Anti-Ischemic Effect of a Novel Cardioprotective Agent, JTV519, Is Mediated Through Specific Activation of δ-Isoform of Protein Kinase C in Rat Ventricular Myocardium

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Background—A new 1,4-benzothiazepine derivative, JTV519, has a strong protective effect against Ca$^{2+}$ overload–induced myocardial injury. We investigated the effect of JTV519 on ischemia/reperfusion injury in isolated rat hearts.

Methods and Results—At 30 minutes of reperfusion after 30-minute global ischemia, the percent recovery of left ventricular developed pressure was improved, and the creatine phosphokinase and lactate dehydrogenase leakage was reduced in a concentration-dependent manner when JTV519 was administered in the coronary perfusate both at 5 minutes before the induction of ischemia and at the time of reperfusion. The myocardial protective effect of JTV519 was completely blocked by pretreatment of the heart with GF109203X, a specific protein kinase C (PKC) inhibitor. In contrast, the effect of JTV519 was not affected by $\alpha_1$, $A_1$, and $B_2$-receptor blockers that couple with PKC in the cardiomyocyte. Both immunofluorescence images and immunoblots of JTV519-treated left ventricular myocardium and isolated ventricular myocytes demonstrated that this agent induced concentration-dependent translocation of the δ-isoform but not the other isoforms of PKC to the plasma membrane.

Conclusions—The mechanism of cardioprotection by JTV519 against ischemia/reperfusion injury involves isozyme-specific PKC activation through a receptor-independent mechanism. This agent may provide a novel pharmacological approach for the treatment of patients with acute coronary diseases via a subcellular mechanism mimicking ischemic preconditioning. (Circulation. 2000;101:797-804.)

Key Words: ischemia ■ JTV519 ■ reperfusion ■ pharmacology ■ immunohistochemistry

Identification of novel therapeutic targets for the prevention of ischemia-induced cardiac injury has been an area of intensive investigation in this decade. First described in a canine model,1 preconditioning of cardiac tissue with ≥1 brief episodes of ischemia remains one of the most potent experimental means of reducing irreversible tissue injury during subsequent prolonged ischemia.

In regard to the intrinsic mechanisms of ischemic preconditioning, accumulating lines of evidence indicate the essential role of the activation of protein kinase C (PKC) as the subcellular hub element of the series of self-protective responses by the ventricular myocardium.2 PKC, a ubiquitous Ser-Thr kinase with multiple isoforms, is associated with a variety of receptors. The agonistic stimulation of $\alpha_1$-adrenergic, adenosine, or bradykinin receptors has been shown to induce preconditioning states via PKC activation.3-5 More recently, among several isoforms of PKC, activation of the $\delta$-isoform and its translocation to the plasma membrane have been shown to be critical steps for the induction of preconditioning.3 However, pharmacological agents that can effectively induce such isozyme-specific activation of PKC and exert anti-ischemic effects in clinical settings have not yet been obtained.

A new 1,4-benzothiazepine derivative, JTV519, which was developed by Kaneko,6 has a protective effect against Ca$^{2+}$ overload–induced myocardial injury. In this study, we showed potent anti-ischemic effects of this drug in isolated rat hearts. By further exploring the subcellular mechanisms, we found that the anti-ischemic effect of JTV519 is related to its activation of the specific isofrom of PKC. Because the anti-ischemic effect of JTV519 is exerted even with a de novo administration at the time of postischemic reperfusion, JTV519 may open the frontier to the clinical application of the benefit of preconditioning.

Methods

Isolated Whole-Heart Preparations

The preparation used in this study is described elsewhere.7 In brief, the isolated rat heart was perfused at 80 mm Hg by the Langendorff method with an oxygenated Krebs-Henseleit solution containing (in mmol/L) NaCl 120, KCl 5.8, NaHCO$_3$ 25, NaH$_2$PO$_4$ 1.2, MgCl$_2$ 1.2, CaCl$_2$ 1.0, and dextrose 10, pH 7.40, at 37°C. With a latex
balloon inserted into the left ventricular (LV) cavity, the LV volume was kept constant at an end-diastolic pressure of 5 to 10 mm Hg. Then, the heart was paced at 3.33 Hz by a pair of pacing electrodes in the right ventricle. The coronary effluent was time-collected to measure the coronary flow rate and to determine the creatine phosphokinase (CPK) and the lactate dehydrogenase (LDH) effluxes. All hearts were allowed to stabilize for 20 minutes before each protocol.

Whole-Heart Experimental Protocol
Essentially, each protocol consisted of a 30-minute global ischemia (abrupt cessation of the coronary perfusion), which was followed by a 30-minute full reperfusion. The control group was subjected solely to this set of 30-minute ischemia and 30-minute reperfusion. In the second group, the hearts were perfused with a randomly selected concentration of JTV519 (0.3, 1.0, and 3.0 μmol/L, respectively) for 5 minutes, followed by a 5-minute washout period before the onset of the ischemia/reperfusion intervention. In the third group, the hearts were pretreated with a relatively specific PKC inhibitor, GF109203X, 0.5 μmol/L (IC50 0.01 μmol/L for PKC, 2 μmol/L for PKA, 0.7 μmol/L for phosphorylase kinase). The treatment was started 5 minutes before administration of JTV519 (0.3 μmol/L) and was continued until the end of the JTV519 perfusion. In the fourth group, hearts were perfused with the β1-blocker prazosin (1.0 μmol/L), the adenosine A1 blocker 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 30 nmol/L), or the bradykinin B2 blocker HOE140 (10 nmol/L), each of which was started 5 minutes before the administration of JTV519 (0.3 μmol/L) and was continued until the end of the JTV519 perfusion. In the fifth group, the hearts were perfused with GF109203X, prazosin, DPCPX, or HOE140 without JTV519 for 10 minutes, followed by a 5-minute washout period before the control ischemia/reperfusion protocol. In the sixth group, at reperfusion, the perfusate contained JTV519 (1.0 μmol/L) or diltiazem (0.3 μmol/L), respectively, for 5 minutes. The perfusate was then switched to the control solution for the remaining 25-minute reperfusion period.

Immunofluorescence Microscopy
Subcellular localization and translocation studies of PKC isoforms in hearts were performed by immunofluorescence staining. At the end of a 5-minute coronary perfusion with the vehicle alone, phorbol 12-myristate 13-acetate (PMA) (1 μmol/L), JTV519 (0.3 μmol/L), or diltiazem (0.3 μmol/L) (n = 4 each), ventricular tissue was rapidly excised, embedded in OCT compound, and snap-frozen in dry ice–cooled acetone. Transverse 5-μm cryosections were prepared with a cryostat and collected on poly-L-lysine–coated slides. All sections were fixed for 10 minutes in a 70% acetone/30% methanol mixture at 20°C. After normal goat serum (10% in PBS) was applied as a blocking agent, the sections were incubated for 1 hour with rabbit polyclonal anti–PKC-d and anti–PKC-e antibodies (dilution 1:100) at room temperature. They were then incubated with Cy-3–conjugated goat anti-rabbit IgG (Biological Detection Systems) for 1 hour and were viewed and photographed with a

TABLE 1. Hemodynamic Measures and CPK and LDH Leakage in Rat Hearts That Received Preischemic Treatment With JTV519

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>%LVDP</th>
<th>EDP, mm Hg</th>
<th>CVR, mm Hg · min · mL⁻¹</th>
<th>CPP, mm Hg</th>
<th>CPK, IU/g</th>
<th>LDH, IU/g</th>
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<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>49.3±8.7</td>
<td>43.0±6.8</td>
<td>8.10±0.83</td>
<td>103±3</td>
<td>15.3±2.5</td>
<td>14.4±2.8</td>
</tr>
<tr>
<td>JTV519 0.3 μmol/L</td>
<td>5</td>
<td>72.6±3.8*</td>
<td>30.0±7.0</td>
<td>8.55±1.20</td>
<td>113±12</td>
<td>7.0±1.1*</td>
<td>6.4±1.2*</td>
</tr>
<tr>
<td>JTV519 1.0 μmol/L</td>
<td>5</td>
<td>77.8±1.0*</td>
<td>26.2±3.7</td>
<td>8.20±0.50</td>
<td>129±9</td>
<td>4.6±1.5*</td>
<td>3.0±1.1*</td>
</tr>
<tr>
<td>JTV519 3.0 μmol/L</td>
<td>5</td>
<td>86.4±4.1*</td>
<td>24.8±6.8</td>
<td>8.29±1.00</td>
<td>104±3</td>
<td>3.2±1.0*</td>
<td>1.9±0.5*</td>
</tr>
</tbody>
</table>

EDP indicates end-diastolic pressure; Cvr, coronary vascular resistance (CPP/coronary flow); and CPP, coronary perfusion pressure. Values are mean±SEM. *P<0.05 vs control.
microscope equipped with fluorescence optics (Fluophot 300, Nikon).

**Immunoblot Analysis of PKC Isoforms**

Heart preparations were treated with the vehicle alone, PMA, JTV519 (0.03, 0.3, and 3.0 μmol/L), or diltiazem as described above (n=4, respectively). Specimens were also obtained from hearts after 5 minutes of reperfusion with or without JTV519 (0.3 μmol/L) after 30 minutes of control ischemia (n=4 and 3, respectively). Soluble and particulate protein fractions were prepared from the freeze-clamped LV myocardium by the method of Rybin and Steinberg. The soluble and particulate samples were electrophoresed on an 8% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond ECL, Amersham). The immunoblotting was performed with anti–PKC-α, anti–PKC-β, anti–PKC-γ, anti–PKC-δ, anti–PKC-ε, and anti–PKC-ζ rabbit polyclonal antibodies at 1:1000 dilution. The protein was visualized on X-OMAT AR x-ray film (Kodak) by the enhanced chemiluminescence method (ECL, Amersham International).

**Ventricular Myocyte Preparation and Confocal Laser Microscopy**

The adult rat ventricular myocytes were enzymatically dissociated with collagenase as described previously. The cells were sedimented on coverslips coated with laminin and then stored for 2 hours at 37°C in a Joklik-modified Eagle’s minimal essential medium. The cells were then transiently permeabilized with saponin (50 μg/mL) with or without 150 μg/mL of rat recombinant PKC fragment dV1–1 or eV1–2 as described previously. The stimulation protocol and the staining protocol were similar to that used for tissue immunofluorescence except that the PMA concentration was reduced to 10 nmol/L. The cells were examined by confocal microscopy (Zeiss LSM410).

**Chemicals**

JTV519 and HOE140 were generously supplied by Japan Tobacco Inc (Takatsuki, Japan) and Hoechst (Frankfurt, Germany), respectively. Rat recombinant PKC fragments dV1–1 and eV1–2 were generous gifts from Daria Mochly-Rosen, PhD, Stanford University.

**Table 2. Effects of GF109203X, Prazosin, HOE140, and DPCPX on Rat Hearts Treated With JTV519 Before Ischemia**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>%LVDP</th>
<th>EDP, mm Hg</th>
<th>CPP, mm Hg</th>
<th>CPK, IU/g</th>
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</tr>
<tr>
<td>JTV519 0.3 μmol/L</td>
<td>5</td>
<td>72.6±3.8</td>
<td>30.0±7.0</td>
<td>113±12</td>
<td>7.0±1.1*</td>
<td>6.4±1.2*</td>
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<tr>
<td>GF</td>
<td>5</td>
<td>47.4±6.9</td>
<td>45.0±6.9</td>
<td>9.29±0.47</td>
<td>115±6</td>
<td>18.4±2.4†</td>
</tr>
<tr>
<td>GF+JTV519</td>
<td>5</td>
<td>50.8±3.8†</td>
<td>48.2±4.1</td>
<td>8.77±0.76</td>
<td>116±8</td>
<td>15.1±2.3†</td>
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<tr>
<td>PRZSN</td>
<td>5</td>
<td>54.8±6.2</td>
<td>40.7±7.8</td>
<td>8.74±0.37</td>
<td>110±4</td>
<td>13.5±0.5</td>
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<td>PRZSN+JTV519</td>
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<td>72.3±9.8*</td>
<td>35.2±10.4</td>
<td>9.43±0.98</td>
<td>115±11</td>
<td>6.6±5.8*</td>
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<tr>
<td>HOE</td>
<td>3</td>
<td>38.7±15.0†</td>
<td>59.0±19.6</td>
<td>8.14±0.27</td>
<td>95±5</td>
<td>21.7±5.2†</td>
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<td>HOE+JTV519</td>
<td>4</td>
<td>72.3±6.3*</td>
<td>32.8±9.2</td>
<td>8.18±0.67</td>
<td>105±5</td>
<td>10.4±7.8</td>
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<td>DPCPX</td>
<td>5</td>
<td>55.4±7.3</td>
<td>39.8±8.5</td>
<td>9.28±0.47</td>
<td>113±5</td>
<td>14.7±3.1</td>
</tr>
<tr>
<td>DPCPX+JTV519</td>
<td>4</td>
<td>80.0±7.0*</td>
<td>33.8±7.4</td>
<td>8.77±0.62</td>
<td>105±7</td>
<td>9.4±6.2</td>
</tr>
</tbody>
</table>

GF indicates GF109203X; PRZSN, prazosin; and HOE, HOE140. Other abbreviations as in Table 1. Values are mean±SEM.

*P<0.05 vs control; †P<0.05 vs JTV519 0.3 μmol/L.
Stanford, Calif. PMA and DPCPX were purchased from Sigma Chemical Co. Rabbit polyclonal isoform-specific anti-PKC antibodies were purchased from Calbiochem-Novabiochem International. Unless otherwise specified, all other chemicals were purchased from Wako Pure Chemical Co.

**Statistical Analysis**

Data are expressed as mean ± SEM. Differences among the various experimental groups for the functional study were tested by 1-way factorial ANOVA. Significant differences among groups (P < 0.05) were detected by Fisher’s protected least significant difference test.

**Results**

**Protective Effects of JTV519 on LV Function of Postischemic Reperfusion**

The administration of JTV519 for 5 minutes before ischemia caused a reduction in the ischemia/reperfusion injury (Figure 1, Table 1). At 30 minutes of reperfusion, both the percent recovery of LV developed pressure (%LVDP) and the CPK and LDH leakage were ameliorated in a concentration-dependent manner. JTV519 showed negative inotropic effects at 3.0 μmol/L, which decreased the %LVDP to 77.6 ± 10.5% of the baseline value (P < 0.05 versus control). However, the negative inotropic effect was not significant at the lower concentrations. The LV end-diastolic pressure tended to be lower in the JTV519-treated hearts, although the reduction did not reach a significant level. There were no significant differences between the control and JTV519-treated hearts.

**Figure 3.** Western blots of PKC-α, -β, -γ, and -ζ isoforms from cardiac homogenates of control hearts (CTL) and JTV519-treated hearts (JTV).

**Figure 4.** Western blots of PKC-δ isoforms from cardiac homogenates of control hearts (CTL) and hearts treated with various concentrations of JTV519 (0.03, 0.3, and 3.0 μmol/L). *P < 0.05 vs CTL.

**Figure 5.** Immunohistofluorescent images of translocation of PKC isoforms in control LV myocardium and after PMA, JTV519, or diltiazem treatment.
relative to the coronary perfusion pressure or coronary vascular resistance.

**Mechanisms of Anti-Ischemic Effects of JTV519**

To examine the PKC activation in relation to the anti-ischemic mechanisms of JTV519, we tested the interaction with GF109203X (Figure 2, Table 2). The treatment of preparations with GF109203X (0.5 μmol/L) alone did not exert any effects either on the %LVDP after ischemia/reperfusion or on CPK and LDH leakage. However, GF109203X completely blocked the myocardial protection conferred by the pretreatment with JTV519. We further tested the interactions of JTV519 with α1, A1, and B2 receptors by treating the preparations with prazosin, DPCPX, and HOE140. These receptor blockers did not modulate the hemodynamic measures by themselves, nor did they affect the JTV519-induced myocardial protection.

**Specific Translocation of PKC-δ With JTV519**

Figure 3 shows immunoblots of α-, β-, γ-, and ζ-isoforms of PKC in the cytosol and membrane fractions in the presence and absence of JTV519. JTV519 did not affect the subcellular localization of these isoforms. In contrast, JTV519 induced a concentration-dependent translocation of the δ-isoform to the membrane fraction (Figure 4). The translocation of the δ-isoform was further examined in comparison to another major Ca2+-independent PKC isoform, the ε-isoform. Figure 5 (first column) shows the diffuse and homogeneous staining of the δ- and ε-isoforms in a control LV myocardium, indicating the cytosolic localization of these inactive forms. After treatment with PMA, the pattern of PKC staining typically changed to a reticular and inhomogeneous pattern in both the δ- and ε-isoforms (second column), presenting positive controls for the rapid activation and translocation of these PKC isoforms to the plasma membrane and the nucleus.3,4 JTV519 rapidly induced the reticular staining of the anti–PKC-δ antibody. At the same time, however, JTV519 did not affect the distribution pattern of PKC-ε (third column). Hence, the effect of JTV519 on PKC appeared to be specific for the δ-isoform. Figure 6 shows immunoblots of δ- and ε-isoforms from cardiac homogenates.
Of more importance is the fact that the distribution consistently shifted in the presence of either PMA or JTV519.

...PKC- and PKC-ε isoforms (by 90.0%, P<0.05, respectively) in the membrane fraction only in the δ isoform (by 90.0%, P<0.05), a finding that is consistent with the immunofluorescent histology. The diltiazem treatment did not affect the PKC distribution in either the immunohistochemistry (Figure 5, last column) or the immunoblot (Figure 6, DIL).

The immunocytochemical examination was reassessed in isolated ventricular myocytes with confocal laser microscopy (Figure 7). Again, PMA but not JTV519 clearly induced an increase in the surface-to-cytosol fluorescence ratio for the δ isoform (Figure 7). The fluorescence of the δ isoform consistently shifted in the presence of either PMA or JTV519. Of more importance is the fact that the δ isoform translocation by JTV519 was abolished when the cells were pretreated with δV_{1,1} but not with εV_{1,2} peptides, which were specific for the V_{1} region of PKC-δ and PKC-ε, respectively.11

Myocardial Protection by JTV519 Administration at Postischemic Reperfusion

We further examined the protective effect of JTV519 on the postischemic myocardium by reperfusing hearts with a solution containing 1.0 μmol/L of this drug for the initial 5 minutes of reperfusion (Figure 8, Table 3). With JTV519, the %LVDP was markedly improved, with reductions of CPK and LDH leakage. In contrast, the administration of diltiazem (0.3 μmol/L) in the postischemic reperfusion did not have any effect on the myocardium with postischemic reperfusion. The immunoblot study demonstrated that both the δ- and ε-isoforms of PKC translocated to the plasma membrane after the control ischemia/reperfusion (Figure 9, Isc.). Administration of JTV519 at the time of reperfusion augmented the membrane shift of the δ- but not the ε-isoform (Isc.+JTV).

Discussion

Ischemic Preconditioning and PKC Activation

Although the precise mechanism has not been fully elucidated, many investigations have implicated PKC in the cellular cascade leading to ischemic preconditioning.2,3,12 Using neonatal rat cardiomyocytes, Zhao et al13 reported that transfection of constitutively active PKC-δ increased cell tolerance to simulated ischemia. These observations not only stressed the potentially unifying role of PKC in the preconditioning but also suggested that the activation of PKC-δ might be critical for this signal cascade in the preconditioning. However, current evidence did not indicate that the translocation of PKC-δ is necessary or has a direct link to the final activation of the putative effector proteins. Other investigators have suggested the critical role of the ε-isoform of PKC.11 Zhao et al also showed the transmission of the protective effect of the constitutively active PKC-δ to cocultured nontransfected myocytes. They suggested that the activation of PKC-ε might eventually be triggered by the PKC-δ-mediated reoccupation of adenosine A_{1} receptors.13 Thus, the translocation of PKC-δ could be a major but not mandatory step that is positioned upstream of this complex subcellular cascade of preconditioning.

Anti-Ischemic Effects of JTV519

JTV519 is a benzothiazepine derivative and shares an analogous chemical structure with the dihydropyridine-binding Ca^{2+} channel blocker diltiazem. Because the cardioprotective effects of the Ca^{2+} channel blocker in the ischemic myocardium have been attributed to the reduction of the cytosolic Ca^{2+} overload that occurs during ischemia and reperfusion,14,15 we preliminarily

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**TABLE 3.** Hemodynamic Measures and CPK and LDH Leakage in Rat Hearts That Received Postischemic Treatment With JTV519 and Diltiazem

<table>
<thead>
<tr>
<th>Group</th>
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<td>JTV519</td>
<td>5</td>
<td>74.8±8.8*</td>
<td>27.8±7.4</td>
<td>7.42±0.55</td>
<td>105±11</td>
<td>8.2±1.4</td>
<td>6.1±0.9*</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>4</td>
<td>49.0±7.4</td>
<td>44.3±5.5</td>
<td>8.36±0.24</td>
<td>106±3</td>
<td>12.7±4.6</td>
<td>11.1±2.2</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1. Values are mean±SEM. *P<0.05 vs control.
tested the interaction of this drug with the L-type Ca\textsuperscript{2+} channel (I_{Ca}) in isolated rat ventricular myocytes using the standard patch-clamp method with the whole-cell configuration. The percent inhibition of the peak current of I_{Ca} was 6.2% at 0.3 \textmu mol/L (P=0.683), 22.0% at 1.0 \textmu mol/L (P<0.01), and 59.6% at 3.0 \textmu mol/L (P<0.01). This concentration-dependent inhibition of I_{Ca} is consistent with the negative inotropic effect demonstrated in the isolated heart study (Figure 1) and indicates that JTV519 functions as a Ca\textsuperscript{2+} channel blocker. However, most of the cardioprotective effects of this drug are not attributed to this Ca\textsuperscript{2+} channel blocking, for the following reasons: (1) the marked cardioprotection by JTV519 was observed at a concentration of 0.3 \textmu mol/L, at which the negative inotropic effect and the inhibition of I_{Ca} were negligible; (2) in indo 1–loaded isolated heart preparations from the rat, 0.3 \textmu mol/L JTV519 did not affect the peak or resting levels of cytosolic Ca\textsuperscript{2+} transients as quantified by LV surface fluorometry (peak R_{405}/R_{500} in control, 1.557 versus 1.482 at the 5-minute perfusion of JTV519, P=0.182; resting R_{405}/R_{500}, 1.229 versus 1.125, P=0.343); and (3) as confirmed in our diltiazem study, Ca\textsuperscript{2+} channel blockers have been reported not to have the ability to reduce myocardial injury when they are administered at the time of ischemic reperfusion, an aspect that is considered a serious limitation to their use in a clinical setting. This is contrasted to the beneficial effect of JTV519 administered de novo at reperfusion.

**Specific PKC-δ Activation and JTV519**

In addition to the above observations, JTV519 appeared not to directly affect the Na\textsuperscript+/Ca\textsuperscript{2+} and Na\textsuperscript+/H\textsuperscript{+} exchangers or radical scavengers (personal communications, Dr J. Kimura, Fukushima, Japan; Dr H. Kusuoka, Osaka, Japan; and Biological/Pharmacological Research Laboratories, Japan Tobacco Inc, Takatsuki, Japan). Thus, we focused our survey on mechanisms relating to ischemic preconditioning. Although JTV519 did not show interactions with \alpha\textsubscript{1}-adrenergic, A\textsubscript{1}, or B\textsubscript{1} receptors, which might mediate the endogenous preconditioning effects, the drug showed an ability to activate PKC. This ability was confirmed by 2 independent methods: (1) a specific PKC inhibitor, GF109203X, completely blocked the myocardial protective effect of JTV519; and (2) a 5-minute perfusion of JTV519 translocated the key isoform, PKC-δ, in the cardiomyocytes, as demonstrated by both the immunohistochemistry and the immunoblot analysis. Under the same conditions, diltiazem did not show such PKC activation, further indicating that the effect was independent of the properties of JTV519 as a Ca\textsuperscript{2+} channel blocker.

In the present study, it remained unresolved how JTV519 activates PKC-δ. JTV519 may interact with membrane receptor sites that were not examined in this study. The candidates include muscarinic M\textsubscript{3} receptors, opioid receptors, angiotensin II receptors, and endothelin A receptors.\textsuperscript{17–20} Alternatively, JTV519 may pass through the sarcolemma and interact with PKC or G-coupled proteins in the cytosolic space. In the isolated myocytes, the JTV519-induced translocation of PKC-δ was abolished by the specific blocking peptide \delta V\textsubscript{1,1} but was not affected by the PKC-\epsilon–specific peptide \epsilon V\textsubscript{1,2}. Thus, the action of JTV519 on PKC-δ was not mediated by a mutual interaction of δ- and \epsilon-isofoms. It is interesting that Kaneko\textsuperscript{6} and others\textsuperscript{21} suggested the binding of JTV519 with annexin V, a component of the cytoskeleton structure connecting the sarcoleminal phospholipid to the contractile element F-actin. Thus, JTV519-induced PKC activation may occur by its interaction with annexin V at the inner site of the sarcolemma. Because annexin V is a binding protein of PKC and plays an inhibitory role on PKC translocation,\textsuperscript{22} JTV519 may facilitate the translocation process by modulating the conformation of this receptor for activated C-kinase (RACK)–like\textsuperscript{23} cytoskeleton molecule.
Effects of Postischemic JTV519 Administration and Clinical Implications

We observed that the administration of JTV519 at the time of reperfusion induced substantial myocardial protection. This effect not only differentiates JTV519 from the Ca<sup>2+</sup> channel antagonists but also indicates its clinical potential to salvage acute ischemic myocardium from subsequent stunning and irreversible cell loss. PKC translocation to the membrane fraction has been shown in hearts with sustained ischemia; however, the translocation may not be associated with phosphorylation/activation in the ischemic environment and may merely result in its breakdown. During the rapid recovery of the intracellular environment at the early phase of reperfusion, JTV519 may induce the additional translocation of PKC-δ or may unlock the inhibited phosphorylation/activation process. Mitchell et al. demonstrated that PKC-δ translocation after either transient ischemia or phenylephrine exposure is so rapid that it is evident after 2-minute interventions, which is consistent with our observation.

When induced within the proper chronological window, ischemic preconditioning exerts the most effective myocardial protection of any known intervention. Enormous efforts have been made to explore agents that could reproduce such intrinsic protection by pharmacological means. The clinical applications of the known receptor-mediated interventions, however, have been limited by their adverse effects on the central nervous system, systemic circulation, cardiac contractility, or coronary perfusion. The postischemic myocardial injury may be aggravated by a certain type of PKC activation, such as via endothelin A receptors. In addition, broad-spectrum PKC isoform activation by phorbol esters could be complicated by growth-promoting effects. Thus, JTV519 may be a novel and clinically applicable pharmacological agent that substantially ameliorates myocardial injury by intracoronary or systemic administration at the time of coronary revascularization.

Study Limitations

Potential limitations of the present study are as follows. (1) In this study, we did not differentiate PKC translocation from activation/phosphorylation. These 2 steps may not coincide, as we hypothesized in the setting of sustained ischemia. Recently, Cohen and Downey and Ytrehus et al. proposed that the PKC translocated during the preconditioning window awaits activation by the reoccupation of adenosine A<sub>1</sub> receptors, which may occur during sustained ischemia. Thus, the PKC translocation we demonstrated in this study may not be the step sufficient for its phosphorylation. (2) Activated PKC cascades and then phosphorylates the target proteins, which might induce virtual protective effects. In this study, we did not explore the downstream mechanisms, including the final effector.

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References

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