

PKC ϵ modulates NF- κ B and AP-1 via mitogen-activated protein kinases in adult rabbit cardiomyocytes

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Li, Richard C. X., Peipei Ping, Jun Zhang, William B. Wead, Xinan Cao, Jiuming Gao, Yuting Zheng, Shuang Huang, Jiahuai Han, and Roberto Bolli. PKC ϵ modulates NF- κ B and AP-1 via mitogen-activated protein kinases in adult rabbit cardiomyocytes. *Am J Physiol Heart Circ Physiol* 279: H1679–H1689, 2000.—We have previously shown that protein kinase C (PKC)- ϵ , nuclear factor (NF)- κ B, and mitogen-activated protein kinases (MAPKs) are essential signaling elements in ischemic preconditioning. In the present study, we examined whether activation of PKC ϵ affects the activation of NF- κ B in cardiac myocytes and whether MAPKs are mediators of this signaling event. Activation of PKC ϵ (+108% above control) in adult rabbit cardiomyocytes to a degree that has been previously shown to protect myocytes against hypoxic injury increased the DNA-binding activity of NF- κ B (+164%) and activator protein (AP)-1 (+127%) but not that of Elk-1. Activation of PKC η did not have an effect on these transcription factors. Activation of PKC ϵ also enhanced the phosphorylation activities of the p44/p42 MAPKs and the p54/p46 c-Jun NH₂-terminal kinases (JNKs). PKC ϵ -induced activation of NF- κ B and AP-1 was completely abolished by inhibition of the p44/p42 MAPK pathway with PD98059 and by inhibition of the p54/p46 JNK pathway with a dominant negative mutant of MAPK kinase-4, indicating that both signaling pathways are necessary. Taken together, these data identify NF- κ B and AP-1 as downstream targets of PKC ϵ , thereby establishing a molecular link between activation of PKC ϵ and activation of NF- κ B and AP-1 in cardiomyocytes. The results further demonstrate that both the p44/p42 MAPK and the p54/p46 JNK signaling pathways are essential mediators of this event.

protein kinase C ϵ ; activator protein-1; nuclear factor- κ B

PROTEIN KINASE C (PKC) is a family of serine-threonine kinases that may be classified into three subfamilies (29), which include the classical isoforms (α , β , γ), the novel isoforms (ϵ , δ , η , θ), and the atypical isoforms (ζ , λ , ι). Activation of PKC has been shown to be an important signaling step in various biological processes (13, 29, 35), including the development of ischemic preconditioning (PC) (24, 27, 52). Recent stud-

ies from our laboratory (32, 33) and others (15, 23) have demonstrated that PKC-mediated cardioprotection is isoform specific and that the ϵ -isoform of PKC plays an essential role in the development of PC in rabbit myocardium. Among the 10 PKC isoforms expressed in the rabbit heart (32), the ϵ -isoform is translocated and activated during ischemic PC (32, 33). Inhibition of this isoform completely blocks the delayed cardioprotection (33), supporting the concept that activation of this signaling molecule is necessary for late PC to become manifest.

Although PKC ϵ appears to play an essential role in ischemic PC (15, 23, 32, 33), the downstream signaling events triggered by the activation of this specific isozyme during the development of the late phase of PC remain largely unknown. Considerable evidence indicates that the development of late PC involves the synthesis of new proteins (34), the upregulation of stress-responsive genes (16, 20), and the activation of transcription factors (25, 48). In noncardiac cells, PKC isoforms are known to promote the activation of various transcription factors, such as nuclear factor (NF)- κ B (3, 4, 10, 12, 19, 45), activator protein (AP)-1 (8, 39, 43), and Elk-1 (38). In cardiac tissue, activation of NF- κ B has been shown to be a necessary event during ischemic PC (25, 48), and activation of AP-1 has been observed after brief ischemia (5). However, the molecular link between the ϵ -isozyme of PKC and ischemia-activated transcription factors has never been established in cardiac cells. We hypothesized that transcription factors are downstream signaling targets of PKC ϵ in cardiomyocytes. Accordingly, in this study, we examined whether selective activation of the ϵ -isozyme of PKC via transfection with recombinant adenovirus encoding this enzyme (30, 31) leads to targeted activation of transcription factors implicated in late PC, i.e., NF- κ B and AP-1.

Mounting evidence indicates that the three subfamilies of mitogen-activated protein (MAP) kinases

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(MAPKs), the p44/p42 MAPKs, the p38 MAPKs, and the p54/p46 c-Jun NH₂-terminal kinases (JNKs), are important upstream regulators for the induction of various transcription factors (12, 37, 44). The role of each MAPK subfamily in the activation of transcription factors appears to be cell-type specific. In noncardiac cells, it has been shown that p44/p42 MAPKs activate AP-1, Elk-1, and NF- κ B (7, 8, 18, 44, 53) and that p54/p46 JNKs activate AP-1 (18, 44, 47). Recent studies have shown that various subfamilies of MAPKs are activated in the ischemic myocardium (28, 41). Interestingly, MAPKs have also been implicated as downstream signaling targets of PKC in late PC (30, 31); specifically, activation and subsequent nuclear translocation of the p44 and p42 MAPKs were observed in the same rabbit model of late PC, where activation of both PKC ϵ (32) and NF- κ B (48) has been demonstrated. Moreover, activation of the ϵ -isozyme of PKC has been found to result in increased phosphorylation activity of both the p44/p42 MAPKs and the p54/p46 JNKs in adult rabbit cardiac cells (30, 31). Nevertheless, whether MAPKs function as intermediate molecules transducing signals from PKC ϵ to transcription factors has not been established. Therefore, we further investigated whether activation of MAPKs is a necessary signaling step for the PKC ϵ -mediated activation of transcription factors in cardiac myocytes.

The rabbit was selected to study the role of MAPKs in PKC ϵ -triggered activation of transcription factors because this is the species in which activation of PKC ϵ and NF- κ B during ischemic PC has been demonstrated (30–32, 48). To determine whether this signaling event is specific to the activation of the ϵ -isoform of PKC or is shared by other PKC isoforms, we also examined PKC η , another isoform in the novel PKC subfamily. The results demonstrate that PKC ϵ (but not PKC η) activates the transcription factors NF- κ B and AP-1 in adult rabbit cardiac myocytes and that both the p44/p42 MAPK and the p54/p46 JNK signaling pathways mediate the activation of these two factors.

MATERIALS AND METHODS

Materials and Reagents

M199 medium, fetal calf serum (FCS), penicillin, and streptomycin were from GIBCO/BRL (Gaithersburg, MD). Mouse monoclonal antibodies for the p42/p44 MAPKs, the p38 MAPKs, NF- κ B p65, NF- κ B p50, c-Jun, and Jun-D were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal phosphor-antibodies for Tyr 204 p44/42 MAPK, Tyr 180/182 p38 MAPK, and Thr 183/184 JNK were from New England Biolab (Beverly, MA). Horseradish peroxidase-labeled sheep anti-mouse secondary antibody and enhanced chemiluminescence (ECL) detecting reagent were from Amersham (Princeton, NJ). Reagents for SDS-polyacrylamide gel were from Bio-Rad. Poly (dl/dc) and T4 polynucleotide kinase was from Pharmacia Biotech (Piscataway, NJ). Type II collagenase was from Worthington Biochemical (Lakeview, NJ). Double-stranded oligonucleotides containing AP-1 consensus sequences, oligonucleotides containing NF- κ B consensus sequences, and oligonucleotides containing Elk-1 consensus sequences were from Promega (Madison, WI).

[γ -³²P]ATP was purchased from DuPont New England Nuclear (Boston, MA). PD98059 and GF109203X were from Calbiochem (San Diego, CA). All other reagents were from Sigma Chemical (St. Louis, MO).

Isolation of Adult Rabbit Cardiac Myocytes

Adult rabbit cardiac myocytes were isolated by use of a modification of the method of Haddad et al. (17). This method yielded 80–85% rod-shaped cardiac cells, which generated an average total of 4–6 $\times 10^7$ cells per rabbit heart. This is the same method that we have previously used to study PKC ϵ -induced activation of MAPKs in rabbit cardiomyocytes (30, 31). Briefly, the myocytes were plated onto laminin-coated 100-mm dishes at 37°C at subconfluency (2 $\times 10^6$ cells/100-mm dish); incubated in M199 medium with 2% FCS, penicillin, and streptomycin; and cultured overnight. The medium was replaced with serum-free M199 medium supplemented with taurine (5 mM), creatine (5 mM), and carnitine (5 mM) for 24 h before adenovirus transfection.

Determination of PKC ϵ Isoform-Selective Phosphorylation Activity

PKC ϵ phosphorylation activity in total cardiac cell lysates was determined as previously described (31). Briefly, cardiac proteins were extracted by use of glass-glass homogenization in buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1% Nonidet NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 16 μ g/ml benzamidine hydrochloride, 10 μ g/ml phenanthroline, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin A. Total cardiac cell protein samples were immunoprecipitated with PKC ϵ antibodies and then subjected to a phosphorylation assay with the use of a PKC ϵ -selective substrate (ERM₁PRKRQGSVRRRV). We found that the basal PKC ϵ activity of rabbit cardiac myocytes is 27.24 \pm 1.52 pmol \cdot min⁻¹ \cdot mg protein⁻¹ ($n = 6$, where n is the no. of rabbits).

Construction of Recombinant Adenoviruses

Recombinant adenoviruses encoding rabbit active PKC ϵ , dominant negative PKC ϵ , and active PKC η were constructed in our laboratory as previously described (30, 31). The hemagglutinin (HA) epitope enabled us to determine the expression of transgenic proteins. Preliminary data showed that the HA epitope, consisting of a nine-amino acid sequence, had no effect on the protein expression or on the enzymatic activity of rabbit PKC ϵ and PKC η . Recombinant adenovirus encoding a dominant negative mutant of MAPK kinase (MKK)-4 (DN-MKK4) was generated in the laboratory of Huang and Han. This HA-tagged DN-MKK4 has been proven to be effective in blocking activation of the JNKs in vitro (51) and in vivo (49). Positive recombinant adenoviruses were isolated by plaque purification and propagated in H293 cells that had been transformed with E1 genes (26). The recombinant viral cell lysates were purified by double CsCl gradient. The integrity of transgene expression was confirmed by PCR and Southern blotting.

Transfection of Cardiac Cells with Adenoviruses

In all groups, 10 plaque-forming units (pfu) of recombinant adenovirus per cardiac myocyte were used for transfection. Transfection efficiency was assessed with the use of recombinant adenovirus encoding the green fluorescence protein (GFP). We found that 10 pfu/cell consistently yielded 85–90% transfection efficiency in adult rabbit cardiac myocytes. The

following experimental groups were studied: a control group, in which cardiac myocytes received recombinant adenovirus encoding a null transgene; a FL-PKC ϵ group, in which cardiac myocytes received recombinant adenovirus encoding rabbit full-length active PKC ϵ (30); and a DN-PKC ϵ group, in which cardiac myocytes received recombinant adenovirus encoding a dominant negative mutant of rabbit PKC ϵ (30).

To block PKC ϵ -induced activation of p44/p42 MAPKs, one group of cardiac myocytes received the MAP or extracellular signal-regulated kinase (ERK) kinase (MEK)-1/2 inhibitor PD98059 (10 μ M) 4 h before incubation with recombinant adenovirus encoding FL-PKC ϵ . To block PKC ϵ -induced activation of p54/p46 JNKs, another group of myocytes received both the recombinant adenovirus encoding FL-PKC ϵ and that encoding DN-MKK4.

The expression of the PKC ϵ transgene in cardiac cells (and thus the activity of this enzyme) is also dependent on the time interval after transfection. In pilot experiments, cardiac cells were collected 8, 12, 16, 18, and 24 h after receiving 10 pfu/cell of FL-PKC ϵ . We found that 18 h was the optimal expression time, because it produced an increase in PKC ϵ phosphorylation activity [$208 \pm 5\%$ of control (null vector)], similar to that observed in conscious rabbits after ischemic PC in our previous studies (32, 33). Thus, in all experiments related to PKC ϵ , 18 h after transfection with PKC ϵ adenoviruses, cells were washed twice with warm PBS, harvested, frozen immediately in liquid nitrogen, and stored at -80°C . Our pilot experiments also showed that activation of PKC η after ischemic PC in vivo was best mimicked after a 16-h transfection with PKC η adenovirus. Specifically, cardiac cells that received FL-PKC η exhibited a level of PKC η phosphorylation activity ($228 \pm 17\%$ of control) 12 h posttransfection that was similar to that induced by ischemic PC in conscious rabbits (in separate studies in three rabbits, we found that cardiac PKC η activity 30 min after an ischemic PC protocol consisting of six 4-min coronary occlusions and reperfusion was $198 \pm 23\%$ of that in control rabbits; Refs. 32–34). Thus, in all experiments related to PKC η , cells were collected 16 h after transfection with PKC η adenovirus, washed twice with warm PBS, harvested, frozen immediately in liquid nitrogen, and stored at -80°C .

Preparation of Nuclear Extracts

Nuclear extracts of myocytes were prepared by a modified detergent treatment method as previously described (48). Briefly, the myocytes were homogenized in ice-cold *buffer A* [in mmol/l: 10 HEPES with pH 7.9, 10 KCl, 0.1 EDTA, 0.1 EGTA, 1.0 dithiothreitol (DTT), 0.5 PMSF, 1 NaF, and 1 Na_3VO_4] and incubated on ice for 15 min, followed by centrifugation at 3,800 rpm at 4°C for 10 min. A value of 0.5% Nonidet NP-40 was added to the reaction, followed by brief vigorous vortexing and incubation on ice for an additional 10 min. The isolated nuclear pellets were resuspended in ice-cold *buffer B* (in mmol/l: 20 HEPES with pH 7.9, 400 NaCl, 1.0 EDTA, 1.0 EGTA, 1.0 DTT, 1.0 PMSF, 1 NaF, and 1 Na_3VO_4), homogenized with a glass-glass homogenizer at 4°C , and incubated on ice for an additional 30 min. The nuclear proteins were extracted by collecting the supernatant of an 8,000-rpm spin of the nuclear homogenates. The Bradford system (Bio-Rad, Hercules, CA) was used to determine the protein content of the nuclear extracts.

Electrophoretic Mobility Shift Assay

Double-stranded synthetic oligonucleotides were 5' end labeled with [γ - ^{32}P]ATP and T4 polynucleotide kinase. The oligonucleotide sequences used for labeling the probes were

as follows: AP-1, 5'-CGCTTGATGACTCAGCCGGAA-3'; NF- κ B, 5'-AGTTGAGGGGACTTTCCC-AGGC-3'; and Elk-1, 5'-GGGGTCCTTGAGGAAGTATAAGAAGAAT-3'.

Standard DNA-binding reactions were carried out in 20 μ l of mixture containing (in mmol/l) 25 HEPES with pH 7.6, 50 KCl, 1 EDTA, 1 DTT, 0.5 spermidine, 0.5 PMSF, 10% glycerol, 0.1 $\text{mg}\cdot\text{mmol}^{-1}\cdot\text{l}^{-1}$ poly (dI-dC), and 6 μ g of the extracted nuclear protein. The DNA probe (40,000–60,000 counts/min) was added, and the reaction was carried out on ice for 20 min. The reaction samples were then loaded onto 4% native polyacrylamide gels made in a $0.5\times$ TBE buffer, and electrophoresis was performed at a constant voltage of 140 V for 90 min. After electrophoresis, the gels were vacuum dried and autoradiographed by use of an intensifying screen at -80°C .

To verify the specificity of the DNA-binding activity, competition assays were performed to identify the specific binding signal for AP-1 and NF- κ B by addition of a 100-fold molar excess of unlabeled double-stranded oligonucleotides. Super-shift assays were performed by incubation of the nuclear extracts with the corresponding antibodies.

Western Blotting Analysis

Standard Western immunoblotting techniques (32, 48) were used to assess the protein expression of AP-1, NF- κ B, and MAPKs. Briefly, the protein content was measured by the Bradford assay. A quantity of 100 μ g of either total cellular proteins or nuclear proteins was separated with a 12% SDS-PAGE, and standard ECL methods were used to visualize the protein signal.

Statistical Analysis

Data are expressed as means \pm SE of five or six experiments, each from a different rabbit heart. To facilitate comparisons, measurements of nuclear DNA-binding activity and protein expression in each experiment were expressed as a percentage of the average value for the control group. Differences among groups were tested by one-way ANOVA. If the *F* test showed an overall significance, comparisons between two groups were performed by unpaired Student's *t*-test.

RESULTS

Activation of PKC ϵ Enhances AP-1 and NF- κ B DNA-Binding Activity

We found that 18 h after transfection with FL-PKC ϵ adenovirus, PKC ϵ phosphorylation activity increased to $208 \pm 5\%$ of control (null vector), a level similar to that observed in conscious rabbits after ischemic PC in our previous studies (32, 33). This level of activation has also been previously shown to protect adult rabbit cardiac myocytes against hypoxic injury (30). To determine whether PKC ϵ modulates the transcription factors AP-1, NF- κ B, and Elk-1 in cardiac myocytes, recombinant adenovirus encoding active PKC ϵ (FL-PKC ϵ) was used (Figs. 1, 2, and 3). Cardiac cells transfected with the active PKC ϵ adenovirus exhibited increased DNA-binding activity for both AP-1 [$227 \pm 19\%$ of control (null vector); Figs. 1A and 2] and NF- κ B [$264 \pm 21\%$ of control (null vector); Figs. 1B and 2] but not for Elk-1 [$127 \pm 13.2\%$ of control (null vector); Fig. 1C]. To determine whether the increased DNA-binding activity was dependent on the activation of PKC ϵ ,

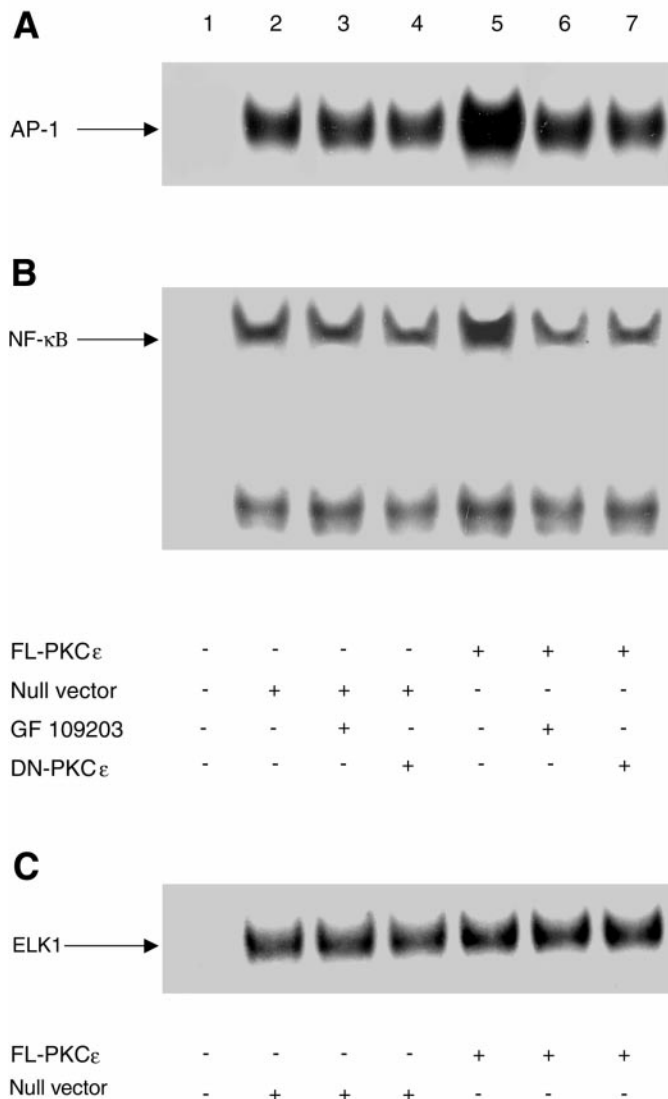


Fig. 1. Representative gels demonstrating the effect of protein kinase C (PKC)- ϵ activation on the nuclear factor (NF)- κ B, activator protein (AP)-1, and Elk-1 DNA-binding activity in adult rabbit cardiac myocytes. *A*: a representative electrophoretic mobility shift assay (EMSA) for AP-1. *B*: a representative EMSA for NF- κ B. *C*: a representative EMSA for Elk-1. Lane 1 is a negative control (reactions without nuclear proteins). In lanes 2–7, cardiac myocytes were treated with (+) or without (–) recombinant adenovirus encoding rabbit full-length active PKC ϵ (FL-PKC ϵ ; lanes 5–7), recombinant adenovirus encoding a null transgene (control; lanes 2–4), the PKC inhibitor GF109203 (lanes 3 and 6), or recombinant adenovirus encoding the dominant negative mutant of rabbit PKC ϵ (DN-PKC ϵ ; lanes 5 and 7). Activation of PKC ϵ resulted a robust increase in the DNA-binding activity of AP-1 (*A*, lane 5) and NF- κ B (*B*, lane 5). In contrast, activation of PKC ϵ did not affect the DNA-binding activity of Elk-1 (*C*).

cardiac cells were either pretreated with GF109203 (1 μ M), a selective PKC inhibitor, or received recombinant adenovirus encoding the dominant negative mutant of PKC ϵ (DN-PKC ϵ) in conjunction with that encoding the FL-PKC ϵ . Separate experiments showed that the concentration of GF109203 used (1 μ M) effectively blocked the increase in PKC ϵ -selective phosphor-

ylation activity induced by transfection with FL-PKC ϵ adenovirus in adult rabbit cardiac myocytes ($107 \pm 5\%$ of control, $n = 5$). The ability of DN-PKC ϵ adenovirus transfection to inhibit PKC ϵ activation after transfection with FL-PKC ϵ has been previously demonstrated (30). Both GF109203 and DN-PKC ϵ completely abrogated PKC ϵ -induced activation of AP-1 (Figs. 1A and 2) and NF- κ B (Figs. 1B and 2).

The identity of the proteins bound to either the AP-1 or the NF- κ B consensus probes was verified by supershift analysis and competition assays. Antibodies to c-Jun and Jun-D or to the p50 and p65 subunits of NF- κ B were used to characterize these protein-probe complexes. Figure 3, *A* and *B*, shows that the c-Jun and Jun-D antibodies supershifted the protein-probe complex of AP-1 and that the p50 and p65 antibodies supershifted the protein-probe complex of NF- κ B, indicating that these complexes were specific for AP-1 and NF- κ B, respectively. Furthermore, competition assays performed by adding a 100-fold molar excess of unlabeled double-stranded AP-1 or NF- κ B consensus probes completely blocked the binding, further confirming the specificity of these signals (Fig. 3, *A* and *B*).

In contrast, cardiac cells transfected with recombinant adenovirus encoding active PKC η exhibited increased PKC η phosphorylation activity ($228 \pm 17\%$ of control), an activation of PKC η that is similar to that evoked by ischemic PC in vivo, as indicated above, but no significant change in the DNA-binding activity of AP-1 ($112 \pm 8\%$ of control), NF- κ B ($121 \pm 13\%$ of control), or Elk-1 ($109 \pm 9\%$ of control), indicating that activation of PKC η does not lead to AP-1-, NF- κ B-, or Elk-1-dependent transcriptional regulation in cardiac cells.

Overexpression of PKC ϵ Results in Nuclear Translocation of NF- κ B and c-Jun Proteins

To determine whether the increased DNA-binding activity of AP-1 and NF- κ B was due to nuclear translocation of AP-1 or NF- κ B subunits, we analyzed the nuclear protein content of AP-1 and NF- κ B with the use of Western immunoblotting. Five experiments (each from a different rabbit heart) were used to assess c-Jun and Jun-D, and five were used to assess p65. As shown in Fig. 4, there was a significant increase in the nuclear expression of c-Jun [$241 \pm 16\%$ of null vector (control)] and Jun-D [$176 \pm 9\%$ of null vector (control)]. Cardiac cells transfected with PKC ϵ also exhibited increased nuclear p65 protein expression [$204 \pm 13\%$ of null vector (control); Fig. 5]. The expression of AP-1 (data not shown) and NF- κ B (Fig. 5) in the total cellular lysates was unaltered. These data indicate that activation of PKC ϵ induces nuclear translocation of the p65 subunit of NF- κ B as well as the c-Jun and Jun-D subunits of AP-1, which results in enhanced DNA-binding activity of NF- κ B and AP-1.

To verify whether the effect of PKC ϵ transfection with FL-PKC ϵ on the nuclear translocation of these transcription factors is dependent on the activation of PKC ϵ , we treated cardiac myocytes with GF109203 or

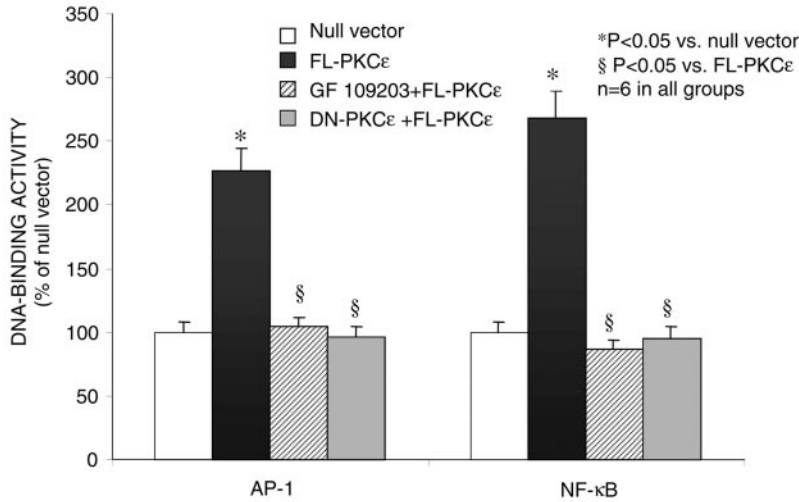


Fig. 2. Effect of the PKC inhibitors GF109203 and DN-PKCε on PKCε-induced DNA-binding activity of NF-κB and AP-1. Four groups of adult rabbit cardiac myocytes received recombinant adenovirus encoding a null transgene (control), recombinant adenovirus encoding active PKCε (FL-PKCε), GF109203 and FL-PKCε, or DN-PKCε and FL-PKCε. Each group included six experiments, each from a different rabbit heart. EMSAs were performed to determine the DNA-binding activity. Data are expressed as a percentage of the null vector (means ± SE); n = no. of rabbits.

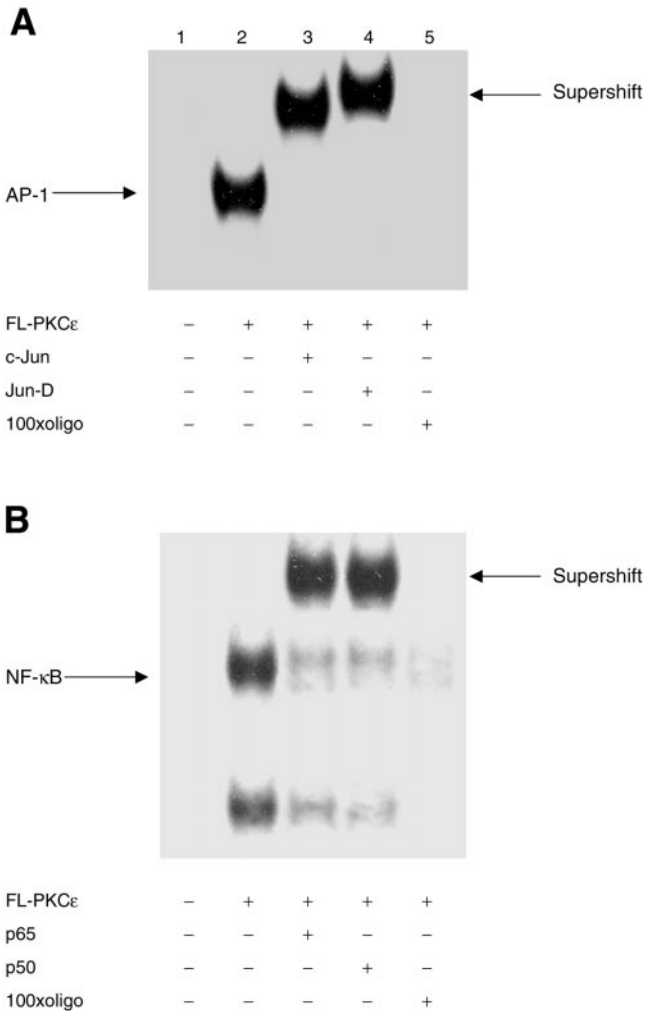


Fig. 3. Representative gels demonstrating the specificity of EMSAs. The proteins bound to the AP-1 and NF-κB probes were identified by supershift assays. The specificity of DNA binding was determined by competition assays. A: nuclear proteins were preincubated with antibodies against c-Jun (lane 3) or Jun-D (lane 4) or with a 100-fold molar excess of unlabeled cold AP-1 oligonucleotides (100×oligo; lane 5) before EMSA. B: nuclear proteins were preincubated with antibodies against p65 (lane 3) or p50 (lane 4) or with a 100-fold molar excess of unlabeled cold NF-κB oligonucleotides (lane 5) before EMSA.

DN-PKCε. Western immunoblotting showed that GF109203 (1 μM) and DN-PKCε abrogated the nuclear translocation of both c-Jun/Jun-D (Fig. 4) and the p65 subunits of NF-κB (Fig. 5), confirming that such translocation was caused by the activation of PKCε.

Activation of MAPKs Mediates PKCε-Dependent Activation of NF-κB and AP-1

After establishing the molecular link between PKCε and AP-1 and NF-κB, we investigated the role of MAPKs in mediating this signaling pathway. To address this question, we performed three experiments. 1) We determined which of the three MAPK pathways was activated by PKCε with the use of antibodies against phosphorylated p44/p42 MAPKs, p54/p46

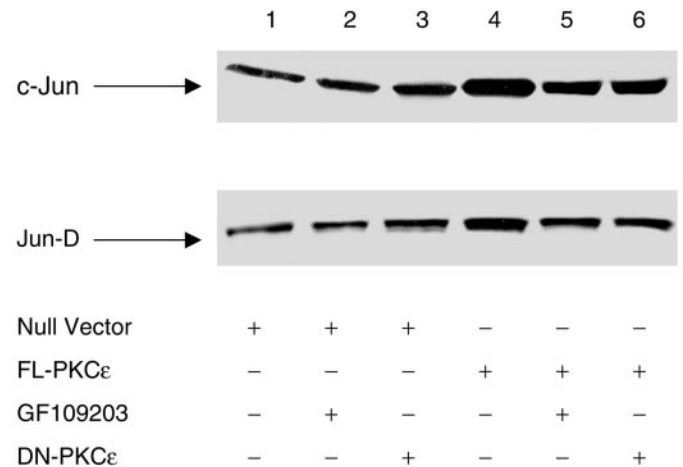


Fig. 4. Representative Western blots for the analysis of AP-1. Lanes 1-3 are protein extracts from myocytes transfected with null transgene (control), whereas lanes 4-6 are protein extracts from myocytes transfected with FL-PKCε recombinant adenovirus. In addition to recombinant adenovirus, cardiac cells in lanes 2 and 5 also received GF109203. Lanes 3 and 6 were cotransfected with DN-PKCε. Protein extracts were probed with antibodies against c-Jun (top) or Jun-D (bottom). Activation of PKCε significantly increased the expression of c-Jun (lane 4) and Jun-D (lane 4) proteins in the nuclei. Both increases were abolished by inhibition of PKCε (lanes 5 and 6).

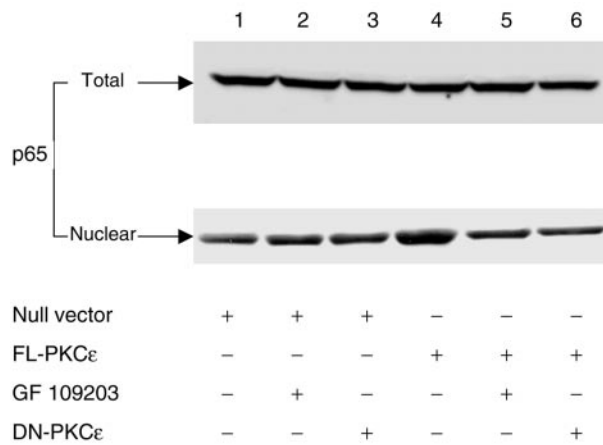


Fig. 5. Representative Western blots for the analysis of NF- κ B expression in total cell lysates and in nuclear extracts. Lanes 1–3 are from cardiac cells transfected with a null transgene (control), whereas lanes 4–6 are from myocytes transfected with FL-PKC ϵ recombinant adenovirus. In addition to recombinant adenovirus, cardiac cells in lanes 2 and 5 also received GF109203. Lanes 3 and 6 were cotransfected with DN-PKC ϵ . Total cell lysates and the nuclear protein extracts were probed with antibodies against the p65 subunit of NF- κ B. Activation of PKC ϵ significantly increased the expression of p65 (lane 4) protein in the nuclear fraction, indicating nuclear translocation of the p65 subunit of NF- κ B. Inhibition of PKC ϵ completely abolished the nuclear translocation of the p65 subunit of NF- κ B (lanes 5 and 6).

JNKs, and the p38 MAPKs. 2) To confirm that phosphorylation of MAPKs was induced by PKC, we tested whether the PKC inhibitor GF109203 abolished the phosphorylation of MAPKs. 3) To identify which MAPK signaling pathway(s) is necessary for PKC ϵ -induced activation of these two transcription factors, we selectively blocked the p44/p42 MAPK pathway with PD98059 and the p54/p46 JNK pathway with a DN-MKK4.

PKC ϵ -induced activation of MAPKs. Using in-gel kinase assays, we have previously shown that overexpression of PKC ϵ leads to increased phosphorylation activities of the p44/p42 MAPKs (30) and the p54/p46 JNKs (31). To confirm PKC ϵ -mediated activation of these MAPKs, we used specific phosphor-antibodies against the p44/p42 MAPKs, the p54/p46 JNKs, and the p38 MAPKs. Increased phosphorylation of p44/p42 MAPKs and p54/p46 JNKs was detected in cardiac myocytes transfected with PKC ϵ (Fig. 6A). In contrast, the signal for phosphorylated p38 MAPKs was unaffected (Fig. 6A), although these antibodies do not differentiate the individual subtypes within the p38 MAPK subfamily. Western immunoblotting analysis confirmed no change in total protein expression for all MAPKs (Fig. 6A). These results corroborate our previous findings (30, 31) that both p44/p42 MAPKs and p54/p46 JNKs are downstream signaling targets of PKC ϵ in rabbit cardiac myocytes.

To determine whether the phosphorylation of p44/p42 MAPKs and p54/p46 JNKs in myocytes was caused by PKC ϵ activation, we pretreated myocytes with GF109203 (1 μ M) or with DN-PKC ϵ together with the active PKC ϵ (FL-PKC ϵ). The results (Fig. 6A) demon-

strate that GF109203, a specific inhibitor of PKC, blocked the enhanced phosphorylation of p44/p42 MAPKs and p54/p46 JNKs. Similar results were observed when cells were pretreated with DN-PKC ϵ (Fig. 6A). These data demonstrate that the enhanced phosphorylation of p44/p42 MAPKs and p54/p46 JNKs was dependent on the activation of PKC ϵ .

To elucidate the role of MEK1/2 and MEK4 in the PKC ϵ -induced phosphorylation of p44/p42 MAPKs and p54/p46 JNKs, we pretreated myocytes with PD98059, a specific inhibitor of the p44/p42 MAPK signaling pathway, or with DN-MKK4, which selectively blocks the p54/p46 JNK signaling pathway, before transfection with FL-PKC ϵ . Both PD98059 and DN-MKK4, at the doses used in this study, inhibited basal phosphorylation of p44/p42 MAPKs and JNKs, respectively (data not shown). As expected, PD98059 and DN-MKK4 completely blocked PKC ϵ -induced phosphorylation of p44/p42 MAPKs and p54/p46 JNKs, respectively (Fig. 6B). Thus, in adult rabbit cardiac myocytes, PKC ϵ induces the activation of p44/p42 MAPKs via a MEK1/2 signaling pathway and the activation of p54/p46 JNKs via a MKK4 signaling pathway.

Involvement of p44/p42 MAPKs and p54/p46 JNKs in PKC ϵ -induced activation of NF- κ B and AP-1. To test whether PKC ϵ -induced activation of AP-1 and NF- κ B involves the p44/p42 MAPK signaling pathway, the p54/p46 JNK signaling pathway, or both, we treated cardiac cells with PD98059 or with recombinant adenovirus encoding DN-MKK4.

Pretreatment of cells with either PD98059 or DN-MKK4 abolished PKC ϵ -induced activation of AP-1 (FL-PKC ϵ , 295 \pm 32% of null vector control; PD98059, 120 \pm 14%; DN-MKK4, 105 \pm 7%) (Fig. 7A). Combined pretreatment with both PD98059 and DN-MKK4 did not result in inhibition greater than with either agent alone (Figs. 7A and 8). These results indicate that PKC ϵ utilizes both the p44/p42 MAPK and the p54/p46 JNK signaling pathways to activate AP-1 and that neither pathway is sufficient for PKC ϵ -induced activation of AP-1 to occur. Thus both the p44/p42 MAPKs and the p54/p46 JNKs are necessary downstream signaling elements in PKC ϵ -dependent regulation of AP-1 induction.

Similar to AP-1, pretreatment of cells with either PD98059 or DN-MKK4 completely blocked PKC ϵ -induced activation of NF- κ B (FL-PKC ϵ , 210 \pm 25% of null vector control; PD98059, 33 \pm 4.1%; DN-MKK4, 22 \pm 3.2%) (Figs. 7B and 8). These data support the concept that both the p44/p42 MAPK and the p54/p46 JNK signaling pathways are necessary for PKC ϵ -induced activation of NF- κ B and that neither pathway is sufficient to mediate this response.

DISCUSSION

There are two important findings in this study. First, we found that selective activation of PKC ϵ induces activation of the transcription factors AP-1 and NF- κ B, demonstrating that these two factors serve as downstream signaling targets of the ϵ -isoform of PKC in

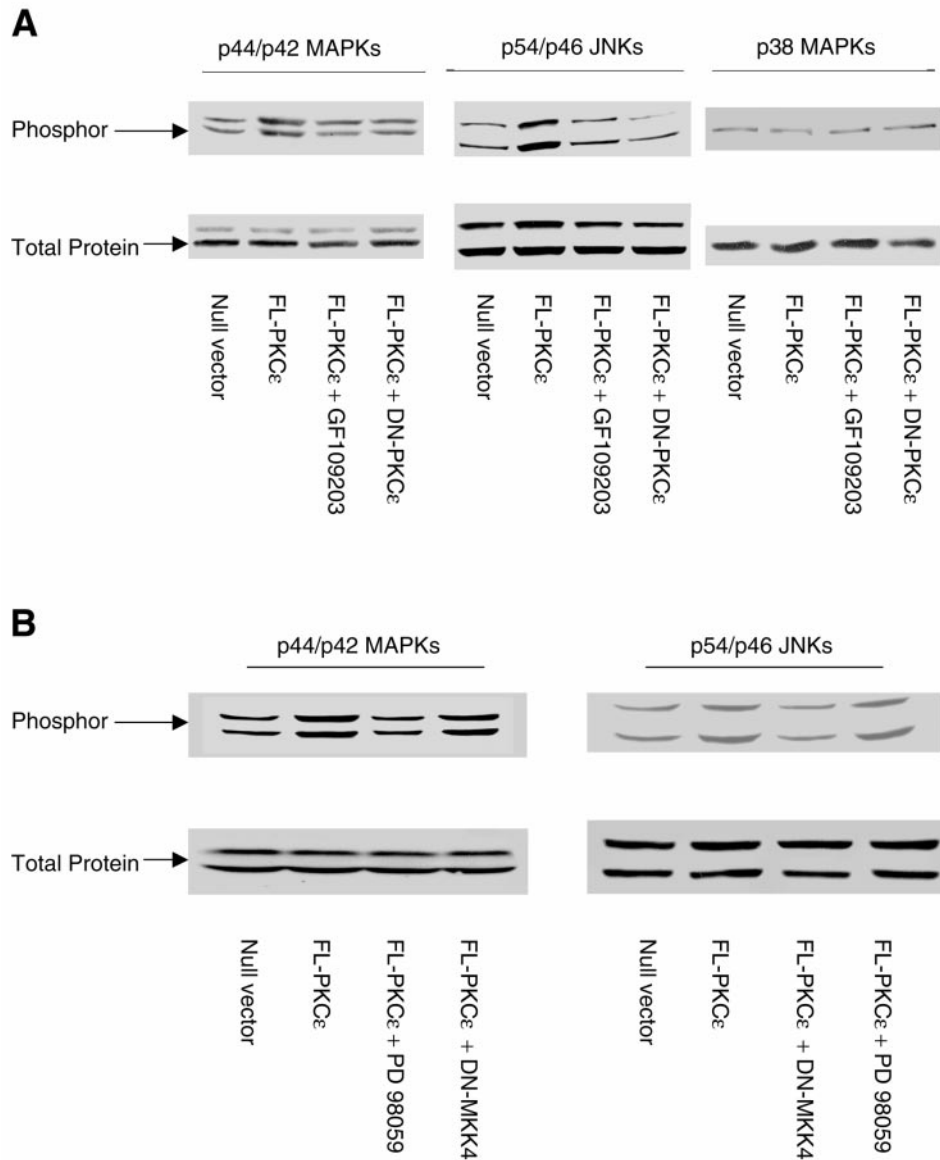


Fig. 6. Representative Western blots demonstrating the effect of PKC ϵ on mitogen-activated protein (MAP) kinase (MAPK) phosphorylation. **A**: cardiac myocytes received recombinant adenovirus encoding a null transgene (control), recombinant adenovirus encoding active PKC ϵ (FL-PKC ϵ), GF109203 before FL-PKC ϵ , or recombinant adenoviruses encoding both DN-PKC ϵ and FL-PKC ϵ . Total cell lysates were analyzed by Western blotting by use of antibodies against nonphosphorylated or phosphorylated p44/p42 MAPKs (*left*), p54/p46 c-Jun NH $_2$ -terminal kinases (JNKs; *middle*), and p38 MAPKs (*right*). Activation of PKC ϵ increased phosphorylation of the p44/p42 MAPKs and p54/p46 JNKs but had no effect on the phosphorylation activity of total p38 MAPKs. Inhibition of PKC ϵ with either the inhibitor GF109203 or a dominant negative mutant of PKC ϵ completely abolished the enhanced phosphorylation signals. Total protein expression of all MAPKs detected by nonphosphor antibodies was not altered by PKC ϵ activation. **B**, *left*: cardiac myocytes were transfected with recombinant adenovirus encoding either a null transgene (control) or active PKC ϵ (FL-PKC ϵ), were pretreated with PD98059 and transfected with active PKC ϵ , or were cotransfected with both active PKC ϵ and a dominant negative mutant of MAPK kinase (MKK)-4 (DN-MKK4). Phosphor-antibodies to p44/p42 MAPKs were used to determine the activation of these kinases. Activation of PKC ϵ increased phosphorylation of the p44/p42 MAPKs, which was completely abolished by inhibition of MAP or extracellular signal-regulated kinase (ERK) kinase (MEK)-1/2 with PD98059. **B**, *right*: cardiac myocytes were transfected with recombinant adenovirus encoding either a null transgene (control) or active PKC ϵ (FL-PKC ϵ), were cotransfected with active PKC ϵ and DN-MKK4, or were pretreated with PD98059 and transfected with active PKC ϵ . Phosphor-antibodies to p54/p46 JNKs were used to determine the activation of these kinases. Activation of PKC ϵ increased phosphorylation of the p54/p46 JNKs, which was completely abolished by inhibition of MKK4 with DN-MKK4. Total protein expression of all MAPKs detected by nonphosphor antibodies was not altered by PKC ϵ activation.

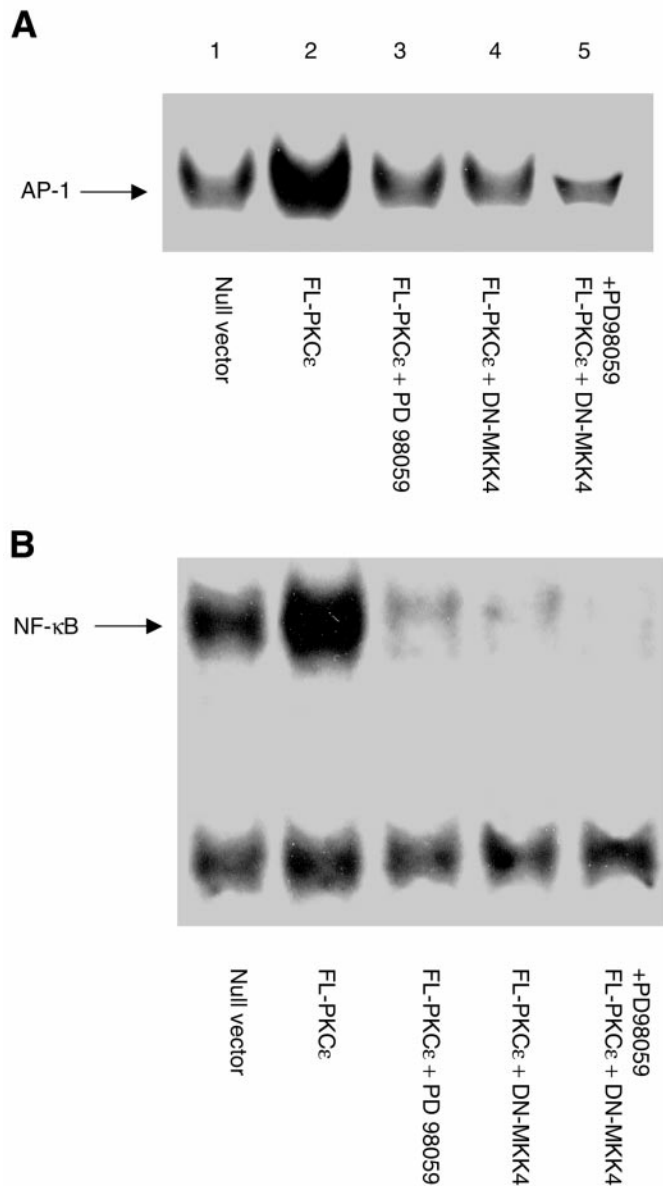


Fig. 7. Representative EMSAs for the effects of PD98059 and DN-MKK4 on DNA-binding activity of AP-1 (A) and NF- κ B (B). Lane 1 is nuclear protein extracts from cardiac cells transfected with a null transgene (control). Lane 2 is with active PKC ϵ (FL-PKC ϵ). Lane 3 was pretreated with PD98059 before receiving active PKC ϵ (FL-PKC ϵ). Lane 4 was cotransfected with both DN-MKK4 and PKC ϵ (FL-PKC ϵ). Lane 5 was pretreated with PD98059 and cotransfected with DN-MKK4 and PKC ϵ (FL-PKC ϵ). Activation of PKC ϵ (lane 2) increased the DNA-binding activity of AP-1 (A) and NF- κ B (B). Activation of AP-1 and NF- κ B was abolished by inhibition of the p44/p42 MAPK signaling pathway with PD98059, by inhibition of the p54/46 JNK signaling pathway with DN-MKK4, and by inhibition of both pathways with combined treatment with PD98059 and DN-MKK4.

adult rabbit cardiac myocytes. Second, we found that activation of the p44/p42 MAPK and the p54/p46 JNK pathways is a critical intermediate signaling step during PKC ϵ -induced activation of AP-1 and NF- κ B and that both of these pathways are necessary to produce the activation of the transcription factors. Taken to-

gether with our previous results demonstrating the role of the PKC ϵ isoform in the development of the late phase of PC (32, 33), these data indicate that activation of PKC ϵ not only can contribute to the posttranslational modulation of intracellular signaling molecules but also can participate in the AP-1- and NF- κ B-dependent transcriptional regulation required for the delayed phase of cardioprotection. To our knowledge, this is the first report that PKC ϵ governs the activity of NF- κ B and AP-1 in the heart, further underscoring the role of this PKC isoform in cardiovascular pathophysiology.

PKC comprises a large family of kinases with 11 known isoforms. Increasing evidence suggests that each individual PKC isoform possesses a unique biological function (29). The molecular structure of PKC isoforms dictates the regulatory mechanism for activation. Both the PKC ϵ and the PKC η isoforms belong to the novel PKC subfamily and share very similar molecular structure domains (29). We have previously reported that both of these isoforms are translocated by ischemic PC (32) but appear to play distinct roles during the development of the cardioprotection: activation of PKC ϵ is essential for late PC (33), whereas translocation of PKC η is not (33). In the present study, we found that PKC ϵ and PKC η appear to be coupled to distinct downstream signaling pathways. Activation of PKC ϵ resulted in activation of the transcription factors NF- κ B and AP-1, consistent with a role of this isoform of PKC in PC. In contrast, activation of PKC η did not have any significant effect on any of the three transcription factors examined (AP-1, NF- κ B, and Elk-1). Taken together with our previous finding that activation of NF- κ B is necessary for the late phase of ischemic PC to occur (48), these results provide a molecular basis for the differential roles of PKC ϵ and PKC η in late PC by indicating that the η -isoform of PKC may not participate in the transcriptional regulation of new genes involved in this cardioprotective phenomenon.

MAPKs have been shown to be important mediators of signal transduction from the cell surface to the nucleus. Transcriptional regulation by MAPKs has been implicated in many cellular processes such as proliferation, differentiation, and apoptotic death (8, 18, 36, 42). In mammals, MAPKs are divided into at least three subfamilies: the ERKs, also known as the p44/p42 MAPKs; the JNKs, which include the p54/p46 JNKs; and the p38 family of MAPKs (11, 41). MAPKs are involved in multiple intracellular signaling cascades and are activated by the stimulation of a variety of cell surface receptors such as receptor tyrosine kinases, G protein-linked receptors, cytokine receptors, and so forth (11, 18, 41). Mounting evidence implicates PKC in signaling pathways leading to the activation of various MAPKs (2, 6, 14, 36). On activation, MAPKs can phosphorylate and activate their target proteins and transcription factors (12, 37), thereby initiating the transcription of new genes and the expression of new proteins. In noncardiac cells, it has been well documented that p44/p42 MAPKs can activate c-Fos and Elk-1 and that p54/p46 JNKs can activate c-Jun

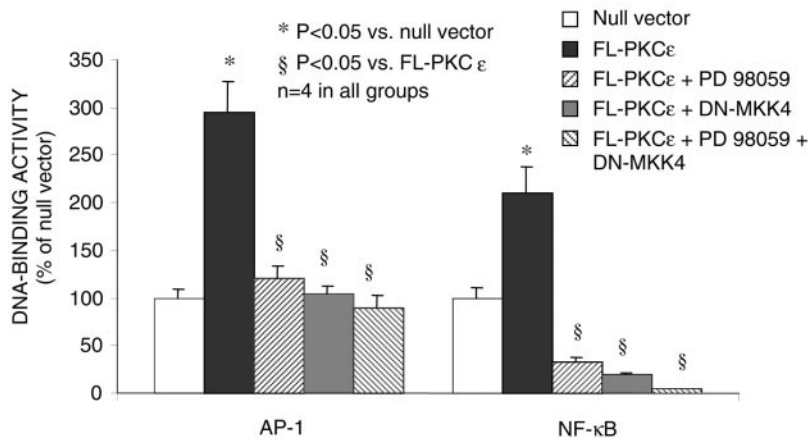


Fig. 8. Role of p44/p42 MAPKs and p54/p46 JNKs in PKC ϵ -induced activation of NF- κ B and AP-1. Five groups of adult rabbit cardiac myocytes received recombinant adenovirus encoding a null transgene (control), recombinant adenovirus encoding active PKC ϵ (FL-PKC ϵ), PD98059 before receiving FL-PKC ϵ , recombinant adenovirus encoding DN-MKK4 in conjunction with FL-PKC ϵ , or PD98059 combined with DN-MKK4 and FL-PKC ϵ . Each group included four experiments, each from a different rabbit heart. EMSAs were performed to determine the DNA-binding activity. Activation of AP-1 and NF- κ B was abolished by inhibition of the p44/p42 MAPK signaling pathway with PD98059, by inhibition of the p54/46 JNK signaling pathway with DN-MKK4, and by inhibition of both pathways with combined treatment of PD98059 and DN-MKK4. Data are expressed as a percentage of control (null transgene) (means \pm SE); n = no. of rabbits.

(18, 36). Recently, it has also been reported that MAPKs are involved in the activation of NF- κ B and AP-1 (12, 37). However, the role of MAPKs in PKC-dependent AP-1 and NF- κ B activation in the heart is unknown. Our data demonstrate for the first time that both p44/p42 MAPKs and p54/p46 JNKs are necessary for PKC ϵ -dependent modulation of these transcription factors in the myocardium.

Interestingly, the effect of p44/p42 MAPKs and p54/p46 JNKs on the basal activity of NF- κ B and AP-1 appears to be very different. Neither PD98059 nor DN-MKK4 had an appreciable effect on the basal activity of AP-1 (Figs. 7A and 8). In contrast, inhibition of p44/p42 MAPKs with PD98059 or inhibition of p54/p46 JNKs with DN-MKK4 in the presence of PKC ϵ activation not only abolished the PKC ϵ -induced activation of NF- κ B but also resulted in a reduced NF- κ B activity that was lower than that observed under control conditions (Figs. 7B and 8). These data suggest that both p44/p42 MAPKs and p54/p46 JNKs may be important in determining the basal activity of NF- κ B in cardiac myocytes, independent of PKC ϵ activation, whereas basal AP-1 activity does not receive input from these MAPK signaling pathways.

The precise molecular mechanisms for MAPK-mediated activation of AP-1 and NF- κ B are unknown. NF- κ B is activated by phosphorylation of inhibitory κ B- α (I κ B- α), either on serine residues 32 and 36 or on tyrosine residue 42 (1, 22, 51). Such phosphorylation leads to dissociation of I κ B- α from NF- κ B and subsequent translocation of NF- κ B to the nucleus (1). The NF- κ B family consists of five different members, p50, p52, p65, c-Rel, and RelB, which can form various homodimers and heterodimers. The active form of NF- κ B usually consists of heterodimers composed primarily of p50/p65 (1, 2). The AP-1 family is subdivided into three main subgroups: the Jun proteins (such as c-Jun and Jun-D), the Fos proteins, and the activating transcription factors. AP-1 activity is regulated at two levels: abundance of AP-1 proteins and posttranslational modification that dictates the DNA-binding activity of AP-1 (46). Because serine 32 and 36 and tyrosine 42 of I κ B- α do not appear to be direct phos-

phorylation sites for p44/p42 MAPKs or JNKs, it seems likely that these kinases activate NF- κ B indirectly via other, as yet unidentified, downstream kinases. Further studies will be necessary to decipher the signaling pathway that links MAPKs to NF- κ B and AP-1. Regardless of the precise mechanism, our data show that the activity of MAPKs results in nuclear translocation of NF- κ B and AP-1, because activation of PKC ϵ enhanced the DNA-binding activities of these two transcription factors (Figs. 1 and 2) and, at the same time, caused translocation to the nucleus of the p65 subunit and the c-Jun and Jun-D subunits (Figs. 4 and 5).

The information provided by this investigation expands our understanding of the signaling mechanisms that underlie late PC. Previous *in vivo* studies have shown that both ischemia-induced and nitric oxide (NO)-induced late PC are associated with activation of PKC ϵ (32) and NF- κ B (48) and that ischemia-induced activation of NF- κ B can be blocked with inhibitors of PKC (48). Although these results imply that recruitment of NF- κ B during late PC is PKC dependent, they cannot discern whether the ϵ -isoform of PKC (rather than PKC η , which is also recruited during late PC, or possibly other PKC isoforms; Ref. 32) is specifically involved in this phenomenon, because chelerythrine is a general PKC inhibitor. The present finding that increased PKC ϵ activity is sufficient to recruit NF- κ B in cardiac myocytes supports the concept that ϵ is the PKC isoform responsible for activation of NF- κ B after ischemia-induced or NO donor-induced late PC. However, further *in vivo* studies using isoform-specific approaches to inhibit PKC ϵ (e.g., transgenesis of dominant negative mutants) will be necessary to firmly establish an obligatory role of PKC ϵ in NF- κ B activation during late PC. The role of AP-1 in this process will also require further investigation, because the ability of PKC ϵ to recruit this transcription factor does not, in itself, signify that AP-1 contributes to the delayed cardioprotection. Nevertheless, the notion that both NF- κ B and AP-1 are downstream targets of PKC ϵ in cardiac myocytes has broad implications that transcend the specific process of late PC and may involve a variety of cardiovascular phenomena. In addition, our

finding that p44/p42 MAPKs and p54/p46 JNKs are required for PKC ϵ -dependent activation of NF- κ B and AP-1 in cardiac myocytes is consistent with the hypothesis that these subfamilies of MAPKs participate in the genesis of late PC. Thus the present study provides a rationale for future investigations aimed at assessing the effect of in vivo inhibition of p44/p42 MAPKs and p54/p46 JNKs on the acquisition of delayed cardioprotection.

In conclusion, although PKC has been implicated in the activation of NF- κ B (3, 4, 7, 10, 19) and AP-1 (8, 39, 43) in a variety of cells, the signaling pathways linking PKC to the activation of transcription factors are poorly characterized, especially in cardiac myocytes. In the present study, we demonstrate that PKC ϵ activates the transcription factors AP-1 and NF- κ B via the p44/p42 MAPK and the p54/p46 JNK signaling pathways. Recruitment of both of these signaling pathways is necessary for the activation of AP-1 and NF- κ B to occur. These findings provide new insights into the role of PKC ϵ in the development of ischemic PC and, more generally, in the modulation of AP-1- and NF- κ B-dependent genes. The identification of the PKC ϵ -MAPK-AP-1/NF- κ B signal transduction pathways in the heart may have significant implications for our understanding of the signaling mechanisms underlying the delayed response of the heart to brief ischemic stresses as well as other pathophysiological events dependent on PKC signaling.

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