0.2 \(\mu\)g/kg per minute and in 5 dogs the effects of postextrasystolic potentiation were examined before and 1 day after pacing. In 4 of these dogs, the recovery from 1 day of pacing was also monitored, and the full return of physiological responses to mechanical restitution, postextrasystolic potentiation, isoproterenol, and ryanodine receptor density was confirmed.

Membrane Preparation

After the dogs were anesthetized with sodium pentobarbital (40 to 50 mg/kg), their hearts were excised immediately and placed in iced saline. The left ventricle and septum were weighed, trimmed of fat and connective tissue, and a crude membrane fraction was prepared as previously described.\textsuperscript{3}

Receptor Binding Studies

To optimize the binding conditions for \(^3\)H-ryanodine binding, 12 concentrations of membrane protein (10 to 200 \(\mu\)g) were assayed with and without unlabeled ryanodine (10 \(\mu\)M) and with 50 mmol/L \(^3\)H-ryanodine (61.5 Ci/mmol). Incubation was at 37°C for 75 minutes. All assays were performed in triplicate and terminated by rapid filtration through Whatman GF/F filters washed with 4 mL cold buffer (150 mmol/L KCl, 10 mmol/L Tris HCl, pH 7.4). Filters were vortexed in 5 mL Ecoscint and counted in a beta scintillation counter for 5 minutes. Specific binding with 100 \(\mu\)g protein was >90%. The optimal time for incubation when the reaction reached equilibrium was determined by incubating 100 \(\mu\)g of membrane protein times for 5 minutes and 1 hour.
protein and 8 nmol/L \(^3\)H-ryanodine for increasing amounts of time from 1 to 120 minutes at 37°C. The samples were filtered and counted as above (Fig 2). Optimal calcium concentration was determined by incubating 100 µg of membrane protein with 5 mmol/L \(^3\)H-ryanodine in HEPES buffer (10 mmol/L HEPES, 150 mmol/L KCl, 3 mmol/L AMP, pH 7.4) with increasing concentrations of CaCl\(_2\), 10\(^{-6}\) to 10\(^{-2}\) mol/L (Fig 3). Incubation was carried out at 37°C for 90 minutes.

Ryanodine binding studies were performed using 12 concentrations of \(^3\)H-ryanodine (0.5 to 40 nmol/L) and 100 µg of membrane protein in HEPES buffer (with 0.3 mmol/L CaCl\(_2\)) in a total volume of 150 µL. Unlabeled ryanodine (10 µM) was used to determine nonspecific binding (Fig 4). The samples were filtered and counted as before. Because of the biphasic nature of the equilibrium binding curve (Fig 2), ligand-membrane incubations were carried for 50 minutes in one series of experiments and for 90 minutes in another set of experiments on a separate day. All assays were standardized by protein content. Protein concentrations were determined using the method of Lowry. All binding data were analyzed by the interactive ligand computer program of Munson and Rodbard. A linear regression was performed on the amount bound versus bound/free ligand. An \(r^2\) value \(>0.85\) was the criterion used for acceptability of the data. Since ryanodine binding results were similar using both incubation times, data were averaged from the two experiments.

The optimal concentration of membrane protein for dihydropyridine binding was determined by assaying varying membrane protein concentrations (10 to 200 µg) in dihydropyridine buffer (145 mmol/L NaCl, 5 mmol/L KCl, 1.25 mmol/L MgCl\(_2\), 1.25 mmol/L CaCl\(_2\), 20 mmol/L Tris HCl, 0.05% bovine serum albumin, pH 7.1) with 0.2 nmol/L \(^3\)H-PN200-110 (86 Ci/ mmol), with and without 1 µM nifedipine. Assays were incubated for 60 minutes at 37°C, filtered, and counted as described above. Nonspecific binding was determined with the addition of 1 µM nifedipine. At 40 µg of membrane protein, specific binding was \(>60\%\). Dihydropyridine receptor binding studies were performed using eight concentrations (0.05 to 1.2 mmol/L) of \(^3\)H-PN200-110 and 40 µg of membrane protein in dihydropyridine buffer, with an assay volume of 150 µL. Assay tubes were wrapped in foil to protect the \(^3\)H-PN200-110 and nifedipine from UV light. An additional series of experiments were performed in a dark room with a sodium lamp providing illumination, and the results were similar. Incubation, filtration, and counting were performed as described above.

**Analysis**

Data were expressed as mean±SEM. Data were analyzed using the SAS program (SAS Institute, Inc) on an IBM-PC 486. For multiple comparisons, Student’s \(t\) test with Bonferroni (Dunn) correction was used to determine statistical significance of the differences among the three or four groups; thus, when three comparisons were made, a value of \(P<.017\) was used to determine significance. The significance of changes (\(P<.05\)) in the mechanical restitution and postextrasystolic potentiation responses was tested with a paired \(t\) test.

**Results**

**Hemodynamics**

The hemodynamics for the 1-day pacing group, the 4- to 7-week pacing group, and sham-operated controls are shown in the Table. After 1-day pacing, LV systolic pressure fell to 104±3 mm Hg (\(P<.01\)) and fell further to 97±3 mm Hg at 4 to 7 weeks after pacing. LV end-diastolic pressure rose to 18±1 mm Hg (\(P<.01\)) and after 4 to 7 weeks rose further to 25±2 mm Hg. LV \(dP/dt\) fell after 1 day of pacing to 1933±58 mm Hg/s (\(P<.01\)) and after 4 to 7 weeks fell further to 1348±54 mm Hg/s. Baseline hemodynamics before pacing were similar in all three groups studied. At 1 day of pacing, neither preload (LV end-diastolic diameter) nor afterload (LV end-systolic stress) was different from base-
Effects of Pacing on Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Sham Operated (n=9)</th>
<th>Control for Both Pacing Groups (n=27)</th>
<th>1-Day Pacing (n=17)</th>
<th>4 to 7 Weeks of Pacing (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV systolic pressure, mm Hg</td>
<td>118±3</td>
<td>118±2</td>
<td>104±3*</td>
<td>97±3*</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mm Hg</td>
<td>6±1</td>
<td>6±1</td>
<td>10±1*</td>
<td>25±2*</td>
</tr>
<tr>
<td>LV dP/dt, mm Hg/s</td>
<td>2930±69</td>
<td>2946±56</td>
<td>1933±58*</td>
<td>1348±54*</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>94±2</td>
<td>96±1</td>
<td>84±2*</td>
<td>80±2*</td>
</tr>
<tr>
<td>Heart rate, beats per minute</td>
<td>92±3</td>
<td>92±2</td>
<td>113±5*</td>
<td>136±7*</td>
</tr>
</tbody>
</table>

LV indicates left ventricular.

*P<.01, difference from before pacing.

In contrast, after 4 to 7 weeks, LV end-diastolic diameter was increased by 9±3%, as was LV end-systolic stress (27±6%).

Mechanical Restitution

The mechanical restitution response was depressed significantly after 1 day of pacing. However, since baseline contractility was also depressed, it was important to compare normalized data. The normalized data for mechanical restitution are plotted in Fig 5. Mechanical restitution was significantly depressed (P<.05) after 1 day of pacing. At a test pulse interval of 350 milliseconds, the normalized response was depressed by 18±6% (P<.05) at 1 day and by a similar amount (24±4%) at 4 to 7 weeks after initiating pacing.

In sham-operated control dogs, neither increasing nor decreasing preload and afterload affected the mechanical restitution response significantly (Fig 6). In sham-operated control dogs (n=4), LV dP/dt was significantly depressed from 3059±173 mm Hg (intact state) to 1849±73 mm Hg (ganglionic blockade plus diltiazem), which is similar to the depression in LV dP/dt observed 1 day after pacing. This maneuver, unlike 1 day of ventricular pacing, did not change the initial phase of mechanical restitution (Fig 6).

Postextrasystolic Potentiation

The postextrasystolic potentiation response was depressed (P<.05) after 1 day of pacing (by 14±3%) in the plateau phase (Fig 5), an amount similar to the depression observed with mechanical restitution.

Isoproterenol

Before pacing, isoproterenol 0.2 μg/kg per minute increased LV dP/dt by 2599±409 from 2929±95 mm Hg/s. After 1 day of pacing, the response to this dose of isoproterenol was only an increase in LV dP/dt of 1176±201 from 1974±64 mm Hg/s. This response was depressed significantly more (P<.05), i.e., by 52±7%, than either the response to mechanical restitution or postextrasystolic potentiation.

Recovery From 1 Day of Pacing

At 3 days after cessation from 1 day of continuous pacing, baseline hemodynamics remained depressed but were fully recovered by 5 days after cessation of pacing (Fig 7). At this time, not only did baseline hemodynamics fully recover, but responses to mechanical restitution, postextrasystolic potentiation, and isoproterenol also returned to prepacing baseline levels (Fig 8).

Ryaznedine Receptors

There was a significant decrease (P<.017) in ryanodine receptor binding (808±42 fmol/mg) in the animals after 1 day of rapid ventricular pacing as compared with the sham-operated control animals (1013±25 fmol/mg) (Fig 9). Ryanodine receptor binding was also significantly depressed similarly (P<.017) in the animals with 4 to 7 weeks of pacing-induced heart failure (782±61 fmol/mg).
fmol/mg) (Fig 10). The affinity ($K_d$) for $^3$H-ryanodine binding was not significantly different among the three groups (sham operated, 6.5±0.4 nmol/L; 1 day of pacing, 7.4±0.9 nmol/L; 4 to 7 weeks of pacing, 7.2±0.6 nmol/L). In the 4 dogs in which it was measured, $^3$H-ryanodine binding density was no longer significantly depressed at 5 days after cessation from 1 day of pacing (Fig 8).

**Dihydropyridine Receptors**

$^3$H-PN200-110 binding was not significantly different among the three groups (sham operated, 149±16 fmol/mg; 1-day pacing, 130±8 fmol/mg; 4 to 7 weeks of pacing, 164±9 fmol/mg) (Fig 10). The $K_d$ was also not significantly different among the three groups (sham operated, 0.11±0.03 nmol/L; 1-day pacing, 0.12±0.01 nmol/L; 4 to 7 weeks of pacing, 0.09±0.02 nmol/L).

**Discussion**

In the present investigation, conscious dogs demonstrated a significant depression in myocardial contractility at 1 day after initiating pacing, ie, LV $dP/dt$ fell by 36±2%. At this time, ryanodine receptor binding was also significantly reduced by 20%. Thus, loss of ryanodine receptor binding may be one mechanism initiating the decline in myocardial contractility. Ryanodine has been shown to specifically bind to the SR foot process calcium channel, which is involved in the release of calcium.27,28 In myocardial tissue, ryanodine binds with both high and low affinity.29 Nanomolar concentrations of ryanodine bind tightly to the high-affinity receptor and lock the native calcium release channel in the open state.30,31 Ryanodine binding in micromolar concentrations to a low-affinity site is responsible for closure of

**Fig 6.** Curve shows effects of reduced and increased preload and afterload on the mechanical restitution responses (left panel). Altering loading conditions did not affect mechanical restitution in normal conscious dogs. No effect of diltiazem in the presence of ganglionic blockade (GB) on the mechanical restitution response was observed despite the reduced level of contractility (right panel). MAP indicates mean arterial pressure; LV, left ventricular; and LVEDP, left ventricular end-diastolic pressure.

**Fig 7.** Graphs show baseline hemodynamics in 4 conscious dogs monitored before pacing, 1 day after rapid ventricular pacing, and at 3 and 5 days after cessation from rapid ventricular pacing. The changes in hemodynamics observed at 1 day after pacing had almost recovered by 3 days but had completely recovered by 5 days after cessation from pacing. AP indicates arterial pressure; LV, left ventricular; and LVEDP, left ventricular end-diastolic pressure.
the calcium release channel and an increase in the calcium uptake rate of the SR. Ryanodine binding is thought to provide a sensitive guide to the degree of activation of the calcium release channel. In this study, only high-affinity ryanodine binding was analyzed.

Although little prior work has been done on ryanodine receptors in heart failure, Brillantes et al. found a decrease in mRNA for the calcium release channel in myocardia from patients with ischemic cardiomyopathy and an insignificant increase in patients with idiopathic dilated cardiomyopathy. However, receptor density was not examined. More recently, Cory et al. found that ryanodine receptors fell in two different models of canine heart failure. However, this study did not examine the initial changes in cardiac dysfunction before heart failure, which was the major thrust of the current investigation. The dihydropyridine receptor has been examined more extensively, but the results of these studies have been conflicting. While some investigators found a decrease in dihydropyridine binding and mRNA in heart failure, other studies found either an increase or no change in binding. In the present study, we also observed no change in dihydropyridine binding, suggesting that if this occurs, it is rather a later event in the evolution of the heart failure process.

The physiological significance of the changes in calcium release channel by ryanodine binding is complex. We chose to assess the physiological significance of the changes in calcium release channel by studying the responses to postextrasystolic potentiation and mechanical restitution, which provide a rough physiological correlate of calcium release from the SR in vitro. Our study evaluated this in vivo in conscious dogs before and after 1 day of pacing. If the 20% reduction in ryanodine binding sites at 1 day after pacing were of physiological significance, we would predict that the postextrasystolic potentiation and mechanical restitution relations would be depressed. Indeed, we observed such a depression after 1 day of pacing (Fig 5), suggesting impaired calcium release.

![Graph showing effects of mechanical restitution](image1)

![Graph showing postextrasystolic potentiation](image2)

![Scatchard analyses of left ventricular 3H-ryanodine binding](image3)

![Bar graph showing average ± SE values for ryanodine binding and dihydropyridine binding](image4)
An important issue is whether the mechanical restitution relation is load dependent. Burkhoff et al. showed that at test pulse intervals less than the priming frequency, there was little effect of load on the time constant or slope in isolated, ejecting dog hearts. In the present study, direct assessment of preload (LV end-diastolic diameter) and afterload (LV systolic wall stress) demonstrated no change after 1 day of pacing, at a time when both ryanodine receptors and the physiological response to mechanical restitution were reduced. Finally, the effects of alterations in preload and afterload on the mechanical restitution response were examined. There was no significant effect over the ranges of preload and afterload examined (Fig 6).

The current data may help to understand more fully the recent observations on alterations in β-adrenergic signaling in this model, which are apparent at 1 day after pacing but become more severe during the development of heart failure. Interestingly, the response to catecholamines is depressed out of proportion to the deficit in β-adrenergic signaling mechanisms after 1 day of pacing in this model, ie, at this time the response of LV dP/dt to a dose of isoproterenol was depressed by 52±7% in the face of only modest alterations in β-adrenergic receptor–adenyl cyclase coupling.1 The data in the current investigation demonstrating alterations in excitation-contraction coupling at 1 day after pacing could reconcile these findings and provide further physiological support to the concept that altered ryanodine receptor binding early in the development of heart failure contributes to the depressed contractility not only at baseline but also in response to inotropic stimulation.

While these data are consistent with the observed decrease in ryanodine binding, they do not exclude other potential abnormalities in the gating function of the calcium release channel. In this regard, D’Agnolo et al. observed a marked increase in caffeine threshold in skinned fiber preparations from humans with dilated cardiomyopathy, although the rate and extent of caffeine-induced calcium release were not different.

An additional feature of this investigation involved monitoring recovery from 1 day of pacing. Interestingly, baseline hemodynamics remained slightly depressed at 3 days after cessation of pacing but had fully recovered by 5 days after cessation of pacing. Furthermore, responses to isoproterenol, postextrasystolic potentiation, and mechanical restitution completely recovered at 5 days after pacing. Thus, the significant alterations in excitation-contraction coupling and β-adrenergic receptor stimulation are fully reversible after 1 day of pacing.

In summary, the present investigation demonstrated that changes in excitation-contraction coupling and potentially the SR calcium release channel occur early in the development of heart failure and therefore may be important in the pathogenesis of the contractile abnormalities in this disease state. These conclusions are based on (1) inference from the observation that physiological responsiveness to β-adrenergic receptor stimulation is depressed out of proportion to the alterations in β-adrenergic receptor signaling, (2) biochemical determinations of reduced ryanodine receptor density, and (3) physiological determination of depressed responses to postextrasystolic potentiation and mechanical restitution.

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