Infarct limitation by a protein kinase G activator at reperfusion in rabbit hearts is dependent on sensitizing the heart to A2b agonists by protein kinase C

By Proprote kinase C,5 guanosine 3',5'-cyclic monophosphate (cGMP) is a key signaling step between the receptors and mitoKATP opening (13, 17, 32, 36). Costa et al. (12) further observed that activated PKG opens mitoKATP in isolated mitochondria.

PKG not only triggers protection against infarction when activated before ischemia, but also it can protect when activated immediately following an ischemic insult. It has recently been found that natriuretic peptides, which activate PKG by stimulating particulate guanylyl cyclase, limited infarct size when administered during early reperfusion (8, 44). Other pharmacological interventions during reperfusion that increase cGMP including the natriuretic peptide urodilatin (20, 33) and phosphodiesterase-5 inhibitors (37) also protect against infarction. The cell-permeant cGMP analog 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (CPT) is a potent and selective activator of PKG, and it also protected against infarction when administered at reperfusion (44). However, little is known about the signaling pathway by which PKG protects hearts during reperfusion. Abdallah et al. (1) recently reported that in adult rat cardiomyocytes CPT prevented hypercontracture by improving calcium handling through PKG-dependent phosphorylation of phospholamban during reoxygenation. On the other hand, we (44) showed that atrial natriuretic peptide at reperfusion protects by a phosphatidylinositol 3-kinase (PI3K)- and ERK-dependent mechanism. These survival kinases are also involved in protection from IPC (5, 18) as well as ischemic postconditioning (35, 40, 43, 45), suggesting a common mechanism. It is believed that survival kinases protect hearts by inhibiting formation of mitochondrial permeability transition pores (7, 14).

Activation of adenosine receptors (22, 35, 38, 43) and PKC (23, 34, 35, 46) during early reperfusion is also required for the protection of both IPC and postconditioning. We (23, 38) recently found that the activation of survival kinases during early reperfusion in IPC hearts depends on occupancy of adenosine receptors and PKC. Our observation that an A2b-selective adenosine receptor antagonist at reperfusion could block protection from IPC and postconditioning (35, 38) suggests that the receptor subtype involved is the low-affinity A2b receptor. PKC is located upstream of A2b receptors in this pathway (23, 35), and PKC activation markedly enhanced the ability of an adenosine agonist 5'-((N-ethylcarboxamido)adeno- sine (NECA) to increase the A2b receptor-dependent phosphorylation of both Akt (a reporter of PI3K activity and

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probably a survival kinase itself) and ERK1/2 (23). Therefore, we proposed that in the IPC heart PKC increases the sensitivity of the heart to adenosine at reperfusion so that endogenous adenosine can activate A2b-dependent signaling, which then protects the heart through activation of survival kinases. We hypothesize that it is the increased sensitivity to adenosine signaling that is responsible for the protected phenotype in the CPT-treated heart as well. We therefore tested whether CPT, like IPC, could lower the threshold of the heart to an A2b agonist. In addition, we tested whether inhibiting any of the known components of the protective signaling of IPC could block the reduction in infarct size by CPT.

METHODS

Isolated heart model. All animal care satisfied published guidelines (29), and procedures were approved by institutional committees. New Zealand White rabbits were anesthetized with sodium pentobarbital (30 mg/kg) and ventilated with 100% oxygen. Hearts were exposed through a left thoracotomy, and a suture was passed around a branch of the left coronary artery. The heart was removed and perfused on a Langendorff apparatus with modified Krebs-Henseleit bicarbonate buffer that contained (in mM) 118.5 NaCl, 24.7 NaHCO3, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, and 10.0 glucose. The buffer was gassed with 95% O2-5% CO2. A fluid-filled latex balloon was inserted into the left ventricle to measure pressure. All hearts were allowed to equilibrate for 20 min before the protocol was started.

Protocol for infarct studies. Twelve groups of hearts were studied (Fig. 1). All hearts were subjected to 30 min of regional ischemia and 120 min of reperfusion. Control hearts received no treatment. The 2nd group of hearts was treated with CPT (10 μM), a membrane-permeable cGMP analog, starting 5 min before reperfusion and continuing for 20 min. In the 3rd through 10th groups, CPT and either wortmannin (100 nM), an inhibitor of PI3K, PD-98059 (10 μM), a blocker of MEK1/2 and therefore of downstream ERK1/2, 8-dipropyl-1,3-cyclopentylxanthine (DPCPX; 20 nM), an adenosine A1-selective receptor antagonist, MRS 1754 (20 nM), an A2b-selective antagonist, 5-hydroxydecanoate (5-HD; 200 μM), a selective mitoKATP closer, glibenclamide (5 μM), a nonselective KATP channel blocker, N-(2-mercaptopropionyl)glycine (MPG; 300 μM), a ROS scavenger, or chelerythrine (2.8 μM), a PKC inhibitor, were simultaneously infused for 20 min beginning 5 min before release of the coronary occlusion. In group 11, CPT infusion was combined with 100 nM NECA, administered for 60 min from 5 min before the onset of reperfusion. In the 12th group, glibenclamide was infused alone at reperfusion.

Measurement of infarct size. At the end of the experiment, the coronary artery was reoccluded, and 2–9 μm fluorescent microspheres (Microgenics, Fremont, CA) were infused to delineate the ischemic zone (region at risk) as the area of tissue without fluorescence. The heart was cut into 2-mm-thick slices. The slices were incubated in 1% triphenyltetrazolium chloride in sodium phosphate buffer (pH 7.4) at 37°C for 10 min. The slices were then immersed in 10% formalin to preserve the stained (viable) and unstained (necrotic) tissue. The risk zone was identified by illuminating slices with ultraviolet light. The areas of infarct and risk zone were determined by planimetry of each slice, and volumes were calculated by multiplying each area by slice thickness and summing them for each heart. Infarct size is expressed as a percentage of the risk zone.

Biochemical studies. We tested to see if CPT given at reperfusion would cause increased phosphorylation of the survival kinases Akt and ERK1/2 and of GSK-3β in the first minutes of reperfusion as is the case with IPC. After a baseline left ventricular biopsy was obtained with a motorized biopsy tool from the isolated heart, a coronary branch was occluded for 30 min. A second biopsy was obtained after the heart had been reperfused for 10 min. Three hearts received CPT at reperfusion, and three more hearts received no drug and served as controls.

Four additional groups of hearts were used to test whether CPT sensitizes the heart to A2b signaling. After equilibration in the first group of normally perfused, isolated rabbit hearts, 10 μM CPT was included in the perfusate for 10 min. Previously, we had shown that 2.5 nM NECA, a nonselective but A2b-potent adenosine receptor agonist, was just below the threshold of NECA concentrations that could increase phosphorylation of Akt and ERK1/2 in the nonischemic heart (23). To reconfirm that observation, 2.5 nM NECA was infused in a second group of nonischemic hearts for 10 min. In the third group, 2.5 nM NECA was coinjected with 10 μM CPT for 10 min. In the fourth group, chelerythrine (2.8 μM) was given together with the combination of 10 μM CPT and 2.5 nM NECA. Biopsies were obtained from the left ventricle at baseline and after 10 min of treatment.

Tissue samples were immediately frozen in liquid nitrogen. Myocardium was homogenized with a Polytron in cell lysis buffer supplemented with PMSF and centrifuged for 15 min at 13,000 g. Protein content of the homogenized samples was measured with a Bradford assay, and an equal amount of protein was loaded in each lane. Electrophoresis of supernatant on 10% SDS-PAGE was followed by transfer to a nitrocellulose membrane. The membrane was probed with monoclonal antibodies to phospho-Akt (Ser473) and phospho-ERK1/2 and in the six ischemic hearts also to phospho-GSK-3β and -GSK-3α linked anti-mouse IgG antibody from Santa Cruz Biotechnology (Santa Cruz, CA). We used to dissolve the drugs and to prepare stock solutions. The final DMSO concentration was kept below 0.1%.

Phospho-Akt antibody to Ser473, phospho-GSK-3β antibody, cell lysis buffer, and Lumiglo were purchased from Cell Signaling Technology (Beverly, MA), monoclonal anti-phospho-ERK1/2 from Upstate (Lake Placid, NY), and horseradish peroxidase-linked anti-mouse IgG antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

Data analysis. All data are expressed as means ± SE. One-way ANOVA with Student-Newman-Keuls post hoc test was performed on baseline hemodynamic variables, risk zone, infarct size, and Western blot band densities at 10 min after treatment. Changes in hemodynamics in a given group and changes in phospho-Akt, -ERK, and -GSK-3β levels were evaluated by one-way ANOVA for repeated
measures with Tukey post hoc testing. \( P < 0.05 \) was considered significant.

**RESULTS**

**Hemodynamics.** No group differences in heart rate, developed pressure, or coronary flow were observed at baseline (Table 1). CPT, wortmannin, PD-98059, DPCPX, MRS 1754, 5-HD, MPG, chelerythrine, and NECA had little effect when infused, but glibenclamide caused a depression of coronary flow (data not shown). Coronary occlusion produced an expected decrease in left ventricular developed pressure and coronary flow in all groups. There was partial recovery of both parameters following reperfusion.

**Infarct size.** Our experimental design was to coadminister CPT and a number of agents that are known to block the protection of IPC. If any agent would block the protection of IPC, we could then conclude that the inhibited component was part of the mechanism by which CPT protected and that the component resided downstream of the site of CPT action (presumably activation of PKG).

There was no significant difference in body weight, heart weight, or risk zone volume among the groups except that body weight was higher in CPT + NECA group and heart weight or risk zone volume among the groups except that body weight was higher in CPT + NECA group and heart weight was higher in CPT + PD-98059 group than the control group (Table 2). Control hearts undergoing 30 min of regional ischemia and 2 h of reperfusion had 33.0 ± 3.0% infarction of the risk zone (Fig. 2). CPT started 5 min before reperfusion and continued for 20 min decreased infarction to 14.5 ± 2.2% (\( P < 0.05 \) vs. control). Both wortmannin and PD-98059 blocked this protective effect (38.4 ± 1.8 and 38.0 ± 3.4%, respectively). The infarct-limiting effect of CPT was also aborted by the highly selective adenosine receptor antagonists DPCPX and MRS 1754 (43.7 ± 2.4 and 35.7 ± 4.1%, respectively). Both the nonselective (glibenclamide) and the mitochondrial-selective (5-HD) K\(_{ATP}\) blockers also abolished protection from CPT (34.0 ± 4.7 and 28.3 ± 3.2%, respectively; Fig. 3). Finally, the free radical scavenger MPG and the PKC inhibitor chelerythrine blocked the infarct-sparing effect of CPT (24.7 ± 3.0 and 30.3 ± 4.7%, respectively). Glibenclamide by itself had no effect on infarct size. In previous studies, we had found that neither wortmannin (43), PD-98059 (42), DPCPX (38), MRS 1754 (38), 5-HD (10), nor MPG (10) administered alone at reperfusion had any effect on infarction.

The nonselective but \( A_{2b} \)-potent adenosine receptor agonist NECA has been shown to limit infarct size when administered just before reperfusion (42). We tested whether protection from NECA could be added to that from CPT. Confusion of CPT and NECA was protective but not more so than CPT alone (Fig. 3), suggesting similar mechanisms are involved in the protection by both CPT and NECA.

**Biochemical studies.** IPC increases phosphorylation (and thus activation) of Akt and ERK 1/2 in the first minutes of reperfusion. Since inhibiting Akt or ERK at reperfusion blocks the protection of IPC, it has been assumed that these kinases are involved in the protective signaling (18). To test whether CPT activates these kinases, we measured their phosphorylation in biopsies obtained from ischemic hearts 10 min after reperfusion. Figure 4 reveals that kinase phosphorylation was decreased relative to the baseline sample in untreated hearts but markedly increased in hearts receiving CPT as is the case in IPC hearts (5, 18, 38). We also measured phosphorylation of GSK-3\( \beta \), another kinase thought to be involved in the protection of IPC (21). Although there was a trend for an increase, the change did not achieve significance, presumably because of the small sample size.

We next tested whether CPT treatment might sensitize the heart to \( A_{2b} \) agonists as did brief preconditioning ischemia and PKC (23). To do that, we tested whether CPT would lower the threshold of the heart for kinase phosphorylation by the nonselective \( A_{2b} \) receptor agonist NECA. Treatment of isolated hearts with 10 \( \mu \)M CPT for 10 min did not cause any significant change in Akt and ERK1/2 phosphorylation (Fig. 5). We (23) previously reported that treatment with 2.5 nM NECA did not increase phosphorylation of the kinases, indicating that this dose was below the threshold for activation. To confirm that observation, we again treated four hearts with 2.5 nM NECA.

**Table 1. Hemodynamic data**

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>30' Occlusion</th>
<th>15' Reperfusion</th>
<th>30' Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR, beats/min</td>
<td>LVDP, mmHg</td>
<td>CF, ml·min(^{-1})·g(^{-1})</td>
<td>HR, beats/min</td>
</tr>
<tr>
<td>Control</td>
<td>210±8</td>
<td>113±7</td>
<td>9.0±0.2</td>
<td>195±6</td>
</tr>
<tr>
<td>CPT</td>
<td>214±5</td>
<td>112±4</td>
<td>8.6±0.2</td>
<td>203±6</td>
</tr>
<tr>
<td>CPT + Wort</td>
<td>236±15</td>
<td>106±5</td>
<td>9.5±0.2</td>
<td>233±15</td>
</tr>
<tr>
<td>CPT + PD-98059</td>
<td>237±15</td>
<td>105±4</td>
<td>8.6±0.5</td>
<td>213±7</td>
</tr>
<tr>
<td>CPT + DPCPX</td>
<td>222±7</td>
<td>113±4</td>
<td>10.6±0.6</td>
<td>213±7</td>
</tr>
<tr>
<td>CPT + MRS</td>
<td>1754</td>
<td>214±8</td>
<td>109±3</td>
<td>10.1±0.6</td>
</tr>
<tr>
<td>CPT + 5-HD</td>
<td>204±2</td>
<td>121±3</td>
<td>10.1±0.4</td>
<td>199±10</td>
</tr>
<tr>
<td>CPT + Glibenclamide</td>
<td>212±6</td>
<td>107±3</td>
<td>8.2±0.6</td>
<td>205±9</td>
</tr>
<tr>
<td>CPT + Gilberclamide</td>
<td>218±8</td>
<td>109±6</td>
<td>9.1±0.6</td>
<td>205±9</td>
</tr>
<tr>
<td>CPT + MPG</td>
<td>202±5</td>
<td>117±2</td>
<td>7.7±0.3</td>
<td>195±4</td>
</tr>
<tr>
<td>CPT + Chelerythrine</td>
<td>213±11</td>
<td>110±3</td>
<td>9.2±0.3</td>
<td>206±4</td>
</tr>
<tr>
<td>CPT + NECA</td>
<td>204±8</td>
<td>113±4</td>
<td>9.7±0.3</td>
<td>228±2</td>
</tr>
</tbody>
</table>

Values are means ± SE. CF, coronary flow; CPT, 8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate; DPCPX, 8-dipropyl-1,3-cyclopentylxanthine; 5-HD, 5-hydroxydecanoate; HR, heart rate; LVDP, left ventricular developed pressure; MPG, N-(2-mercaptopropionyl)glycine; NECA, 5'-((N-ethylcarboxamido)adenosine; Wort, wortmannin. *\( P < 0.05 \) vs. baseline.
and not surprisingly found no significant increase in either Akt or ERK phosphorylation (Fig. 5). On the other hand, both phospho-Akt and phospho-ERK1/2 were significantly increased from baseline after 10-min coinfusion of 2.5 nM NECA and 10 μM CPT indicating that CPT had lowered the threshold of NECA for kinase phosphorylation. The ability of CPT and NECA to increase phosphorylation of Akt and ERK1/2 was significantly attenuated by coadministration of the PKC inhibitor chelerythrine. Thus, just as we (23) previously saw with a preconditioning ischemia or a PKC activator, CPT lowered the threshold required for an adenosine agonist to activate the survival kinases and did so in a PKC-dependent manner.

DISCUSSION

The present study demonstrated that infarct size limitation by administration of CPT at reperfusion is dependent on PI3K, ERK1/2, adenosine A1/A2b receptors, mitoKATP, ROS, and PKC. All of these components are associated with the protection of IPC, suggesting that CPT protects through the same signaling pathway. We recently reported that in IPC hearts PKC lowers the threshold of agonists of the A2b adenosine receptor, and we proposed that this sensitization is the fundamental difference between an ischemically preconditioned and nonpreconditioned heart. Here, we have found that CPT also enhances the ability of the adenosine agonist NECA to phosphorylate Akt and ERK1/2 in a PKC-dependent manner. These experiments did not attempt to determine the relative order of the signaling steps involved in the protection of CPT. However, based on what we know about the role of PKG in the trigger pathway of IPC (9), the simplest explanation is that PKG activated PKC through mitoKATP opening and ROS production. Blockade of the protection of CPT by 5-HD, glibenclamide, and MPG supports that hypothesis. Activated PKC then would increase the sensitivity of the adenosine A2b receptor-dependent signaling, which would allow endogenous adenosine to protect the heart by activating survival kinases PI3K and ERK. Western blots confirmed that PI3K and ERK were activated in hearts treated with CPT at reperfusion. In the untreated heart, the affinity of the A2b receptors would be too low to be occupied by endogenous adenosine even during ischemia.

![Fig. 2](http://ajpheart.physiology.org/)

![Fig. 3](http://ajpheart.physiology.org/)

Table 2. Infarct size data

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, kg</th>
<th>Heart Weight, g</th>
<th>Risk Zone Volume, cm³</th>
<th>Infarct Volume, cm³</th>
<th>I/R, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>2.0 ± 0.1</td>
<td>6.9 ± 0.1</td>
<td>1.42 ± 0.06</td>
<td>0.48 ± 0.07</td>
<td>33.0 ± 3.9</td>
</tr>
<tr>
<td>CPT</td>
<td>14</td>
<td>2.0 ± 0.0</td>
<td>7.1 ± 0.2</td>
<td>1.26 ± 0.07</td>
<td>0.20 ± 0.04*</td>
<td>14.5 ± 2.2*</td>
</tr>
<tr>
<td>CPT + Wort</td>
<td>6</td>
<td>2.2 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td>1.59 ± 0.27</td>
<td>0.62 ± 0.12</td>
<td>38.4 ± 1.8</td>
</tr>
<tr>
<td>CPT + PD-98059</td>
<td>6</td>
<td>2.1 ± 0.1</td>
<td>7.9 ± 0.3*</td>
<td>1.57 ± 0.10</td>
<td>0.59 ± 0.06</td>
<td>38.0 ± 3.4</td>
</tr>
<tr>
<td>CPT + DPCPX</td>
<td>6</td>
<td>2.0 ± 0.1</td>
<td>6.2 ± 0.3</td>
<td>1.39 ± 0.14</td>
<td>0.61 ± 0.07</td>
<td>43.7 ± 2.4</td>
</tr>
<tr>
<td>CPT + MRS 1754</td>
<td>6</td>
<td>2.1 ± 0.1</td>
<td>6.7 ± 0.2</td>
<td>1.35 ± 0.08</td>
<td>0.49 ± 0.07</td>
<td>35.7 ± 4.1</td>
</tr>
<tr>
<td>CPT + 5-HD</td>
<td>7</td>
<td>2.0 ± 0.0</td>
<td>6.6 ± 0.2</td>
<td>1.12 ± 0.12</td>
<td>0.33 ± 0.07</td>
<td>28.3 ± 3.2</td>
</tr>
<tr>
<td>CPT + Glibenclamide</td>
<td>6</td>
<td>2.1 ± 0.0</td>
<td>6.5 ± 0.3</td>
<td>1.06 ± 0.11</td>
<td>0.39 ± 0.09</td>
<td>34.0 ± 4.7</td>
</tr>
<tr>
<td>CPT + NECA</td>
<td>6</td>
<td>2.5 ± 0.1*</td>
<td>7.7 ± 0.2</td>
<td>1.19 ± 0.09</td>
<td>0.38 ± 0.08</td>
<td>30.3 ± 4.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. I/R, infarction as a percentage of risk zone; n, number of animals. Statistical significance of difference between experimental and control groups: *P < 0.05.
We did not measure tissue adenosine in these studies, and thus we cannot eliminate the possibility that CPT may have raised endogenous adenosine to the point that the latter along with the exogenous NECA was enough to elicit a response. However, a number of factors make this suggestion unlikely. First, we studied nonischemic hearts, which would have had very low endogenous adenosine levels before treatment. Second, we know of no data that suggest PKG stimulation might raise adenosine levels. Third, in our previous report (23), low-dose NECA administered 20 min after a preconditioning protocol also initiated signaling, and we know that endogenous tissue adenosine in rabbit hearts returns to baseline within 5 min following brief preconditioning ischemia (16). Finally, because our findings reveal that CPT protects through signal transduction elements identical to those used by preconditioning, it seems likely that CPT also lowered the threshold for \( \text{A}_2\text{b} \) signaling.

In this study, we relied on pharmacological activators and inhibitors to reveal the pathways. We tried to use highly selective agents, but, of course, we cannot completely exclude an error in interpretation caused by an unknown nonspecific effect. We first tested the effect of inhibitors of PI3K and ERK on protection from CPT. Hausenloy et al. (18) found that inhibition of either kinase following a prolonged ischemic insult would abolish the anti-infarct effect of IPC. Sensitivity to these inhibitors has been regarded as a signature for interventions that use the mechanism of protection of IPC. As expected, either wortmannin or PD-98059 completely blocked the protection of CPT. Because the protection of CPT was also blocked by both a mitoK\(_{\text{ATP}}\) blocker and a ROS scavenger, it would appear that CPT acted to cause mitochondria to generate ROS by opening their K\(_{\text{ATP}}\) channels as was the case in the trigger pathway of IPC (32).

An obvious shortcoming of these studies is that we have not confirmed that CPT, putatively a very selective PKG activator, acted through PKG. Unfortunately, we could not find a PKG inhibitor that is inexpensive enough for use in the isolated heart to directly test if PKG was the target of CPT. Biochemical assays are also not useful since PKG activity in tissue is dependent on the cGMP level in the assay mixture, which would have little relationship to that in the original tissue. Normally, we would measure cGMP levels to estimate PKG activity, but in this case the tissue would have been rich in the cGMP analog CPT. However, since PKG is known to open the mitochondrial potassium channel (12), and CPT a very selective and potent activator of PKG, it seems highly likely that CPT protected by a PKG-dependent mechanism. Recently, Maas et al. (26) observed that vardenafil, which raises tissue cGMP by inhibiting phosphodiesterase-5, reduced infarct size in rat hearts when given at reperfusion. They could block that protection by coadministration of KT-5823, a reasonably selective PKG inhibitor.
The protection of CPT was blocked by either DPCPX or MRS 1754, suggesting that binding of endogenous adenosine to both A1 and A2b receptors at early reperfusion is required for protection by CPT administration. We saw a similar requirement for these two adenosine receptors for protection in the IPC heart at the time of reperfusion (38). We recently demonstrated that during reperfusion A2b receptors control survival kinase activation. MRS 1754 given at reperfusion abolished the protective effect of IPC (38), postconditioning (35), and NECA (42). Furthermore, treatment of hearts with a highly selective A2b receptor agonist BAY60-6583 in the early minutes of reperfusion reduced infarction, and this agonist increased phosphorylation of Akt and ERK1/2 in an MRS 1754-dependent manner (23). Thus stimulation of A2b receptors alone is sufficient for protection. The importance of A2b receptors for cardioprotection has been further supported by the finding that IPC failed to elicit cardioprotection in mice with genetic deletion of A2b receptors although IPC did protect hearts in A1−, A2a−, and A3-deficient mice (15). The exact role of the A1 receptor at reperfusion is unknown, but the above knockout studies suggest that it is not critical. Unlike the noted protective effect of A2b agonists, administration of an A1-selective agonist at reperfusion has not been found to be protective (4). Furthermore, protection from neither postconditioning nor the A1/A2 adenosine agonist AMP579 was affected by DPCPX in rabbit hearts (35, 41). The A1 receptors could possibly reinforce the PKC activation and be additive to the stimulation from ROS.

In the present study, the protective effect of CPT was blocked by the PKC inhibitor chelerythrine. PKC activation is necessary during early reperfusion for IPC and postconditioning to be protective (23, 34, 35, 46). In IPC, PKC is needed to recruit the survival kinases during reperfusion (23). The infarct-limiting effect of direct activation of PKC with phorbol ester in the first minutes of reperfusion was blocked by an A2b receptor blocker (23, 35), whereas the PKC blocker chelerythrine could not abort protection from the A2b-potent agonist NECA given at reperfusion (35), suggesting that PKC is located upstream of A2b receptors.

We believe that PKC causes activation of the A2b receptors by lowering the threshold for their signaling. We were able to show that NECA activates the survival kinases through the A2b receptors (23). We demonstrated sensitization of A2b signaling in rabbit hearts by showing that after PKC had been activated a subthreshold dose of NECA was then able to cause phosphorylation of the survival kinases (23). In the present study, CPT alone also did not affect kinase phosphorylation itself, but rather, like IPC, it sensitized the heart to the subthreshold concentration of NECA. Furthermore, this potentiation was blocked by coadministration of the PKC blocker chelerythrine. These results support our hypothesis that CPT protected by lowering the threshold for A2b-receptor-dependent activation of the survival kinases through a PKC-dependent pathway. The reperfused heart has high levels of adenosine resulting from breakdown of ATP. Unfortunately, this level is not likely to be high enough to significantly populate the protective A2b receptors because of their low affinity (KD ~16 μM). After PKC facilitates this signaling pathway, however, endogenous adenosine (1 to 5 μM) would be sufficient to activate the survival kinases and protect the heart.

The present study did not reveal the mechanism by which PKC enhanced A2b-dependent protective signaling. Adenosine A2b receptors couple to both Gs and Gq proteins (24). Agents that activate PKC have been shown to enhance NECA-induced cAMP production in various cell types (2, 28, 30). PKC activation also potentiated cAMP production induced by isoproterenol, forskolin (28), and cholera toxin (30), suggesting that the target for PKC is not the adenosine receptor but perhaps adenyl cyclase or one of the coupling proteins.

Redox signaling provides important second messengers in the trigger phase of IPC (3, 39). On the other hand, it has long been thought that at the time of reperfusion a burst of ROS production contributes to cell killing. However, the ROS scavenger MPG at reperfusion blocked the anti-infarct effect of CPT. The same was seen in the case of postconditioning (34) and IPC (19, 25). These findings imply that a “protective” redox signaling pathway operates independently of other “harmful” radical species during reperfusion. This may be explained by either localization or by specificity of the signaling ROS. We have hypothesized that ROS in the trigger phase of IPC activate PKC. They may well do that at reperfusion also. In a recent study, MPG at reperfusion blocked protection from IPC but not from direct PKC activation with phorbol ester indicating that the ROS site is upstream of the PKC site (25). The source of protective ROS in this study might well be mitochondria as was the case in the trigger phase of IPC. MPG reportedly concentrates inside cardiac mitochondria (27). The ROS involved is unknown, but MPG has been reported to selectively scavenge hydroxyl radical and peroxynitrite but neither superoxide nor hydrogen peroxide (6). Obviously, more work is required to elucidate this pathway.

Abdallah et al. (1) noted that CPT at reperfusion could prevent contracture in cardiac cells subjected to simulated ischemia. The documented mechanism appeared to be prevention of Ca2+ overload by increasing sarcoplasmic reticulum (SR) uptake of Ca2+ following phosphorylation of phospholamban. These observations may be relevant to the present study. The survival kinases are thought to protect against infarction by preventing formation of lethal permeability transition pores in the mitochondria at reperfusion (21), and formation of those pores is promoted by elevated calcium, which also occurs at reperfusion. We did not test for SR involvement, nor did Abdallah et al. (1) test for any of the components identified in our study. Thus the two studies may well be describing the same phenomenon.

Conclusion. The present study reveals that the protective effect of PKG activation with CPT during early reperfusion is dependent on mitoKATP. ROS, PKC, adenosine A1/A2b receptors, PI3K, and ERK. CPT treatment enhanced the ability of an adenosine agonist to activate PI3K and ERK in a PKC-dependent manner. Our results suggest that activation of PKG by CPT during early reperfusion opens mitoKATP channels causing the mitochondria to produce ROS, which, in turn, activate PKC just as occurs in the preconditioned heart. Activated PKC then lowers the threshold for A2b receptor-dependent activation of the survival kinases so that endogenous adenosine can elicit protection during the first minutes of reperfusion.

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