Protein Kinase C Isoform Activation and Endothelin-1 Mediated Defects in Myocyte Contractility After Cardioplegic Arrest and Reperfusion

Rupak Mukherjee, PhD; Kimberly A. Apple, MD; Christina E. Squires, BS; Brooke S. Kaplan, BS; Julie E. McLean, BS; Stuart M. Saunders, BS; Robert E. Stroud, MS; Francis G. Spinale, MD, PhD

Background—Endothelin-1 (ET-1) is released after hyperkalemic cardioplegic arrest (CA) and reperfusion and may contribute to contractile dysfunction. ET-1 receptor transduction causes activation of protein kinase C (PKC) isoforms, which can cause differential intracellular events. The goal of this study was to determine which PKC isoforms contribute to myocyte contractile dysfunction with ET-1 and CA.

Methods and Results—Percent shortening (PERSHORT) and the time to 50% relaxation (T50) were measured in porcine (n=22) left ventricular myocytes, randomized (minimum: 30 cells/group) to normothermia: (cell media for 2 hours/37°C), and CA: (2 hours/4°C, 24 mEq K+ solution followed by reperfusion in cell media), ET-1/CA: (100 pM ET-1 during CA). Studies were performed in the presence and absence of PKC inhibitors (500 nM) against the classical (Beta-I, Beta-II, Gamma) and novel (Epsilon, Eta) isoforms (myocytes from a minimum of 3 pigs per inhibitor). CA reduced PERSHORT by 35% from normothermia (P<0.05), which was further reduced with ET-1. PKC-Beta-II or PKC-Gamma inhibition increased PERSHORT from ET-1/CA as well as CA only (P<0.05). CA prolonged T50 by 19% from normothermia (P<0.05) and was further prolonged with ET-1. Inhibition of the classical PKC isoforms reduced T50 from ET-1/CA (P<0.05). Inhibition of novel PKC isoforms did not yield similar effects on either PERSHORT or T50 with ET-1/CA.

Conclusions—Inhibition of the classical PKC isoforms relieved the negative inotropic and lusitropic effects of ET-1 after CA. These findings provide mechanistic support for developing targeted inhibitory strategies with respect to ET-1 signaling and myocyte contractile dysfunction with cardioplegic arrest and reperfusion. (Circulation. 2006;114[suppl I]:I-308–I-313.)

Key Words: active relaxation • cardioplegia • endothelin • kinases • myocyte contractility

Hypothermic, hyperkalemic cardioplegic arrest (CA) remains a common means of providing a quiescent heart during cardiac surgery. Transient left ventricular (LV) dysfunction occurs after CA1 and is associated with increased levels of bioactive peptides, including the vasoactive peptide, endothelin-1 (ET-1).2–4 Moreover, elevated ET-1 levels in patients during and after cardiac surgery requiring CA may contribute to a complex postoperative course.3 Although the effects of ET-1 on LV pump function after CA are likely to be multifactorial, ET-1 has been demonstrated to modulate myocyte contractile function,5 which in turn can influence LV function. Accordingly, the overall goal of this study was to identify specific components of ET-1–mediated signaling using an isolated myocyte model of simulated CA.

ET-1 receptor activation, through specific G-protein-coupled receptors, leads to the activation of protein kinase C (PKC).9 The PKC family of kinases have been classified into 3 broad subfamilies: classical, novel, and atypical. Activation of the classical PKC isoforms is dependent on increased cytosolic Ca2+ levels.9,10 Novel PKC isoforms, however, are activated through Ca2+-independent pathways.10 Activation and translocation of the PKC isoforms to an effector target site are dependent on isoform-specific intracellular receptors for activated PKC (RACK proteins).9,11 Identification of these RACK proteins has led to the development of peptide fragments that target specific PKC isoforms, resulting in loss of function of the targeted isozymes.9 Using RACK peptides, divergent roles for specific PKC isoforms in preconditioning-mediated prevention of ischemic injury have been demonstrated.12,13 Therefore, these RACK fragments were used to identify the potential role of PKC isoform activation with respect to modulating myocyte contractile function after ET-1 exposure with CA.
Methods

Experimental Overview and Rationale
An in vitro model of simulated CA and rewarming using isolated porcine myocytes was used.5–2 Myocyte function was measured under normothermic conditions or after simulated CA in the presence and absence of ET-1. In addition, these measurements were performed after incubation with inhibitors of specific PKC isoforms. Because past studies have identified potential roles for both the classical (Beta-I, Beta-II, Gamma) and novel (Eta, Epsilon) PKC isoforms within the myocardium,9,10,14 this study focused on dissecting out the role of these PKC isoforms with respect to ET-1 signaling after CA.

Myocyte Isolation and Contractile Function Measurements
Animals were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC, 1996). Myocytes were isolated from the left ventricular free wall of pigs (Hambone Farms, Orangeburg, SC) (Yorkshire; n = 22; 25 to 30 kg) using collagenase digestion.5–7 Viable myocytes, as described previously5–7 (yield >60%), were plated onto coverslips previously coated with a basement membrane substrate (Matrigel, Collaborative Research Inc). These were stabilized at 37°C in oxygenated media for 60 minutes and then randomized to the experimental protocols described below. Contractile measurements were performed using computer-assisted video-microscopy.5–7 Extent of myocyte shortening, expressed as a percentage of resting length (%), time to peak contraction (ms), relengthening velocity (μm/s), and time to 50% relaxation (ms) were determined as the average value obtained from 20 consecutive contractions.

PKC Isoform Inhibition
Specific RACK proteins that corresponded to the specific binding domains for the activated PKC isoform were used.9,11 RACK inhibitory peptides (KAI Pharmaceuticals, San Francisco, Calif) for all classical PKC isoforms (pan-classical, KIC1–1), for each of the classical PKC isoforms: Beta-I (KIBI31–1), Beta-II (KIBI31–1), Gamma (KIG31–1), and the novel PKC isoforms: Eta (KIEI1–1) and Epsilon (KAEI1–1) were used. Based on past reports using these inhibitory peptides and initial titration studies,12,13 it was determined that a final concentration of 500 nM would provide inhibition of each PKC isoform without a change in myocyte viability.

Experimental Design
Myocytes were randomly assigned to be maintained under normothermic control conditions (incubation in a physiological solution (Medium 199, Gibco at 37°C) or subjected to simulated CA (CA: incubation in Ringer’s solution at 4°C containing 24 mM Eq/L K+ and 30 mM Eq/L HCO3−) and stored at 4°C for 2 hours. The partial O2 pressure in both incubating media was between 120 and 170 torr, with an O2 saturation of 100%. Myocytes were randomized to be exposed to ET-1 (100 μM) throughout the incubation and reperfusion period or to be coincubated with ET-1 and the respective PKC isoform inhibitor. The effects of pan-classical PKC inhibition with ET-1 and CA as well as the effects of specific PKC isoform inhibition on contractility following CA, but without exogenous ET-1, were examined in subsets of myocytes (minimum of 16 myocytes per group using a minimum of 3 preparations for each of the inhibitors).

Data Analysis
The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written. Myocytes were successfully isolated from all animals (>100 myocytes per preparation) and contractile function from 3126 individual myocytes, randomized to the different treatment groups (Table 1), were analyzed. Distribution of contractile parameters in each group conformed to a Gaussian pattern, which allowed the use of parametric tests. Initial data analysis was performed on 22 independent experiments by using a multi-way analysis of variance (ANOVA), constructed using a split-plot design to identify differences in baseline myocyte contractility between the normothermia and CA-only groups, with or without ET-1. Each experiment was thus considered a complete block for the ANOVA design, and the number of myocytes from each preparation was considered repeated observations within these treatment blocks. Sample size computations (NQuery, α: 0.05, β: 0.2) using the 2-way ANOVA test (treatment effects: incubation condition [normothermia, CA] and the presence or absence of ET-1) revealed that a minimum of 19 observations would be required per group. Therefore, the number of observations of myocyte contractile function included per group allowed statistical testing with acceptable power (>80%). A multi-way ANOVA was then performed to identify specific treatment effects on myocyte contractile performance. In this split-plot design, the first level was normothermia or CA. The second level was the

<table>
<thead>
<tr>
<th>Isolated Myocyte Contractile Parameters With Simulated CA and Rewarming: Effects of ET-1 and Selective Inhibition of PKC Isoforms</th>
<th>Normothermic</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Shortening (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.7±1.0</td>
<td>3.7±1.9*</td>
</tr>
<tr>
<td>ET-1</td>
<td>4.3±1.8*</td>
<td>2.9±1.5†</td>
</tr>
<tr>
<td>ET-1/Beta I</td>
<td>5.0±2.9</td>
<td>4.4±3.2*</td>
</tr>
<tr>
<td>ET-1/Beta II</td>
<td>5.3±4.1</td>
<td>4.8±1.7†‡</td>
</tr>
<tr>
<td>ET-1/Gamma</td>
<td>4.5±5.1*</td>
<td>5.2±2.8‡</td>
</tr>
<tr>
<td>ET-1/Eta</td>
<td>4.3±3.1*</td>
<td>4.1±3.6*‡</td>
</tr>
<tr>
<td>ET-1/Epsilon</td>
<td>4.6±5.1*</td>
<td>3.8±2.8*</td>
</tr>
<tr>
<td>Time to Peak Contraction (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>201.3±59.0</td>
<td>165.7±49.6*</td>
</tr>
<tr>
<td>ET-1</td>
<td>213.1±57.4</td>
<td>177.2±50.5</td>
</tr>
<tr>
<td>ET-1/Beta I</td>
<td>205.5±116.4</td>
<td>192.5±57.4</td>
</tr>
<tr>
<td>ET-1/Beta II</td>
<td>224.0±79.7</td>
<td>207.0±81.3</td>
</tr>
<tr>
<td>ET-1/Gamma</td>
<td>200.9±99.4</td>
<td>185.3±74.6</td>
</tr>
<tr>
<td>ET-1/Eta</td>
<td>228.5±109.5</td>
<td>194.9±104.0</td>
</tr>
<tr>
<td>ET-1/Epsilon</td>
<td>205.6±121.6</td>
<td>193.8±63.8</td>
</tr>
</tbody>
</table>

Relengthening Velocity (μm/s)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>ET-1</th>
<th>ET-1/Beta I</th>
<th>ET-1/Beta II</th>
<th>ET-1/Gamma</th>
<th>ET-1/Epsilon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>66.4±8.9</td>
<td>57.9±17.7*</td>
<td>55.8±42.4*</td>
<td>60.0±51.8</td>
<td>49.5±44.8*</td>
<td>56.0±29.5*</td>
</tr>
<tr>
<td>ET-1</td>
<td>72.4 (4)</td>
<td>36 (4)</td>
<td>1.7*‡</td>
<td>94.5 (5)</td>
<td>68 (3)</td>
<td>46 (3)</td>
</tr>
</tbody>
</table>

Values presented as Mean±SD.
*P<0.05 vs normothermic baseline.
†P<0.05 vs CA baseline.
‡P<0.05 vs ET-1–only (normothermic or CA matched).
presence or absence of ET-1. Finally, the third level was the presence or absence of the inhibitor for each of the PKC isoforms. If ANOVA revealed significant differences, mean separation was performed using pair-wise adjusted Bonferroni probabilities. Results are presented as mean±standard deviation. \( P<0.05 \) was considered statistically significant. All statistical procedures were performed using STATA statistical software (STATA Intercooled v8.0; College Station, Tex).

Results

Effects of CA, Endothelin-1, and Broad Inhibition of Classical PKC Isoforms on Myocyte Contractility

Myocyte resting length was 140.2±20.5 \( \mu \)m and was not different between groups. Myocyte shortening was reduced after CA and rewarming. Indices of myocyte contractility after incubation with ET-1 alone or coincubation with ET-1 and inhibitors of specific PKC isoforms are presented in Table 1. Incubation with ET-1 reduced myocyte shortening with normothermia and after CA. The presence of ET-1 with CA and reperfusion caused a further decline in shortening when compared with CA only. In the presence of the inhibitor for the classical PKC isoforms (pan-classical), myocyte shortening (5.8±1.1%) was not different from normothermia-only values (\( P=0.41 \)). In contrast, in myocytes incubated with the pan-classical PKC inhibitor during and after CA, shortening was increased when compared with CA-only values (4.6±0.9\%, \( P<0.05 \) versus CA-only). Coincubation with both ET-1 and the pan-classical PKC inhibitor increased myocyte shortening from respective ET-1–only values under both normothermic conditions (5.3±0.7\%, \( P<0.05 \)) and after CA (3.3±1.0\%, \( P<0.05 \)).

Relengthening velocity (Table 1) was reduced with CA compared with normothermic values and the time to 50% relaxation (Figures 1 and 2) was increased in the presence of CA compared with normothermia alone and further increased with ET-1. Coincubation of myocytes with both ET-1 and the pan-classical PKC inhibitor attenuated the prolongation in the time to 50% relaxation under normothermic conditions (106.4±18.8 ms, \( P<0.05 \)), as well as with CA (122.2±16.0 ms, \( P<0.05 \)).
Effects of Inhibiting Specific PKC Isoforms on Myocyte Shortening and Relaxation With CA

In the absence of exogenously added ET-1, incubation of myocytes subjected to CA with PKC–Beta-II or Gamma inhibitory peptides improved percent shortening compared with CA alone (4.0±0.6% and 4.3±1.0%, respectively; P<0.05), but was lower than normothermia only values (P<0.05 for both). Myocyte shortening with the PKC–Beta-I inhibitory peptide in CA was 3.9±1.2% and not different from CA-only values (P=0.12). Myocyte shortening was similar to CA only values with coincubation with either of the novel PKC inhibitory peptides (Eta: 3.8±0.8%; Epsilon: 3.4±0.8%; P>0.26). Time to 50% relaxation was less prolonged than CA only in myocytes coincubated with PKC–Beta-I and Beta-II inhibitory peptides (124.3±17.8 ms and 119.7±16.7 ms, respectively; P<0.05) but was similar to CA only values for the other PKC inhibitory peptides.

Effects of Coincubation With Inhibitors of Specific PKC Isoforms and ET-1 on Myocyte Shortening and Relaxation Properties With CA

In the presence of PKC–Beta-I or Beta-II inhibitory peptides under normothermic conditions, myocyte shortening was similar to those for normothermia alone (Table 1). However, with CA, shortening in myocytes coincubated with ET-1 and the PKC–Beta-II inhibitory peptide was significantly higher than CA only. Under normothermic conditions, coincubation with ET-1 and the PKC–Gamma inhibitor yielded similar results to that obtained with ET-1 only. With CA, coincubation with PKC–Gamma inhibitor improved myocyte shortening when compared with CA only. Compared with myocytes subjected to specific inhibition of each of the classical PKC isoforms with CA only, myocyte shortening was greater (P<0.05) in the concomitant presence of ET-1. Under normothermic conditions, PKC–Eta inhibition did not affect shortening when compared with ET-1 only values. In contrast, with PKC–Epsilon inhibition under normothermic conditions, there were no differences in myocyte shortening between normothermia alone or CA alone. After CA, coincubation with ET-1 and inhibition of the novel PKC isoforms yielded myocyte shortening values similar to CA-only values. However, with PKC–Eta inhibition, myocyte shortening was higher than ET-1 and CA values.

With CA, coincubation with inhibitors of the classical PKC isoforms (Beta-I, Beta-II, and Gamma; Figure 1) caused a reduction in the ET-1-mediated increase in the time to 50% relaxation. However, time to 50% relaxation with inhibition of the PKC–Beta-I and Beta-II isoforms remained longer than normothermia only values. After CA, the prolongation of the time to 50% relaxation in myocytes coincubated with inhibitors of PKC–Eta and Epsilon was similar to that of myocytes incubated with ET-1 alone.

Discussion

Increased levels of bioactive peptides, such as ET-1, have been implicated in negatively modulating LV pump performance after CA and rewarming.2–4 ET-1, which is released within the myocardial interstitium during and after CA,10 causes receptor-mediated changes in myocyte contractility.5,6 Because the intracellular signaling pathway for ET-1 involves activation of PKC,8 this study determined the relative contribution of specific PKC isoforms in ET-1-mediated contractile dysfunction with CA. There are several unique and important findings of the present study. First, the recruitment of specific PKC isoforms was condition-specific in that myocyte contractile performance was different under normothermic conditions and that after simulated CA. Second, the negative contractile effects of ET-1 in the context of CA were ameliorated, at least in part, with inhibition of the classical PKC isoforms (Beta-II, Gamma). Finally, inhibition of the novel PKC isoforms (Eta, Epsilon) did not provide similar effects on myocyte contractile and relaxation characteristics in the setting of CA. Taken together, these findings provide mechanistic support for developing targeted PKC inhibitory strategies with respect to ET-1 signaling and LV pump dysfunction after CA and reperfusion.

ET-1 is a potent effector of myocyte contractility and both positive and negative inotropic effects of ET-1 stimulation have been reported.5–7,17 Differences in findings with respect to the inotropic effects of ET-1 have been attributed to species-dependence and experimental conditions, such as the concentration of ET-1 used, as well as the duration of ET-1 exposure.17,18 Despite these differences, the role of ET-1 receptor activation (through ET-1 receptor antagonists6,19,20) and subsequent activation of PKC (through PKC inhibition6,15,21) in modulating myocyte contractile response has been established. For example, coincubation of myocytes with ET-1 and chelerythrine, a broad-spectrum PKC inhibitor, eliminated the inotropic effects of ET-1.6 In the present study, prolonged exposure to ET-1 resulted in negative inotropic and lusitropic effects in normothermic myocytes. Coincubation of the myocytes with the inhibitory peptide against the classical PKC isoforms at ET-1 normalized myocyte contractile parameters under normothermic conditions. Taken together, these findings suggest that the activation of the classical PKC subfamily occurs with myocyte exposure to ET-1, and that activation of the classical PKC isoforms may play a major role in the determination of the myocyte contractile response to ET-1.

Consistent with a past report,5 myocyte contractile function was reduced CA, which was further exacerbated in the presence of ET-1. Inhibition of the classical PKC isoforms (without exogenous ET-1) during CA improved myocyte shortening from CA only values. Because past studies have provided evidence that ET-1 levels increase within the myocardial interstitium during cardiopulmonary bypass5,16 and that myocytes can synthesize and release ET-1,7 incubation of myocytes with CA may have likely resulted in ET-1 production and subsequent PKC activation. Nevertheless, concomitant inhibition of each of the classical PKC isoforms in the presence of exogenous ET-1 and CA resulted in a marked improvement in myocyte shortening when compared with CA only. Intriguingly, myocyte shortening was improved with coincubation of classical PKC isoforms with ET-1 when compared with...
myocytes incubated with the same classical PKC isoform inhibitors in CA only (without exogenous ET-1). Taken together, these findings suggest that inhibition of the classical PKC isoforms in the context of ET-1 stimulation and CA may have unmasked a positive inotropic effect of ET-1. Inhibition of the novel PKC isoforms provided modest effects on myocyte contractility after CA with or without exogenous ET-1. Because intracellular Ca\(^{2+}\) levels increase in this model of simulated CA and that the classical PKC isoforms require changes in intracellular Ca\(^{2+}\) for activation,\(^9\) it is likely that the negative inotropic effects of ET-1 during CA were caused by the activation of the classical PKC isoforms.

Myocyte relaxation requires resequestration of cytosolic Ca\(^{2+}\) into sarcoplasmic reticular stores using an energy-dependent process that involves the sarcoplasmic reticular Ca\(^{2+}\) pump and phospholamban.\(^{14}\) Increased sensitivity of the myofilaments to Ca\(^{2+}\), which can occur after ET-1 receptor activation,\(^{22}\) and alterations in the phosphorylation state of phospholamban can prolong myocyte relaxation. Consistent with past findings,\(^3\) time to 50% relaxation was prolonged in isolated myocytes exposed to ET-1. inhibition of the classical PKC isoforms (Beta-I, Beta-II, Gamma) attenuated the ET-1–mediated prolongation of the time to 50% relaxation. In contrast, inhibition of the novel PKC isoforms did not provide similar effects on myocyte lusitropy with ET-1 stimulation and CA. Further, coinubation of the myocytes with ET-1 and the PKC-Epsilon inhibitor under normothermic conditions prolonged the time to 50% relaxation. Because activated PKC-Epsilon may influence Ca\(^{2+}\) resequestration through phosphorylation of phospholamban,\(^{14}\) these findings suggest that PKC-Epsilon inhibition may not confer beneficial effects with respect to ET-1–mediated changes in myocyte relaxation.

There are several limitations of this study that must be recognized. First, an isolated myocyte model of CA was used to control the extracellular milieu and to deliver PKC isoform inhibitors. In light of the findings of this study, further research will be required to determine the in vivo effects of PKC isoform inhibition on changes in LV performance after CA and reperfusion. Second, this study focused on representative PKC isoforms from the classical and novel subfamilies. Practical considerations, such as peptide availability or incomplete characterization of the effects of atypical PKC isoform activation, precluded inclusion of all PKC isoforms. Finally, only single concentrations of ET-1 and PKC inhibitory peptides were used, precluding the determination of dose-dependent effects. Moreover, effects of PKC isoform inhibition on myocyte contractility were assessed at only one time point after simulated CA. Therefore, whether inhibition of these PKC isoforms would alter myocyte contractile recovery after CA could not be determined. These limitations notwithstanding, the findings of the present study demonstrate that ET-1–mediated activation of specific PKC isoforms, particularly those of the classical PKC subfamily, contribute to the negative inotropic and lusitropic effects with CA and reperfusion. Thus, a more selective strategy to modulate the negative effects of ET-1 with CA and reperfusion would be to target specific post-receptor transduction pathways.

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**Disclosures**

None.

**References**


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