Gene Expression and In Situ Localization of Diacylglycerol Kinase Isozymes in Normal and Infarcted Rat Hearts

Effects of Captopril Treatment

Morihiko Takeda, Yutaka Kagaya, Jun Takahashi, Tadashi Sugie, Jun Ohta, Jun Watanabe, Kunio Shirato, Hisatake Kondo, Kaoru Goto

Abstract—Diacylglycerol (DG) kinase (DGK) terminates signaling from DG, which serves as an activator of protein kinase C (PKC), by converting DG to phosphatidic acid. DGK is thus regarded as an attenuator of the PKC activity. In rats, five DGK isozymes have been cloned, but little is known about their role in the heart. In this study, the spatiotemporal expression of DGK isozymes was investigated in rat hearts under a normal condition and after myocardial infarction (MI) by in situ hybridization histochemistry and immunohistochemistry. In normal left ventricular myocardium, DGKα, DGKε, and DGKζ mRNAs were expressed evenly throughout the myocardium, although the DGKα expression was very low. In infarcted hearts, the expression of DGKζ was enhanced in the peripheral zone of the necrotic area and at the border zone 3 and 7 days after MI, and to a lesser extent in the middle layer of the granulation tissue 21 days after MI. The enhanced DGKζ expression in the infarcted and border areas could be attributed to granulocytes and macrophages. In contrast, the expression of DGKε in the infarcted and border areas was lower than that in the viable left ventricle (LV) throughout the postoperation period. Furthermore, DGKε expression in the viable myocardium 21 days after MI decreased significantly compared with left ventricular myocardium in the sham-operated rats and was completely restored by treatment with captopril. Our results demonstrate that three DGK isozymes are expressed in the heart and that each isozyme might have different functional characteristics in the healing and LV remodeling after MI. (Circ Res. 2001;89:265-272.)

Key Words: diacylglycerol kinase ■ myocardial infarction ■ phagocytes ■ ventricular remodeling ■ angiotensin converting enzyme inhibitor

The phosphoinositide (PI) cycle and the resulting two second messengers, 1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP₃), have been shown to mediate a variety of cellular signaling triggered initially by hormones and neurotransmitters. DG serves as an activator of phospholipid-dependent protein kinase C (PKC), which mediates many cellular responses including growth, differentiation, apoptosis, and other events such as secretion in a variety of cell types, whereas IP₃ mobilizes calcium ions from the endoplasmic reticulum. This is also true in cardiac myocytes, in which angiotensin II and endothelin are known to trigger the PI cycle through phospholipase C (PLC), and the consequent activation of PKC has been reported to mediate inotropic responses, promote myocardial hypertrophy, and mediate the protection of cardiac cells in ischemic preconditioning. All of these processes are observed in the acute stage of myocardial ischemia and/or in the chronic stage after myocardial infarction (MI). Therefore, because DG may play a pivotal role in these processes by controlling the PKC activity, the cellular level of DG must be strictly regulated in various cells of the heart.

DG kinase (DGK) is an enzyme that is responsible for controlling the cellular level of DG by converting DG to phosphatidic acid (PA),8 thus acting as a regulator of PKC. Furthermore, PA, the product of DGK, may also serve as a second messenger and has been reported to activate PKCζ,9 polyphosphoinositide kinase,10 PLC-γ,11 and some other enzymes. It has been shown in cardiac myocytes that PA stimulates IP₃ production,12 increases intracellular free calcium,13 and further induces the phosphorylation of 30-kDa cardiac proteins.14 Considering all of these reports on DG and PA, DGK may play a crucial role not only through the attenuation of DG but also through the production of a possible second messenger, PA, in the heart.

Recent studies have reported15–21 the molecular structures of several DGK isozymes from mammalian species, indicat-
ing that DGK represents a large gene family of isozymes differing remarkably in their structures, their modes of tissue expression, and their enzymological properties, as can be seen in other signaling molecules such as PKC and PI-specific PLC. Five DGK isozymes, DGKα, -β, -γ, -ε, and -ζ, have been cloned from a rat cDNA library by our group and others, and all five isozymes show distinct tissue distributions and cellular localizations. In addition, we have reported the detailed cellular expression of mRNAs for these isozymes and their functional implications in the central nervous system. Therefore, it is highly conceivable that DGK isozymes show differential expression in cardiac tissue and play a unique role in normal and diseased conditions.

The aims of the present experiments were (1) to determine whether DGK isozymes are expressed in rat heart and (2) to localize and quantify the expression of these isozymes in rat heart at different time points after infarction. In addition, we also investigated the effects of an angiotensin converting enzyme inhibitor, captopril, on the expression of DGK isozymes in the infarcted heart. The present study provides important clues for revealing their putative roles in the wound-healing process, including removal of damaged tissue and scar formation at the border and infarcted region as well as in the development of myocyte hypertrophy in the surviving myocardium.

Materials and Methods

Northern Blotting

Total RNA was extracted (ISOGEN, Nippon Gene) from normal brain and heart ventricles of three adult male Wistar rats. Northern blots for rat DGKα, -β, -γ, -ε, and -ζ were carried out as previously described. Brain was included as a positive control. GAPDH was used as an internal control.

Animal Models

The experiments were performed according to the guidelines for the care and use of laboratory animals of Tohoku University. Left coronary artery ligation was performed in 8-week-old male Wistar rats as previously described. Animals were killed and their hearts were isolated 3, 7, and 21 days after the induction of MI (n=8 per time point both in MI rats and in sham-operated rats). In another protocol, rats with MI were assigned to either a captopril-treated group (n=5) (captopril was provided by Sankyo) or an untreated group (n=5). The administration of captopril (200 mg/kg body weight per day, 2 g/L of drinking water) was initiated on the day after coronary ligation and was continued for 21 days.

In Situ Hybridization Histochemistry

Probes were constructed from each isozyme cDNA outside the highly conserved catalytic domain to avoid background cross-hybridization on the sections. After linearization, [35S]UTP-labeled riboprobe was synthesized using a MAXIscriptTM in vitro transcription kit (Ambion). Heart sections (7 μm) were cut on a cryostat from infarcted hearts as well as from sham-operated hearts, 3, 7, and 21 days after MI (n=3 per time points). Sections were processed for in situ hybridization as previously described.

Immunohistochemistry

For the localization of different cell types in the border and infarct area, immunohistochemical analysis was performed in sections adjacent to those used for in situ hybridization. Procedures were performed according to the avidin-biotin-peroxidase method (Vector Laboratories). The primary antibodies were anti-α-smooth muscle actin (anti-ASMA) (Dako), a marker for myofibroblasts and vascular smooth muscle cells; anti-factor VIII (Dako), a marker for endothelial cells; anti-ED1 (Pharmingen), a marker for macrophages; anti-HIS48 (Pharmingen), a marker for granulocytes; anti-OX19 (Pharmingen), a marker for pan-T cells; and anti-OX33 (Pharmingen), a marker for B cells. The color was developed with diaminobenzidine.

Competitive Polymerase Chain Reaction (PCR)

Infarcted left ventricles (LVs) isolated 3, 7, and 21 days (n=5 per time points) after the surgery were cut into noninfarcted and infarcted areas by visual inspection. The border zone was included in the infarcted area. Total RNA was extracted (ISOGEN), and first-strand cDNA was synthesized. Competitive PCR was performed by titration of the sample cDNA with known amounts of nonhomologous rat DGKε and DGKζ competitor DNA produced using PCR (Competitive DNA Construction Kit, TaKaRa). PCR products were separated by gel electrophoresis and quantified using a charge-coupled device image sensor (Densitograph AE-6900-F, Atto) (Figure 1). The concentration of mRNA was expressed as attomoles of DGKε or DGKζ mRNA per microgram total RNA.

Statistical Analysis

Statistical analysis was performed using 1-way ANOVA. Values are mean±SEM. A value of P<0.05 was considered significant.
Results

Northern Blot Analysis
The hybridization signals for the three DGK isozymes ($\alpha$, $-\varepsilon$, and $-\zeta$) were detected in heart ventricles (Figure 2A). The positions of the hybridization bands for each of the three isozymes in the ventricles were the same as those in the brain and as those originally reported.15–19 Among those three isozymes, the expression level of DGK$\zeta$ mRNA was the highest and that for DGK$\alpha$ very weak in heart ventricles. Two hybridization bands for DGK$\varepsilon$ and DGK$\zeta$ were observed in brain and ventricles, suggesting alternatively spliced transcripts in these tissues as previously reported in the nervous system.18,19

In Situ Hybridization
The expression signals for DGK$\varepsilon$ and DGK$\zeta$ were detected ubiquitously in normal rat ventricular myocardium (data not shown). At higher magnification, the signals were deposited more densely on cells with large ovoid nuclei rather than on cells with small slender nuclei (Figure 2B), suggesting that the expression signals could be attributed predominantly to cardiac myocytes. The signal for DGK$\zeta$ was more intense than that for DGK$\varepsilon$, which is consistent with the findings of Northern blot analysis. The signal for DGK$\alpha$ was also ubiquitous, although very faint (data not shown). No significant hybridization signal was detected with control sense probes for the three DGK isozymes.

On day 3 after experimental MI, the expression signal for DGK$\zeta$ was intensified greatly in an area around the necrosis and at the border zone between the viable myocardium and infarcted area (Figure 3D). No signal was detected in the necrotic area. On day 7 after MI, the elevated DGK$\zeta$ signal was still visible in the same area although less prominently than in the preceding stage (Figure 3E). On day 21 the

Figure 2. Expression of DGK isozymes in heart ventricles. A, Northern blot analysis of rat brain (B) and heart ventricles (H). Blots were sequentially hybridized to $^{32}$P-labeled cDNA probes for DGK$\alpha$, $-\beta$, $-\gamma$, $-\varepsilon$, and $-\zeta$. A GAPDH probe was used as internal control. Size markers (arrowheads) are 28S and 18S rRNAs. B, In situ hybridization analysis of DGK$\varepsilon$ (a, b, e, and f) and DGK$\zeta$ (c, d, g, and h) in normal rat ventricular myocardium with $^{35}$S-labeled antisense (a, c, e, and g) and sense (b, d, f, and h) riboprobes. The sets of dark- and bright-field images for individual isozymes are from the same zones. Note that signals for DGK$\varepsilon$ and DGK$\zeta$ are deposited more densely on cells with large ovoid nuclei (arrowheads) rather than on cells with small slender nuclei. Also note that there was no significant hybridization signal with the control sense probe. Bar=25 $\mu$m.

Figure 3. Dark-field micrographs showing changes in gene expression for DGK$\varepsilon$ (A through C) and DGK$\zeta$ (D through F) in transverse sections of hearts in rats 3 days (A and D), 7 days (B and E), and 21 days (C and F) after left coronary artery ligation. Note that there was a marked decrease in the hybridization signal for DGK$\varepsilon$ in the infarct region whereas there was an increase in the signal for DGK$\zeta$ at postoperative day 3 as compared with signals in the viable region at any stages or in the lesion at postoperative day 21. RV indicates right ventricle.
necrotic area was almost absorbed, and the slightly elevated DGKζ signal remained in the middle layer of the infarcted ventricular wall (Figure 3F). On the other hand, the signal for the DGKe decreased in the infarcted area and at the border zone on day 3 compared with the viable myocardium, and the decreased expression was continued throughout the experiment (Figures 3A through 3C). The DGKe signal was very faint in both the viable myocardium and the infarcted area (data not shown). No hybridization signals were detected for DGKβ and DGKγ (data not shown).

Immunohistochemistry
MI is followed by an inflammatory reaction. 23 Immune cells infiltrate and accumulate around the necrotic area to be intensively engaged in the healing process. Therefore, we performed immunohistochemical analysis on serial sections to determine which types of cells the elevated DGKζ signal could be ascribed to. On day 3, abundant HIS48 and ED1 immunoreactivities were detected around the necrotic area. This area precisely corresponded to the one where the enhanced signal for DGKζ was observed (Figure 4), suggesting that the enhanced signal for DGKζ may be predominantly ascribed to the migrated granulocytes (HIS48 positive) and macrophages (ED1 positive). On day 7, the ED1 immunoreactivity increased greatly around the necrotic area and was colocalized with the elevated DGKζ signal, whereas HIS48 immunoreactivity decreased considerably (Figures 5A through 5C). This observation suggests that the elevated DGKζ signal was mostly from the macrophages at this stage. On day 21, when necrotic myocytes were almost scavenged, the elevated DGKζ signal was still observed in the middle layer of the infarcted ventricular wall and was colocalized with the ED1 immunoreactivity (Figures 6A and 6B). The distribution of the elevated signal for DGKζ was markedly different from that of ASMA immunoreactivity (myofibroblasts and vascular smooth muscle cells; Figures 5D and 6C) or factor VIII immunoreactivity (endothelial cells; data not shown) throughout the postoperative stages. OX19- and OX33-immunoreactive cells (T and B cells, respectively) were very few in number on day 3 and almost absent on days 7 and 21 (data not shown). These immunohistochemical observations strongly suggest that the elevated hybridization signal for DGKζ in the infarcted area was for the most part from infiltrating macrophages and granulocytes, with the latter detected only in the acute phase of infarction.

Quantitative Analysis of the mRNAs in the Infarcted Hearts and the Effect of Captopril
To quantitatively determine the mRNA levels for DGKe and DGKζ in the infarcted hearts in comparison with sham-operated hearts (n=5 per time points both in MI rats and in sham-operated rats), we performed competitive reverse transcriptase (RT)–PCR analysis (Figure 7). On postoperative day 3, the DGKζ mRNA level increased by ~30% in the infarcted area compared with the viable and sham-operated LVs. The increase at this acute phase might be ascribed to the infiltrating macrophages and granulocytes as revealed by our immunohistochemistry. The DGKζ mRNA level in the infarcted area returned to the level of the sham-operated LV on days 7 and 21. In contrast, the DGKe mRNA level in the infarcted area was reduced to ~20% of the level in the sham-operated LV on day 3, 40% on day 7, and 50% on day 21. On the other hand, the expression of DGKe mRNA was significantly downregulated by 29% in the viable LV of untreated MI rats compared with sham-operated rats 21 days after MI (P<0.05).

The ratio of the heart weight/body weight after MI increased significantly compared with that of the sham-operated rats (Table). In rats treated with captopril for 21 days, this ratio was significantly reduced to the level of the sham-operated hearts (Table). As shown in Figure 7, captopril treatment did not alter the DGKζ mRNA level significantly in either the infarcted area or the viable LV. On the other hand, the treatment with captopril for 21 days completely normalized the DGKe gene expression in the viable LV of the infarcted heart (P<0.05) to a level quite comparable with that in the sham-operated groups.
The protocol used in this study could not address the effects of captopril on the gene expression of DGK\(\zeta\) and DGK\(\epsilon\) in sham-operated rats. Thus, a separate experiment was performed in which first-strand cDNA was prepared from sham-operated rats with and without captopril treatment for 21 days. Sham-operated rats treated with captopril demonstrated a significant reduction in systolic blood pressure measured by the tailcuff method compared with untreated sham-operated rats (113±2 versus 121±2 mm Hg, \(n=6\) for each, \(P<0.02\)). The heart weight/body weight ratio was significantly decreased in the captopril-treated sham-operated rats compared with untreated sham-operated rats (2.34±0.05 versus 2.52±0.04 mg/g, \(P<0.02\)). Interestingly, semiquantitative RT-PCR analysis demonstrated that the expression level of DGK\(\epsilon\) was significantly increased by 104% in the hearts of captopril-treated sham-operated rats compared with those of untreated sham-operated rats, whereas that of DGK\(\zeta\) was comparable between the two groups.

**Discussion**

We identified for the first time that DGK isozymes are expressed in cardiac tissue and suggested that some of them may play roles in its diseased condition. Recently, the PI cycle and related cascades were shown to be closely involved in the ventricular remodeling after MI\(^{24,25}\). In addition,
molecular biological studies have revealed several isozymes of PI-related signal transduction molecules, such as PI-PLC and PKC, and have identified the specific isozymes that are involved in heart disease.6,25,26 Our data provided new insights into the importance of the DG-mediated PKC cascade and its regulatory mechanism in the heart at the cellular level.

Elevated DGKz Signal in the Infarcted Area
A specific elevation of the DGKz signal was observed in the acute phase of MI in the area surrounding the necrosis. Histological changes in the process of infarct healing in rats have been well established.23,27 At the acute inflammatory phase of healing after MI, the infarcted area is composed of degenerating necrotic myocytes and focally dense infiltrated granulocytes. Thereafter, the disappearance of granulocytes coincides with the onset of the chronic inflammatory and reparative phases of infarct healing. At the border of the ingrowing granulation tissue and the necrotic muscle, numerous macrophages create a resorption front and gradually remove the residual debris. Our immunohistochemical data revealed that the increased signal for DGKz may be ascribed to the infiltrated macrophages and granulocytes. Lennartz28 described that phospholipases play an integral role in phagocytosis by generating essential second messengers such as DG and PA, and it is suggested that DG is required as an activator of PKC in phagocytosis and that PA plays a role in actin polymerization to control cellular motility and the activation of the p47phox subunit of NADPH oxidase. DGK, therefore, may play a key role in phagocytosis in the acute phase of MI. Our data from in situ hybridization strongly suggest that the DGKz is responsible for these phagocytic reactions by granulocytes (mostly neutrophils) and macrophages in the infarct healing. Whether granulocytes and macrophages intrinsically express DGKz at a high level or the elevated signal observed in the present study resulted from the activation of the phagocytic reaction in these cells remains to be elucidated.

The upregulation of DGKz in the infarcted area was most evident 3 days after MI, and the expression became less prominent 7 and 21 days later. Ju et al25 showed that the Gqa/PLCβ pathway, which triggers the PI cycle, is upregulated predominantly in scar tissue 8 weeks after MI in rats. At chronic stages, the sustained upregulation of PLCβ, which may increase DG production, and relatively downregulated DGK expression revealed by the present study might elicit a sustained DG level and subsequent long-lasting PKC activation in the scar tissue.

DGKe Gene Expression and the Effects of Captopril
DGKe is unique among isozymes insofar as it acts specifically on species containing arachidonate at the sn-2 position,21,29 whereas the other isozymes act on DG irrespective of the fatty acyl composition.15–18,20 This strongly suggests the direct involvement of DGKe in the PI cycle, because it is known that PI has a characteristic fatty acid composition of 1-stearoyl-2-arachidonoyl.30 With regard to its functional aspect, one possibility is that the arachidonoyl-specific DGKe may attenuate specifically the signal of arachidonoyl DG derived mostly from the breakdown of PI, although the other isozymes are also capable of catalyzing arachidonoyl DG nonspecifically. The other possibility is that if the DGKe works specifically on the arachidonate-containing species of DG, then multiple cycles would progressively enrich PI with arachidonate. Glomset31 reported that arachidonoyl-specific DGK is located in the endoplasmic reticulum, an organelle responsible for the synthesis of various lipids, and may be engaged in de novo synthesis of sn-1-stearoyl-2-arachidonoyl PI. In either case, it is clear that DGKe may reflect an activity of the cellular PI cycle.

McCluskey et al32 reported that, in infarcted canine hearts, the activity of arachidonate metabolism in the microsomes from infarcted tissue increases compared with that from noninfarcted LV. Furthermore, Weber et al33 reported that cultured fibroblasts from infarcted canine ventricle can metabolize more arachidonate than those from normal ventricle. These observations suggest that enhanced arachidonate metabolism might result from the inflammatory cell invasion and/or fibroblast activation that accompanies the healing process after MI. In phagocytes, arachidonate-containing phospholipid may be preferentially cleaved by phospholipase...

<table>
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<tr>
<th>Days After Surgery</th>
<th>Body Weight, g</th>
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<th>Heart Weight/Body Weight, mg/g</th>
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<tr>
<td>3 days</td>
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<tr>
<td>Sham</td>
<td>169±4</td>
<td>468±9</td>
<td>2.78±0.02</td>
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<tr>
<td>MI</td>
<td>154±6</td>
<td>495±25</td>
<td>3.23±0.19</td>
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<tr>
<td>7 days</td>
<td></td>
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<tr>
<td>Sham</td>
<td>176±6</td>
<td>463±11</td>
<td>2.64±0.04</td>
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<tr>
<td>MI</td>
<td>151±5*</td>
<td>456±13</td>
<td>3.02±0.10*</td>
</tr>
<tr>
<td>21 days</td>
<td></td>
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<tr>
<td>Sham</td>
<td>226±4</td>
<td>535±13</td>
<td>2.36±0.03</td>
</tr>
<tr>
<td>MI untreated</td>
<td>210±13</td>
<td>628±16*</td>
<td>3.02±0.15*</td>
</tr>
<tr>
<td>MI captopril</td>
<td>189±10*</td>
<td>495±25†</td>
<td>2.63±0.11†</td>
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Values are mean±SEM obtained from 5 rats.
*P<0.05 vs sham.
†P<0.05 vs MI untreated.

Figure 7. Quantitative analysis of expression levels for DGKe and DGKz. Value at each time point is mean±SEM for 5 rats. C in columns represents rats treated with captopril. *P<0.05 compared with sham-operated group; #P<0.05 compared with viable LV.
A<sub>2</sub> to release arachidonate, a substrate for prostaglandins and leukotrienes that mediate the inflammatory response. The possible decrease in de novo synthesis of sn-1-stearoyl-2-arachidonoyl PI caused by the downregulation of the DGK ε may facilitate the supply of arachidonate to the inflammatory and healing process after MI.

In addition to the expression in the infarcted area, it should also be noted that our competitive RT-PCR analysis revealed the decreased expression of DGK ε, but not that of DGK ζ, in the noninfarcted myocardium compared with that in LV myocardium in sham-operated rats. It is well known that experimental MI brings about cardiac myocyte hypertrophy in viable myocardium remote from the infarcted area. 7,34,35 Bowling et al. 26 reported that the increased expression of PKCα and PKCβ was associated with this condition in humans. In this regard, a PKC β inhibitor was shown to attenuate cardiac hypertrophy in PKCβ2 isozyme transgenic mice. 36 Furthermore, Pettit and Wakelam 17 reported that the overexpression of DGK ε, but not that of DGK ζ, in porcine aortic endothelial cells induced the translocation of PKCα and PKCβ from the membrane to the cytosol, suggesting that DGK ε is a physiological terminator of DG signaling to activate PKC. It is possible that the decreased DGK ε level in the noninfarcted myocardium increases the PKC activity through the arachidonoyl-containing DG level, which may accelerate the hypertrophic response in the viable myocardium.

It is well known that captopril attenuates the hypertrophic response in viable myocardium after MI. 7,35 In the present study, it should be noted that captopril normalized the expression of DGK ε in the noninfarcted area to the control level and was associated with a 13% reduction in the heart weight/body weight ratio. Therefore, it is highly conceivable that cardiac hypertrophy and the level of DGK ε expression are closely related in terms of the mechanism of PKC activation, although much remains to be elucidated. The cascade of angiotensin II receptor and its downstream pathway might have some regulatory effect on the transcription of the DGK ε. However, it is also possible that the decrease in the LV systolic pressure and/or the sustained elevation of the bradykinin level through an inhibitory action of captopril on its breakdown might play a role in the gene expression of DGK ε.

Interestingly, captopril reduced the heart weight/body weight ratio and enhanced the gene expression of DGK ε in the hearts of sham-operated rats as well as in the viable LVs of the infarcted hearts. The decrease in the heart weight elicited by captopril treatment in the sham-operated rats is consistent with earlier reports by Rossi et al. 38 The results of the present study suggest the possibility that DGK ε is involved in the signal transduction of the renin-angiotensin system in cardiac myocytes in terms of regulating the normal growth of the LV.

**Limitation**

We do not know the relative contribution of each DGK isozyme to the total DGK activity that controls the DG levels in phagocytes and cardiac myocytes in the healing and hypertrophic responses after MI. The individual involvement of each DGK isozyme in the pathophysiological development of heart diseases has to be elucidated. Furthermore, recent studies have shown that DG is derived not only from phosphatidylinositol 4,5-bisphosphate but also from phosphatidylcholine by a sequential action of phospholipase D and PA phosphatase. 39 Therefore, it may be necessary to examine the origin of DG to elucidate the regulatory mechanism of the DG level and subsequent PKC activation. To date there are some reports that address the contribution of DGK in the cardiovascular system. 40,41 These studies are based on the effects of the DGK inhibitors R59022 and R59949 on the DG and PA levels in their model systems. Jiang et al. 12 reported that both reagents selectively inhibit Ca<sup>2+</sup>-activated DGKs. DGK ε and DGK ζ, which are implicated as having important roles in cardiac pathophysiology as was also shown in the present study, are both Ca<sup>2+</sup>-insensitive isozymes. Therefore, the present study might be focused on different functional aspects of DGK in cardiac tissue from the earlier studies. 40,41

In conclusion, the present study suggests that several DGK isozymes are involved in cardiac pathophysiology and have distinct functional properties. Such differences may be reflected in the specific localization of the isozymes in particular areas or cell types in post-MI heart. Further studies are needed to elucidate the role of DGK isozymes in the cardiovascular system.

**Acknowledgments**

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