Protein Kinase C, but Not Tyrosine Kinases or Ras, Plays a Critical Role in Angiotensin II-induced Activation of Raf-1 Kinase and Extracellular Signal-regulated Protein Kinases in Cardiac Myocytes*

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Angiotensin II (AngII) induces cardiac hypertrophy through activating a variety of protein kinases. In this study, to understand how cardiac hypertrophy develops, we examined AngII-evoked signal transduction pathways leading to the activation of extracellular signal-regulated protein kinases (ERKs), which are reportedly critical for the development of cardiac hypertrophy, in cultured cardiac myocytes isolated from neonatal rats. Inhibition of protein kinase C (PKC) with calphostin C or down-regulation of PKC by pretreatment with a phorbol ester for 24 h abolished AngII-induced activation of Raf-1 and ERKs, and addition of a phorbol ester conversely induced a marked increase in the activities of Raf-1 and ERKs. Pretreatment with two chemically and mechanistically dissimilar tyrosine kinase inhibitors, genistein and tyrphostin, did not attenuate AngII-induced activation of ERKs. In contrast, genistein strongly blocked insulin-induced ERK activation in cardiac myocytes. Although pretreatment with manumycin, a Ras farnesyltransferase inhibitor, or overexpression of a dominant-negative mutant of Ras inhibited insulin-induced ERK activation, neither affected AngII-induced activation of ERKs. Overexpression of a dominant-negative mutant of Raf-1 completely suppressed ERK2 activation by AngII, endothelin-1, and insulin. These results suggest that PKC and Raf-1, but not tyrosine kinases or Ras, are critical for AngII-induced activation of ERKs in cardiac myocytes.

Clinical studies have demonstrated that cardiac hypertrophy is not only an adaptive state before cardiac failure, but is also an independent risk factor of cardiac morbidity and mortality (1). Thus, it has become even more important to understand how cardiac hypertrophy develops. Cardiac hypertrophy is induced by mechanical load (2–4) and humoral factors such as angiotensin II (AngII) (5), endothelin-1 (ET-1) (6), phenylephrine (PHE) (7), and peptide growth factors (8). These humoral factors activate a variety of second messenger systems through various receptors, such as tyrosine kinase receptors and heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors, which regulate the expression of specific genes and increase protein synthesis. Among the above-mentioned factors, AngII has recently attracted great attention not because of its potency (by comparison with ET-1, PHE etc.), but because of its established importance in vivo as well as in vitro (5). AngII directly induces cardiac myocyte hypertrophy, independently of an increase in vascular resistance or cardiac afterload (5, 9). There is a growing body of evidence suggesting that locally produced AngII, more than circulating AngII, is a potent stimulator of cardiac hypertrophy (5, 9). Moreover, it has been demonstrated that mechanical stress stimulates the secretion of AngII from cardiac myocytes and that AngII induces cardiac hypertrophy in cultured cardiac myocytes, possibly by autocrine mechanisms (9–11).

After binding to the AngII type 1 receptor, AngII rapidly stimulates phosphatidylinositol-specific phospholipase C through a heterotrimeric G protein, leading to the generation of inositol triphosphate and diacylglycerol, which are involved in intracellular Ca2+ mobilization and protein kinase C (PKC) activation, respectively (5, 12). Recently, many laboratories have reported that AngII also activates a variety of protein kinases and signaling molecules such as tyrosine kinases, including Src family tyrosine kinases, Ras, Raf-1 kinase (Raf-1), and extracellular signal-regulated protein kinases (ERKs), in various cell types (11–17). Many lines of evidence have indicated that ERKs function as integrators for mitogenic and differentiation signals originating from several distinct classes of cell-surface receptors such as receptor tyrosine kinases and G protein-coupled receptors (18–20). In cardiac myocytes, the activation of ERKs is also required for PHE-induced expression of specific genes such as atrial natriuretic factor, c-fos, and myosin light chain 2 genes (21). Although ERK activation is not sufficient to fully promote cardiac hypertrophy (22, 23), recent evidence using an antisense oligodeoxynucleotide has shown that ERKs are necessary for PHE-induced sarcenomerogenes and increased cell size (24). Stimulation of receptor tyrosine kinases usually results in the activation of the Raf-1/MEK/ERK cascade through Ras in many cell types (18–20, 23). The pathway that leads to the activation of ERKs from G protein (espe-
 activation of Ras through sequential activation of Shc, Grb2, and Sos (15). Ras has been reported to cause hypertrophic changes in cultured cardiac myocytes both in terms of cell morphology and gene expression (28). On the other hand, there have been reports indicating that the activation of the Raf-1/ERK cascade by AngII in cultured vascular smooth muscle cells and kidney cells depends on PKC (29, 30). Moreover, in cardiac myocytes, AngII activates PKC, which in turn induces a variety of hypertrophic responses (11, 12, 31–33). We have observed that mechanical stretch activates Raf-1 and ERKs partly through secreted AngII and that the activation of these kinases depends on PKC (11, 32, 33). Thus, in this study, we examined which pathway, the PKC-dependent pathway or the tyrosine kinase/Ras-dependent pathway, is critical for the AngII-induced ERK activation observed in cardiac myocytes. Using a variety of inhibitors, we demonstrate that in cardiac myocytes, AngII activates Raf-1 and ERKs through the PKC-dependent pathway, but not through the pathway of tyrosine kinases or Ras.

**EXPERIMENTAL PROCEDURES**

**Materials—**[γ-^32P]ATP was purchased from DuPont NEN. Dulbecco’s modified Eagle’s medium, fetal bovine serum, tyrothrin, and genistein were from Life Technologies, Inc. Calphostin C and ET-1 were from Funakoshi (Tokyo). Syntide-2 and polyclonal antibody against Raf-1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-hemaggullatin (HA) polyclonal antibody was from Mitsubishi Biochemical Laboratories (Tokyo). AngII, 12-O-tetradecanoylphorbol-13-acetate (TPA), myelin basic protein (MBP), and other reagents were from Sigma.

**cDNA Plasmids—**HA-tagged ERK2 with the SV40 promoter (HA-ERK2) was a kind gift from M. Karin (34). The dominant-negative mutant (Ann-17) of Ras (D.N.Ras) and the dominant-negative mutant (Ala-375) of Raf-1 (D.N.Raf-1), both of which are driven by the cytomegavirustransgene, were provided by Y. Takai (35) and T. Kadokawa (36), respectively. The constructs of all expression plasmids have been described elsewhere (34–36). All plasmid DNA was prepared using QIAGEN plasmid DNA preparation kits.

**Cell Culture—**Primary cultures of cardiac myocytes were prepared from ventricles of 1-day-old Wistar rats according to the method of Simpson (7) as described previously (3). In brief, cardiomyocytes were plated at a field density of $1 \times 10^5$ cells/cm² on 35-mm culture dishes in 2 ml of culture medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum). Twenty-four hours after seeding, the culture medium was changed to serum-free Dulbecco’s modified Eagle’s medium, and the cells were cultured for 48 h before stimulation.

**Transfection—**Twenty-four hours after plating the cells on culture dishes, DNA was transfected with the calcium phosphate method as described previously (3). For each dish, 2.5 μg of HA-ERK2 plasmid DNA was transfected with or without 7.5 μg of the other relevant plasmids, such as D.N.Ras or D.N.Raf-1. Transfected DNA content was kept constant in each experiment. After 20 h of transfection, the culture medium was removed, and the cells were washed with phosphate-buffered saline twice and were maintained in Dulbecco’s modified Eagle’s medium without fetal bovine serum for 48 h before treatment with AngII or other reagents. The transfection efficiency of each experiment was ~3–5% as assessed by LacZ staining after transfection of a LacZ-containing expression plasmid.

**Assay of ERK Activity—**ERK activities were measured by “in-gel” assay using MBP-containing gel as described previously (32). In brief, cells were lysed with buffer A (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 0.4% SDS, and 1% 2-mercaptoethanol) and cell lysates were electrophoresed on SDS-polyacrylamide gels containing 0.5 mg/ml MBP. ERKs in the gels were denatured in 6 M guanidine HCl and renatured in 50 mM Tris-HCl, pH 8.0, containing 0.04% Triton X-100 and 5 mM 2-mercaptoethanol. The phosphorylative activity of ERKs was assayed by incubating the gel with [γ-^32P]ATP. After incubation, the gel was thoroughly washed, dried, and subjected to autoradiography. A representative autoradiogram from three independent experiments is shown.

**RESULTS**

**AngII-induced ERK Activation Is Dependent on PKC in Cardiac Myocytes—**It has been reported that AngII induces the activation of phospholipase C through the AngII type 1 receptor and Gα protein, which results in PKC activation in cardiac myocytes (11–13). PKC has been shown to activate ERKs in many cell types (20, 29, 30, 32, 38). Thus, we first examined whether PKC is involved in AngII-induced ERK activation in cardiac myocytes. After down-regulation of PKC by pretreatment with 10−7 M TPA for 24 h or after pretreatment with 10−6 M calphostin C for 60 min, TPA did not activate ERKs in cardiac myocytes (data not shown). AngII markedly increased the activities of 42- and 44-kDa ERKs as described previously (11, 13), and activation was completely blocked by down-regulation of PKC or by calphostin C (Fig. 1). These results suggest that PKC plays a critical role in AngII-induced ERK activation in cardiac myocytes.

**AngII-induced Raf-1 Activation Is Dependent on PKC in Cardiomyocytes—**It has been shown that ERKs are activated by phosphorylation of both threonine and tyrosine residues cata-

![Fig. 1. Role of PKC in AngII-induced ERK activation in cardiac myocytes](image-url)
Angiotensin II-evoked Signaling Pathway in Cardiomyocytes

Many lines of evidence have suggested that Ras plays a key role in a variety of cell functions partly through a sequential activation of Raf-1 and ERKs (18, 19, 25). Ras has been reported to be required for PHE-induced hypertrophic responses (28) and to be activated by AngII through a member of the Src family tyrosine kinases in cardiac myocytes (15). To elucidate whether the activation of Ras is required for AngII-induced ERK activation, we transfected an HA-ERK2 expression plasmid with or without D.N.Ras into cultured cardiomyocytes and treated cells with AngII or insulin. Transfected DNA content was kept constant, and the transfection efficiency was not changed with or without the D.N.Ras plasmid. Although cotransfection of D.N.Ras abolished insulin-induced activation of transfected ERK2, which is in agreement with a previous report (41), AngII-induced ERK2 activation was not affected by cotransfection with D.N.Ras (Fig. 4A). We further examined the role of Ras in AngII-induced ERK activation pharmacologically using manumycin, which is a Ras farnesyltransferase inhibitor and effectively suppresses Ras biological functions (42). We preincubated cardiomyocytes with 10−6 M manumycin for 60 min and stimulated the cells with AngII or insulin. Treatment with manumycin completely suppressed AngII-induced ERK activation (Fig. 4B). On the contrary, AngII-induced activation of ERKs was not inhibited by the manumycin treatment (Fig. 4B). From these results, we conclude that unlike insulin, AngII may activate ERKs through Ras-independent pathways in cardiac myocytes.

AngII-induced ERK Activation Is Independent of Ras in Cardiomyocytes—Many tyrosine kinases, including receptor- and non-receptor-type tyrosine kinases, have been shown to activate ERKs in many cell types (18–20). Recently, AngII has been reported to activate tyrosine kinases in cardiac myocytes (13, 15). We further examined whether the activation of tyrosine kinases is required for AngII-induced activation of ERKs in cardiomyocytes. Thirty minutes after pretreatment with two chemically and mechanistically dissimilar tyrosine kinase inhibitors, tyrphostin (5 × 10−5 M) (39) and genistein (2 × 10−5 M) (40), cardiomyocytes were stimulated with AngII (10−6 M) or insulin (10−6 M) for 8 min. Pretreatment with tyrphostin or genistein had no effect on AngII-induced ERK activation (Fig. 3). Although the levels of ERKs activated by insulin were almost the same as those activated by AngII, insulin-induced ERK activation was abolished by genistein (Fig. 3). These results suggest that unlike insulin, AngII may activate ERKs through tyrosine kinase-independent pathways in cardiac myocytes.

AngII-induced ERK Activation Is Independent of Raf-1—ERKs are activated by the dual protein kinase MEK, and MEK is in turn activated by serine/threonine kinases including Raf-1 (18–20). Since AngII activates Raf-1 in cardiac myocytes (11) and PKC has been reported to directly activate Raf-1 in other cell types (19, 20, 38), we next examined the role of PKC in AngII-induced Raf-1 activation in cardiac myocytes. After pretreatment with TPA for 24 h or with calphostin C for 60 min, cardiac myocytes were stimulated with AngII for 2 min and then subjected to filter assay for Raf-1 activity. In the absence of pretreatment, AngII increased the activity of Raf-1 by ~2-fold (Fig. 2) as reported previously (33). After preincubation with TPA or calphostin C, however, Raf-1 was not activated by AngII, suggesting that AngII induces the activation of Raf-1 via PKC-dependent pathways in cardiac myocytes.

FIG. 2. Role of PKC in AngII-induced Raf-1 activation in cardiomyocytes. Cardiomyocytes were preincubated with 10−6 M calphostin C for 60 min or with 10−7 M TPA for 24 h. Cells were stimulated with 10−6 M AngII for 2 min. Raf-1 was immunoprecipitated with anti-Raf-1 polyclonal antibody, and the immunocomplex was incubated with syntide-2 as a substrate for Raf-1 and [γ-32P]ATP. After terminating the reaction, aliquots of the reaction mixture were spotted on Whatman P-81 paper. The incorporation of 32P into syntide-2 was measured by Cerenkov counting. The data are presented as the means ± S.E. of three independent experiments and are compared with controls (100%). * p < 0.05 versus control.

Effects of tyrphostin or genistein on AngII- or insulin-induced ERK activation in cardiac myocytes. Cardiomyocytes were pretreated with 5 × 10−5 M tyrphostin or 2 × 10−5 M genistein for 30 min and exposed to 10−6 M AngII or 10−6 M insulin for 5 min. ERK activations were assayed using the so-called in-gel assay as described under "Experimental Procedures." A representative autoradiogram from three independent experiments is shown.
examined the activities of Raf-1 and ERKs, respectively. TPA strikingly activated Raf-1 (Fig. 6A) and ERKs (Fig. 6B), suggesting that Raf-1 and ERKs can be activated through the activation of PKC-dependent pathways in cardiac myocytes.

DISCUSSION

We and others have previously reported that AngII activates phosphorylation cascades of protein kinases such as PKC, tyrosine kinases, Raf-1, ERKs, and 90-kDa ribosomal S6 kinase (11–13, 15, 33). The activation of protein kinases generally plays a key role in cell growth and gene expression (18–20). Especially ERKs could be a convergence point for growth signals originating from different G protein-coupled receptors as well as tyrosine kinase receptors (18–20, 24). It has been reported that in cardiac myocytes, ERK activation is important for PHE-induced cardiac hypertrophy and gene expression (21, 24), although there are reports showing the existence of dissociation between ERK activation and cardiac hypertrophy (20, 23). In this study, we examined AngII-induced signaling pathways leading to the activation of Raf-1 and ERKs in cultured cardiomyocytes of neonatal rats. Using various kinds of inhibitors, we have elucidated that PKC, but not tyrosine kinases or Ras, is critical for AngII-induced activation of ERKs in cardiac myocytes.

Many reports have suggested that PKC is involved in the control of a number of cell functions and that PKC activation is an important step that mediates the transduction of external stimuli into the nucleus through the activation of downstream protein kinase cascades (11, 29, 30, 38). It has also been shown that Raf-1, which is a key component in the signal transduction pathways leading to the activation of ERKs induced by a variety of growth factors and vasoconstrictors such as insulin, AngII, and ET-1 (11, 37, 43), is directly phosphorylated and activated by PKC in cultured vascular smooth muscle cells and kidney cells (29, 30, 38). In cardiomyocytes, we have demonstrated that mechanical stress, partly through secreted AngII and ET-1, activates the Raf-1/MEK/ERK cascade through PKC (11, 33, 37). Another line of evidence has shown that the activation of PKC is necessary for immediate-early gene induction and ERK activation during recovery from metabolic inhibition (44). Consistent with these observations, we demonstrated in this study that the activation of Raf-1 and ERKs by AngII is completely suppressed by down-regulation or inhibition of PKC and that a PKC activator, TPA, strongly activates Raf-1 and ERKs in cardiac myocytes, suggesting that PKC plays a pivotal role in AngII-induced activation of the Raf-1/ERK cascade in these cells. It has previously been shown that AngII-induced activation of ERKs is suppressed by chelating Ca$^{2+}$, but not by
down-regulation of PKC in cardiac myocytes (13). We have also observed that Ca$^{2+}$ plays an important role in AngII-induced ERK activation in cardiac myocytes, but this study demonstrates that PKC is critical for the activation of ERKs by AngII. We cannot find an explanation for the discrepancies between the results of these studies at present. However, the same group has reported that PKC and a binding site for the nuclear transcription factor p62TCF, but not the Ca$^{2+}$/cAMP response element in the c-fos promoter, are critical for AngII-induced c-fos expression in cardiac myocytes (12). Since p62TCF has been reported to be phosphorylated and activated by ERKs (45), these results suggest that PKC is involved in AngII-induced c-fos expression through ERK activation. We obtained the same results in this study using other PKC inhibitors such as H-7, staurosporine, and GF109203X (data not shown). Thus, we are confident that PKC is critical for AngII-induced ERK activation under the culture conditions employed in this study.

A lot of information has been accumulated suggesting that stimulation of G protein-coupled receptors activates tyrosine kinases (15, 24–27). Moreover, it has recently been reported that receptor tyrosine kinases such as epidermal growth factor and platelet-derived growth factor receptors are activated by stimulation of G protein-coupled receptors (26, 27). AngII has also been reported to activate Src family tyrosine kinases, resulting in the activation of Ras in cardiac myocytes and smooth muscle cells (14–17). A recent report has shown that AngII-induced ERK activation is mediated by Ras through a currently unidentified tyrosine kinase that lies downstream of G$_c$-coupled Ca$^{2+}$/calmodulin signals in smooth muscle cells (17). In cardiac myocytes, AngII has been reported to activate Fyn, one of the Src family tyrosine kinases (15). On the other hand, there have been reports suggesting that AngII activates ERKs via PKC-dependent, but not tyrosine kinase-dependent, pathways in smooth muscle cells and kidney cells (29, 30). We therefore examined whether tyrosine kinase activation is involved in AngII-induced ERK activation in cardiomyocytes. Although insulin-induced ERK activation was suppressed by tyrosine kinase inhibitors such as genistein, as expected, AngII-induced ERK activation was not affected by these agents. Furthermore, overexpression of Csk (C-terminal Src kinase, which can inhibit the function of Src family tyrosine kinases (46), had no effect on AngII- or ET-1-induced ERK activation in cardiac myocytes. Although there is a possibility that tyrosine kinases that are insensitive to the tyrosine kinase inhibitors used in this study are involved in AngII-induced ERK activation, the results of this study suggest that tyrosine kinases including Src family tyrosine kinases, if activated, may not play critical roles at least in AngII-evoked ERK activation.

Ras, the small G protein, controls an extraordinarily wide variety of cellular processes including growth and differentiation of many cell types (25). A growing body of evidence has suggested that Ras is activated by a variety of stimuli for growth and differentiation and that the activated Ras evokes the phosphorylation cascade of protein kinases including Raf-1 and ERKs (18–20, 24, 25). It has been reported that AngII activates Raf in cardiac myocytes (15), Ras was found not to be required for AngII-induced ERK activation in this study. Although it is not clear why activated Ras was not involved in AngII-induced ERK activation in cardiac myocytes at present, it has been reported that TPA activates Ras, but inhibition of Ras function does not affect TPA-induced ERK activation in Rat-1 fibroblasts (41). It is possible that AngII activates two signaling pathways such as PKC and Ras pathways and that the PKC pathway somehow predominantly leads to ERK activation in cardiac myocytes.

AngII has been reported to evoke a variety of signals and to induce proliferation of cardiac fibroblasts (49). In cardiac fibroblasts of neonatal rats, AngII-induced ERK activation was partly suppressed by tyrosine kinase inhibitors, but was not affected by PKC down-regulation. Although inhibition of G$_i$ protein functions by pretreatment with pertussis toxin did not suppress AngII-induced ERK activation in cardiac myocytes, AngII-induced ERK activation was sensitive to pertussis toxin in cardiac fibroblasts. It has been reported that ERK activation is mediated by $\beta$-subunits of heterotrimeric G$_i$ proteins acting through a Src/Ras-dependent pathway (20, 24, 50). Collectively, AngII may activate ERKs through $\beta$-subunits derived from G$_i$ proteins and through the activation of tyrosine kinases in cardiac fibroblasts. In smooth muscle cells, AngII activates Src without a direct interaction between Src and the AngII type 1 receptor, and the activation of Src, but not Fyn or Yes, is critical for Ras activation and Ras-Raf-1 complex formation (14, 16). In cardiac myocytes, AngII activated the Raf-1/ERK cascade mainly through PKC-dependent pathways and tyrosine kinases including Src family tyrosine kinases, and Ras may have little effect on AngII-induced activation of Raf-1 and ERKs. Taken together, these results suggest that AngII-evoked signal transduction pathways are highly divergent among cell types.

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