Influence of sarcoplasmic reticulum calcium loading on mechanical and relaxation restitution

BRIAN D. HOIT,1 VIVEK J. KADAMBI,2 DANIEL A. TRAMUTA,1 NANCY BALL,1 EVANGELIA G. KRANIAS,2 AND RICHARD A. WALSH3

1Division of Cardiology and 2Department of Pharmacology and Cell Biophysics, University of Cincinnati Medical Center, Cincinnati 45267-0575; and 3Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Hoit, Brian D., Vivek J. Kadambi, Daniel A. Tramuta, Nancy Ball, Evangelia G. Kranias, and Richard A. Walsh. Influence of sarcoplasmic reticulum calcium loading on mechanical and relaxation restitution. Am. J. Physiol. Heart Circ. Physiol. 278: H958–H963, 2000.—Mechanical and relaxation restitution represent the restoration of contractile force and relaxation, respectively, in premature beats having progressively longer extrasystolic intervals (ESI); these phenomena are related to intracellular activator Ca2+ by poorly defined mechanisms. We tested the hypothesis that the level of phospholamban (which modulates the affinity of the sarcoplasmic reticulum (SR) Ca2+-ATPase for Ca2+, and thus the SR Ca2+ load) may be an important determinant of both mechanical and relaxation restitution. Five mice with ablation of the phospholamban (PLB) gene (PLBKO), eight isogenic wild-type controls (129SvJ), eleven mice with PLB overexpression (PLBOE), and nine isogenic wild-type (FVB/N) controls were anesthetized and instrumented with a 1-Fr Millar catheter in the left ventricle and a 1-Fr pacemaker in the right atrium. At a cycle length of 200 ms, extrastimuli with increasing ESI were introduced, and the peak rates of left ventricular isovolumic contraction (∆dP/dtmax) were normalized and fit to monoeponential equations. In a subset, the protocols were repeated after ryanodine (4 ng/g) was administered to deplete SR Ca2+ stores. The time constant of mechanical restitution in PLBKO was significantly shorter (6.3 ± 1.2 (SE) vs. 47.7 ± 7.6 ms) and began earlier (50 ± 10 vs. 70 ± 19 ms) than in 129SvJ. In contrast, the time constant of mechanical restitution was significantly longer (80.3 ± 7.6 vs. 54.1 ± 9.2 ms) in PLBOE than in FVB/N. The time constant of relaxation restitution was less in PLBKO than in 129SvJ (26.2 ± 9.9 vs. 44.6 ± 3.3, P < 0.05) but was similar in PLBOE and FVB/N (21.1 ± 6.3 vs. 20.5 ± 5.7 ms). Intravenous ryanodine decreased significantly the time constants of mechanical restitution in PLBOE, 129SvJ, and FVB/N but was lethal in PLBKO. In contrast, ryanodine increased the time constant of relaxation restitution. Thus 1) the phospholamban level is a critical determinant of mechanical restitution and (to a lesser extent) relaxation restitution in these transgenic models, and 2) ryanodine differentially affects mechanical and relaxation restitution. Furthermore, our data suggest a dissociation of processes within the SR that govern contraction and relaxation.

MANIFESTATIONS of the force-interval relation, (force-frequency, mechanical restitution, postextrasystolic potentiation) have been studied extensively in isolated muscle preparations (31), the isolated heart (32), and to a lesser extent in the intact animal (1, 7); changes in these parameters have been reported after congestive heart failure (23) and thyroid hormone administration (15). Processes related to force-interval behavior are important insofar as they are fundamental physiological control mechanisms, are used as indices of myocardial function, and play a role in the response to exercise and the development and maintenance of heart failure (9, 21). Although we (11) have recently shown that force-frequency relations are dependent on the level of the sarcoplasmic reticulum (SR) protein phospholamban, and therefore, activity of the SR Ca2+-ATPase pump, the physiochemical mechanisms responsible for mechanical restitution are poorly defined. Moreover, the force-frequency relation integrates all SR Ca2+ cycling processes (i.e., uptake, release, and recirculation); thus one cannot assume a priori that restitution of contraction and relaxation are dependent on similar processes. In this regard, ryanodine accelerated mechanical restitution and augmented the force-frequency relation but slowed relaxation restitution in an instrumented canine model (22).

Mechanical restitution represents the increase in force of contraction associated with progressively longer extrasystolic intervals (ESI) and is linearly related to time-dependent increases in intracellular activator Ca2+ cycling processes (i.e., uptake, release, and recirculation). Intravenous ryanodine decreased significantly the time constants of mechanical restitution in PLBOE, 129SvJ, and FVB/N but was lethal in PLBKO. In contrast, ryanodine increased the time constant of relaxation restitution. Thus 1) the phospholamban level is a critical determinant of mechanical restitution and (to a lesser extent) relaxation restitution in these transgenic models, and 2) ryanodine differentially affects mechanical and relaxation restitution. Furthermore, our data suggest a dissociation of processes within the SR that govern contraction and relaxation.

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critical determinant of both mechanical and relaxation restitution. The effects of SR loading were isolated by studying transgenic mice with phospholamban overexpression (PLBOE) and gene-targeted mice with phospholamban ablation (PLBKO). A major premise of the current investigation is that the fidelity and precision of the transgenic mouse models as reagents are adequate to uniquely reflect SR Ca$^{2+}$ loading and restitution in vivo relatively independent of preload and neurohormonal reflexes. In this regard, studies in these mice have demonstrated that the magnitude of the Ca$^{2+}$ transient and the mechanical activity (isolated myocytes, isolated working heart, in vivo echocardiography, and catheterization) are increased in PLBKO and decreased in PLBOE hearts (12).

**MATERIALS AND METHODS**

**Instrumentation**

Mice were anesthetized with a mixture of ketamine (100 mg/kg), xylazine (5 mg/kg), and morphine (2.5 mg/kg) given as 0.25 ml intraperitoneally, and were taped to a warmed operating table in the supine position. A tracheotomy was performed with a 24-gauge catheter, and animals were ventilated at 100 cycles/min using a Harvard respirator. The right carotid artery was isolated, and a 1.4-F Millar high-fidelity catheter was introduced into the vessel and advanced into the left ventricle. The right jugular vein was isolated, and a 1-Fr bipolar pacemaker (NuMED) was placed in the right atrium.

**Animals**

The phospholamban knockout mice were produced using gene-targeting technology as previously described (17). Wild-type mice of identical genetic background were bred simultaneously with the phospholamban knockout animals. Mice with cardiac-specific overexpression of phospholamban were generated as previously described (13).

Inasmuch as the genetic background of the phospholamban knockout differs from that of the phospholamban overexpression mice, it was important to compare hemodynamic data from each animal model with the appropriate wild-type controls (11). Thus the phospholamban knockout mice were processed in parallel with age-matched wild-type littermates (129SvJ), whereas the phospholamban overexpression mice were compared with their isogenic wild-type littermates (FVB/N).

Studies were performed in 33 animals (19.9–31.3 g) of either sex. There were eleven phospholamban overexpression and nine FVB/N controls, and five phospholamban knockouts (FVB/N). There were eleven phospholamban overexpression mice, it was important to compare hemodynamic data generated as previously described (13).

**Protocols**

A Medtronic programmable stimulator (model 5325) was used for the restitution protocol. Determinations of pacing thresholds were made, and stimulation was performed with a 1.0-ms pulse width at twice the capture threshold.

Restitution protocol. The right atrium was stimulated at a basic cycle length of 200 ms (300 beats/min). The sinus node fuzzy channel (I$_f$) inhibitor DKAH-0269 (0.1 ml iv; a generous gift from Boehringer Ingelheim, Ridgefield, CT) was given to slow the heart rates when necessary. This agent is free of inotropic effects (11), and a similar I$_f$ inhibitor UL-FS49 did not alter the time constant (TC) of mechanical restitution (18). After a series of beats ($\approx 15$), an atrial extrastimulus was introduced in the refractory period of the atrioventricular node, and extrastimuli intervals (ESI) were increased at 10-ms intervals (producing beats with progressively increased cycle length) until the basic cycle length was achieved.

Ryanoine infusion. Protocols were repeated 30 min after an intravenous infusion of ryanoine at a rate of 0.5 ng.g$^{-1}$.min$^{-1}$ for a total dose of 4 ng/g. This dose produces a serum concentration in the nanomolar range that binds specifically to high-affinity sites, locking the SR Ca$^{2+}$ channel in a subconductance state, and has been shown to depress indices of LV isovolumic contraction and relaxation (26).

**Data Analysis**

The micromanometer was electronically calibrated in vitro by submerging the tip of the catheter in warm saline with the reference zero taken at the mid chest. The analog LV first derivative of pressure (dP/dt) signal was obtained by electronic differentiation of the high-fidelity LV pressure signal. The electrocardiogram and the intracardiac recordings were displayed with the hemodynamic analog data on a Gould WindowGraf four-channel recorder. Data were taken from the original records.

To analyze mechanical restitution, dP/dt$_{max}$ was normalized to the dP/dt from beats having the basic cycle length and expressed as a percentage (7, 23). Normalized dP/dt = Y$_{max}$[1- exp (ESI$_0$/ESI)]/TC, where Y$_{max}$ is the maximal or plateau response, ESI$_0$ is the largest ESI that fails to produce a response, and TC is the time constant.

Relaxation restitution was calculated by normalizing peak –dP/dt of the extrastimulus beat to the peak –dP/dt of the basic cycle length and fitting the relation between normalized dP/dt and the ESI to a monoexponential function (23, 24).

**RESULTS**

**Baseline Hemodynamics**

Steady-state hemodynamics were measured at an atrially paced heart rate of 300 beats/min. LV systolic pressure was significantly less, and LV diastolic pressure tended to be greater in PLBOE than in FVB/N controls. In contrast, LV systolic and diastolic pressure tended to be reduced in PLBKO compared with their isogenic 129SvJ controls (Table 1).

As seen in Table 1, both +dP/dt$_{max}$ and –dP/dt$_{max}$ were significantly less in PLBOE than in FVB/N controls and significantly greater in PLBKO than in 129SvJ controls.

**Mechanical Restitution**

A representative recording of three extrastimuli (80, 150, and 190 ms) at a basic cycle length of 200 ms and the relation between normalized +dP/dt$_{max}$ and ESI for a single animal are shown in Fig. 1. As the extrastimulus interval increased from the first appearance of mechanical activity to the basic cycle length (200 ms),...
respectively.

FLBKO, phospholamban overexpression; FLBKO, phospholamban knockout; FVB/N and 129SvJ are their wild-type isogenic controls, respectively.

PLBOE, phospholamban overexpression; PLBKO, phospholamban knockout; FVB/N and 129SvJ are their wild-type isogenic controls, respectively.

Table 1. Baseline hemodynamics at an atrially paced rate of 300 beats/ min

<table>
<thead>
<tr>
<th></th>
<th>LVSP, mmHg</th>
<th>LVDP, mmHg</th>
<th>+dP/dt max, mmHg/s</th>
<th>−dP/dt max, mmHg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB/N</td>
<td>9 109 ± 6</td>
<td>1.8 ± 0.4</td>
<td>6,521 ± 512</td>
<td>5,618 ± 642</td>
</tr>
<tr>
<td>PLBOE</td>
<td>11 129 ± 7</td>
<td>3.5 ± 0.8</td>
<td>4,947 ± 244</td>
<td>3,880 ± 212*</td>
</tr>
<tr>
<td>129SvJ</td>
<td>8 100 ± 6</td>
<td>5.7 ± 1.8</td>
<td>7,008 ± 527</td>
<td>6,086 ± 219</td>
</tr>
<tr>
<td>PLBKO</td>
<td>5 98.5 ± 9</td>
<td>3.1 ± 2.9</td>
<td>18,250 ± 1,794*</td>
<td>12,500 ± 1,439*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n is no. of mice/group. LVSP, left ventricular (LV) systolic pressure; LVDP, LV diastolic pressure; PLBOE, phospholamban overexpression; PLBKO, phospholamban knockout; FVB/N and 129SvJ are their wild-type isogenic controls, respectively. +dP/dt max, rate of pressure development; −dP/dt max, rate of pressure relaxation. *P < 0.05 vs. isogenic control.

there was an exponential increase in +dP/dt max. The TC, time to one-half restitution (t1/2), and plateau (Y max) of the exponential curve fit were the parameters used to characterize mechanical restitution.

Compared with isogenic FVB/N controls, PLBOE mice had a longer TC and t1/2, indicating a decreased rate of mechanical restitution. PLBKO tended to achieve a higher plateau response (Y max), suggesting incomplete mechanical restitution at the paced cycle length (Table 2). On the contrary, compared with isogenic 129SvJ controls, PLBKO mice had a markedly shorter TC and t1/2, indicating a faster rate of mechanical restitution. There were no significant differences in Y max, suggesting that restitution was complete in both PLBKO and their controls at the paced cycle length.

Relaxation Restitution

The relation between 1/normalized −dP/dt max and ESI for a single animal is shown in Fig. 2. As the extrastimulus interval increased from the first appearance of mechanical activity to the basic cycle length, there was an exponential increase in −dP/dt max (and thus a decrease in the reciprocal, 1/normalized −dP/dt max). The TC of the exponential decay was used to characterize relaxation restitution (23, 24).

Both the TC of relaxation restitution and ESI 0 (the latter identical in mechanical restitution and relaxation restitution) were similar in PLBOE and FVB/N controls (Table 3). In contrast, the TC of relaxation restitution in PLBKO was markedly shorter than 129SvJ controls, indicating a faster rate of relaxation restitution in the absence of phospholamban. ESI 0 was less in PLBKO than in 129SvJ, suggesting an earlier onset of relaxation restitution in PLBKO, but this difference failed to achieve statistical significance (Table 3).

Effect of Ryanodine

Ryanodine infusion produced the expected decreases in LV systolic pressure and ±dP/dt max in PLBOE, 129SvJ, and FVB/N mice (Table 4) but was uniformly fatal (even at half dose) in PLBKO. As shown in Fig. 3, in both the control mice (FVB/N and 129SvJ) and PLBOE, ryanodine decreased the TC of mechanical restitution, although this failure to achieve statistical significance in the two groups of control mice. However, in PLBOE, the TC of mechanical restitution and the
plateau response ($Y_{\text{max}}$) both significantly decreased after ryanodine, indicating a faster, more complete restitution of contraction (Fig. 3). In contrast, ryanodine increased the TC of relaxation restitution in PLBOE and both wild-type strains, although the change failed to achieve statistical significance in 129SvJ mice. Because PLBKO mice did not tolerate ryanodine, only a few of the 129SvJ isogenic controls were studied; thus statistical comparisons in this subgroup have limited power. ESI$_0$ was unaltered by ryanodine infusion (Fig. 3).

**DISCUSSION**

The principle findings of this study are threefold. First, the rate of mechanical restitution is inversely related to the level of phospholamban, reflecting the important influence of phospholamban on the affinity of the SR Ca$_{\text{2+}}$-ATPase pump for Ca$_{\text{2+}}$ and confirming the critical role of the SR Ca$_{\text{2+}}$ load on mechanical activity in the intact animal. Second, whereas relaxation restitution is enhanced in mice with ablation of phospholamban, the kinetics of relaxation restitution are unaffected in mice with overexpression of phospholamban. Third, ablation of SR function with ryanodine enhances the restoration of contraction but impairs the restoration of relaxation. Together with a recent study (11) in which the critical heart rate of the force-frequency relation was decreased in mice with phospholamban overexpression and increased in mice with ablation of phospholamban, these data indicate a dissociation of the SR effects on force-interval behavior and confirm the findings of a recent study in instrumented dogs (22).

Recovery of the mechanical response to increasing ESI has been described mathematically as a monoeponential function in muscle strips (31), the isolated heart (4), and intact animals (7). Investigators hypothesize that mechanical restitution is a function of Ca$_{\text{2+}}$ release channel kinetics and propose a compartmentalized model of Ca$_{\text{2+}}$ transport (3, 7, 32), but direct evidence for this construct is lacking. Our study is the first to unambiguously demonstrate the dependence of mechanical restitution on phospholamban level, and there-

**Table 3. Parameters of relaxation restitution**

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>Time Constant, ms</th>
<th>$ESI_0$, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB/N</td>
<td>9</td>
<td>20.5 ± 5.7</td>
<td>86 ± 5</td>
</tr>
<tr>
<td>PLBOE</td>
<td>11</td>
<td>21.1 ± 6.3</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>SVJ</td>
<td>8</td>
<td>44.6 ± 3.3</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>PLBKO</td>
<td>5</td>
<td>14.4 ± 8.3*</td>
<td>50 ± 4†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n is no. of mice/group. $ESI_0$, largest ESI that fails to produce a response. *$P < 0.05$ vs. isogenic control, †$P = 0.06$ vs. isogenic control.

**Table 4. Hemodynamics after ryanodine infusion**

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>LVSP, mmHg</th>
<th>LVDP, mmHg</th>
<th>$+\frac{dP}{dt}_{\text{max}}$</th>
<th>$-\frac{dP}{dt}_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB/N</td>
<td>5</td>
<td>60 ± 2</td>
<td>1 ± 0</td>
<td>1,867 ± 743</td>
<td>1,667 ± 667</td>
</tr>
<tr>
<td>PLBOE</td>
<td>6</td>
<td>64 ± 5</td>
<td>5 ± 1</td>
<td>2,554 ± 124</td>
<td>2,539 ± 118</td>
</tr>
<tr>
<td>SVJ</td>
<td>3</td>
<td>65 ± 8</td>
<td>2 ± 1</td>
<td>2,466 ± 649</td>
<td>1,967 ± 650</td>
</tr>
</tbody>
</table>

Values are means ± SE; n is no. of mice/group.

Fig. 3. Bar graphs depicting influence of ryanodine on plateau response $Y_{\text{max}}$ (A) and time constant (TC) of mechanical restitution (B) and on TC (C) and $ESI_0$ (D) of relaxation restitution. ESI of relaxation and mechanical restitution are identical. Hatched bars, baseline data; filled bars, data after ryanodine. *$P < 0.05$ vs. baseline, †$P = 0.10$ vs. baseline.
fore the affinity of the SR Ca$^{2+}$-ATPase for Ca$^{2+}$; mice with ablation of phospholamban had faster restitution, and mice with overexpression of phospholamban had slower restitution of LV + dP/dt$\text{max}$ than their respective isogenic controls. Thus in the intact mouse, the rate of Ca$^{2+}$ sequestration by the SR, and not simply the recovery of the SR Ca$^{2+}$ release channel, is responsible for mechanical restitution. It is possible that the rate of SR Ca$^{2+}$ uptake is primarily responsible for mechanical restitution and that the resultant SR Ca$^{2+}$ load influences the TC of the ryanodine (or Ca$^{2+}$ release) channel. In support of this concept, cross signaling between Ca$^{2+}$ channels and the ryanodine receptor have recently been demonstrated with confocal microscopy (6).

Recovery of relaxation (−dP/dt$\text{max}$) also occurs as a monoexponential function and can be described by a TC (23, 24). Although relaxation restitution is most rigorously described by two concatenated monoexponentials, we examined only the first, rapid phase of restitution, because −dP/dt$\text{max}$ was not measured in ESI beyond the basic cycle length. Because SR uptake of intracellular Ca$^{2+}$ is a determinant of myocardial relaxation and is indirectly related to the level of phospholamban, it is expected that relaxation restitution is enhanced in the PLBKO versus control mice, consistent with the greater Ca$^{2+}$ uptake rates for the same ESI in the former. However, the finding that the TCs of relaxation restitution are similar in PLBOE and their controls is more difficult to understand and suggests that recovery of relaxation in extrastimuli is not simply dependent on the rate of relaxation measured at steady state. Prabhu and Freeman (23) studied mechanical and relaxation restitution in dogs with rapid pacing heart failure, and although heart failure prolonged mechanical restitution TCs, relaxation restitution TCs were unchanged. Their data suggest that pacing heart failure affects mechanical, but not relaxation, restitution kinetics, despite the fact that SR Ca$^{2+}$-ATPase expression and function are depressed in this model (8, 20). In an analogous fashion, relaxation restitution did not change with a twofold overexpression of phospholamban in our transgenic mice. Additional possible explanations include the load dependence of −dP/dt$\text{max}$ [although similar results were reported with load independence indices (23)], a relative insensitivity of the relaxation restitution assay, or saturation of the relaxation response; evidence against the latter is provided by the response of the TC of relaxation restitution to ryanodine.

Effects of Ryanodine

Ryanodine selectively binds to the ryanodine channel in a dose-dependent manner; at low (nanomolar to low micromolar) concentrations, ryanodine binding depletes SR Ca$^{2+}$ by maintaining the channel in the open state (26). The effects of ryanodine in vitro include impaired contractility and relaxation and reduced intracellular Ca$^{2+}$ transients, SR content, and force-interval behavior (16, 31). Although there is consensus regarding the negative inotropic and lusitropic effects of ryanodine in vivo (14, 25), there are few data that examine ryanodine effects on restitution kinetics. Kalthof et al. (14) found that infusion of 0.5–4 g/kg ryanodine in conscious dogs depressed mechanical restitution, whereas others have observed faster mechanical restitution kinetics after ryanodine (2, 25, 31). Experimental design and preparation, ryanodine dosage, and species variability may account for some of the reported differences (27). We found that ryanodine accelerated the kinetics of mechanical restitution in both PLBOE and FVB/N; our data are consistent with the hypothesis that with loss of a functioning SR, the rate of recovery from the inactivation of sarcenmal Ca$^{2+}$ channels may become the rate-limiting factor for mechanical restitution. In this regard, isolated myocyte studies suggest that recovery of the Ca$^{2+}$ current during the action potential (l) is two to three times faster than recovery of twitch force (10, 29). In our study, both PLBOE and their isogenic FVB/N controls had similar parameters of mechanical restitution after SR function was ablated with ryanodine, suggesting a change to the same rate-limiting determinant of contractile recovery. Interestingly, PLBKO mice did not survive ryanodine infusion (even at reduced doses), which may be caused by the leak of the high SR load and resultant intracellular Ca$^{2+}$ overload.

In summary, mechanical restitution is increased although the absolute quantity of Ca$^{2+}$ cycled is less and baseline function is reduced in the presence of ryanodine. In contrast, relaxation restitution is prolonged after ryanodine, which suggests that recovery of relaxation is more dependent on SR Ca$^{2+}$-ATPase function (i.e., that the kinetics of sarcenmal Ca$^{2+}$ disposal are slower than SR uptake mechanisms). These data are consistent with preliminary reports in a dog model (25).

Limitations

There are several potential problems that should be acknowledged. First, Ca$^{2+}$ regulation involves the interplay of several overlapping and potentially confounding compensatory mechanisms; we recognize that the assumption that these mice represent unambiguous reagents may not be entirely correct. Although the ryanodine receptor is downregulated in PLBKO (5), this would be expected to slow mechanical restitution. In addition, Na$^{+}$/Ca$^{2+}$ exchanger activity is increased nearly fourfold in PLBOE (7a); however, this more likely to influence late (i.e., restitution occurring at intervals greater than the basic cycle length) mechanical restitution kinetics (19). Nevertheless, caution will need to be applied in the interpretation and extrapolation of our results. Second, we have observed considerable species variability in the hemodynamic and force-frequency parameters (11). Therefore, it is important that isogenic controls are employed in these studies. Third, the isovolumic indices of contraction and relaxation that we used are dependent on loading conditions. However, the effect of load on mechanical restitution was abrogated in the isolated, ejecting dog heart (4) and was not significant in the conscious dog (30) with qualitatively similar results. Finally, although ryanodine is a specific drug, there is a dose-dependent effect, and there is a possibility of nonspecific effects; these issues are a focus of ongoing studies in our laboratory.
Unfortunately, the use of pharmacological manipulation of the ryanodine receptor is necessary, because ryanodine receptor knockouts are lethal and the large size of this protein has made genetic manipulation of this protein difficult.

In conclusion, the level of phospholamban is a critical determinant of mechanical and, to a lesser extent, relaxation restitution. Experiments with ryanodine in these animals suggest that the kinetics of mechanical restitution are rate limited by the SR and that those of relaxation restitution are enhanced by processes in the SR.

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