Effects of \(\kappa\)-opioid receptor activation on myocardium

W. G. PYLE, J. W. LESTER, AND P. A. HOFMANN

Department of Physiology, University of Tennessee, Memphis, Tennessee 38163

Received 1 June 2000; accepted in final form 16 April 2001

Pyle, W. G., J. W. Lester, and P. A. Hofmann. Effects of \(\kappa\)-opioid receptor activation on myocardium. Am J Physiol Heart Circ Physiol 281: H669–H678, 2001.—\(\kappa\)-Opioid receptor stimulation of the heart transiently increases twitch amplitude and decreases \(\text{Ca}^{2+}\)-dependent actomyosin \(\text{Mg}^{2+}\)-ATPase activity through an undetermined mechanism. One purpose of the present study was to determine if the increase in twitch amplitude is due to changes in myofilament \(\text{Ca}^{2+}\) sensitivity. We also wanted to determine if \(\kappa\)-opioid receptor activation alters maximum actin-myosin ATPase activity and \(\text{Ca}^{2+}\) sensitivity of tension in a way consistent with protein kinase A or protein kinase C (PKC) action. Rat hearts were treated with U50,488H (a \(\kappa\)-opioid receptor agonist), phenylephrine plus propranolol (\(\alpha\)-adrenergic receptor stimulation), isoproterenol (a \(\beta\)-adrenergic receptor agonist), or phorbol 12-myristate 13-acetate (PMA, receptor independent activation), isoproterenol (a \(\alpha\)-adrenergic receptor agonist), or phorbol 12-myristate 13-acetate (PMA, receptor independent activator of PKC) or were untreated (control), and myofibrils were isolated. U50,488H, phenylephrine plus propranolol, and PMA all decreased maximum \(\text{Ca}^{2+}\)-dependent actomyosin \(\text{Mg}^{2+}\)-ATPase activity, whereas isoproterenol treatment increased maximum \(\text{Ca}^{2+}\)-dependent actomyosin \(\text{Mg}^{2+}\)-ATPase activity. Untreated myofibrils exposed to exogenous PKC-\(\epsilon\), but not PKC-\(\delta\), decreased maximum actomyosin \(\text{Mg}^{2+}\)-ATPase activity. Langendorff-perfused hearts treated with U50,488H, phenylephrine plus propranolol, or isoproterenol had significantly higher ventricular ATP levels compared with control hearts. PKC inhibitors abolished the effects of U50,488H on \(\text{Ca}^{2+}\)-dependent actomyosin \(\text{Mg}^{2+}\)-ATPase activity and myocardial ATP levels. U50,488H and PMA treatment of isolated ventricular myocytes increased \(\text{Ca}^{2+}\) sensitivity of isometric tension compared with control myocytes at pH 7.0. The U50,488H-dependent increase in \(\text{Ca}^{2+}\) sensitivity of tension was retained at pH 6.6. Together, these findings are consistent with the hypotheses that (1) the positive inotropy associated with \(\kappa\)-opioid receptor activation may be due in part to a PKC-mediated increase in myofilament \(\text{Ca}^{2+}\)-sensitivity of tension and (2) the \(\kappa\)-opioid receptor–PKC pathway is a modulator of myocardial energy status through reduction of actomyosin ATP consumption.

Calcium sensitivity of tension; pH; ATP; protein kinase C; actomyosin \(\text{Mg}^{2+}\)-ATPase

THE MAMMALIAN MYOCARDIUM EXPRESSES \(\mu\), \(\delta\), and \(\kappa\)-opioid receptors. Expression of \(\mu\)-opioid receptors decreases postnataally to undetectable levels by day 7, whereas the \(\delta\)- and \(\kappa\)-opioid receptors continue to be expressed in the adult (46). Myocardial \(\kappa\)-opioid receptor activation causes a transient increase in twitch amplitude followed by a negative inotropic effect in adult rats (43). Opioid receptor activation before ischemia can also protect the heart from posts ischemic contractile dysfunction (33) and necrosis (23, 34, 35, 36) via a protein kinase C (PKC)-dependent pathway. The overall goal of the present study was to investigate the cellular mechanism(s) responsible for the effects of \(\kappa\)-opioid receptor activation on the heart. An understanding of the basic underlying mechanism will help in the characterization of a possible therapeutic role for \(\kappa\)-opioid receptor stimulation in diseased myocardial states.

The observed positive inotropic effects of \(\kappa\)-opioid receptor stimulation of the heart (43) might involve one or several mechanisms. These include an increase in intracellular \([\text{Ca}^{2+}]\) (43), activation of the sarcolemmal Na\(^+\)/H\(^+\) exchanger [leading to intracellular alkalosis and a resulting increase in myofilament force on contraction at a given \([\text{Ca}^{2+}]\) concentration (42)], and/or a direct increase in the \([\text{Ca}^{2+}]\) sensitivity of myofilament tension generation. A direct effect of \(\kappa\)-opioid receptor activation on myofilament \([\text{Ca}^{2+}]\) sensitivity has not been previously investigated. Therefore, the first objective of the present study was to examine the effects of \(\kappa\)-opioid receptor stimulation on the relationship between \([\text{Ca}^{2+}]\) and isometric tension in agonist-treated and subsequently skinned ventricular myocytes. \(\kappa\)-Opioid receptor activation may improve posts ischemic myocardial function (33) by decreasing the sensitivity of the myofilaments to ischemia-induced acidosis. As such, the second aim of this study was to determine if the \([\text{Ca}^{2+}]\) sensitivity of tension and maximum isometric tension are equally influenced by decreased pH in \(\kappa\)-opioid receptor agonist-treated compared with untreated and subsequently skinned ventricular myocytes.

A PKC-dependent decrease in maximum actin-myosin ATPase activity was previously observed in hearts pretreated with a \(\kappa\)-opioid receptor agonist (33). However, this decrease in actin-myosin ATPase could be due to the combined effects of \(\kappa\)-opioid receptor activation and ischemia. Alternatively, a PKC-dependent decrease in actin-myosin ATPase may be an effect due solely to \(\kappa\)-opioid receptor activation of the heart. Thus the third objective of the present study was to determine if nonischemic perfused hearts treated with a \(\kappa\)-opioid agonist demonstrate (1) increases in whole

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heart [ATP], 2) myofibrils with decreased maximum Ca$^{2+}$-dependent actin-myosin ATPase, and 3) a PKC dependency on any observed $\kappa$-opioid-induced changes. Finally, $\kappa$-opioid receptor activation of ventricular myocytes has been shown to activate PKC (42). Thus the fourth objective of the present study was to establish which PKC isoforms, if any, decreases maximum actin-myosin ATPase in cardiac myofibrils.

**MATERIALS AND METHODS**

*Enzymatic isolation of cardiac myocytes.* Ventricular myocytes were obtained by enzymatic digestion of hearts from female Wistar rats according to the method of Lester et al. (19). In brief, the heart was excised, and the aorta was cannulated for suspension on a Langendorff perfusion apparatus. The heart was perfused with oxygenated Ca$^{2+}$-containing Ringer solution (Ca$^{2+}$-Ringer solution) to rinse out residual blood and then with Ca$^{2+}$-Ringer solution containing collagenase (1 mg/ml; Worthington). During collagenase perfusion, three sequential additions of CaCl$_2$ were done to yield a final Ca$^{2+}$ of 0.75 mM. The ventricles were cut into small pieces and agitated in a flask containing recycled enzyme solution with CaCl$_2$. Ventricular pieces were dissociated by gentle aspiration through a large-bore pipette tip, and cells were washed in Ca$^{2+}$-Ringer solution. Washed cells were incubated for 2 min with norbinaltorphimine (norBNI; 1 $\mu$M, a $\kappa$-opioid receptor antagonist) or Ca$^{2+}$-Ringer solution (control). Cells were then treated for 5 min with various receptor agonists/antagonists dissolved in Ca$^{2+}$-Ringer solution (see Results and Inhibitor dose rationale). After centrifugation and the decanting of the supernatant, myocytes were exposed to a relaxing solution (see Solutions) containing 0.6% Triton X-100 for 5 min to chemically remove lipid membranes. Cells were then washed three times in a relaxing solution without Triton X-100 and stored on ice.

*Effects of isometric tension as a function of [Ca$^{2+}$].* Isolated cardiac myocytes were attached via glass micropipettes to a force transducer (model 403, Cambridge Technology; Watertown, MA) and piezoelectric translator (model 173, Physik Institute; Waldbronn, Germany) with Great Stuff adhesive (Insta-Foam; Marietta, GA). Sarcomere length was adjusted to 2.1–2.3 $\mu$m, and cell length and width were measured. A tension-pCa relationship was obtained by initially measuring force during maximal activation (pCa 4.5), followed by contractions at randomly chosen submaximal pCa solutions, and again at pCa 4.5 to assess any decline in the performance of the cell. Active tension was calculated as the difference in measured total tension (P) and resting tension (RT) obtained in a pCa 9.0 solution. For each submaximal contraction, active tension was normalized to maximum active tension ($P_0$) generated by the cell, i.e., ($P - RT)/P_0$. Tension-pCa relationships were characterized by Hill plot analysis and curve fit (15). Data from individual cells were included in the cumulative tension-pCa analysis if the following criteria were met: >80% of maximum isometric tension was retained from initial to final pCa 4.5 contraction, initial contraction in pCa solution exceeded 1.3 mg, striae were visible for two-thirds of the cell length in photomicrographs obtained at both pCa 9.0 and 4.5, sarcomere length was between 1.90 and 2.30 $\mu$m, and the curve fit of the individual cell tension-pCa relationship had a >90% goodness of fit to the data.

*Langendorff-perfused heart preparation.* Hearts were removed from female Wistar rats anesthetized by Metofane inhalation. The isolated hearts were mounted on a Langendorff perfusion apparatus and paced at 300 beats/min, and a balloon was inserted in the left ventricle and inflated until end-diastolic pressure (EDP) was 5–15 mmHg (33). All hearts were perfused for a total of 25 min. U50,488H, phenylephrine plus propranolol, phenylephrine, phorbol 12-myristate 13-acetate (PMA), and $\alpha$-PMA-treated groups differed from the control group only in that hearts were treated with agonists/antagonists for 2 min. Propranolol was given simultaneously with phenylephrine. Perfusion with the $\kappa$-opioid receptor antagonist norBNI was started 2 min before U50,488H treatment and continued during U50,488H treatment. Preischemic left ventricular developed pressure (LVDP) was taken as the average LVDP for the first 10 min (control) or 8 min (agonist/antagonist-treated hearts) of baseline perfusion. LVDP and EDP were stable during baseline perfusion (data not shown). LVDP and EDP were altered by U50,488H, phenylephrine, and phenylephrine plus propranolol treatment but returned to baseline values before the onset of global ischemia. norBNI did not alter baseline LVDP or EDP by itself.

*Myofibrillar ATPase measurements.* ATPase buffers with [Ca$^{2+}$] of pCa 4.0 and 9.0 were used (see Solutions). Myofibrils containing regulated actin were added to the 32°C buffers. After 2 min of incubation, the reaction was quenched with 2 ml of 20% trichloroacetic acid. Inorganic phosphate levels were determined according to the method of Fiske and SubbaRow (12). Inorganic phosphate production was found to be linear with respect to time under conditions of 32°C with a final protein concentration of 1.0–2.0 mg/ml (data not shown).

*Ventricular ATP.* ATP was quantified using the luciferin-luciferase enzyme technique (21). Hearts were perfused as described under Langendorff-perfused heart preparation and were removed after 25 min. The ventricles were cut from the hearts, quickly frozen in liquid nitrogen, and homogenized in a modified Krebs-Henseleit solution with a pestle and cold mortar. The homogenate was used to measure ventricular ATP with a luciferin-luciferase assay kit (Sigma; St. Louis, MO). The light produced by ATP plus luciferin is used to calculate unknown ATP concentrations of samples. A small amount of homogenized ventricle was used to determine protein concentration with a Biuret assay. Ventricular ATP levels were expressed as nanomoles of ATP per milligram of protein in the homogenate.

*Exogenous PKC treatment.* Myofibrils from isolated ventricular myocytes were treated with exogenous PKC according to a modified protocol of Noland and Kuo (28). Briefly, myofibrils from isolated ventricular myocytes were incubated for 5 min at $37^\circ$C in a reaction mixture (see Solutions) plus recombinant human PKC-C or -$\delta$ (PanVera; Madison, WI). The amount of recombinant PKC added was equal to the myofibrillar Ca$^{2+}$-independent PKC activity previously measured (data not shown).

AJP-Heart Circ Physiol • VOL 281 • AUGUST 2001 • www.ajpheart.org
**Solutions.** The standard phosphate buffer contained 60 mM KCl, 30 mM imidazole (pH 7.0), 2 mM MgCl₂, 4 μM aprotoninin, 15 μM pepstatin A, and 20 μM leupeptin hemisulfate. The resuspension buffer contained 10 mM EGTA, 8.2 mM MgCl₂, 14.4 mM KCl, 60 mM imidazole (pH 7.0), 5.5 mM ATP, 12 mM creatinine phosphate, 10 U/ml creatinine phosphokinase, 100 nM calyculin A, and 1% Triton X-100. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 30 mM β-mercaptoethanol, 0.9 mM CaCl₂, 10 mM MgCl₂, 0.5 mM EGTA, 1 mM ATP, 100 nM calyculin A, and 50 mM KCl. The pCa 4.0 buffer contained 23.48 mM KCl, 5 mM MgCl₂, 3.22 mM ATP, 2 mM EGTA, 20 mM imidazole, and 5.15 mM CaCl₂ (pH 7.0). The pCa 9.0 buffer contained 25.96 mM KCl, 5.13 mM MgCl₂, 3.16 mM ATP, 2 mM EGTA, 20 mM imidazole, and 4.86 μM CaCl₂ (pH 7.0). The free \([\text{Ca}^{2+}]\) was calculated using the program of Fabiato (10). The modified Krebs-Henseleit solution was composed of 4.7 mM KCl, 118 mM NaCl, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 25 mM NaHCO₃, 11 mM glucose, 1.2 mM KH₂PO₄, 0.05 mM EDTA, and 2 mM lactic acid (pH 7.4).

**Agonist/antagonist/inhibitor dosage rationale.** The concentration of 1 μM for U50,488H was chosen to selectively activate κ-opioid receptors (7). Concentrations of 10 μM phenylephrine plus 3 μM propranolol have previously been shown to produce maximal increases in intracellular \([\text{Ca}^{2+}]\) and twitch amplitude in myocardium (3). Phenylephrine plus propranolol was included as a positive control for PKC activation. The concentration of 100 nM isoproterenol maximally increases troponin I phosphorylation (29) and was included as a positive control for protein kinase A (PKA) activation. PMA (1 μM) activates the conventional and novel PKC isoforms found in the rat heart (39).

Chelerythrine chloride was dissolved in DMSO and diluted with modified Krebs-Henseleit or Ca²⁺-Ringer solution. The final concentration of DMSO was <0.0001%. Chelerythrine chloride (2 μM) and bisindolylmaleimide (100 nM) specifically inhibit PKC activity but no other known kinases (1, 6). A concentration of 1 μM norBNI has been shown to selectively block κ-opioid receptor activation by U50,488H (45).

**Statistical analysis.** All values are reported as means ± SE, and \(P < 0.05\) was chosen to indicate statistical significance. For tension-pCa²⁺ relationships, a two-way analysis of variance and a Student's \(t\)-test were used to determine significance. All other data were analyzed by two-way analysis of variance and Fisher's least-significant difference post hoc test.

**RESULTS**

**Characterization of ventricular myocytes.** Photomicrographs of cardiac myocytes attached to micropipettes from control and agonist-treated myocytes were indistinguishable (Fig. 1). Sarcomere lengths of myocytes were not significantly different in relaxing solution and during contraction between any of the groups tested. Average sarcomere lengths, total cell lengths and other characteristics were similar across all conditions.

**Fig. 1.** Light photomicrographs of skinned cardiac myocytes while relaxed in a pCa 9.0 solution and during maximum activation at pCa 4.5 solution at pH 7.0. Average distance between striations are 2.10 and 2.16 μM for all photos.
The pCa50 decreased by 0.59 pCa units and shifted myocytes at pH 6.6 compared with data obtained at pH 7.0. The cumulative tension-pCa relationships for cardiac myocytes at pH 6.6 in all myocytes was lower than at pH 7.0. The maximum tension developed at pCa 4.5, pH 7.0, or pH 6.6. Maximum tension developed at pCa 4.5, pH 7.0, or pH 6.6. For each preparation, the tension values were normalized to the maximum active tension (P0) generated by the cell, i.e., total tension (P) and resting tension (RT) obtained in a pCa 9.0 solution. For each submaximal contraction, active tension was normalized to the mean relative tension (P/RT/P0).

### Table 1. Characteristics of agonist-treated and subsequently skinned ventricular myocytes as a function of pH

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Isoproterenol</th>
<th>Phenylephrine and Propranolol</th>
<th>U50,488H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcomere length at pH 7.0, μm</td>
<td>2.07 ± 0.05</td>
<td>2.18 ± 0.03</td>
<td>2.03 ± 0.02</td>
<td>2.13 ± 0.05</td>
</tr>
<tr>
<td>pCa 9.0</td>
<td>2.07 ± 0.07</td>
<td>2.12 ± 0.03</td>
<td>2.10 ± 0.07</td>
<td>2.07 ± 0.05</td>
</tr>
<tr>
<td>Cell length, μm</td>
<td>80.1 ± 7.5</td>
<td>79.8 ± 5.9</td>
<td>67.9 ± 6.8</td>
<td>73.1 ± 4.8</td>
</tr>
<tr>
<td>Cell width, μm</td>
<td>24.7 ± 1.4</td>
<td>24.3 ± 2.0</td>
<td>25.0 ± 3.0</td>
<td>23.6 ± 1.5</td>
</tr>
<tr>
<td>Maximum tension, g/mm²</td>
<td>4.91 ± 0.60</td>
<td>4.65 ± 0.51</td>
<td>5.79 ± 1.36</td>
<td>5.02 ± 0.62</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>3.30 ± 0.37</td>
<td>2.85 ± 0.39</td>
<td>3.81 ± 0.99</td>
<td>3.27 ± 0.34</td>
</tr>
<tr>
<td>pH 6.6</td>
<td>5.92 ± 0.03</td>
<td>5.80 ± 0.03†</td>
<td>5.92 ± 0.03</td>
<td>6.00 ± 0.02†</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>5.33 ± 0.03a</td>
<td>5.33 ± 0.04a</td>
<td>5.36 ± 0.05a</td>
<td>5.43 ± 0.03†</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>1.88 ± 0.19</td>
<td>1.73 ± 0.08</td>
<td>1.77 ± 0.28</td>
<td>1.65 ± 0.07</td>
</tr>
<tr>
<td>pH 6.6</td>
<td>2.13 ± 0.14</td>
<td>2.02 ± 0.11†</td>
<td>2.23 ± 0.28</td>
<td>1.77 ± 0.13</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of cells. *P < 0.05 compared with values from the same cell at pH 7.0; †P < 0.05 compared with control values at the same pH.

Receptor agonist effects on isometric tension as a function of [Ca²⁺] at pH 7.0. Cumulative tension-pCa relationships at pH 7.0 for control and agonist-treated cardiac myocytes are shown in Fig. 2A. Maximum tension was not significantly affected by any of the agonist treatments (Table 1). The β-adrenergic receptor agonist isoproterenol induced a significant decrease in the Ca²⁺ sensitivity of tension for pCa values between 5.6 and 6.2. Tensions at submaximal [Ca²⁺] were 10–15% lower in myocytes treated with isoproterenol compared with untreated myocytes. The k-opioid receptor agonist U50,488H induced a significant increase in the Ca²⁺ sensitivity of tension for pCa values between 5.8 and 6.2. Tensions at submaximal [Ca²⁺] were 5–8% higher in myocytes treated with U50,488H compared with untreated myocytes. α-Adrenergic receptor stimulation with phenylephrine plus the β-adrenergic receptor antagonist propranolol did not alter the Ca²⁺ sensitivity of tension. The pCa values of half-maximum tension generation, i.e., pCa₅₀, for all treatments are shown in Table 1. The slopes of the tension-pCa relationships were not significantly different between any of the agonist-treated and control groups.

Receptor agonist effects on isometric tension as a function of [Ca²⁺] at pH 6.6. The cumulative averages of maximum tension at pH 6.6 for the various agonist treatments are presented in Table 1. Maximum tension at pH 6.6 in all myocytes was lower than at pH 7.0. The cumulative tension-pCa relationships for cardiac myocytes at pH 6.6 are shown in Fig. 2B. For control myocytes at pH 6.6 compared with data obtained at pH 7.0, the pCa₅₀ decreased by 0.59 pCa units and shifted the tension-pCa relationship rightward. At pH 6.6, neither the β-adrenergic receptor agonist isoproterenol nor α-adrenergic receptor stimulation with phenylephrine plus propranolol significantly altered the Ca²⁺ sensitivity of tension compared with control myocytes at pH 6.6. The k-opioid receptor agonist U50,488H at pH 6.6 significantly altered the Ca²⁺ sensitivity of tension compared with control myocytes at pH 7.0. The sensitivity of tension compared with control myocytes at pH 7.0 significantly different between any of the agonist treatments (Table 1). The pCa₅₀, for all treatments are shown in Table 1. The mean relative tensions ± SE as a function of pCa at pH 7.0 (A) and pH 6.6 (B) in ventricular myocytes. Myocytes were treated with 100 nM isoproterenol (Iso), 10 μM phenylephrine (Phen) plus 3 μM propranolol (Prop), and 1 μM U50,488H or were untreated (control). After 5 min of agonist treatment, myocytes were skinned. For each preparation, the tension values were normalized to the maximum tension developed at pCa 4.5, pH 7.0, or pH 6.6. Maximum tension, pCa₅₀, and the Hill coefficient can be found for these cells in Table 1. Active tension was calculated as the difference in measured total tension (P) and resting tension (RT) obtained in a pCa 9.0 solution. For each submaximal contraction, active tension was normalized to maximum active tension (P₀) generated by the cell, i.e., (P – RT/P₀).
**Maximum tension and pCa\textsubscript{50} of treated and subsequently skinned ventricular myocytes**

<table>
<thead>
<tr>
<th></th>
<th>Maximum Tension, g/mm\textsuperscript{2}</th>
<th>pCa\textsubscript{50}</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>Effect of (\kappa)-opioid receptor antagonist and agonist treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.28 ± 0.34</td>
<td>5.69 ± 0.03</td>
<td>11</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>5.58 ± 0.50</td>
<td>5.55 ± 0.03*</td>
<td>7</td>
</tr>
<tr>
<td>U50,488H</td>
<td>5.49 ± 0.44</td>
<td>5.76 ± 0.02†</td>
<td>8</td>
</tr>
<tr>
<td>norBNI</td>
<td>5.21 ± 0.60</td>
<td>5.68 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>norBNI + U50,488H</td>
<td>5.06 ± 0.68</td>
<td>5.68 ± 0.05</td>
<td>5</td>
</tr>
<tr>
<td>Effect of direct activation of PKC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.68 ± 0.90</td>
<td>5.71 ± 0.02</td>
<td>5</td>
</tr>
<tr>
<td>PMA</td>
<td>4.67 ± 0.59</td>
<td>5.86 ± 0.01‡</td>
<td>5</td>
</tr>
<tr>
<td>Effect of PKC inhibition and (\kappa)-opioid receptor activation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chel</td>
<td>4.57 ± 0.49</td>
<td>5.64 ± 0.06</td>
<td>9</td>
</tr>
<tr>
<td>U50,488H + Chel</td>
<td>4.98 ± 0.54</td>
<td>5.65 ± 0.05</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\) = no. of cells. norBNI, norbinaltorphimine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Chel, chelerythrine chloride. For the effect of \(\kappa\)-opioid receptor agonist and antagonist treatment, see Fig. 3. For the effect of direct activation of PKC, see Fig. 4. *\(P < 0.05\) (paired analysis) compared with control; †\(P < 0.05\) (paired analysis) compared with control, norBNI, and norBNI + U50,488H; ‡\(P < 0.05\) (paired analysis) compared with control.

Antagonist norBNI abolished the U50,488H-dependent increase in the Ca\textsuperscript{2+} sensitivity of tension. norBNI had no effect on Ca\textsuperscript{2+} sensitivity of tension by itself.

**Effect of PMA on the tension-pCa relationship at pH 7.0.** Additional experiments were done to determine the effects of receptor-independent activation of PKC. For these experiments, enzymatically isolated myocytes were exposed to either 1 \(\mu\)M PMA plus 1% DMSO or to 1% DMSO alone (paired control). After exposure, the cells were chemically skinned, and isometric tension as a function of [Ca\textsuperscript{2+}] was determined at pH 7.0. Figure 4 presents the cumulative tension-pCa relationships for paired control and PMA-treated myocytes. Maximum tension was not affected, whereas the Ca\textsuperscript{2+} sensitivity of tension increased after PMA exposure compared with controls (Table 2). PMA exposure caused a significant 10–17% increase in isometric tension for pCa values between 5.6 and 6.2. The slope of the tension-pCa relationships were not significantly different between control and PMA-treated myocytes.

**Effects of PKC inhibitor on U50,488H-dependent changes in isometric tension as a function of [Ca\textsuperscript{2+}] at pH 7.0.** Cumulative tension-pCa relationships at pH 7.0 are shown in Fig. 5. Maximum tension was not significantly affected by either treatment (Table 2). Treatment of myocytes with the PKC inhibitor chelerythrine chloride before U50,488H exposure inhibited the \(\kappa\)-opioid receptor-dependent increase in the Ca\textsuperscript{2+} sensitivity of tension. Chelerythrine chloride alone did not alter Ca\textsuperscript{2+} sensitivity.

**Actomyosin Mg\textsuperscript{2+}-ATPase activity.** Maximum Ca\textsuperscript{2+}-dependent actomyosin Mg\textsuperscript{2+}-ATPase activity was determined from myofibrils isolated from whole hearts transiently treated with agonists, antagonists, and/or PKC inhibitors (Fig. 6). \(\kappa\)-Opioid or \(\alpha\)-adrenergic receptor agonists had significantly lower mean Ca\textsuperscript{2+}-dependent actomyosin Mg\textsuperscript{2+}-ATPase activity compared with myofibrils from untreated control hearts (Table 3). norBNI abolished the effects of \(\kappa\)-opioid receptor activation but had no effect by itself. Mean Ca\textsuperscript{2+}-dep
Adrenergic, or β-adrenergic agonists had significantly increased after treatment with the β-adrenergic receptor agonist isoproterenol. The receptor-independent PKC activator PMA reduced Ca\(^{2+}\)-dependent actomyosin Mg\(^{2+}\)-ATPase, whereas α-PMA, the inactive form of PMA, had no effect on Ca\(^{2+}\)-dependent actomyosin Mg\(^{2+}\)-ATPase activity.

Chelerythrine chloride, a PKC inhibitor, abolished the α-adrenergic and k-opioid receptor-dependent decreases in maximum Ca\(^{2+}\)-dependent actomyosin Mg\(^{2+}\)-ATPase activity but did not abolish the effects of β-adrenergic receptor activation. Bisindolymaleimide, a second PKC inhibitor, also inhibited k-opioid receptor-dependent reduction in maximum Ca\(^{2+}\)-dependent actomyosin Mg\(^{2+}\)-ATPase activity. Neither chelerythrine chloride nor bisindolymaleimide alone had any effect on Ca\(^{2+}\)-dependent actomyosin Mg\(^{2+}\)-ATPase activity.

Ventricular ATP. Hearts treated with k-opioid, α-adrenergic, or β-adrenergic agonists had significantly higher ventricular ATP compared with untreated control hearts (Fig. 7). Both norBNI (a k-opioid receptor antagonist) and chelerythrine chloride (a PKC inhibitor) abolished the effects of k-opioid receptor activation.

**Effect of recombinant PKC on actomyosin Mg\(^{2+}\)-ATPase.** Maximum Ca\(^{2+}\)-dependent actomyosin Mg\(^{2+}\)-ATPase activity of myofibrils from isolated untreated ventricular myocytes (control) was 164.0 ± 18.9 nmol P\(_i\)·min\(^{-1}\)·mg protein\(^{-1}\) (Fig. 8). Treatment of myocytes with U50,488H before myofibril isolation significantly reduced the maximum Ca\(^{2+}\)-dependent actomyosin Mg\(^{2+}\)-ATPase activity to 129.4 ± 8.0 nmol P\(_i\)·min\(^{-1}\)·mg protein\(^{-1}\). The maximum Ca\(^{2+}\)-dependent actomyosin Mg\(^{2+}\)-ATPase activity of control myofibrils incubated with recombinant PKC-ε was 136.4 ± 8.0 nmol P\(_i\)·min\(^{-1}\)·mg protein\(^{-1}\). Incubation with PKC-δ resulted in a maximum Ca\(^{2+}\)-dependent actomyosin Mg\(^{2+}\)-ATPase activity of 113.4 ± 7.4 nmol P\(_i\)·min\(^{-1}\)·mg protein\(^{-1}\).

**Table 3. Ca\(^{2+}\)-dependent actomyosin ATPase activity of myofibrils isolated from hearts treated as indicated**

<table>
<thead>
<tr>
<th>Treatment (function)</th>
<th>ATPase Activity, nmol P(_i)·min(^{-1})·mg protein(^{-1})</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>164.0 ± 9.5</td>
<td>4</td>
</tr>
<tr>
<td>U50,488H (k-opioid agonist)</td>
<td>129.4 ± 8.0*</td>
<td>4</td>
</tr>
<tr>
<td>U50, 488H + norBNI (k-opioid antagonist)</td>
<td>159.6 ± 5.5</td>
<td>3</td>
</tr>
<tr>
<td>norBNI</td>
<td>162.1 ± 2.8</td>
<td>3</td>
</tr>
<tr>
<td>U50, 488H + Chel (PKC inhibitor)</td>
<td>173.1 ± 5.3</td>
<td>3</td>
</tr>
<tr>
<td>Chel</td>
<td>168.9 ± 2.9</td>
<td>3</td>
</tr>
<tr>
<td>U50, 488H + Bis (PKC inhibitor)</td>
<td>170.3 ± 4.8</td>
<td>3</td>
</tr>
<tr>
<td>Bis</td>
<td>158.9 ± 6.2</td>
<td>3</td>
</tr>
<tr>
<td>Phenylephrine + Propranolol (α-adrenergic agonist)</td>
<td>126.5 ± 11.8*</td>
<td>3</td>
</tr>
<tr>
<td>Phenylephrine + Propranolol + Chel</td>
<td>164.0 ± 5.0</td>
<td>3</td>
</tr>
<tr>
<td>Isoproterenol (β-adrenergic agonist)</td>
<td>195.1 ± 8.5*</td>
<td>3</td>
</tr>
<tr>
<td>Isoproterenol + Chel</td>
<td>193.5 ± 3.9*</td>
<td>3</td>
</tr>
<tr>
<td>PMA (receptor-independent activation of PKC)</td>
<td>135.8 ± 3.6*</td>
<td>3</td>
</tr>
<tr>
<td>α-PMA (inactive PMA)</td>
<td>166.5 ± 6.1</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of cells. Bis, bisindolymaleimide. *P < 0.05 compared with control.
Mg$^{2+}$-ATPase activity of 174.3 ± 5.9 nmol P$_i$ min$^{-1}$ mg protein$^{-1}$. This was not significantly different than untreated myofibrils. Myofibrillar actomyosin ATPase was unaffected by incubation with heat-inactivated PKC-ε or PKC-δ.

**DISCUSSION**

In the present study, stimulation of κ-opioid receptors increased the Ca$^{2+}$ sensitivity of isometric tension compared with untreated control ventricular myocytes at pH 7.0. This increase in the Ca$^{2+}$ sensitivity of tension was similar to that observed with direct activation of PKC with PMA. Furthermore, the U50,488H-dependent increase in the Ca$^{2+}$ sensitivity of isometric tension was abolished by the κ-opioid receptor antagonist norBNI and the PKC inhibitor chelerythrine chloride. These results are consistent with our hypothesis that the positive inotropy associated with κ-opioid receptor activation (33, 43) is due in part to a PKC-mediated increase in the myofilament Ca$^{2+}$ sensitivity of tension. Decreasing pH from 7.0 to 6.6 resulted in decreased maximum tension and decreased Ca$^{2+}$ sensitivity of isometric tension in control myocytes. Stimulation of κ-opioid receptors attenuated the pH-dependent decrease in the Ca$^{2+}$ sensitivity of tension compared with untreated controls.

κ-Opioid receptor activation induces cytosolic alkalization (42) and increases intracellular calcium (30, 42). It has been suggested that either or both of these effects may account for the initial positive inotropy associated with κ-opioid receptor stimulation. Although the present study did not address the κ-opioid receptor-dependent effects on intracellular calcium or acid-base management, the results suggest that the κ-opioid receptor-dependent increase in tension development may be due in part to changes in myofilament Ca$^{2+}$ sensitivity. The use of chemically demembranated myocytes allows for the experimental control of myofilament calcium and pH. Thus, in the absence of a κ-opioid receptor-dependent decrease in intracellular pH and/or increase in [Ca$^{2+}$], isometric tension development at submaximal [Ca$^{2+}$] was higher in myocytes treated with U50,488H compared with untreated control myocytes. These findings suggest that U50,488H-induced changes in the myofilaments contribute to the κ-opioid receptor-dependent increase in myocardial contractility.

Previous studies have established that acidosis is a useful tool in accentuating differences in myofilament Ca$^{2+}$ sensitivity between experimental groups. For example, an acid challenge more clearly demonstrated changes in the Ca$^{2+}$ sensitivity of tension due to pathological (24) and development (22) changes in the myofilaments. In the present study, we examined the effects of acidosis on the Ca$^{2+}$ sensitivity of isometric tension and found that differences in the Ca$^{2+}$ sensitivity of isometric tension between control and U50,488H-treated cells were not increased at pH 6.6. These findings strongly support the conclusion that a U50,488H-dependent change in cardiac myofilaments can, at most, account for a 8–10% increase in force of contraction at submaximal [Ca$^{2+}$]. In addition, troponin I is an important pH-responsive protein in myocardium (44). We (33) have previously shown that κ-opioid receptor activation increases troponin I phosphorylation levels. Data from our current study show little difference in the pH-induced changes in myofilament Ca$^{2+}$ sensitivity of tension with and without U50,488H treatment. These results are consistent with the hypothesis that the troponin I sites phosphorylated after κ-opioid receptor stimulation are not functionally connected to the pH-sensitive domain of troponin I.

Conflicting reports exist regarding the effect of stimulation of α₁-adrenergic receptors on Ca$^{2+}$ sensitivity of isometric tension in ventricular myocytes. Pucet et al. (31) observed an increase, whereas Strang and Moss (38) saw no change in the Ca$^{2+}$ sensitivity of tension after phenylephrine stimulation of ventricular myocytes. Under the experimental conditions of the present study, we observed no effect of stimulating α₁-adrenergic receptors on Ca$^{2+}$ sensitivity of isometric tension in ventricular myocytes. It is well established that stimulation of the β-adrenergic-PKA pathway decreases the Ca$^{2+}$ sensitivity of isometric tension in myocardium (15, 31, 38). Our current observations are consistent with these past studies. β-Adrenergic-dependent phosphorylation of troponin I is thought to account for the decrease in the Ca$^{2+}$ sensitivity of tension (14).

Our observation of differential effects on Ca$^{2+}$ sensitivity of tension by stimulation of α₁-adrenergic receptors, κ-opioid receptors, and PMA is of interest because activation of PKC is the probable second messenger pathway utilized by each of these agents. This raises the possibility of PKC isoform functional specificity. Others (9, 32) have reported that activation of various neurohormonal receptors in cardiomyocytes selectively induce an increase in activation of different

![Image](http://ajpheart.physiology.org/DownloadedFromhttp://ajpheart.physiology.org)
PKC isoforms. Furthermore, it has been demonstrated that isoforms of PKC can serve discrete functions within a cell (4, 13, 17). Our finding of an increased Ca\(^{2+}\) sensitivity of tension with some but not all purported activators of PKC is consistent with the hypothesis that specific isoforms of PKC have differential effects in cardiac myocytes.

One concern in the present study was the difference in control myocyte pCa\(_{50}\) values between studies. The average pCa\(_{50}\), pH 7.0, for control cells in Fig. 2 was 5.92 ± 0.03 (n = 12). The average pCa\(_{50}\), pH 7.0, for control/DMSO cells in Fig. 3 was 5.71 ± 0.02 (n = 5). One possible cause of the difference is the collagenase used to isolate myocytes. Data presented in Fig. 2 used cells isolated with type IV collagenase, whereas the myocytes in Fig. 3 were isolated with type I collagenase. Problems with Ca\(^{2+}\) contamination of the solutions probably do not account for the differences in control pCa\(_{50}\), because the three sets of pCa solutions, pH 7.0, made with deionized water and salts from three different chemical suppliers all gave pCa\(_{50}\) values of ~5.70 for myocytes isolated with the type I collagenase. In addition, the presence or absence of DMSO did not affect the pCa\(_{50}\) values. Cells isolated with type I collagenase and treated with 1% DMSO had an average pCa\(_{50}\) of 5.71 ± 0.02 (n = 5) compared with 5.74 ± 0.02 (n = 12) for non-DMSO-treated control cells. It should be emphasized that the shift in control pCa\(_{50}\) values between studies does not alter our findings of relative increases in the Ca\(^{2+}\) sensitivity of isometric tension with \(\kappa\)-opioid or PMA treatment compared with control myocytes from the same hearts.

In the present study, the \(\kappa\)-opioid receptor agonist U50,488H also decreased maximum actomyosin Mg\(^{2+}\)-ATPase activity. This effect was abolished by the \(\kappa\)-opioid receptor antagonist norBNI. The U50,488H-dependent decrease in maximum actomyosin Mg\(^{2+}\)-ATPase activity was mimicked by the known PKC activators phenylephrine and PMA and abolished by the PKC inhibitors chelerythrine chloride and bisindolylmaleimide. Exogenous PKC-\(\epsilon\) was also able to reduce maximum actomyosin Mg\(^{2+}\)-ATPase activity. The reduction in actomyosin Mg\(^{2+}\)-ATPase activity was associated with an increase in whole heart ventricular ATP. This effect was also abolished with PKC inhibition. Together, these results suggest that the \(\kappa\)-opioid receptor-dependent reduction in maximum actomyosin Mg\(^{2+}\)-ATPase activity is mediated through PKC-\(\epsilon\)-dependent myofibrillar alterations and that the PKC-dependent slowing of actomyosin Mg\(^{2+}\)-ATPase activity slows the depletion of intracellular ATP stores.

Administration of exogenous PKC-\(\delta\) did not reduce actomyosin Mg\(^{2+}\)-ATPase activity. Jideama et al. (16) have previously demonstrated PKC-\(\delta\) decreases actomyosin Mg\(^{2+}\)-ATPase. This apparent inconsistency may be due to two methodological differences. First, myofibrils isolated from ventricular myocytes were used for the present studies, whereas Jideama et al. (16) used reconstituted myofilaments consisting of tropinin I that had been phosphorylated by PKC-\(\delta\) before reconstitution. Thus it is possible that the PKC-\(\delta\) phosphorylation site on isolated tropinin I is not readily accessible in intact myofilaments. Second, the amount of PKC-\(\delta\) used in the present study was chosen to be approximately equal to the amount of myofibrillar Ca\(^{2+}\)-independent PKC activity found in rat ventricular myocytes. Jideama et al. (16) make no mention of the amount of PKC-\(\delta\) used in their study. A difference in the amount of PKC-\(\delta\) and the subsequent level of tropinin I phosphorylation and/or the myocardial preparations may explain the disparate results.

Clement et al. (8) have reported that exogenous PKC treatment does not alter maximum actomyosin Mg\(^{2+}\)-ATPase activity. This finding contradicts the results of the present study. One possible reason for this discrepancy may be the types of PKC utilized. Clement et al. (8) used PKC isolated from bovine brains, whereas the present study used only PKC-\(\epsilon\) or PKC-\(\delta\). It is possible that the PKC isoforms in addition to PKC-\(\epsilon\) and -\(\delta\) found in the bovine brain may phosphorylate different myofilament proteins. The effects of PKC on actomyosin Mg\(^{2+}\)-ATPase activity depends on which myofilament protein is phosphorylated by PKC (27, 28).

A variety of neurohormonal agents and transient ischemic protocols protect the heart against postischemic dysfunction or necrosis comcomitant with an attenuated decline in intracellular ATP (26). Phosphocreatine reserves may slow ATP depletion, but the rapid decline of their stores in the early stages of myocardial ischemia minimizes the contribution of this mechanism to preserving ATP levels (2). Given that glycolytic abatement is a well-defined characteristic of ischemia, increased ATP production through increased glycolysis is also a doubtful consideration. The results of the present study indicate that in the whole heart a reduction in actomyosin Mg\(^{2+}\)-ATPase activity is associated with higher levels of ventricular ATP. It is generally accepted that myocardial ATP levels remain unchanged in the normoxic heart. Our findings of increased ventricular ATP levels may be dependent on the protocol used. In the Langendorff-perfused heart preparation, glycogen levels may be depleted over the course of the experiment, thereby reducing ATP levels (11). It is conceivable that in the studies reported here ATP levels were reduced during the 20-min perfusion period and treatment with the \(\kappa\)-opioid receptor agonist slowed this ATP depletion (Fig. 7). It should be noted, however, that mean ATP levels from all hearts were within the normal range.

The cardiac myofibrillar protein that may mediate the effects of the receptor agonist-PKC pathway is difficult to identify. Activation of PKC has been associated with in vitro increased phosphorylation of a 15-kDa sarcolemma protein, a 28-kDa cytosolic protein (40), myosin light chain 2 (8, 41), C-protein (20), tropomin I (18), and tropomin T (18). Myosin light chain 2 phosphorylation levels have been associated with an increase in maximum actomyosin Mg\(^{2+}\)-ATPase activity (27). However, myosin light chain 2 may not be an in vivo substrate for activated PKC (26).

The present study demonstrates that \(\kappa\)-opioid receptor activation of normoxic myocardium increased the
$\text{Ca}^{2+}$ sensitivity of isometric tension and decreased maximum $\text{Ca}^{2+}$-dependent actomyosin Mg$^{2+}$-ATPase. Strong support was obtained in indicating that these effects are mediated by PKC, with PKC-$\epsilon$ as the potential isofrom involved. The implications of these findings on whole heart function in normal and diseased states include a $\kappa$-opioid-dependent increase in contractility at a given $[\text{Ca}^{2+}]$ under normal and acidic conditions and an improved energy state of the heart through modulation of myofilament function. Thus we propose that $\kappa$-opioid receptor agonists and other neurohormonal agents that are cardioprotective in fact by decreasing actomyosin ATPase activity, which increases or conserves ATP levels such that critical ATP-dependent pumps and channels remain more fully active during and after ischemia.

This study was supported by National Heart, Lung, and Blood Institute Grant HL-48839 and was done during the tenure of an Established Investigatorship (to P. A. Hofmann) of the American Heart Association.

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