Effect of milrinone on left ventricular relaxation and Ca^{2+} uptake function of cardiac sarcoplasmic reticulum

MASAFUMI YANO, MICHIHIRO KOHNO, TOMOKO OHKUSA, MAMORU MOCHIZUKI, JUTARO YAMADA, MASATERU KOHNO, TAKAYUKI HISAOKA, KAORU ONO, TAKETO TANIGAWA, SHIGEKI KOBAYASHI, AND MASUNORI MATSUZAKI
Second Department of Internal Medicine, Yamaguchi University School of Medicine, Ube, Yamaguchi 755-8505, Japan
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Yano, Masafumi, Michihiro Kohno, Tomoko Ohkusa, Mamoru Mochizuki, Jutaro Yamada, Masateru Kohno, Takayuki Hisaoka, Kaoru Ono, Taketo Tanigawa, Shigeki Kobayashi, and Masunori Matuszaki. Effect of milrinone on left ventricular relaxation and Ca^{2+} uptake function of the cardiac sarcoplasmic reticulum. Am J Physiol Heart Circ Physiol 279: H1898–H1905, 2000.—Milrinone, a phosphodiesterase 3 (PDE3) inhibitor, is known to enhance left ventricular (LV) contractility by an inhibition of the phosphodiesterase 3 (PDE3) inhibitor, is known to enhance left ventricular (LV) contractility by an inhibition of the phosphodiesterase 3 (PDE3) inhibitor, is known to enhance left ventricular (LV) contractility by an inhibition of the phosphodiesterase 3 (PDE3) inhibitor, is known to enhance left ventricular (LV) contractility by an inhibition of the phosphodiesterase 3 (PDE3) inhibitor, is known to enhance left ventricular (LV) contractility by an inhibition of the.


milrinone on left ventricular relaxation and Ca^{2+} uptake function of the cardiac sarcoplasmic reticulum (SR) compared with those of dobutamine, which also elevates the cellular cAMP (7, 25), which in turn activates cAMP-dependent protein kinases with a resultant increase in the transsarcolemmal influx of Ca^{2+} (21) and the rate of Ca^{2+} uptake by the sarcoplasmic reticulum (SR) (9). Although several reports (6, 22) have demonstrated that milrinone improves left ventricular (LV) diastolic property as well as systolic function, the mechanism by which milrinone exerts positive lusitropic effects remains to be elucidated.

At a subcellular level, LV relaxation is closely related to the Ca^{2+} uptake function by the SR. In cardiac muscle, the SR Ca^{2+}-ATPase activity and the Ca^{2+} uptake are enhanced when SR membrane-associated phospholamban is phosphorylated by the cAMP-dependent protein kinase (31).

Recently, a particulate, cGMP-inhibited phosphodiesterase 3 (PDE3) has been shown to exist in association with SR vesicles isolated from the mammalian myocardium (14, 16). The potency of the PDE3 inhibitors as inotropic agents in this tissue are considered to correlate with their potency as inhibitors of the SR membrane-bound PDE3 activity (14, 33). Therefore, it is possible that milrinone interacts with the SR-associated PDE3 and, hence, activates the cAMP-dependent protein kinase, resulting in an acceleration of the SR Ca^{2+} uptake and LV relaxation.

In the present study using dogs, we assessed the positive inotropic and positive lusitropic effects of milrinone compared with those of dobutamine, which also elevates the cytosolic level of cAMP through a mechanism of β-receptor stimulation, and we demonstrated that milrinone substantially improved LV relaxation and was associated with an enhancement of the SR Ca^{2+} uptake function, probably through the direct inhibition of the SR membrane-bound PDE3.
MATERIALS AND METHODS

Milrinone was provided by Yamanouchi Pharmaceutical (Tokyo, Japan). cGMP, rolipram, cAMP, cAMP-dependent protein kinase, and cAMP-dependent protein kinase inhibitor were all purchased from Sigma (St. Louis, MO). One microgram of cAMP-dependent protein kinase has 1.0–2.0 units of phosphorylating activity, where one unit transfers 1.0 picomole of phosphate from \( [\gamma-^{32}P]ATP \) to hydrolyzed and partially dephosphorylated casein per minute at pH 6.5 at 30°C in the presence of 6.0 mM CAMP (Sigma). One microgram of cAMP-dependent protein kinase inhibitor inhibits 0.75–2.0 phosphorylating units of cAMP-dependent protein kinase (Sigma).

Twelve beagle dogs weighing 10–15 kg were sedated with morphine sulfate (15 mg sc) and cromazine maleate (10 mg sc). They were then anesthetized with isoflurane (2%, 1.5 l/min) and a mixture of nitrous oxide and oxygen (50:50), intubated with a cuffed endotracheal tube, and ventilated at a tidal volume of 22 ml/kg and a respiratory rate of 15 breaths/min. LV pressure was measured by means of a high-fidelity 7-Fr micrometer-on-tipped catheter (Millar) inserted from the left carotid artery. Before it was inserted, the catheter was calibrated at 37°C with a mercury manometer. Zero shift of the pressure transducer was checked by simultaneous recording of a fluid-filled transducer, in which the zero reference point was taken at the level of the right atrium. The care of the animals and the protocols used were in accord with guidelines laid down by the Animal Ethics Committee of Yamaguchi University School of Medicine.

Experimental protocol. After the control recording was measured, a stepwise intravenous infusion of dobutamine (1–10 μg·kg⁻¹·min⁻¹) was started in six dogs. 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 dobutamine was infused, premilrinone baseline hemodynamic values were established by waiting at least 30 min. After the full recovery of hemodynamics was confirmed, milrinone was intravenously administered by a stepwise cumulative infusion of 1–20 μg/kg, with repeat hemodynamic measurements. Five to ten minutes were allowed to obtain a steady state at each dose, and hemodynamic measurements were made before increasing each infusion rate. The order of drug administration was not randomized due to the long duration of hemodynamic effects by milrinone.

In six different dogs, milrinone (4–12 μg/kg) was administered until the peak value of the first derivative of LV pressure (+dP/dt) increased by about 50%, followed by a stepwise infusion of phenylephrine hydrochloride (0–2 μg·kg⁻¹·min⁻¹) to elevate LV pressure.

All data were recorded at the end of an expiration on a multichannel recorder digitized at intervals of 2 ms with an online 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 peak value of dP/dt decrease (−dP/dt), obtained from the digital data of the dP/dt signal, was used to estimate end systole. The time constant (τ) of LV pressure decay during isovolumic relaxation period was calculated as the negative inverse slope of the natural log of the pressure-versus-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 (34).

Preparation of LV crude homogenates and SR vesicles. The homogenates and the SR vesicles were prepared as described previously (11, 24). LVs were homogenized in a solution containing 30 mmol/l Tris-maleate, 0.3 mol/l sucrose, 5 mg/l leupeptin, and 0.1 mol/l phenylmethanesulfonyl fluoride (PMSF) at pH 7.0 (solution I). The homogenate was centrifuged at 5,500 g for 10 min, and the resultant supernatant was filtered through four layers of cheesecloth before centrifugation at 12,000 g for 20 min (LV homogenates). The supernatant was then again filtered through cheesecloth and centrifuged at 143,000 g (55,000 rpm; model TLA 100.4, Beckman Optima) for 30 min. The pellet was resuspended in a solution containing 0.6 mol/l KCl, 30 mmol/l Tris-maleate, 0.3 mol/l sucrose, 5 mg/l leupeptin, and 0.1 mol/l PMSF at pH 7.0 (solution II). This suspension was centrifuged at 143,000 g for 45 min. The pellet was resuspended in solution II, homogenized, and centrifuged at 143,000 g as described above. The pellet was suspended in solution I and centrifuged at 143,000 g. The resultant pellet represents the microsomal fraction rich in SR vesicles, and it was suspended in a solution containing 0.1 mol/l KCl, 20 mmol/l Tris-maleate, 0.5 mol/l sucrose, 5 mg/l leupeptin, and 0.1 mol/l PMSF at pH 7.0 to give a final concentration of 10–20 mg protein/ml. This fraction was rapidly frozen in liquid nitrogen and stored at −80°C. An aliquot was retained for determination of protein concentration by the method of Lowry et al. (18).

Ca²⁺-ATPase activity and cAMP assays in LV crude homogenates. The Ca²⁺-ATPase activity in LV crude homogenates was obtained by measuring the amount of P i released during the reaction after adding ATP. The assay mixture had a total assay volume of 500 μl and contained 150 mmol/l KCl, 20 mmol/l MES (at pH 6.8), 0.3 mmol/l MgCl₂, 10 mmol/l Na₃cit, 10 mmol/l NaF, 6 μM of the ionophore A-23187, 0.32 mmol/l CaCl₂, 0.5 mmol/l EGTA (free [Ca²⁺] = 1 μmol/l), and 0.125 mg crude homogenate. To start the reaction, 1.0 mmol/l ATP was added to the above priming solution in the presence or absence of dobutamine (0–0.3 μmol/l) or milrinone (0–1 μmol/l). The amount of reacted P i was calculated by converting nanometers (absorbance of 0.1% malachite green) to nanomoles by means of a standard linear line (31, 33). The above procedures were repeated in the presence of 1 μmol/l thapsigargin. The thapsigargin-sensitive portions of the reacted P i (82.3 ± 7.7% of total Ca²⁺-ATPase activity) were subtracted from the total reacted P i. The thapsigargin-sensitive portions of the reacted P i were then obtained and defined as the SR Ca²⁺-ATPase activity.

The cAMP content in LV crude homogenate was determined with an enzyme immunoassay kit (Biotrak, cAMP enzyme immunoassay system, Amersham International) according to the kit instructions.

Ca²⁺⁺ uptake assay in purified SR vesicles. The SR vesicles (0.6 mg/ml) were preincubated in a solution containing 0.15 mol/l KCl, 1 mmol/l MgCl₂, 10 mmol/l Na₃cit, 20 mmol/l MES (at pH 6.8), 5 mmol/l oxalate, 0.2 mmol/l EGTA, 0.09 mmol/l CaCl₂ (free [Ca²⁺] = 0.1 μmol/l), and 2.5 μmol/l fluo 3 as a Ca²⁺ indicator. ATP (1 mmol/l) was then added to the above priming solution to load the SR with Ca²⁺⁺. The SR Ca²⁺⁺ uptake was measured by the change in the fluorescence intensity of fluo 3, recorded in a cuvette with an excitation wavelength of 480 nm and an emission wavelength of 530 nm using a spectrophotometer (model F2000, Hitachi, Tokyo, Japan). The Ca²⁺⁺ uptake (nmol/mg) was calculated from the fluorescence intensity of fluo 3 after determining the coefficient of fluo 3 signal divided by the change in [Ca²⁺⁺] at each [Ca²⁺⁺] in the range of 0.03–0.3 μmol/l adjusted with the EGTA-Ca²⁺⁺ buffer (13, 35).

The effect of cAMP-dependent phosphorylation on the SR Ca²⁺⁺ uptake was determined by addition of cAMP to the SR vesicles in the presence or absence of 5 μg/ml cAMP-depen-
Table 1. Effect of dobutamine or milrinone on hemodynamics

<table>
<thead>
<tr>
<th>Dobutamine, g/kg min⁻¹</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
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<td>HR, beats/min</td>
<td>84</td>
<td>84</td>
<td>86</td>
<td>86</td>
<td>90</td>
<td>93</td>
<td>99</td>
<td>103</td>
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<tr>
<td>LVPSP, mmHg</td>
<td>123</td>
<td>128</td>
<td>135</td>
<td>138</td>
<td>142</td>
<td>148</td>
<td>151</td>
<td>151</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>5.7</td>
<td>5.7</td>
<td>6.7</td>
<td>7.3</td>
<td>7.2</td>
<td>7.8</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>peak +dP/dt, mmHg/s</td>
<td>2.15</td>
<td>2.36</td>
<td>2.76</td>
<td>3.07</td>
<td>3.51</td>
<td>4.03</td>
<td>4.56</td>
<td>4.86</td>
</tr>
<tr>
<td>τ, ms</td>
<td>29.1</td>
<td>28.7</td>
<td>28.0</td>
<td>27.2</td>
<td>25.7</td>
<td>24.3</td>
<td>23.0</td>
<td>21.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 dogs. HR, heart rate; LVPSP, left ventricular (LV) peak systolic pressure; LVEDP, LV end-diastolic pressure; peak +dP/dt, peak value of the first derivative of LV pressure; τ, time constant of LV pressure decay during isovolumic relaxation period. *P < 0.05 vs. baseline.

...dient protein kinase. PDE3 inhibitors (milrinone and cGMP) were also added in the presence of 0.1 μmol/l cAMP and 5 μg/ml cAMP-dependent protein kinase. To evaluate the effect of phosphodiesterase 4 (PDE4) inhibition on the SR Ca²⁺ uptake, rolipram was also added to quantify the extent of cAMP-dependent activation of the SR Ca²⁺ uptake mediated through cAMP-dependent protein kinase.

**Statistics.** Data are presented as means ± SE or SD. Changes within the same group were analyzed by one-way analysis of variance (ANOVA) for repeated measures and subsequent Fisher's protected least-significant difference. Differences between two groups were analyzed by two-way ANOVA and subsequent paired Student's t-test. Statistical significance was defined by P < 0.05.

**RESULTS**

Hemodynamic data before and after administration of milrinone or dobutamine. Hemodynamics are summarized in Table 1. After dobutamine (1–10 μg·kg⁻¹·min⁻¹) was administered, the heart rate tended to increase, and peak LV pressure gradually increased. LV end-diastolic pressure tended to increase. The peak +dP/dt of LV pressure significantly increased, and τ was shortened. Although the peak +dP/dt increased to a lesser extent than dobutamine, τ was shortened significantly more than dobutamine. Figure 1 shows the relationship between percent change in τ and that in +dP/dt after milrinone or dobutamine administration. At a similar percent increase in +dP/dt, τ was shortened significantly more by milrinone than by dobutamine.

Table 2 summarizes the hemodynamic data after the addition of milrinone and phenylephrine hydrochloride. τ, which was shortened by preinfusion of milrinone, was not prolonged after phenylephrine hydrochloride, although the peak LV pressure increased by about 25%. Figure 2 shows a representative example of dP/dt-pressure loops of LV during the administration of milrinone or milrinone plus phenylephrine hydrochloride. The slope of the linear relation between dP/dt and pressure during the isovolumic relaxation period that indicates LV relaxation function (5, 10) became steeper with milrinone, and it was not significantly influenced by a small rise in LV pressure by phenylephrine hydrochloride.

**Effects of dobutamine and milrinone on SR Ca²⁺-ATPase activity and cAMP level in LV crude homogenates.** Figure 3 shows the relationship between the SR Ca²⁺-ATPase activity and the cAMP level after the addition of milrinone or dobutamine in LV crude homogenates. At a given increase in the SR Ca²⁺-ATPase activity, the cAMP level was significantly less increased by milrinone than by dobutamine, indicating the higher sensitivity of Ca²⁺-ATPase activity on cAMP in the case of milrinone.

![Fig. 1. Relationship between the percent change in the time constant (τ) of left ventricular (LV) pressure decay and that in peak value of the first derivative of LV pressure over time (+dP/dt) during the infusion of milrinone (●; 3, 6, 10, or 20 μg/kg) or dobutamine (○; 1, 2, 3, or 4 μg·kg⁻¹·min⁻¹). At the similar percent increase in peak +dP/dt, τ was shortened significantly more with dobutamine. Data are means ± SE. *P < 0.05 vs. baseline; #P < 0.05 vs. baseline.](http://ajpheart.physiology.org/Downloadedfromhttp://ajpheart.physiology.org/by10.22033.5onJune29,2017)
Effects of milrinone, cGMP, and rolipram on SR Ca\(^{2+}\) uptake in purified SR vesicles. Figure 4, A–C, shows the dose-dependent effect of cAMP on the SR Ca\(^{2+}\) uptake in the absence or presence of milrinone, cGMP, or rolipram. The addition of cAMP increased the SR Ca\(^{2+}\) uptake in a dose-dependent fashion, and the half-maximum effect was obtained at ~0.3 μmol/l cAMP. Both 10 μmol/l milrinone and 30 μmol/l cGMP shifted the curves upward and to the left, indicating the stimulation of the SR Ca\(^{2+}\) uptake by milrinone and cGMP. In contrast, rolipram had virtually no effect on the cAMP dependence of the SR Ca\(^{2+}\) uptake. Figure 5, A–C, shows the dose-dependent effect of milrinone, cGMP, or rolipram on the SR Ca\(^{2+}\) uptake in the presence of 0.1 μmol/l cAMP and 5 μg/ml cAMP-dependent protein kinase. Both milrinone and cGMP stimulated the SR Ca\(^{2+}\) uptake in a dose-dependent fashion. The half-maximum stimulating effect was elicited at ~0.5 μmol/l by milrinone and 2 μmol/l by cGMP. However, rolipram again had no dose-dependent effect on the SR Ca\(^{2+}\) uptake.

Figure 6 shows the effect of cAMP, cAMP-dependent protein kinase, or cAMP-dependent protein kinase inhibitor on the SR Ca\(^{2+}\) uptake. cAMP or cAMP-dependent protein kinase alone did not enhance the SR Ca\(^{2+}\) uptake. Only when cAMP was added together with the cAMP-dependent protein kinase was the SR Ca\(^{2+}\) uptake significantly enhanced. The cAMP-dependent protein kinase inhibitor partially inhibited the baseline Ca\(^{2+}\) uptake (without cAMP and protein kinase) and completely inhibited the increase in the SR Ca\(^{2+}\) uptake by the addition of cAMP plus protein kinase up to the level below the baseline Ca\(^{2+}\) uptake. Both milrinone and cGMP also did not increase Ca\(^{2+}\) uptake without cAMP (Fig. 4) or cAMP-dependent protein kinase (data not shown). Both milrinone and cGMP increased Ca\(^{2+}\) uptake only when both the protein kinase and cAMP were added together with the SR vesicles. The protein kinase inhibitor completely inhibited the augmentation of the SR Ca\(^{2+}\) uptake after the addition of milrinone or cGMP to the level below baseline (data not shown).

**DISCUSSION**

The major findings of this study are as follows. First, the PDE3 inhibitor milrinone accelerated LV relaxation much more than dobutamine, as evidenced by the decrease in τ of LV pressure decay. Second, milrinone increased the SR Ca\(^{2+}\)-ATPase activity in LV crude homogenates and the SR Ca\(^{2+}\) uptake in dose-dependent fashion and also stimulated the response of cAMP-induced augmentation of the SR Ca\(^{2+}\) uptake.

**Table 2. Afterloading effect of phenylephrine hydrochloride on hemodynamics**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Milrinone</th>
<th>Milrinone + Phenylephrine (first group)</th>
<th>Milrinone + Phenylephrine (second group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>76 ± 7</td>
<td>80 ± 7</td>
<td>78 ± 8</td>
<td>79 ± 8</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>133 ± 7</td>
<td>131 ± 7</td>
<td>147 ± 8†</td>
<td>163 ± 10‡</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>8.1 ± 0.3</td>
<td>4.6 ± 0.4*</td>
<td>5.6 ± 0.6*</td>
<td>6.5 ± 1.4†</td>
</tr>
<tr>
<td>peak +dP/dt, mmHg/s</td>
<td>2,284 ± 49</td>
<td>3,408 ± 56*</td>
<td>3,594 ± 123*</td>
<td>3,617 ± 93*</td>
</tr>
<tr>
<td>τ, ms</td>
<td>29.9 ± 2.9</td>
<td>22.5 ± 2.4*</td>
<td>22.8 ± 1.5*</td>
<td>23.9 ± 1.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 dogs. *P < 0.05 vs. baseline; †P < 0.05 vs. milrinone; and ‡P < 0.05 vs. milrinone + phenylephrine hydrochloride (first group).
Third, cGMP also increased the SR Ca\textsuperscript{2+} uptake in a dose-dependent fashion, whereas the PDE4 inhibitor rolipram had no effect on the cAMP-dependent augmentation of the SR Ca\textsuperscript{-} uptake.

**Different hemodynamic effects between milrinone and dobutamine.** Milrinone, a PDE3 inhibitor, is known to enhance LV contractility by an inhibition of the breakdown of cAMP through the mechanism inhibiting PDE3 (2, 3, 7, 25). However, it remains to be elucidated whether milrinone also exerts positive lusitropy, like dobutamine. Although both dobutamine and milrinone elevate the cytosolic level of cAMP, the present results show the clear difference in positive

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**Fig. 5.** Dose-dependent effect of milrinone (A; ●), cGMP (B; ▲), or rolipram (C; ■) on the SR Ca\textsuperscript{2+} uptake in the presence of 0.1 μmol/l cAMP and 5 μg/ml cAMP-dependent protein kinase. Data represent means ± SD of 5 experiments from 3 different preparations. Both milrinone and cGMP stimulated the SR Ca\textsuperscript{2+} uptake in a dose-dependent fashion, whereas rolipram had no effect on the Ca\textsuperscript{2+} uptake. *P ≤ 0.05 vs. baseline (0 μmol/l milrinone or cGMP).
lusitropic action between dobutamine and milrinone. The effect was decreased to a greater extent by milrinone than by dobutamine when compared at a dose by which a similar increase of +dP/dt was elicited. Therefore, in the case of milrinone, an additional mechanism for the improvement of LV relaxation should be considered.

Inhibition of SR membrane-bound PDE3 by milrinone or cGMP. Low Michaelis-Menten constant cGMP-inhibited PDE3 activity has been identified in both cytosolic and SR-enriched microsomal fractions of the mammalian myocardium (14, 16). There are two reported genes for the PDE3 family, PDE3A and PDE3B, and four genes for PDE4 (PDE4A–PDE4D) (4). Some molecular probes have recently been used to define which phosphodiesterase genes are in the canine heart. PDE4D and PDE3A mRNAs (28) and several PDE3A proteins are present in canine ventricles (30). It is not known whether PDE3B is also present. An antibody to human platelet PDE3A cross reacts on Western blots with canine ventricular proteins in both the cytoplasm and SR-enriched fractions (30). However, Liu and Maurice (17) suggested that the microsomal forms of PDE3 are PDE3B (125–135 kDa) in cardiovascular tissues, whereas PDE3A represents the alternatively spliced cytosolic forms (e.g., 80–120 kDa).

Evidence has accumulated to suggest that certain cardiotonic agents (milrinone, imazodan, and amrinone) inhibit SR membrane-bound PDE3 (27, 32, 33) and exert their contractile effects through subtle alterations in the metabolism of cAMP (15, 16). With regard to this, functional compartmentalization of cAMP and protein kinases has previously been proposed for cardiac muscle (1, 12), and, hence, intracellular Ca^{2+} mobilization might be affected by cAMP located in the particulate compartment of canine cardiac myocytes (12).

The present findings showed that SR Ca^{2+} uptake was accelerated by cAMP in the presence of cAMP-dependent protein kinase, and both milrinone and cGMP raised the sensitivity of cAMP on the SR Ca^{2+} uptake. The half-maximum stimulating effect of milrinone on the SR Ca^{2+} uptake was elicited at 0.3–0.5 μmol/l, which is very similar to the concentration range of milrinone by which half-maximum phosphodiesterase inhibition is elicited. Moreover, in crude LV homogenates, milrinone hypersensitized the SR Ca^{2+}-ATPase activity on cAMP compared with dobutamine. Taken together, it is strongly suggested that milrinone specifically binds the SR membrane-bound PDE3, and inhibition of PDE3 could lead to localized increases in cAMP with a resultant activation in cAMP-dependent protein kinase, followed by an increase in the SR Ca^{2+} uptake through phosphorylation of phospholamban.

However, milrinone has been reported to have no effect on the phosphorylation of phospholamban in the SR vesicles isolated from guinea pig hearts (26). The reduced inotropic response to the PDE3 inhibitors in guinea pig myocardium has been attributed to an absence of SR-associated activity in these species (32, 33). More recently, Smith et al. (29) demonstrated that, like dogs, humans, and rabbits, guinea pig ventricular SR vesicles contain a 135-kDa PDE3 that is a substrate itself for cAMP-dependent protein kinase. These authors argued that the lack of in vivo inotropic effects of PDE3 inhibitors in rodents cannot be explained by the absence of PDE3 in the SR fraction, as was previously suggested by Weishaar et al. (33). Endogenous levels of endogenously phosphorylated phospholamban, phosphatase, and/or cAMP-dependent protein kinase may differ between guinea pig and dog SR vesicles.

In the present study, because exogenous cAMP (with or without milrinone and cGMP) did not increase the Ca^{2+} uptake in the absence of added cAMP-dependent protein kinase, endogenous phosphorylation of phospholamban might not blunt an additional effect of cAMP-dependent protein kinase in dog SR vesicles.

Effect of PDE4 inhibition on SR Ca^{2+} uptake. The SR microsomes largely contain cGMP-inhibited PDE3, whereas the cGMP-insensitive PDE4 is present in the sarcolemmal fraction (20). However, depending on the
purity of the microsomal preparation, the sarcolemmal fraction containing the cGMP-insensitive PDE4 may be contaminated. In this regard, we evaluated the effect of the PDE4 inhibitor rolipram on the SR Ca\(^{2+}\) uptake. As a result, rolipram had no effect on the cAMP-dependent activation of the SR Ca\(^{2+}\) uptake, unlike milrinone or cGMP, suggesting that the inhibition of particulate PDE3 indeed mediates cAMP-dependent activation of the SR Ca\(^{2+}\)-ATPase.

**Afterload reduction by milrinone and LV relaxation**

Milrinone is known to exert a vasodilating effect as well as positive inotropic and lusitropic effects. Therefore, afterload reduction by this drug may induce acceleration of LV relaxation. However, when LV pressure was increased by about 25% (mean pressure 30 mmHg) by adding phenylephrine hydrochloride together with milrinone, \(\tau\) was not significantly influenced. With regard to this, we (36) previously showed that \(\tau\) was not significantly increased by a small increase (~20–30 mmHg) in peak LV pressure unless systolic loading sequence is dramatically changed, i.e., by early or late systolic loading. In the present study, because the time to peak systolic pressure did not significantly change (data not shown) after administration of milrinone, it seems unlikely that vasodilatation by milrinone could account for the changes in \(\tau\). In our study, LV end-diastolic pressure decreased in association with the shortening of \(\tau\). Because LV preload itself does not influence the isovolumic relaxation rate (8), the shortening of \(\tau\) after milrinone administration might be provided by the direct effect of this drug, which in turn may partly contribute to the decreasing tendency of LV end-diastolic pressure.

**Direct effect of milrinone on LV relaxation.**

In the clinical setting, both dobutamine and milrinone have been shown to lead to a significant improvement in the hemodynamic state of patients with acute heart failure. However, much of the research in heart failure has been shown to lead to a significant improvement in the SR Ca\(^{2+}\) uptake, probably through an inhibition of SR membrane-bound, not cytosolic, PDE3.

In conclusion, milrinone substantially improved LV relaxation in association with an acceleration of Ca\(^{2+}\) uptake by SR. This acceleration might be due to an inhibition of membrane-bound PDE3 in SR, which might induce a local elevation of cAMP.

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