Inotropic Responses to Isoproterenol and Phosphodiesterase Inhibitors in Intact Guinea Pig Hearts: Comparison of Cyclic AMP Levels and Phosphorylation of Sarcoplasmic Reticulum and Myofibrillar Proteins

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The influence of selective (milrinone: 10, 50, 100 μM) and nonselective phosphodiesterase (isobutylmethylxanthine: 0.1, 10, 100 μM) inhibitors and β-adrenergic stimulation (isoproterenol: 0.01, 0.1 μM) on phospholamban and myofibrillar protein phosphorylation was studied in guinea pig hearts perfused with [32P]orthophosphate. Changes in protein phosphorylation were compared to alterations in tissue cyclic AMP (cAMP) levels and positive inotropic effects induced by these agents. Isoproterenol (0.01 μM), milrinone (50 μM), and isobutylmethylxanthine (100 μM) all produced similar, twofold increases in dP/dt and −dP/dt but only stimulation with isobutylmethylxanthine and isoproterenol was associated with significant increases in phospholamban phosphorylation. At these equipotent doses, the effects of isobutylmethylxanthine were associated with higher increases (3.1-fold) in cAMP than those observed with isoproterenol (twofold). Milrinone (50 μM) produced a 2.5-fold increase in cAMP levels but failed to change phospholamban phosphorylation. Higher doses of milrinone (100 μM) resulted in relatively high (4.1-fold) cAMP levels, and this was associated with increased (1.5-fold) phosphorylation of phospholamban. Phosphorylation of troponin I was significantly increased at 0.01 and 0.1 μM isoproterenol, while phosphorylation of C protein was observed only at 0.1 μM isoproterenol. Isobutylmethylxanthine and milrinone did not significantly increase phosphorylation of either troponin I or C protein at any of the doses studied. These findings indicate that cardiotonic agents acting via the cAMP pathway may produce similar inotropic responses at different levels of cAMP and phosphorylation of sarcoplasmic reticulum and myofibrillar proteins. (Circulation Research 1989;64:104–111)

Changes in the level of cyclic AMP (cAMP) during the response of hearts to inotropic interventions may be due to alterations in the rates of separate processes regulating synthesis and breakdown of cyclic nucleotides. Activation of adenylate cyclase, for example, by β-receptor stimulation, is one pathway which leads to the increased formation of cAMP.1 An alternative pathway to elevate cellular cAMP is by the inhibition of phosphodiesterase(s) (PDE) which catalyze its hydrolysis.2 Apart from the mechanisms for increases in cAMP, it is generally accepted that there is activation of cAMP-dependent protein kinases, which in turn may catalyze the phosphorylation of various intracellular proteins.3 In cardiac cells, such phosphorylations may alter the function of proteins resulting in an increased Ca²⁺ influx through the sarcolemmal Ca²⁺ channels4 as well as increased rate of Ca²⁺ uptake and thus, storage of Ca²⁺ in the sarcoplasmic reticulum.5 Ultimately, there is an increase in contractility associated with a rise in Ca²⁺ available to the myofibrillar proteins, which are also phosphorylated.6

Previously, we have shown that β-adrenergic stimulation of isolated beating hearts results in increased phosphorylation of phospholamban in sarcoplasmic reticulum (SR) and troponin I (TnI) and C protein in the myofibrils.7–9 Phosphorylation of phospholamban was associated with increases in...
SR Ca\(^{2+}\) transport\(^9\) and Ca\(^{2+}\)-ATPase activity\(^10\) in agreement with in vitro studies.\(^{11,12}\) Phosphorylation of myofibrillar proteins is also associated with a decreased Ca\(^{2+}\) sensitivity of the myofibrillar Mg\(^{2+}\)-ATPase activity.\(^9\) These findings support the hypothesis that stimulation of SR Ca\(^{2+}\) uptake and reduction of Ca\(^{2+}\) sensitivity by the myofibrillar proteins, mediated by cAMP-dependent phosphorylation of phospholamban, Tnl, and C protein, may partially underly the myocardial effects of agents that increase cAMP.

The present study tests the hypothesis that increases in cAMP levels mediated by PDE inhibition are associated with in vivo protein phosphorylation similar to that observed with \(\beta\)-adrenergic stimulation of the heart. The nonspecific PDE inhibitor isobutylmethylxanthine (IBMX),\(^{13,14}\) along with the specific PDE inhibitor milrinone,\(^{13,14}\) which has been recently shown to inhibit selectively the PDE type IV associated with canine cardiac SR,\(^{15}\) were used. Studies were designed to answer whether PDE inhibition by IBMX and milrinone is associated with 1) increases in tissue cAMP levels and positive inotropic and enhanced relaxation responses and 2) increases in phosphorylation of phospholamban, Tnl, and C protein.

**Materials and Methods**

**Heart Perfusion**

Hearts from anesthetized (30 mg/kg sodium pentobarbital) and heparinized (5,000 units/kg) Hartley guinea pigs (600–700 g) were rapidly excised and immediately cannulated for retrograde aortic perfusion of the coronary arteries with Krebs-Henseleit balanced salt solution.\(^{16,17}\) The Krebs-Henseleit solution contained (mM) 118 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 25 NaHCO\(_3\), 0.5 Na\(_2\) EDTA, 0.26 KH\(_2\)PO\(_4\), and 5.5 glucose, yielding a total Ca\(^{2+}\) concentration of 2.0 mM and a K\(^+\) concentration of 4.9 mM. The buffer solution was saturated with 95% O\(_2\)-5% CO\(_2\), which maintained a pH of 7.4; perfusion temperature was set at 37±0.2°C. The hearts were perfused at a constant aortic pressure of 65 mm Hg, initially for 20–30 minutes in a nonrecirculating flow to allow the preparations to stabilize. The perfusion circuit was switched to a recirculating flow containing 120 ml of the same buffer and to which 1–2 mCi of \(^{32}\)Porthophosphate (\(^{32}\)P, in H\(_2\)O; NEN Research Products, Boston, Massachusetts) was added for 30 minutes. After this labeling period, the circuit was returned to a nonrecirculating flow with nonradioactive buffer for 2 minutes, and drugs of interest were then introduced into the buffer flow line, at a point just before the perfusion fluid entered the coronary system. At the peak of the positive inotropic response to each cardiotonic agent (approximate perfusion times: 1 minute with isoproterenol, 4 minutes with IBMX and milrinone), the hearts were freeze-clamped with precooled (~196°C) Wolffenberger clamps, powdered, and stored under liq-
Pended in inhibiting buffer containing 1% Triton X-100 and were centrifuged at 3,500g for 10 minutes. The pellets were subsequently washed three times as above with standard buffer (60 mM KCl, 30 mM Imidazole, and 2 mM MgCl₂, pH 7.0) containing 10 mM NaF. The resulting pellet was resuspended in a final volume (5 ml) of standard buffer containing 10 mM NaF. The yield was 30–40 mg of myofibrillar protein/heart as determined according to the Lowry method.¹⁸

**Gel Electrophoresis**

Polyacrylamide gel electrophoresis under denaturing conditions was performed by the procedures described by Laemmli with a 5–18% gradient acrylamide slab gel. Samples of ³²P-labeled SR or ³²P-labeled myofibrils were solubilized by addition of an equal volume of sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 6% sodium dodecyl sulfate, 20% glycerol, 5% β-mercaptoethanol, and a trace of bromophenol blue as a tracking dye. Generally, 150–200 μg of sample protein were applied to each lane and electrophoresis was conducted at 35 mA/gel. In each set of experiments, two identically loaded gels were run with one gel being placed into fixing (20% trichloroacetic acid; 5% methanol; 1% glycerol) for 24 hours after electrophoresis. The second gel was dried, and autoradiography was performed by placing the dried gel in contact with Kodak OG-1 film in Kodak Lanex Regular cassettes (with intensifying screens; Rochester, New York) at −70°C for 24–72 hours. The radioactive bands corresponding to phospholamban, TnI, and C protein were identified on the appropriate autoradiograms and then cut from the fixed gel for counting in nonaqueous scintillation fluid. Phosphate incorporation into the various proteins was quantified by dividing the ³²P-incorporation in each band by the specific activity of [γ-³²P]ATP determined for that heart, and expressed as picomoles ³²P per milligram protein.

**Cyclic AMP Assay**

Tissue cAMP content in samples from ³²P-perfused hearts was determined with a radioimmunoassay kit (Rianen cAMP [¹²⁵I]RIA Kit, DuPont Specialty Diagnostics, Wilmington, Delaware). These assays were performed approximately six months after perfusion to allow for sufficient decay of ³²P-radioactivity. Samples (50 μg) of frozen powdered tissue were homogenized in 6% (wt/vol) trichloroacetic acid, extracted in ether, acetylated and assayed in triplicate. Results were corrected for recoveries, which were monitored with [¹H]cAMP and routinely determined to be 70–80%.

**Other Assays**

The specific activity of [γ-³²P]ATP in each heart was determined from the specific radioactivity of [³²P]-phosphocreatine extracted and assayed as described by Kopp and Barany.²² Generally, 200 μg samples of powdered tissue were used for this determination. There was no significant change in the specific activity of [γ-³²P]ATP among hearts perfused on a particular day with the same stock solution of [³²P].

Statistical analysis was performed using the Student’s t test for unpaired observations.

**Results**

**Phospholamban Phosphorylation by Phosphodiesterase Inhibitors and β-Adrenergic Stimulation**

The β-adrenergic agonist isoproterenol and the PDE inhibitors milrinone and IBMX were examined for their ability to increase ³²P-incorporation in SR membrane proteins isolated from perfused guinea pig hearts. Autoradiograms of typical experiments are shown in the left panels of Figures 1 and 2.

In agreement with previous observations, isoproterenol increased ³²P-incorporation into proteins with apparent Mr = 27,000 and 11,000 (left panels of Figures 1 and 2, arrows), the high and low molecular weight forms of phospholamban.⁹,¹⁰ The electrophoretic mobility of the 27,000 Mᵣ form of phospholamban could be altered by varying the solubilization conditions prior to electrophoresis. Boiling of the SR samples in 6% sodium dodecyl sulfate for 5 minutes completely converted the high Mr form of phospholamban into the 11,000 Mr, form (Figure 1, left panel, lanes 5 and 6; Figure 2, left panel, lanes 3 and 4), as has previously been reported.⁹,¹⁰ Treatment with isoproterenol at 0.1 μM resulted in a 4.6-fold increase in ³²P-incorporation into phospholamban over control, while treatment with 0.01 μM isoproterenol resulted in a 2.2-fold increase (left panels of Figures 1 and 2; Table 1). A third major protein, with an apparent Mr of 15,000, also displayed an isoproterenol-induced increase in ³²P-incorporation. This phosphoprotein, whose mobility did not change upon boiling, is probably of sarcolemmal membrane origin.²³

Exposure to the nonspecific PDE inhibitor IBMX at concentrations of 0.1 μM did not appear to alter ³²P-incorporation into phospholamban while concentrations of 10 μM were associated with slight increases (Figure 1, left panel, lanes 3 and 4; Table 1). Elevating the IBMX concentration to 100 μM produced a significant increase (1.6-fold) in phospholamban phosphorylation (Figure 2, left panel, lanes 5 and 6; Table 1). Lindemann and Watanabe have previously reported that IBMX induced larger increases in contractility and ³²P-incorporation into phospholamban in perfused guinea pig hearts compared to those observed in this study. The reason for this discrepancy may be related to differences in experimental conditions including perfusion temperature and cardiac frequency, thereby reflecting differences in the potential for changes in contractility.²⁵,²⁶

When perfused hearts were exposed to the selective PDE type 1V inhibitor, milrinone, at concentrations of 10 μM and 30 μM, no effects on phos-
phospholamban phosphorylation were observed (Figure 1, left panel, lanes 7 and 8; Table 1). Similar to what we observed with IBMX, higher concentrations of milrinone (100 μM) produced a significant increase (1.5-fold) in 32P-incorporation into phospholamban (Figure 2, left panel, lanes 7 and 8; Table 1).

Myofibrillar Protein Phosphorylation by Phosphodiesterase Inhibitors and β-Adrenergic Stimulation

Myofibrils were prepared from the same hearts used in the studies for SR phosphorylation, described above. Isoproterenol stimulation was associated with an increase in the extent of phosphorylation of TnI and a 130,000–140,000 M protein (Figure 1, right panel, lanes 5 and 6; Figure 2, right panel, lanes 3 and 4), most likely C protein. Concentrations of 0.01 μM produced a 2.0-fold increase in TnI phosphorylation, while 0.1 μM isoproterenol produced a 2.5-fold increase (Table 1). However, changes in C protein phosphorylation were demonstrated only with 0.1 μM isoproterenol (1.6-fold increase). Perfusion of guinea pig hearts with either IBMX or milrinone in doses up to 100 μM did not alter TnI or C protein phosphorylation levels. In agreement with data showing a failure of the PDE inhibitors to increase TnI and C protein phosphorylation, there was a lack of effect on Ca2+ activation of myofibrillar Mg2+-dependent ATPase activity (data not shown).

Effects of Phosphodiesterase Inhibitors and β-Adrenergic Stimulation on cAMP Levels and Contraction/Relaxation

Data summarized in Table 1 show that in the presence of 10 μM and 100 μM IBMX, cAMP levels were elevated respectively by 1.5 and 3.7 times compared with control. In response to milrinone there was a 2.5-fold increase in cAMP levels at 50 μM and a 4.1-fold increase at 100 μM. Perfusions of hearts with isoproterenol were associated with a 1.8-fold increase in cAMP levels at 0.01 μM and a 5.4-fold increase at 0.1 μM.

Levels of myocardial activity, as measured by +dP/dt (contraction) and −dP/dt (relaxation), were determined in the same hearts from which SR vesicles and myofibrillar preparations were isolated. Isoproterenol at 0.01 μM produced an approximate 2.0-fold stimulation in +dP/dt and a 2.5-fold increase in −dP/dt, and these responses were similar to responses obtained with 100 μM IBMX and 50 μM milrinone (Table 1). This level of activation by 0.01 μM isoproterenol correlated well with its ability to elevate cAMP levels and phosphorylation of phospholamban and TnI. IBMX at 100 μM elevated 32P-incorporation into phospholamban to levels similar to those obtained with 0.01 μM isoproterenol (Figure 3). Milrinone (50 μM) elevated cAMP to levels similar to those obtained with IBMX and milrinone. For both IBMX and milrinone, the increases in cAMP were associated with a similar increase in phosphorylation of phospholamban and TnI.
FIGURE 2. Autoradiography of sodium dodecyl sulfate (0.1%) polyacrylamide (5–18%) gel electrophoresis of sarcoplasmic reticulum (150 µg) and myofibrils (100 µg) isolated from guinea pig hearts perfused with [32P]orthophosphate. Sarcoplasmic reticulum enriched membrane preparations (left) and myofibrils (right) were isolated from hearts frozen after perfusion with Krebs-Henseleit buffer (CON), 0.01 µM isoproterenol (ISO) for 1 minute, 100 µM isobutylmethylxanthine (IBMX) for 5 minutes, or 100 µM milrinone (MIL) for 4 minutes. Some samples were held at room temperature (−boil) while others were boiled for 5 minutes (+boil) before electrophoresis as described in “Materials and Methods.” PLBH, high molecular weight form of phospholamban; PLBL, low molecular weight form of phospholamban.

demonstrated with 0.01 µM isoproterenol, but there was no change in protein phosphorylation observed. Perfonnsions of hearts with 100 µM milrinone resulted in further elevation in cAMP levels (twofold higher than those observed with 0.01 µM isoproterenol) but produced only a modest increase in 32P-incorporation into phospholamban. With this concentration, there was a decreased positive inotropic response (1.5-fold of control) as compared with 50 µM milrinone (2.1–2.3-fold increase). Compared with 0.01 µM isoproterenol, higher doses of isoproterenol (0.1 µM) were associated with increases in +dP/dt and −dP/dt and cAMP levels, but only phosphorylation of phospholamban increased significantly (Table 1).

Discussion

Our results demonstrate that the PDE inhibitor agents IBMX and milrinone can significantly elevate intracellular cAMP and cardiac contractility and relaxation with little or no stimulation of SR and myofibrillar protein phosphorylation in intact hearts. In contrast, an equipotent concentration of isoproterenol produced similar increases in cAMP levels and in phosphorylation of phospholamban and TnI.

One possible explanation for the demonstrated lack of effect on SR and myofibrillar protein phosphorylation by the PDE inhibitors is that these agents may have an alternative cellular site of action. It is known that β-agonists increase Ca2+ influx via the slow inward channels,28 and evidence indicates that in vivo β-adrenergic stimulation is associated with increased phosphorylation of a sarcolemmal 15,000 M, protein.23 Isobutylmethylxanthine28 and milrinone30 have been shown to increase transsarcolemmal Ca2+ influx, and this may play a role in their positive inotropic effects. It is plausible then that the biochemical mechanisms involved in the positive inotropic effects of these two drugs are through cAMP-dependent phosphorylation of sarcolemmal protein(s), which may increase transsarcolemmal Ca2+ influx. Intracellular Ca2+ so elevated would be available for enhanced myofilament activation and SR Ca2+ uptake and release and may ultimately lead to increased tension development.

A more complex interpretation of our data relates to the possible cellular compartmentation of phosphodiesterase isozymes, cAMP, protein kinases, and their substrates. Functional compartmentation of cAMP and protein kinases has previously been proposed for cardiac muscle.31-33 In relation to this concept, there is evidence that PDEs are also compartmentalized in heart.33 Milrinone is known to specifically inhibit the “low Km” or “cGMP-inhibited” PDE type IV isozyme,13,14 which recently has been demonstrated to be membrane-bound and associated with the SR in dog heart.15 Inhibition of this PDE isozyme could lead to localized increases in cAMP and cAMP-dependent protein kinase pools and thus to increases in phospholamban phosphorylation. However, recent evidence by Weishaar et al34 indicates that most of this PDE isozyme is found in the soluble compartment of guinea pig heart cells rather than in the membrane-bound form. These findings are consistent with our observations that low concentrations of milrinone (≤50 µM), despite elevating cAMP and contraction/relaxation activities, failed to elicit changes in phospholamban phosphorylation. The increase in tension...
under these circumstances could be due to possible cAMP-dependent activation of trans-sarcolemmal Ca\(^{2+}\) influx. The positive inotropic effects of milrinone have been shown to occur with increases in the peak of the intracellular Ca\(^{2+}\) transient and with increases in the rates of Ca\(^{2+}\) release and uptake by the SR.\(^{33}\) At higher concentrations of milrinone (100 \(\mu M\)) we observed low levels of phospholamban phosphorylation, which may be due either to inhibition of the small amounts of SR membrane-bound PDE type IV isozyme or to higher activation of the cAMP-dependent protein kinase(s). Alternatively, phospholamban phosphorylation may be due to Ca\(^{2+}/\)calmodulin-dependent protein kinase, activated by the increased trans-sarcolemmal Ca\(^{2+}\) influx. This is, however, less likely to occur in view of evidence\(^{36}\) demonstrating a lack of effect on phospholamban phosphorylation by changes in extracellular Ca\(^{2+}\).

We observed that the increase in contractility was less at 100 \(\mu M\) milrinone than at 50 \(\mu M\) milrinone, although cAMP levels were 1.5-fold larger at the higher drug concentration. The reasons for this effect are likely to be complex but may be due to direct inhibition of cAMP-dependent protein kinase activity by milrinone.\(^{37}\)

Apparently, milrinone and IBMX were not able to activate a cAMP-dependent compartment(s) associated with the myofibrils, since no changes in Tnl or C protein phosphorylation were observed. This was supported by a lack of effect on the Ca\(^{2+}\)-

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### Table 1. Protein Phosphorylation, Rates of Contractility, and Relaxation and Cyclic AMP Levels in Perfused Guinea Pig Hearts After Phosphodiesterase Inhibition and \(\beta\)-Adrenergic Stimulation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate of contraction (+dP/dt (% Control))</th>
<th>Rate of relaxation (-% Control)</th>
<th>P, Incorporation (pmol (^{32})P/mg protein)</th>
<th>Cyclic AMP (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.8±4.0 (15)</td>
<td>101.4±3.4 (15)</td>
<td>65.3±6.7 (9)</td>
<td>61.6±11.7 (8)</td>
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<td>Isoproterenol</td>
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<tr>
<td>0.01 (\mu M)</td>
<td>210.9±13.1* (7)</td>
<td>218.8±30.9 (9)</td>
<td>142.8±20.5* (7)</td>
<td>126.9±28.1* (6)</td>
</tr>
<tr>
<td>0.1 (\mu M)</td>
<td>305.0±30.0* (12)</td>
<td>291.8±32.4 (12)</td>
<td>300.0±66.0* (4)</td>
<td>158.5±36.8* (4)</td>
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<td>IBMX</td>
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<tr>
<td>0.1 (\mu M)</td>
<td>131.1±10.9* (4)</td>
<td>116.7±11.7 (4)</td>
<td>49.1 (2)</td>
<td>69.9 (2)</td>
</tr>
<tr>
<td>10 (\mu M)</td>
<td>146.7±11.9* (4)</td>
<td>136.2±10.3 (4)</td>
<td>83.0 (2)</td>
<td>ND (2)</td>
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<tr>
<td>100 (\mu M)</td>
<td>197.5±9.4* (5)</td>
<td>159.5±15.0 (5)</td>
<td>104.7±4.1* (3)</td>
<td>56.8±4.1 (3)</td>
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<td>Milrinone</td>
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<tr>
<td>10 (\mu M)</td>
<td>144.6±11.2* (4)</td>
<td>173.5±17.0 (4)</td>
<td>ND (2)</td>
<td>ND (2)</td>
</tr>
<tr>
<td>50 (\mu M)</td>
<td>233.0±25.1* (6)</td>
<td>216.7±21.9 (6)</td>
<td>71.8±6.5 (6)</td>
<td>81.9±10.9 (6)</td>
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<tr>
<td>100 (\mu M)</td>
<td>147.9±14.1* (7)</td>
<td>138.5±11.6 (7)</td>
<td>97.7±9.5* (6)</td>
<td>84.1±10.8 (6)</td>
</tr>
</tbody>
</table>

\(^{32}\)P-Perfused guinea pig hearts were exposed to the indicated agents and processed as described under "Materials and Methods." Results are mean±SEM of \(n\) hearts (parentheses). P, inorganic phosphate; PLB, phospholamban; Tnl, troponin I; IBMX, isobutylmethylxanthine.

\(*p<0.05\) when compared with control. ND, not determined.

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![Figure 3. Effect of equipotent (similar +dP/dt and −dP/dt) doses of milrinone (MIL), isobutylmethylxanthine (IBMX) and isoproterenol (ISO) on cAMP levels and phospholamban phosphorylation in perfused guinea pig hearts. \(^{32}\)P-orthophosphate-perfused hearts were exposed to 50 \(\mu M\) MIL or 100 \(\mu M\) IBMX or 0.01 \(\mu M\) ISO. Cyclic AMP levels and \(^{32}\)P-incorporation in phospholamban were determined as described in "Materials and Methods." Results are the mean±SEM of \(n\) hearts as indicated in Table 1 and are expressed as fold increase over control levels.](image-url)

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![Graph showing effects of different treatments on cAMP levels and phospholamban phosphorylation.](graph-url)
dependence of the Mg^{2+}-ATPase activity of myofibrils isolated from hearts perfused with milrinone and IBMX (data not shown). Moreover, there is no evidence for a direct effect of milrinone on myofibrillar ATPase activity.38

In conclusion, our findings with guinea pig hearts indicate that PDE inhibitors can enhance contraction and relaxation with little or no change in phosphorylation of SR and contractile proteins, despite relatively large increases in tissue cAMP levels. The lack of increase in protein phosphorylation may be due to complex effects on protein kinases and phosphatases regulating SR and myofibrillar function, while the increase in contractility may be due to effects at the level of the sarcolemma.

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