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Regulation of c-Fos and c-Jun gene expression by phospholipase C activity in adult cardiomyocytes

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Abstract This study was undertaken to determine whether gene expression for transcriptional factors such as c-Fos and c-Jun is regulated by phospholipase C (PLC) activity. Norepinephrine (NE) increased PLC β_1, β_3, γ_1, and δ_1 isozyme gene expression, protein contents and their activities in adult rat cardiomyocytes. Increases in PLC β_1, β_3, γ_1, and δ_1 activities and gene expression in response to NE were prevented by prazosin, an α_1-adrenoceptor (AR) antagonist. Furthermore, mRNA levels for c-Fos and c-Jun, unlike other transcriptional factors, were increased by both NE and phenylephrine, a specific α_1-AR agonist. Increases in c-Fos and c-Jun gene expression due to NE were attenuated by both prazosin and a PLC inhibitor, U73122. Activation of protein kinase C (PKC) with phorbol myristate acetate increased c-Fos and c-Jun mRNA, whereas inhibition of PKC with bisindolylmaleimide as well as inhibition of extracellular signal-regulated kinases (ERK) 1/2 with PD98059 abolished the NE-induced increase in c-Fos and c-Jun gene expression. Reduction of c-Jun phosphorylation by SP600125, an inhibitor of JNK activity, was associated with an attenuation of the NE-induced increases in PLC gene expression. It is suggested that c-Fos and c-Jun gene expression is regulated by PLC in adult cardiomyocytes through a PKC- and ERK1/2-dependent pathway.

Keywords Adult cardiomyocytes · Transcription factors · Gene expression · Phospholipase C · Signal transduction · Protein kinase C · Extracellular signal-regulated kinase

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Introduction

Phospholipase C (PLC) is considered to play an important role in the signal transduction mechanisms of cardiac hypertrophy [1, 2]. Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a substrate for PLC and is converted into two messenger molecules, namely 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). These two products participate in cardiomyocyte hypertrophy via downstream signaling mechanisms. A single injection of norepinephrine (NE) and infusion with phenylephrine (PhE) have been shown to result in a transient increase in the level of c-Fos and c-Jun mRNA levels [3, 4]; a response that has been reported to be mediated by the α_1-adrenoceptor (AR) [5], and increase the protein synthesis [6]. While a mechanical overload has also been shown to increase the c-Jun expression [7], transfection of cardiomyocytes with a dominant negative c-Jun was found to inhibit the cardiomyocyte hypertrophic response to PhE; this was evidenced by inhibition of the enhanced protein synthesis as well as gene expression for atrial natriuretic peptide and brain natriuretic peptide, which are markers of cardiac hypertrophy [8]. On the other hand, overexpression of c-Jun resulted in a significant increase in atrial natriuretic factor (ANF) gene promoter activity [9]. Thus, it is evident that c-Fos and c-Jun are intimately involved in cardiac hypertrophy.

However, the role of PLC in the induction of gene expression for c-Fos and c-Jun has not been clearly defined.

Schnabel et al. [10] have reported that preincubation of isolated neonatal rat cardiomyocytes with PLC β_3 antisense oligonucleotides abolished the insulin-like growth factor-1 (IGF-1)-induced upregulation of c-Fos and c-Jun gene. This observation indicated that the PLC isozyme has a role in mediating the signal transduction mechanisms in the regulation of c-Fos and c-Jun gene expression. We have shown that the hypertrophic response to NE, as evidenced by increases in ANF expression and protein synthesis, is due to the activation of PLC via α_1-AR in isolated adult rat cardiomyocytes [11]. The present study was undertaken to test the involvement of PLC in the induction of c-Fos and c-Jun gene expression due to NE. These experiments were carried out using prazosin, an α_1-AR antagonist and U73122, a known PLC inhibitor. Although downstream signaling sites involving protein kinase C (PKC) and extracellular signal-regulated kinases 1/2 (ERK1/2) for the induction of c-Fos and c-Jun have been shown in neonatal preparations using a variety of agonists [5, 12, 13], no such information is available in adult cardiomyocytes in response to α_1-AR stimulation. Thus, some experiments were also conducted to confirm the participation of PKC and ERK1/2 under our experimental conditions.

Materials and methods

Cardiomyocyte isolation

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, in accordance with the guidelines established by the Canadian Council on Animal Care. Left ventricle (LV) cardiomyocytes were isolated from male Sprague Dawley rats (250–300 g) as previously described [11]. The cells were incubated with 5 μM NE or PhE, 1 μM for 2 h (for gene expression) and 5 μM NE for 24 h (for protein contents and activity measurements); these experimental conditions have previously been found to be optimal [11]. Cardiomyocytes were pretreated for 30 min with different inhibitors in some experiments as indicated. Cell viability was determined by estimating LDH activity using the Sigma LDH assay kit. On previous occasions, the rod-shaped quiescent cardiomyocytes comprised more than 95% of the final cell population.

RNA isolation and semi-quantitative PCR

Total RNA was isolated from LV cardiomyocytes using RNA isolation kit (Life Technologies, ON, Canada) according to the manufacturers' procedures. Reverse transcription (RT) was conducted for 45 min at 48°C using the Superscript Preamplification System for First-Strand cDNA Synthesis (Life Technology, ON, Canada) as described previously [11, 14]. Primers used for amplification were synthesized as follows: PLC β_1: 5′-ATTCGG CCAGGC TATCACTA-3′ (forward), 5′-TGCATACGTGTCTGGGA CAT-3′ (reverse); PLC β_3: 5′-TTGGAAATCTTCGAGCG GTT-3′ (forward), 5′-AGGAACTGTTTGTTCG GCTCAT -3′ (reverse); PLC γ_1: 5′-AGCCAAGGACCTGA AGAACA-3′ (forward), 5′-GCAAACTGCCCATAGGTG AT-3′ (reverse); PLC δ_1: 5′-ACACAAGCCCAAGGAGG ATA-3′ (forward), 5′-ACGGACAAAACCATTTCCTG-3′ (reverse); c-Fos: 5′-GGAGCCGGTCAAGAACATTA-3′ (forward), 5′-ATGAT GCCGGAAACAAGAAG-3′ (reverse); c-Jun: 5′-TGACTG CAAAGATGGAAACGA-3′ (forward), 5′-CAGGTTCAA GGTCATGCTCTGT-3′ (reverse); and ANF: 5′-AGATCT GCCCTCTTGAAAAGCA-3′ (forward) and 5′-TCGAGCA GATTTGGCTGTTATC-3′ (reverse). Temperatures used for PCR were as follows: denaturation at 94°C for 30 s, annealing at 62°C for 60 s, and extension at 68°C for 120 s, with a final extension for 7 min; 25 amplification cycles for each individual primer sets were carried out. For the purpose of normalization of the data, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers, 5′-CATGACAACTTTGGCA TCGT-3′ (forward) and 5′-GGATGCAGGGATGATGTT CT-3′ (reverse), were used to amplify GAPDH gene as a multiplex with the target genes. The PCR products were analyzed by electrophoresis in 2% agarose gels. The intensity of each band was photographed and quantified using a Molecular Dynamics STORM scanning system (Amersham Biosciences Corp., PQ, Canada) as a ratio of a target gene over GAPDH.

Real-time PCR

About 500 ng of total RNA was used for RT. The Superscript First-Strand Synthesis System for RT-PCR (Bio-Rad, Hercules, CA, USA) was used according to the instructions of the manufacturer. Primer sequences for PLC β_1, β_3, γ_1, and δ_1, c-Fos, and c-Jun were same as described above. Quantitative real-time PCR (qRT-PCR) was performed using the Bio-Rad iCycler detection System. For analysis, cycle threshold (C_t) values were calculated for each sample; this value represents the value at which the fluorescent signal rises above background levels. Gene expression was further analyzed by the $2^{-\Delta\Delta C_t}$ method [15].

Western blot analysis

Total membrane proteins (20 μg) and high molecular weight marker (Bio-Rad, Hercules, CA, USA) were separated on SDS-PAGE as described previously [14, 16, 17]. Proteins were transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membrane. PVDF membrane was blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% skim milk and probed with monoclonal primary antibodies

for PLC isozymes β_1, γ_1, and δ_1 and c-Fos, and polyclonal primary antibodies for PLC β_3 and c-Jun (Santa Cruz Biotechnology, CA, USA). Primary antibodies were diluted in TBS with 0.1% (vol/vol) Tween 20 (TBS-T) (1:200 for c-Fos, c-Jun, PLC β_1, and β_3; 1:2,000 for PLC γ_1 and 1:10,000 for PLC δ_1) according to the manufacturers' instructions. Horseradish peroxidase-labeled anti-mouse IgG (Bio-Rad) was diluted 1:3,000 in TBS-T and used as secondary antibody for c-Fos, PLC isozymes β_1, γ_1, and δ_1 and 1:2,000 in TBS-T for c-Jun and PLC β_3 isozyme. Protein bands were visualized by enhanced chemiluminescence according to the manufacturers' instructions (Boehringer Mannheim, Laval, PQ, Canada). Band intensities of the Western blot analysis were quantified using a CCD camera imaging densitometer (Bio-Rad GS 800). These data were normalized to loading controls.

Measurement of PLC isozyme activities

The PLC isozyme activities were determined by measuring the hydrolysis of [^{3}H]-PIP$_2$, following immunoprecipitation (IP) as previously described [16, 17]. The IP was conducted overnight at 4°C with monoclonal antibodies to PLC β_1, γ_1, and δ_1 and polyclonal antibodies to β_3 (5 μg of antibody to 350 μg membrane extract). For control experiments, IP and subsequent activity measurements were conducted with non-immune mouse IgG. It is pointed out that the IP of the specific PLC isozymes is complete under the conditions described here [16, 17]. Furthermore, each antibody cross-reacts with its corresponding PLC isozyme and it does not cross-react with the other isozymes [18]. Also, while other proteins have been shown to co-immunoprecipitate with PLC antibodies [19], they do not interfere with activity measurements [17].

Statistical analysis

All values are expressed as mean ± SE. The differences between two groups were evaluated by Student's *t*-test. The data from more than two groups were evaluated by one-way ANOVA followed by Duncan's multiple comparison tests. A probability of 95% or more ($P < 0.05$) was considered significant.

Results

Upregulation of PLC isozymes, c-Fos, and c-Jun in response to NE in the absence and presence of various inhibitors

Treatment of cardiomyocytes with NE (5 μM) for 2 h significantly increased PLC β_1, β_3, γ_1 and δ_1 as well as increased c-Fos and c-Jun mRNA levels when monitored by semi-quantitative PCR (Fig. 1a, b). Similar changes were also seen when mRNA levels were monitored by RT-PCR analysis (Fig. 1c). The protein contents of PLC isozymes, c-Fos, and c-Jun were measured to determine if increases in the mRNA levels translated into increases in their respective proteins. It can be seen from Fig. 2a and b that NE treatment of cardiomyocytes (5 μM for 24 h) increased the protein contents of PLC β_1, β_3, γ_1, and δ_1, and of c-Fos, and c-Jun.

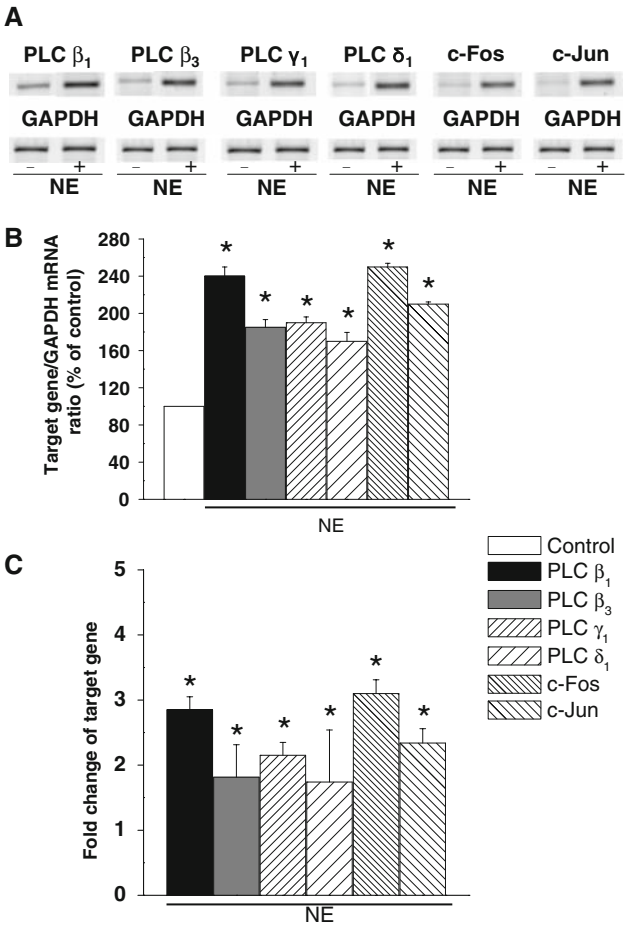

Fig. 1 Norepinephrine-induced increases in PLC isozymes, c-Fos, and c-Jun gene expression in adult cardiomyocytes. Representative blots (**a**) and quantified data (**b**) showing PLC isozyme β_1 (114 bp), β_3 (230 bp), γ_1 (123 bp), δ_1 (190 bp), c-Fos (74 bp), and c-Jun (163 bp) mRNA levels relative to GAPDH (138 bp) mRNA level as determined by RT-PCR analysis in LV cardiomyocytes treated with 5 μM NE for 2 h as described in Materials and methods. Panel (**c**) shows the PLC isozymes, c-Fos, and c-Jun gene mRNA expression as determined by real-time RT-PCR analysis in the presence of NE 5 μM for 2 h as described in Materials and methods. Values are mean ± SE of five experiments performed with five different cardiomyocyte preparations. *Significantly different ($P < 0.05$) versus control. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; NE, norepinephrine

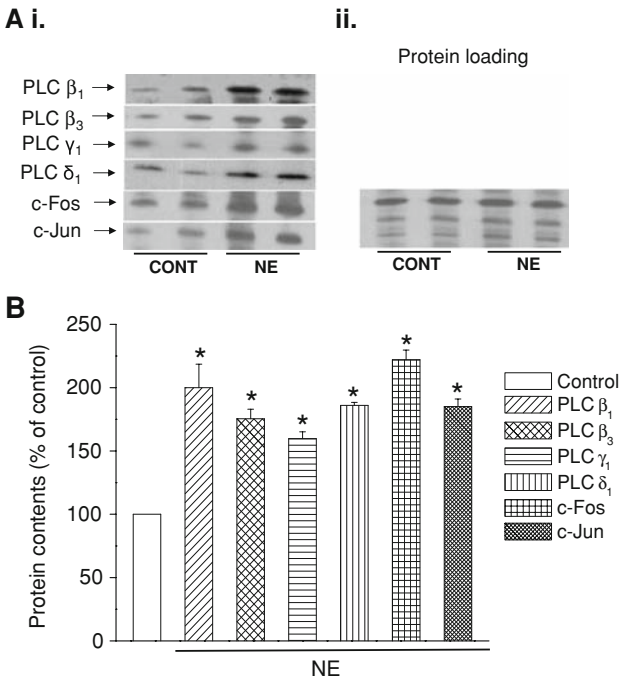

Fig. 2 Increases in cardiomyocyte PLC isozymes, c-Fos, and c-Jun protein content and PLC isozyme activities in response to norepinephrine. Representative blots (**a**(i)) and amido black stained PVDF membrane showing equivalent protein loading (**a**(ii)). Quantified data (**b**) showing PLC β_1 (150 kDa), β_3 (150 kDa), γ_1 (135 kDa), δ_1 (85 kDa), c-Fos (62 kDa), and c-Jun (39 kDa) protein content in a total membrane fraction isolated from cardiomyocytes treated with 5 μM NE for 24 h. Values are mean ± SE of five experiments performed with five different cardiomyocytes preparations. *Significantly different ($P < 0.05$) versus control. CONT, control; NE, norepinephrine

Specificity of the NE-induced nuclear transcription factor expression in adult cardiomyocytes

In order to determine whether increase in the expression of the transcription factors c-Fos and c-Jun was of specific nature, we also examined the expression of several other transcription factors. No changes in NFAT3, NFκB, MEF2C, and MEF2D mRNA levels were observed in response to 5 μM NE in cardiomyocytes after 2 h incubation, whereas increases in c-Fos and c-Jun mRNA levels by NE were detected by semi-quantitative PCR (Fig. 3a, b). The increases in c-Fos and c-Jun gene expression due to NE were verified by employing RT-PCR technique (Fig. 3c). The differences in c-Fos and c-Jun expression were observed to be greater by RT-PCR than by the conventional-PCR technique. Since the NE-induced changes in mRNA levels of PLC β_1, β_3, γ_1, and δ_1, c-Fos, and c-Jun were similar with both techniques, subsequent experiments for the determination of gene expression in adult cardiomyocytes were conducted with semi-quantitative PCR. The specificity regarding the role of α_1-AR in eliciting the NE-induced increases in c-Fos and c-Jun mRNA levels was also examined by determining the effects of PhE, a specific

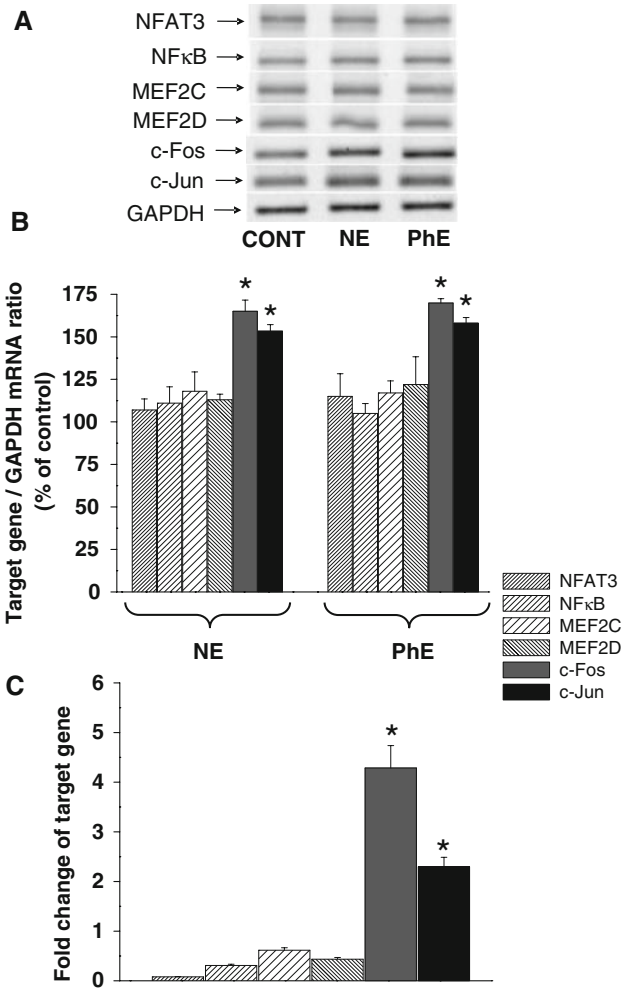

Fig. 3 Changes in nuclear transcription factor mRNA levels in response to norepinephrine and phenylephrine. Representative blots (**a**) and quantified data (**b**) of conventional RT-PCR showing NFAT3 (99 bp), NFkB (124 bp), MEF2C (92 bp), MEF2D (105 bp), c-Fos (74 bp), and c-Jun (163 bp) mRNA levels relative to GAPDH (138 bp) mRNA level in cardiomyocytes treated with 5 μM NE and 1 μM PhE for 2 h as described in Materials and methods. Real-time PCR (**c**) showing transcription factor gene expression in response to 5 μM NE for 2 h. Values are mean ± SE of five experiments performed with five different cardiomyocytes preparations. *Significantly different ($P < 0.05$) versus control. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; CONT, control; NE, norepinephrine; PhE, phenylephrine

α_1-AR agonist. PhE (1 μM), like NE, caused a significant increase in c-Fos and c-Jun mRNA levels (Fig. 3a, b).

Attenuation of NE-induced increases in PLC isozyme gene expression and activities, ANF and c-Fos and c-Jun mRNA levels by different inhibitors

We have previously shown that prazosin attenuated the increase in PLC isozyme gene expression and protein contents due to NE [14]. In the present study, it was observed that the inhibitory effects of prazosin on PLC isozyme gene expression (Fig. 4a, b) were correlated to an

attenuation of PLC isozyme activities due to prazosin (2 μM) (Fig. 4c). Prazosin also prevented the increase in ANF gene expression in response to NE (Fig. 4a, b), this observation served as a positive control for the NE-induced changes in the signal transduction. Since the α_1-AR transduces the signal to PLC, the participation of PLC activities

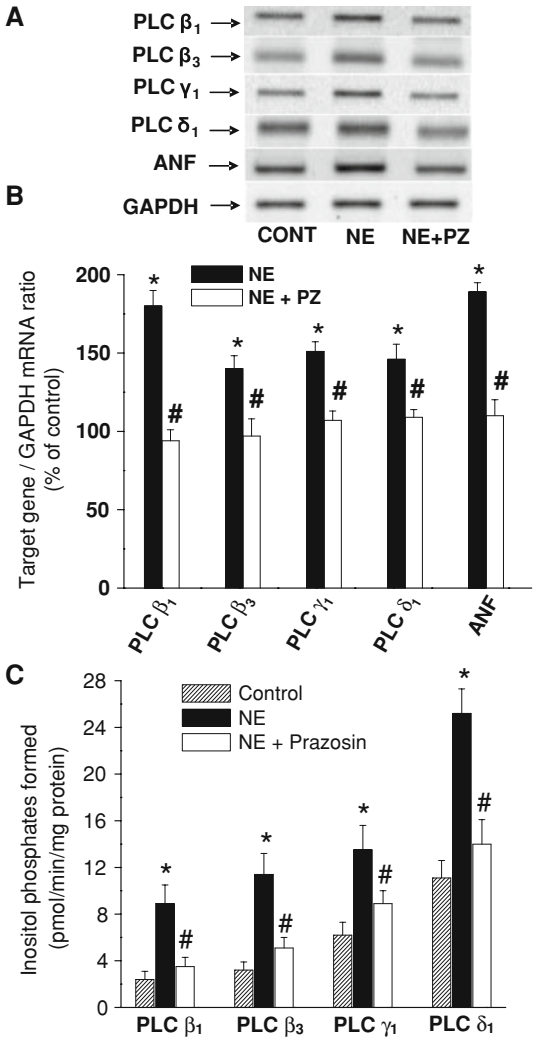

Fig. 4 Attenuation of the norepinephrine-induced increases in PLC isozyme gene expression and activities and ANF mRNA levels in adult cardiomyocytes. Representative blots (**a**) and quantified data (**b**) showing PLC isozymes β_1 (114 bp), β_3 (230 bp), γ_1 (123 bp), δ_1 (190 bp), and ANF (161 bp) mRNA levels relative to GAPDH (138 bp) mRNA level in cardiomyocytes treated with 5 μM NE for 2 h in the absence and presence of prazosin (2 μM). Panel (**c**) shows the PLC isozyme activities in response to treatment of cardiomyocytes with 5 μM NE for 24 h in the absence and presence of prazosin (2 μM). PLC isozymes activities were determined by measuring hydrolysis of [^{3}H]-PIP$_2$ as described in Materials and methods. Values are mean ± SE of five experiments performed with five different cardiomyocyte preparations. *Significantly different ($P < 0.05$) versus control; #significantly different ($P < 0.05$) versus NE. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; CONT, control; NE, norepinephrine; PZ, prazosin; ANF, atrial natriuretic factor

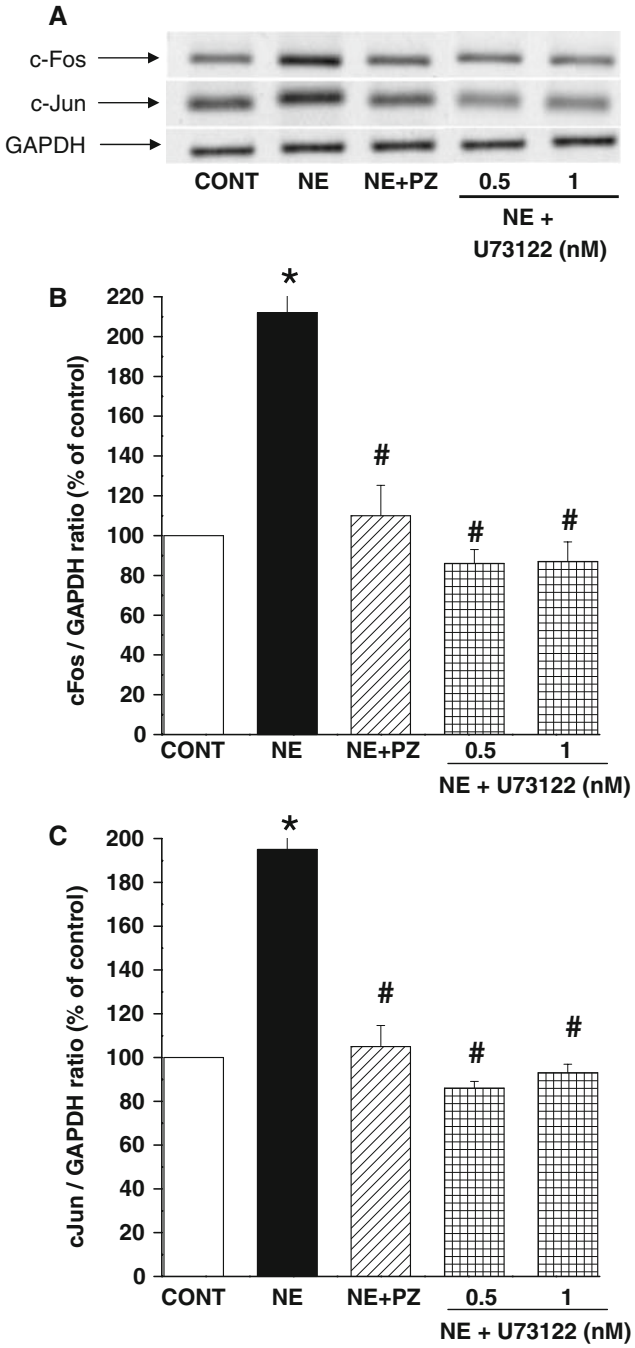

Fig. 5 Attenuation of the norepinephrine-induced increases in c-Fos and c-Jun mRNA levels and by prazosin and U73122. Representative blots (**a**) showing c-Fos (74 bp) and c-Jun (163 bp) mRNA levels relative to GAPDH (138 bp) mRNA level in LV cardiomyocytes. Quantified data (**b**) and (**c**) show c-Fos and c-Jun mRNA levels, respectively, in cardiomyocytes treated with NE (5 μM) in the absence and presence of prazosin (2 μM) and different concentrations of U73122 (0.5 and 1 nM) for 2 h. Values are mean ± SE of five experiments performed with five different cardiomyocyte preparations. *Significantly different ($P < 0.05$) versus control; #significantly different ($P < 0.05$) versus NE. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; CONT, control; NE, norepinephrine; PZ, prazosin

in NE-induced increases in c-Fos and c-Jun mRNA levels was determined by pretreating cardiomyocytes for 30 min with prazosin (2 μM) as well as U73122 (0.5 and 1 nM), a PLC inhibitor, prior to the addition of NE. Figure 5 shows that both prazosin and U73122 prevented the increase in c-Fos and c-Jun mRNA levels induced by NE. It should be noted that prazosin and U73122 alone did not affect PLC isozymes, c-Fos, and c-Jun gene expression (data not shown).

Involvement of PKC and ERK1/2 in the PLC-mediated increases in c-Fos and c-Jun gene expression in response to NE

Although PKC and ERK1/2 have been reported to be involved in the regulation of c-Fos and c-Jun gene expression in neonatal cardiomyocytes [5, 12, 13], there is no such information available in adult cardiomyocytes in response to α_1-AR stimulation. We have previously shown that PKC and ERK1/2 are involved in the PLC-mediated increases in adult cardiomyocyte gene expression [20]. Accordingly, cardiomyocytes treated with a PKC activator, PMA (0.1 to 10 μM) showed a concentration dependent increase in c-Fos and c-Jun mRNA levels (Fig. 6a–c). The role of PKC was further demonstrated by pretreating cardiomyocytes for 30 min with bisindolylmaleimide (Bis) (100 and 200 nM), an inhibitor of PKC activities, prior to the addition of NE (5 μM). It can be seen from Fig. 6d–f that inhibition of PKC attenuated the NE-induced increases in c-Fos and c-Jun mRNA levels. Pretreatment of cardiomyocytes with PD98059 (10 nM), an ERK1/2 inhibitor for 30 min prior to the addition of NE (5 μM), also prevented

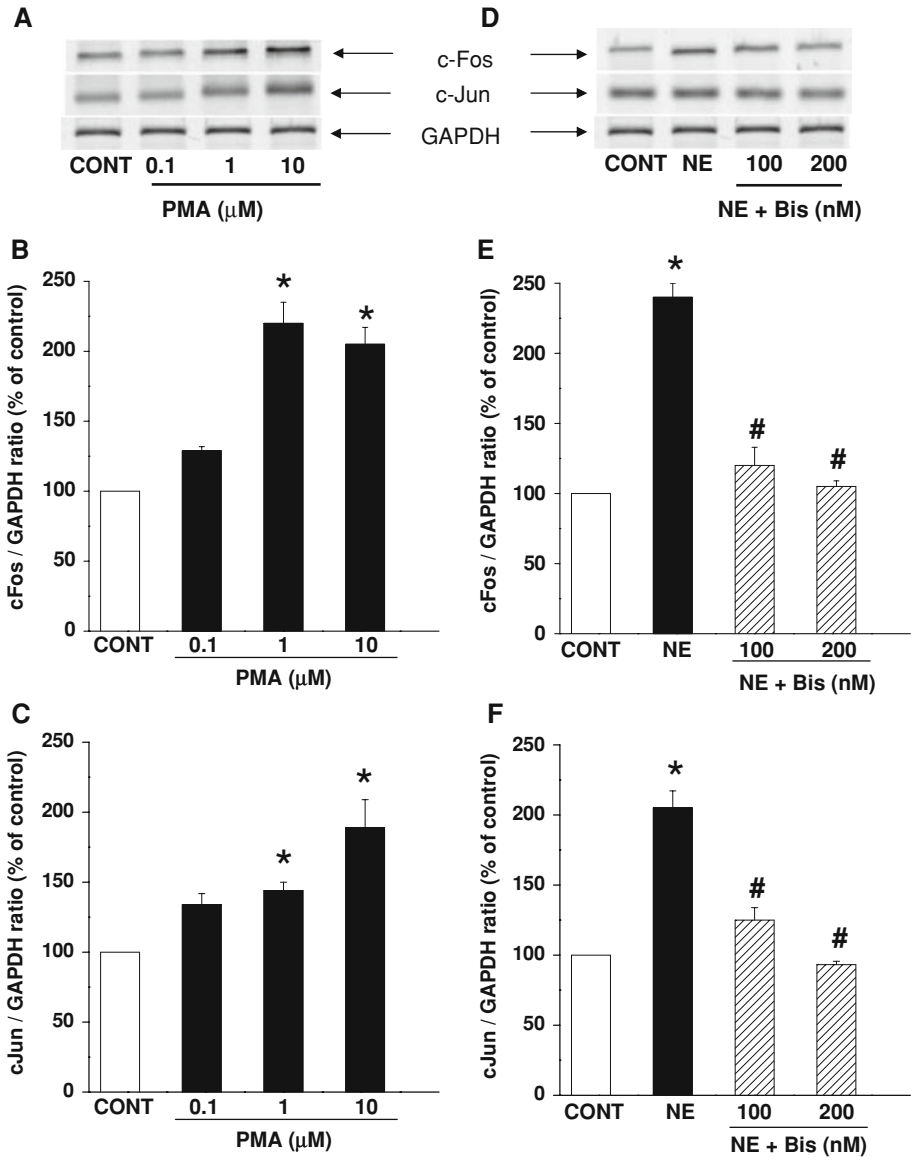

Fig. 6 Phorbol 12-myristate 13-acetate induced increases in c-Fos and c-Jun mRNA levels and inhibition of NE-induced increases in c-Fos and c-Jun mRNA levels by bisindolylmaleimide. Representative blots (**a**) showing c-Fos (74 bp) and c-Jun (163 bp) mRNA levels relative to GAPDH (138 bp) mRNA level in LV cardiomyocytes. Quantified data (**b**) and (**c**) show c-Fos and c-Jun mRNA levels, respectively, in cardiomyocytes treated with different concentrations of PMA (0.1, 1, and 10 μM). Representative blots (**d**) showing c-Fos and c-Jun mRNA levels relative to GAPDH mRNA level in LV cardiomyocytes. Quantified data (**e**) and (**f**) show c-Fos and c-Jun mRNA levels, respectively, in cardiomyocytes pretreated with different concentrations of bisindolylmaleimide (100 and 200 nM) in the presence of NE for 2 h. Values are mean ± SE of five experiments performed with five different cardiomyocyte preparations. *Significantly different ($P < 0.05$) versus control; #significantly different ($P < 0.05$) versus NE. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; CONT, control; PMA, phorbol 12-myristate 13-acetate; Bis, bisindolylmaleimide, and NE, norepinephrine

the increases in c-Fos and c-Jun mRNA due to NE (Fig. 7). Bis or PD98059 alone did not affect c-Fos and c-jun gene expression (data not shown).

Attenuation of NE-induced increases in PLC isozyme gene expression by prevention of c-Jun phosphorylation

The role of c-Jun in the regulation of PLC gene expression was further examined by determining PLC isozyme mRNA levels in cardiomyocytes pretreated for 30 min with SP600125 (10 μM) [21], an inhibitor of JNK activity, prior to the addition of NE (5 μM) for 2 h. Figure 8a–e shows that SP600125 prevented the increase in PLC β_1, β_3, and δ_1 gene expression in response to NE, but did not inhibit the NE-induced increase in PLC γ_1 gene expression. Since the inhibition of PKC and ERK1/2 with Bis and PD98059, respectively, has also been shown to decrease the PLC isozyme gene expression in response to NE [14], it is likely that these effects may be due to the attenuation of c-jun phosphorylation. It can be seen from Fig. 9 that an almost complete inhibition of the phosphorylation of c-Jun was detected with SP600125 (10 μM), Bis (200 nM), and PD98059 (25 nM). It should be mentioned that our preliminary experiments have also revealed similar results on PLC β_1, β_3, and δ_1 gene expression in response to NE with inhibition of c-Fos phosphorylation with 25 nM PD98059 [22] (data not shown).

Discussion

We have previously reported that the adult cardiomyocyte hypertrophic response to NE, as evidenced by an increase in ANF expression and protein synthesis, is due to the activation of PLC via α_1-AR [11]. In the present study, we have shown that NE caused concomitant increases in PLC isozymes as well as c-Fos and c-Jun gene expression in the isolated adult rat cardiomyocytes, both of which were prevented by an α_1-AR antagonist, prazosin. Furthermore, an inhibitor of PLC activity, U73122, attenuated the NE-induced increase in c-Fos and c-Jun gene expression, indicating a role of PLC activities in the NE-induced increase in c-Fos and c-Jun gene expression. Interestingly, the changes in the PLC isozymes, c-Fos, and c-Jun mRNA and protein levels in response to NE were observed to be similar, indicating that the main control in the increase is at the transcriptional level, which would seem to obviate the need to examine the regulation at other levels of expression, an important concept that warrants further investigation.

It is known that α_1-AR agonists, including NE, are stimulants of PLC β isozymes via the α-subunits of Gq subfamily [23]. PLC δ isozyme is also considered to be activated by α_1-AR through the GTP binding protein $G\alpha_h$ (transglutaminase II) [24, 25], while a possible link between Gq and tyrosine kinase provides a mechanism for the α_1-AR mediated activation of PLCγ [26, 27]. Thus, we believe that stimulation of α_1-AR with NE activates the

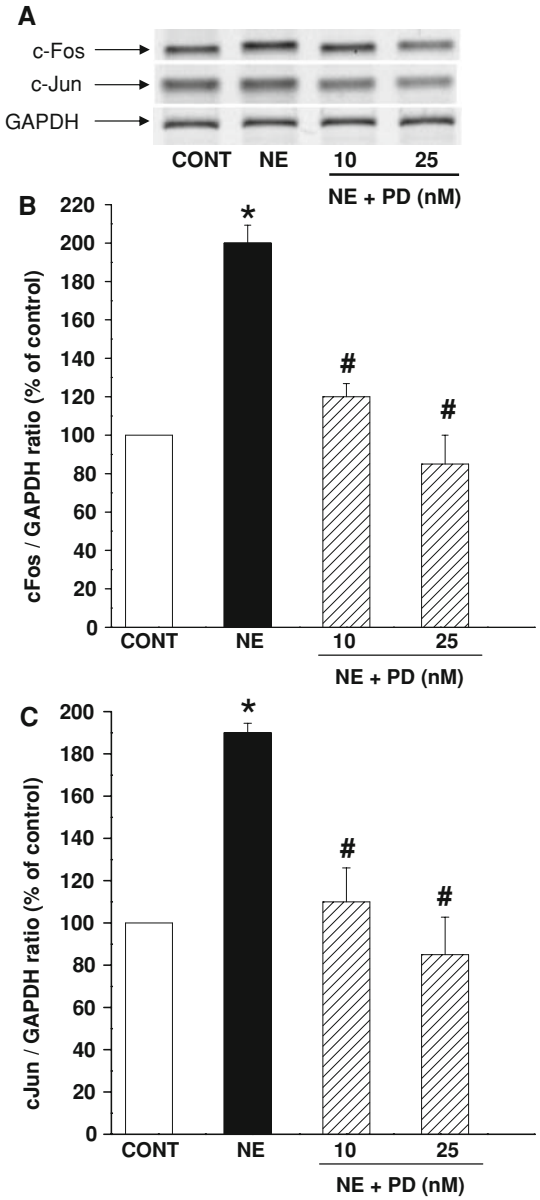

Fig. 7 Norepinephrine-induced increases in c-Fos and c-Jun mRNA expression in cardiomyocytes treated with and without different concentrations of PD98059. Representative blots (**a**) showing c-Fos (74 bp) and c-Jun (163 bp) mRNA levels relative to GAPDH (138 bp) mRNA level in LV cardiomyocytes. Quantified data (**b**) and (**c**) show c-Fos and c-Jun mRNA levels, respectively, in cardiomyocytes treated with NE (5 μM) in the absence and presence of different concentrations of PD98059 (10 and 25 nM) for 2 h. Values are mean ± SE of five experiments performed with five different cardiomyocyte preparations. *Significantly different ($P < 0.05$) vs control; #significantly different ($P < 0.05$) versus NE. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; CONT, control; NE, norepinephrine; PD, PD98059

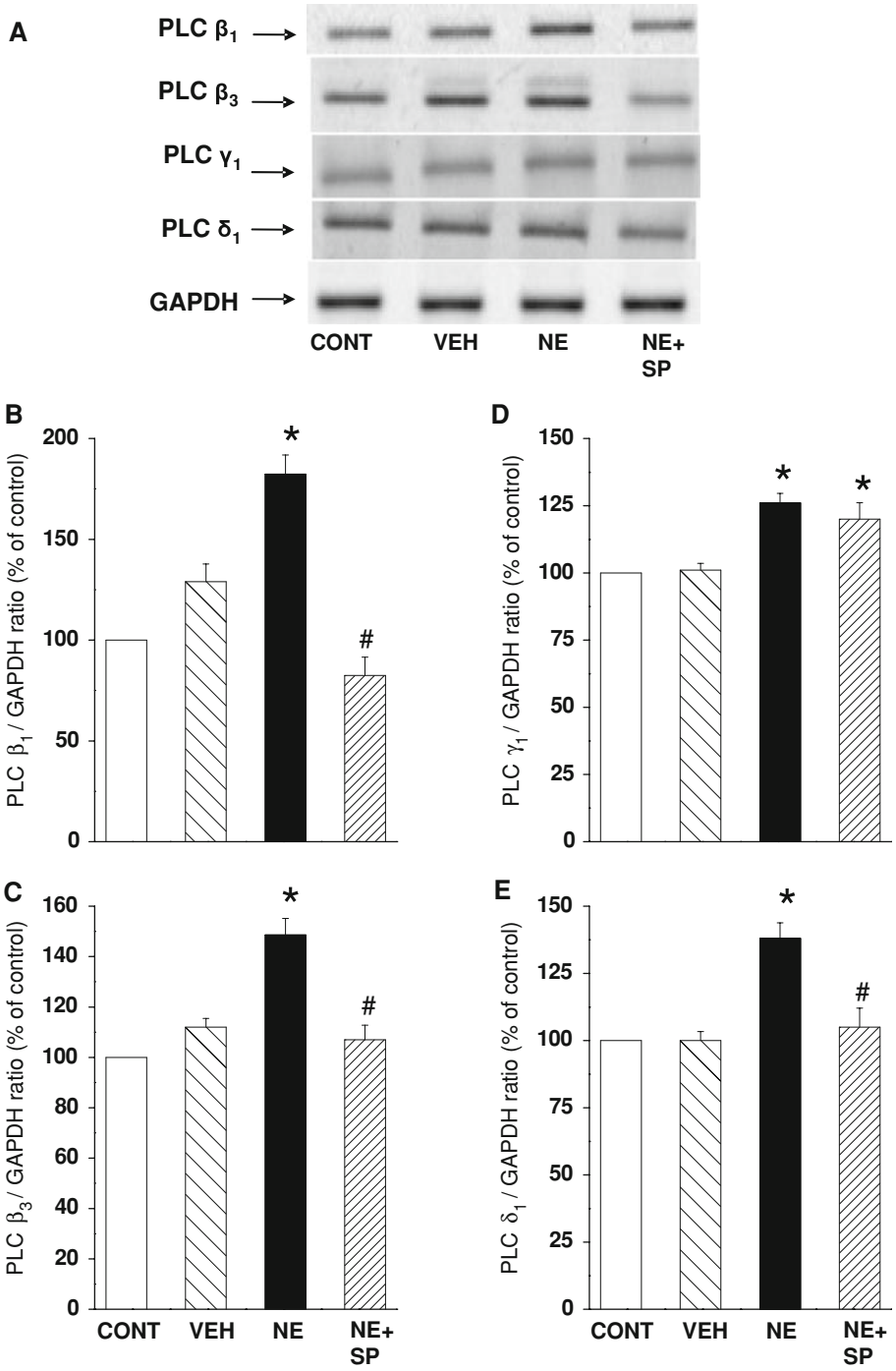

Fig. 8 Prevention of the NE-induced increases in PLC isozyme gene expression and c-Jun phosphorylation in cardiomyocytes treated with JNK inhibitor. Representative blots (**a**) and quantified data showing (**b**) PLC isozyme β_1 (114 bp), (**c**) β_3 (230 bp), (**d**) γ_1 (123 bp), and (**e**) δ_1 (190 bp) mRNA levels relative to GAPDH (138 bp) mRNA level in response to 5 μM NE for 2 h in cardiomyocytes pre-treated with a JNK inhibitor, SP600125 (10 μM) as described in the Materials and methods. Values are mean ± SE of five experiments performed with five different cardiomyocyte preparations. *Significantly different ($P < 0.05$) versus control; #significantly different ($P < 0.05$) versus NE. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; CONT, control; NE, norepinephrine, SP, SP600125; VEH, DMSO vehicle control

PLC isozymes under examination in this study. Supportive evidence is revealed by the prevention of the α_1-AR activation of PLC isozymes in response to NE by prazosin. It should be noted that U73122 has also been reported to exert non-specific actions in other cell types [28–30]; however, these were observed at concentrations that were up between 100- and 10,000-fold greater than used in the present study. It is also pointed out that stimulation of both α- and β-adrenergic receptors has been reported to induce c-Fos and c-Jun gene expression in neonatal rat cardiomyocytes [31]. Since prazosin, under our experimental conditions, prevented the NE-induced increase in c-Fos and c-Jun gene expression, it is likely that induction of c-Fos and c-Jun gene expression is primarily due to the α_1-AR in adult cardiomyocytes. Interestingly, transcription activator protein-1 (AP-1), a complex of c-Fos/c-Jun, has been reported to mediate the α-AR, but not β-AR, hypertrophic growth responses in adult cardiomyocytes [32].

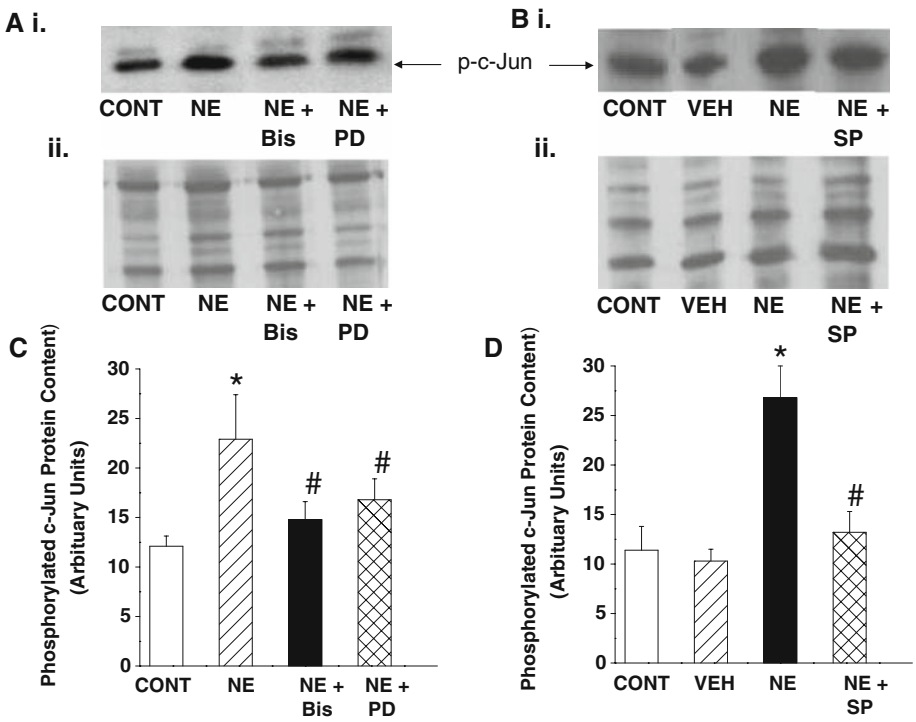

Fig. 9 Prevention of the NE-induced phosphorylation of c-jun in cardiomyocytes treated with different kinase inhibitors. Representative blots (**a** and **b**(i)) and (**a** and **b**(ii)) amido black stained PVDF membrane showing equivalent protein loading. Quantified data (**c**) showing the effect of bisindolylmaleimide (200nM), a PKC inhibitor, PD 98059 (25 nM), an ERK1/2 inhibitor and (**d**) SP600125 (10 μM), a JNK inhibitor, on c-Jun phosphorylation in cardiomyocytes treated with 5 μM NE for 2 h. Values are mean ± SE of five experiments performed with five different cardiomyocyte preparations. *Significantly different ($P < 0.05$) versus control; #significantly different ($P < 0.05$) versus NE. CONT, control; NE, norepinephrine, Bis, bisindolylmaleimide; PD, PD98059; SP, SP600125; VEH, DMSO vehicle control

Since an attenuation of the NE-induced increases in c-Fos and c-Jun gene expression with inhibition of PLC, directly with U73122 and indirectly through blockade of the α_1-AR with prazosin, was observed, it is evident that the downstream signal effectors are involved in the PLC-mediated increases in c-Fos and c-Jun gene expression in response to NE in adult cardiomyocytes. In this regard, inhibition of PKC with Bis and ERK1/2 with PD98059 inhibited the NE-induced increase in c-Fos and c-Jun gene expression in adult cardiomyocytes. While a role for PKC and ERK1/2 in cardiac hypertrophy is known [33, 34], this study has provided evidence for the involvement of PLC in the signal transduction mechanism in the transcriptional regulation of c-Fos and c-Jun in adult cardiomyocytes. Because PKC and ERK1/2 have been reported to be involved in the regulation of c-Fos and c-Jun gene expression in neonatal cardiomyocytes [5, 12, 13], our data demonstrate a similar role for these kinases in the regulation of c-Fos and c-Jun gene expression in adult cardiomyocytes in response to α_1-AR stimulation.

c-Fos and c-Jun proteins form AP-1 which is a transcription factor considered to be involved in cardiac hypertrophy [32, 35, 36]. Taimor et al. [32] reported that PhE promotes the formation of c-Fos/c-Jun AP-1 transcription complex in cardiomyocytes, and that the functional involvement of AP-1 in hypertrophic growth could only be demonstrated for α-adrenergic stimulation in adult cardiomyocytes. Accordingly, it is possible that the AP-1 mediated hypertrophy could involve the activation of PLC isozyme gene expression. It should also be mentioned that the hypertrophic phenotype in dominant negative c-Jun transfected cardiomyocytes has been reported to be inhibited in response to PhE [8]. Our earlier [11, 14] and present data would seem to indicate that this inhibitory response to PhE is likely attributed to an attenuation of specific PLC isozyme gene expression and subsequent activities.

Studies in neonatal cardiomyocytes have shown that preincubation with PLC β_3 antisense oligonucleotides abolished the insulin-like growth factor-1 (IGF-1)-induced upregulation of c-Fos and c-Jun genes [10], indicating that PLC β_3 expression may be required for the induction of immediate early genes by IGF-1. This raises an intriguing possibility that a reciprocal regulation of specific PLC isozymes and c-Fos/c-Jun gene expression may exist in adult cardiomyocytes. In this regard, prevention of c-Jun phosphorylation with SP600125 was observed not to

inhibit the NE-induced increase in PLC γ_1 gene expression, which suggests the involvement of a different transcription factor. A possible candidate could be the early growth response factor-1, the expression of which is known to be induced in response to α_1-AR stimulation [31].

A number of studies have indicated the role of prazosin in mitigating the progression of cardiac hypertrophy to heart failure [32, 37–42]. It is likely that this action is due to the inhibition of PLC activation and subsequent signal transduction events. In addition to the activation of the sympathetic nervous system, activation of the renin-angiotensin system is also known to occur in cardiac hypertrophy [43]. Angiotensin II (ANG II) can initiate cardiac hypertrophy and upregulate PLC β_3 and ERK1/2 [44, 45] as well as increase the expression of c-Fos and c-Jun [46]. Losartan, an ANG II type 1 receptor blocker, has also been reported to regress cardiac hypertrophy [38, 42, 47]; an effect that may, in part, be due to an inhibition of the upregulation of PLC isozymes [45, 48]. Thus, the activation of PLC isozyme signal transduction may be considered as an important step in cardiac hypertrophy and may therefore constitute novel therapeutic targets for the prevention of cardiac hypertrophy.

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