BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 171–175 (1997)
ARTICLE NO. RC977445

Role of Protein Kinase C-α in Activation of Ecto-5′-nucleotidase in the Preconditioned Canine Myocardium

Masafumi Kitakaze,[1] Hiroharu Funaya, Tetsuo Minamino, Koichi Node, Hiroshi Sato, Yasunori Ueda, Yuji Okuyama, Tsunehiko Kuzuya, Masatsugu Hori, and Ken-ichi Yoshida*
*The First Department of Medicine, Osaka University School of Medicine, Suita, Japan; and *The Department of Legal Medicine, Yamaguchi University School of Medicine, Ube, Japan*

Received August 29, 1997

We have reported that activation of protein kinase C (PKC) increases ecto-5′-nucleotidase activity, which may contribute to the infarct size-limiting effect of ischemic preconditioning. Since we have reported that Ca^{2+}- and phospholipid-sensitive PKC is activated due to ischemic preconditioning, we further tested 1) whether PKC-α or -β is translocated to the cellular membrane of the preconditioned canine myocardium, and 2) whether activation of PKC contributes to the increase in ecto-5′-nucleotidase activity via phosphorylation-dependent mechanisms. Four times of 5 minutes coronary occlusion separated by 5 minutes of reperfusion (ischemic preconditioning) translocated PKC-α to the cellular membrane in the canine hearts, although PKC-β, -δ, -ϵ, and -ζ were not translocated. The activity of Ca^{2+}- and phospholipid-sensitive PKC increased, which was attenuated by the removal of either Ca^{2+} or phosphatidylserine. Ecto-5′-nucleotidase was also activated in the preconditioned myocardium compared with control. Inhibition of PKC due to GF109203X blunted the activation of myocardial ecto-5′-nucleotidase. Okadaic acid (an inhibitor of phosphatase) enhanced the increases in ecto-5′-nucleotidase activity due to preconditioning, and this enhancement was blunted by GF109203X. We conclude that ischemic preconditioning activates PKC-α, and thus ecto-5′-nucleotidase.

Infarct size is limited when brief periods of ischemia precede the sustained ischemia, a phenomenon known as "ischemic preconditioning" (IP) (1). The precise mechanisms underlying this phenomenon have been investigated (2,3), and several studies have suggested that the infarct size-limiting effect of IP is attributable to adenosine (3,4). We have previously shown that IP increases the activity of ecto-5′-nucleotidase during ischemia and reperfusion (5), and that the inhibition of ecto-5′-nucleotidase blunts the infarct size-limiting effect of IP (6). Furthermore, we have revealed that activation of ecto-5′-nucleotidase is attributable to phosphorylation via PKC (7-9). However, it is not elucidated what type(s) of PKC is activated and translocated to the cellular membrane in the canine preconditioned myocardium, while PKC-ϵ and δ are involved in the rat cardiomyocytes (10). It is also not elucidated whether activation of protein kinase C contributes to the phosphorylation and activation of ecto-5′-nucleotidase.

The present study was undertaken to examine what subtype(s) of PKC is translocated to the cellular membrane in the preconditioned myocardium. Since OA is known to inhibit phosphatase, we further tested whether OA augmented the increases in ecto-5′-nucleotidase due to ischemic preconditioning.

MATERIALS AND METHODS

Immediately after ninety mongrel dogs of either sex (15-21 kg) were anesthetized with sodium pentobarbital (30 mg/kg, intravenous), the dogs were intubated and ventilated with room air mixed with oxygen (100% O_2 at 1 to 2 L/minute). The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. We cannulated the LAD and perfused it with blood from the left carotid artery through an extracorporeal bypass tube. Systemic hemodynamic parameters, i.e., aortic blood pressure and heart rate were also monitored.

In the open chest dogs, heart rate and systemic blood pressure were measured continuously. Coronary arterial blood was sampled every 60- to 90-minutes for the analysis of blood gas to monitor the condition of the dogs. The IP protocol consisted of 4 cycles of 5 minutes of LAD occlusion and subsequent reperfusion for 5 minutes each. IP procedures were performed in the presence of OA (80 ng/kg/min, the IP+OA group, n=5), GF109203X (300 ng/kg/min, the IP+GF group, n=5), AMP-CP (80 μg/kg/min, the IP+AMP-CP group, n=5) or OA+GF109203X (the IP+OA+GF group, n=5). These chemi-

[1] To whom correspondence and reprint requests should be addressed at The First Department of Medicine, Osaka University School of Medicine, 2-2 Yamadaoka, Suita 565, Japan. Fax: 81-6-879-3645. E-mail: kitakaze@medone.med.osaka-u.ac.jp.

Abbreviations: **PKC,** protein kinase C; **OA,** okadaic acid; **LAD,** left anterior descending coronary artery; **AMP-CP,** α,β-methyleneadenosine 5′-diphosphate.

0006-291X/97 $25.00

cals were administered into the LAD via the bypass tube. The IP protocol was also performed without any pharmacological interventions (the IP group, n=5). Both OA and GF109203X were administered 5 min prior to the IP, and continued until the end of the IP procedure, and AMP-CP was administered 5 min prior to the IP, and continued throughout the study. As controls, we infused either OA (the OA group, n=4), GF109203X (the GF group, n=5) or AMP-CP (the AMP-CP group, n=5) without IP. As control, we also obtained the myocardium without any intervention (the control group, n=5).

At the end of the protocol, a biopsy specimen of the myocardium (1-2 grams) supplied by LAD was obtained. We measured ecto-5′-nucleotidase and PKC activity, and performed immunoblotting to determine the subtypes of PKC. The myocardium was separated into membrane and cytosolic fractions using the following technique: Myocardial tissue was homogenized using a Potter-Elvehjem homogenizer (30 strokes) for 5 minutes in 10 volumes of ice-cold 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid-potassium hydroxide (HEPES-KOH) buffer (pH 7.4) containing 0.25 M sucrose, 1 mM $MgCl_2$, and 1 mM mercaptoethanol at 0°C. The crude homogenate was strained through a double-layered nylon sieve and homogenized again for 1 minute. For the preparation of membrane and cytosolic fractions, the homogenate was centrifuged at 1,000 g for 10 minutes, and the supernatant was centrifuged at 200,000 g for 1 hour. After this procedure, we regarded the pellet and supernatant fractions as the membrane and cytosolic fractions, respectively. The membrane and cytosolic fractions were dialyzed at 4°C for 4 hours against 10 mM HEPES-KOH (pH 7.4) containing 1 mM $MgCl_2$, 1 mM mercaptoethanol, and 0.01% activated charcoal, and divided into aliquots which were frozen immediately and stored at −80°C. The protein concentration was measured by the method of Lowry et al. (11) using bovine serum albumin as the standard.

Antibodies to PKCs-α, β, δ, ϵ, and ζ and their antigenic peptides were obtained from Gibco, the ECL Western blotting detection kit from Amersham, the Vectastain ABC kit from Vector Laboratories, prestaining makers for PAGE from Biorad, the biotim-blocking system from Dako, and OCT compounds from Miles, Pep Tag™ PKC assay kit from Promega. All other reagents were obtained commercially.

The fractions were subjected to SDS-polyacrylamide gel electrophoresis using 7.5% polyacrylamide gels, and then to immunoblotting. The amount of protein applied to the gel varied for each isoform and fraction to obtain on the immunoblot. The blots were blocked with 5% skim mild in buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Tween-20 for at least one hour, incubated with one of 2,000-3,000-fold diluted antibodies against PKC isoforms for one hour at room temperature, and the PKC isoforms were visualized by the ECL Western blotting detection kit. For preadsorption experiments, the antibodies were preincubated with immunizing peptides (10 μg/ml) for 30 minutes or more before application. The amounts of PKC isoforms on the immunoblots were measured by densitometry, and were expressed in arbitrary units.

5′-Nucleotidase activity was assessed by the enzymatic assay technique (12) and was reported using the units of nmol/mg protein/minute. 5′-Nucleotidase activity of the membrane fraction were defined as ecto-5′-nucleotidase activity.

The activity of protein kinase C in the membrane and cytosolic fractions was measured by the enzyme assay using the RPN 77A kit (Amersham), which provides a simple and reliable method of estimating protein kinase C without extensive purification of the samples (5-9). Activity of protein kinase C was expressed as nmol/mg protein/min. Furthermore, to examine the Ca^{2+}- and phospholipid-dependency of activity of protein kinase C, we measured protein kinase C activity adding 0.5mM in excess of EGTA to chylate Ca^{2+} and eliminated phosphatidyl-L-serine from the assay system (8,9).

Data are expressed as mean±SEM. Results were analyzed by analysis of variance. When differences between groups reached statistical significance, data were analyzed using a Bonferroni's test to determine significance at the $p<0.05$ level. A p value <0.05 was considered statistically significant.

RESULTS

Systolic and diastolic blood pressures (∼142/∼85 mmHg), and heart rate (∼136 min^{-1}) did not change throughout the protocol in each group. The IP procedure increased the PKC activity of the membrane fraction, and this increased PKC activity was Ca^{2+}- and phospholipid-dependent (Table 1). The immunoblotting of subtypes of PKC revealed that α-PKC is translocated to the cellular membrane fraction, however, other subtypes of PKC were not translocated to the cellular membrane (Figure 1). Quantitative analysis of the immunoblotting also confirmed this observation (% increases in the density in the preconditioned myocardium compared with the control myocardium, PKC-α: 248±38% ($p<0.01$), PKC-β: 109±21%, PKC-δ: 118±28%, PKC-ϵ: 95±13%, PKC-ζ: 104±12%, n=5 in each). The IP procedure also increased ecto-5′-nucleotidase activity, and this increase was blunted by GF109203X (Table 2). OA further increased ecto-5′-nucleotidase (Table 2), but did not augment the increase in PKC activity (Table 1). AMP-CP did not affect the activity of PKC (Table 1). These results indicate that IP predominantly activates α-PKC, and this increased activity of PKC-α activates ecto-5′-nucleotidase.

DISCUSSION

We have previously reported that IP activates ecto-5′-nucleotidase, and this increase contributes to the cardioprotection of ischemic preconditioning. Furthermore, we have revealed that the activation of ecto-5′-nucleotidase is attributable to PKC, because 1) the activation of ecto-5′-nucleotidase and the infarct size-limiting effect of ischemic preconditioning are attenuated by GF109203X and prazosin, and 2) transient exposures to methoxamine and PMA activate ecto-5′-nucleotidase, and mimic the infarct size-limiting effect in the canine heart.

However, in our previous studies, we did not clarify two important issues. The first issue is the subtypes of protein kinase C. In the rat hearts, ϵ- and δ-PKC are activated and translocated to the cellular membrane after 2 min of coronary occlusion. However, Yoshida et al. reported that a long period of myocardial ischemia in the rat activates PKC-α as well as PKC-ϵ and -δ (13). Furthermore, Ca^{2+} preconditioning activates α- and δ-PKC in the rat heart. On the other hand, there is no report of the subtype(s) of activated PKC after 4 times of 5 min of coronary occlusion in the canine heart. Here we have revealed that 4 times of 5 min of coronary occlusion translocates PKC-α, but not PKC-ϵ and -δ. This agrees to the present biochemical findings that Ca^{2+}- and phospholipid-sensitive PKC is activated in the preconditioned myocardium, since PKC-α is known to be Ca^{2+}- and phospholipid-sensitive. We did not ob-

TABLE 1

Changes in Protein Kinase C Activity (nmol/mg protein/min) of the Control and Preconditioned Myocardium

	Ca^{2+} (+), PL (+)		Ca^{2+} (−), PL (+)		Ca^{2+} (+), PL (−)		Ca^{2+} (−), PL (−)	
	Membrane	Cytosol	Membrane	Cytosol	Membrane	Cytosol	Membrane	Cytosol
Control	5.7±1.2	38.9±5.1	6.0±1.5	10.8±1.6	5.1±1.7	12.6±1.5	4.8±0.9	12.0±0.7
IP	22.7±4.1**	35.8±7.1	7.6±2.1	9.8±1.2	5.1±2.4	11.8±1.3	4.8±0.7	9.8±1.4
IP+OA	20.2±2.8**	42.9±3.5	5.9±2.1	9.1±1.5	6.5±2.1	12.6±1.7	3.9±0.9	9.0±1.2
IP+AMP−CP	24.8±2.9**	34.5±8.4	7.3±2.9	8.8±1.3	6.4±2.0	13.4±0.8	4.4±0.9	9.8±1.4
OA	4.6±1.3	36.9±5.0	6.7±1.3	12.4±2.8	4.2±2.4	11.9±1.2	3.7±0.7	11.3±0.7
AMP-CP	5.2±1.1	40.7±3.4	6.8±2.2	11.6±2.1	5.9±1.8	11.1±1.5	3.9±0.5	12.0±0.7

Values (pmol/mg protein) are mean±SEM. n=6 in each group.
Abbreviations: PL, phospholipid; IP, ischemic preconditioning; OA, okadaic acid; AMP−CP, α,β-methyleneadenosine 5′-diphosphate.
** $P<0.01$ vs. the control group in each condition.

serve the translocation of β-PKC to the cellular membrane. Therefore, the activation of Ca^{2+}- and phospholipid-sensitive PKC may be mainly attributable to PKC-α. The differences between the data of α-PKC in the results of Mitchell et al. (10) and ours may be attributable to the severity of ischemia during preconditioning. Indeed, a long period of ischemia also translocated PKC-α to the cellular membrane in the rat heart. As for PKC-ϵ and -δ, the species differences (rat and dogs) may be involved. On the other hand, Przyklenk

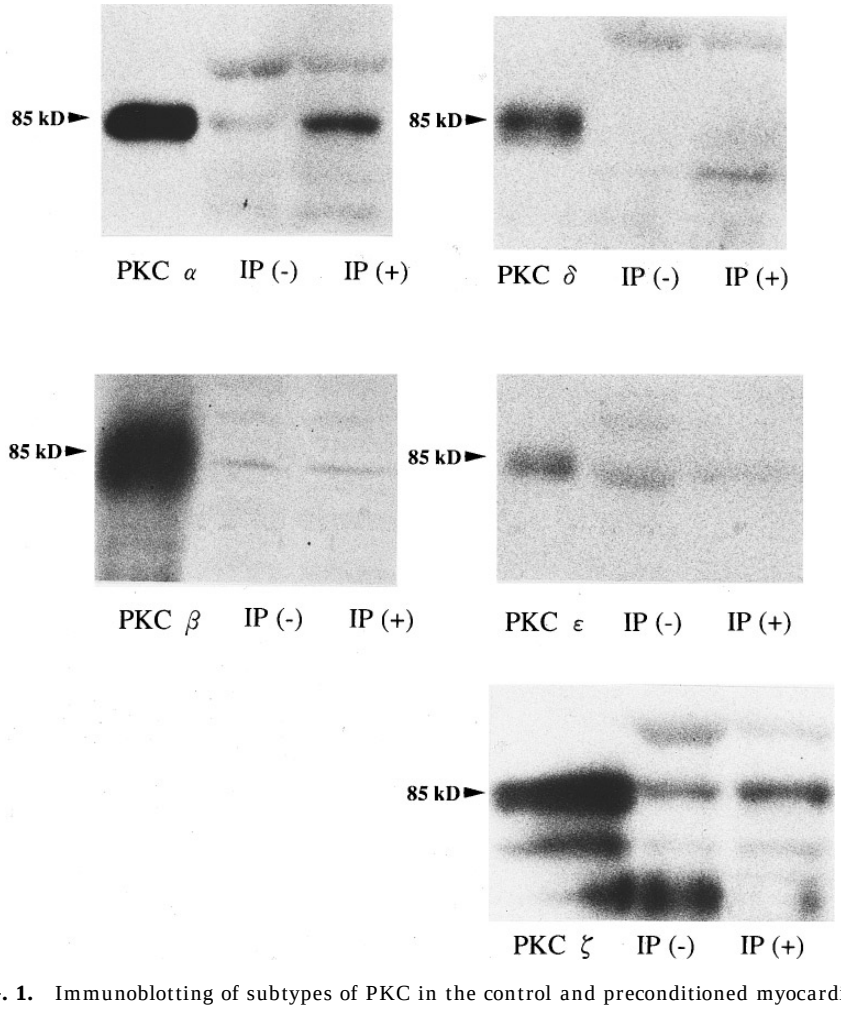

FIG. 1. Immunoblotting of subtypes of PKC in the control and preconditioned myocardium.

TABLE 2

Changes in Ecto-5′-nucleotidase Activity (nmol/mg protein/min) of the Control and Preconditioned Myocardium

	LAD		LCX	
	Endocardium	Epicardium	Endocardium	Epicardium
Control	37.1±5.1†	35.5±5.1†	37.3±3.9	32.5±3.3
IP	64.6±5.4*	60.0±6.6*	37.9±2.1	39.9±2.8
IP+OA	99.2±9.5*†	93.6±7.9*†	39.8±4.2	43.3±3.7
IP+GF109203X	34.9±6.6†	31.4±5.6†	33.8±1.3	34.4±2.0
IP+GF109203X+OA	38.7±1.8†	36.5±1.4†	38.4±3.1	36.4±2.1
OA	42.9±1.6†	39.8±1.1†	37.0±4.4	35.1±3.8
GF109203X	34.3±1.6†	30.6±1.1†	39.5±4.4	35.0±3.6

Values (pmol/mg protein) are mean±SEM.
Abbreviations: IP, ischemic preconditioning; OA, okadaic acid.
* $P<0.05$ vs. the control group in each condition. †$P<0.05$ vs. the IP group in each condition.

et al. (14) reported that protein kinase C is not activated by 4 times of 5 min coronary occlusion in the canine hearts. Furthermore, neither H-7 nor polymyxin B blunted the infarct size limiting effect of ischemic preconditioning. We have previously reported that both GF109203X and polymyxin B into the canine coronary artery blunts the infarct size-limiting effect of IP (8). We did not clarify the differences. One possibility is the differences of the method to detect PKC, because Przyklenk et al. (14) used fluorescent probe for active PKC and we used biochemical and immunoblotting methods. The second possibility is to the differences in the time of myocardial sampling, The activation of PKC seems to be very transient, since we did not observe any increases in PKC activity 3 and 7 min after the ischemic preconditioning procedure (data are not shown). Third, since both H-7 and polymyxin B were administered to the systemic vein in the study of Przyklenk et al. (14), these chemicals may cause systemic hemodynamic changes. This may blunt the cardiac effects of H-7 and polymyxin B, or may be diluted to the less effective concentrations before reaching the heart, when inhibitors of PKC were directly administered to the coronary artery. Indeed, there are several reports that protein kinase C is involved in the cardioprotection of ischemic preconditioning (15-17). We also revealed that α_1-adrenoceptor activation due to the IP procedure increases the activity of PKC in the canine hearts (6), although other factors, e.g., adenosine, bradykinin and endothelin, are proposed.

The second issue is how the activation of PKC-α links activation of ecto-5′-nucleotidase. In the present study, OA, which inhibits protein phosphatase, augmented the increases in ecto-5′-nucleotidase due to ischemic preconditioning, and this augmentation was blunted by the inhibitor of PKC. Therefore, we believe that phosphorylation of this enzyme due to PKC is responsible for its activation. We did not clarify the phosphorylation site of this enzyme, which needs further effort. Furthermore, although we clarified the subtypes of PKC activated by ischemic preconditioning, we did not clarify the specific role of PKC-α. PKC-α may have a strong affinity for the phosphorylation and activation of ecto-5′-nucleotidase in the canine hearts.

The present study suggests that the activator of PKC-α selectively mediates activation of ecto-5′-nucleotidase and the infarct size-limiting effect. Although the specific activator of PKC-α has not been available at present, non-specific activator of PKC, PMA, mimics preconditioning. Another possibility is to find the activator or inducer of ecto-5′-nucleotidase. We have revealed that vesnarinone is the potent activator of ecto-5′-nucleotidase (18).

REFERENCES

1. Murry, C. E., Jennings, R. B., and Reimer, K. A. (1986) *Circulation* **74,** 1124–1136.
2. Murry, C. E., Richard, V. J., Reimer, K. A., and Jennings, R. B. (1990) *Circ. Res.* **66,** 913–931.
3. Liu, G. S., Thornton, J., Van Winkle, D. M., Stanley, A. W. H., Olsson, R. A., and Downey, J. M. (1991) *Circulation* **84,** 350–356.
4. Miura, T., Ogawa, T., Iwamoto, T., Shimamoto, K., and Iimura, O. (1992) *Circulation* **86,** 979–985.
5. Kitakaze, M., Hori, M., Takashima, S., Sato, H., Inoue, M., and Kamada, T. (1993) *Circulation* **87,** 208–215.
6. Kitakaze, M., Hori, M., Morioka, T., Minamino, T., Takashima, S., Sato, H., Shinozaki, Y., Chujo, M., Mori, H., Inoue, M., and Kamada, T. (1994) *Circulation* **89,** 1237–1246.
7. Kitakaze, M., Hori, M., Morioka, T., Minamino, T., Takashima, S., Okazaki, Y., Node, K., Komamura, K., Iwakura, K., Itoh, T., Inoue, M., and Kamada, T. (1995) *Circulation* **91,** 2226–2234.
8. Kitakaze, M., Node, K., Minamino, T., Komamura, K., Funaya, H., Shinozaki, Y., Chujo, M., Mori, H., Inoue, M., Hori, M., and Kamada, T. (1996) *Circulation* **93,** 781–791.
9. Kitakaze, M., Minamino, T., Node, K., Komamura, K., Inoue, M., Hori, M., and Kamada, T. (1986) *J. Mol. Cell Cardiol.* **28,** 1945–1955.
10. Mitchell, M. B., Meng, X., Ao, L., Brown, J. M., Harken, A. H., and Banerjee, A. (1995) *Circ. Res.* **76,** 73–81.

11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
12. Smith, K., Varon, H. H., Race, G. J., Paulson, D. L., Urshel, H. C., and Mallams, J. T. (1965) *Cancer* **19,** 1281–1285.
13. Yoshida, K., Hirata, T., Mizukami, Y., Yamaguchi, K., Sorimachi, Y., Ishihara, T., and Kawashiyama, S. (1996) *Biochim. Biophys. Acta.* **1317,** 36–44.
14. Przyklenk, K., Sussman, M. A., Simkhovich, B. Z., and Kloner, R. A. (1995) *Circulation* **92,** 1546–1557.
15. Ytrehus, K., Liu, Y., and Downey, J. (1994) *Am. J. Physiol.* **266,** H1145–H1152.
16. Liu, Y., Cohen, M. V., and Downey, J. M. (1994) *Circulation* **90,** 1–207.
17. Speechly-Dick, M. E., Mocanu, M. M., and Yellon, D. M. (1994) *Circulation* **90,** 1–208. Abstract
18. Kitakaze, M., Minamino, T., Funaya, H., Node, K., Shinozaki, Y., Mori, H., and Hori, M. (1997) *Circulation* **95,** 2108–2114.