PKCα regulates the hypertrophic growth of cardiomyocytes through extracellular signal–regulated kinase1/2 (ERK1/2)

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Members of the protein kinase C (PKC) isozyme family are important signal transducers in virtually every mammalian cell type. Within the heart, PKC isozymes are thought to participate in a signaling network that programs developmental and pathological cardiomyocyte hypertrophic growth. To investigate the function of PKC signaling in regulating cardiomyocyte growth, adenoviral-mediated gene transfer of wild-type and dominant negative mutants of PKCα, βII, δ, and ε (only wild-type ζ) was performed in cultured neonatal rat cardiomyocytes. Overexpression of wild-type PKCα, βII, δ, and ε revealed distinct subcellular localizations upon activation suggesting unique functions of each isozyme in cardiomyocytes. Indeed, overexpression of wild-type PKCα, but not βII, δ, ε, or ζ induced hypertrophic growth of cardiomyocytes characterized by increased cell surface area, increased [^{3}H]-leucine incorporation, and increased expression of the hypertrophic marker gene atrial natriuretic factor. In contrast, expression of dominant negative PKCα, βII, δ, and ε revealed a necessary role for PKCα as a mediator of agonist-induced cardiomyocyte hypertrophy, whereas dominant negative PKCε reduced cellular viability. A mechanism whereby PKCα might regulate hypertrophy was suggested by the observations that wild-type PKCα induced extracellular signal–regulated kinase1/2 (ERK1/2), that dominant negative PKCα inhibited PMA-induced ERK1/2 activation, and that dominant negative MEK1 (up-stream of ERK1/2) inhibited wild-type PKCα–induced hypertrophic growth. These results implicate PKCα as a necessary mediator of cardiomyocyte hypertrophic growth, in part, through a ERK1/2-dependent signaling pathway.

Introduction

The mammalian myocardium undergoes a period of developmental hypertrophic growth during postnatal maturation that is characterized by the enlargement of individual cardiomyocytes, without cell division. Aspects of developmental hypertrophy are reemployed in the adult heart in response to diverse pathophysiologic stimuli such as hypertension, ischemic heart disease, valvular insufficiency, and cardiomyopathy (for review see Lorell and Carabello, 2000). Although such hypertrophic growth is initially beneficial in adapting cardiac function, prolonged states of adult hypertrophy are a leading predictor for the development of arrhythmias, sudden death, and heart failure (Levy et al., 1990; Ho et al., 1993). Both pathophysiologic and developmental hypertrophy of the myocardium are regulated by endocrine, paracrine, and autocrine growth factors that activate membrane-bound receptors resulting in signal transduction through discrete G-proteins and kinase cascades (for review see Molkentin and Dorn, 2001). In turn, intermediate signal transduction cascades regulate both cytoplasmic and transcriptional machinery to augment gene transcription and protein accumulation as part of the hypertrophic program.

The protein kinase C family of calcium and/or lipid-activated serine-threonine kinases function downstream of nearly all membrane-associated signal transduction pathways (Molkentin and Dorn, 2001). Approximately 12 different isozymes comprise the PKC family, which are broadly classified by their activation characteristics. The conventional PKC isozymes (PKCα, βI, βII, and γ) are calcium- and lipid-activated, whereas the novel isozymes (ε, θ, η, and δ) and atypical isozymes (ζ, ι, ν, and μ) are calcium independent but activated by distinct lipids (for review see Dempsey et al., 2000). Once activated, PKC isozymes translocate to discrete subcellular sites through direct interactions with docking proteins termed receptors for activated C kinases

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(RACKs)* (Mochly-Rosen, 1995), which permit specific substrate recognition and subsequent signal transduction. Different PKC isozymes or broad groups associate with unique RACKs as an important mechanism for regulating differential target specificities in vivo (Mochly-Rosen, 1995).

A number of reports have associated PKC activation with either cardiac hypertrophy, heart failure, ischemic injury, or agonist stimulation. For example, hemodynamic pressure overload stimulation in rodents can promote efficient translocation of PKCα, β, γ, ε, and θ (Gu and Bishop, 1994; Jalili et al., 1999; Takeishi et al., 1999; De Windt et al., 2000). In cultured cardiomyocytes, diverse agonist and stress stimuli are also potent stimulators of PKC isozyme translocation (Clerk et al., 1996; Goldberg et al., 1997; Gray et al., 1997; Sil et al., 1998; Rohde et al., 2000). Using pharmacologic inhibitors, PKC activation has been implicated in regulating molecular events associated with agonist-induced cardiomyocyte hypertrophy (Glembotski et al., 1993; Yamazaki et al., 1995; Schluter et al., 1997; Sil et al., 1998). Isozyme-specific peptide inhibitors have also been employed in cultured cardiomyocytes and in transgenic mice to afford greater specificity of PKC inhibition. Specifically, overexpression of a PKCβ C_2 domain peptide in cardiomyocytes blocked phorbol ester–mediated calcium channel activity (Zhang et al., 1997), while PKCε inhibitory peptide or activating peptide affected inotropy and ischemia-induced cellular injury (Johnson et al., 1996a; Gray et al., 1997; Dorn et al., 1999).

More recently, transgenic mice have been generated with altered PKC isozyme signaling in the heart. Overexpression of either wild-type or a constitutively active deletion mutant of PKCβ in a mouse heart was reported to induce cardiomyopathy (Bowman et al., 1997; Wakasaki et al., 1997), but more recent investigation has suggested that lower levels of expression or adult onset PKCβ activation benefits cardiac contractility and ischemic recovery (Tian et al., 1999; Huang et al., 2001). Three groups have also reported transgenic mice with altered PKCε activity in the heart. Expression of a PKCε-activating peptide in the mouse heart was associated with a physiologic activation of PKCε and an increase in myocyte cell number, but not cellular hypertrophy (Mochly-Rosen et al., 2000). In contrast, overexpression of an activated mutant PKCε in the mouse heart was reported to induce significant cardiac hypertrophy (Takeishi et al., 2000), but such a result is likely dependent on the absolute levels of PKCε overexpression and activity (Pass et al., 2001). Although a number of studies have demonstrated associations between various PKC isozymes and cardiac hypertrophy, the necessary and sufficient functions of specific PKC isozymes in the heart have not been established.

To evaluate potential functional divergence amongst PKC isozymes in regulating cardiomyocyte hypertrophy, cultured neonatal cardiomyocytes were subjected to adenoviral-mediated gene transfer of wild-type and dominant negative mutants of PKCα, βII, δ, ε, and ζ (ζ wild-type only). PKCα was the only isozyme tested that was capable of inducing a hypertrophic response characterized by enhanced sarcomeric organization, increased cell surface area, increased atrial natriuretic factor (ANF) expression, and increased [^{3}H]-leucine incorporation. Similarly, dominant negative PKCα, but not dominant negative βII, δ, and ε, suppressed agonist-

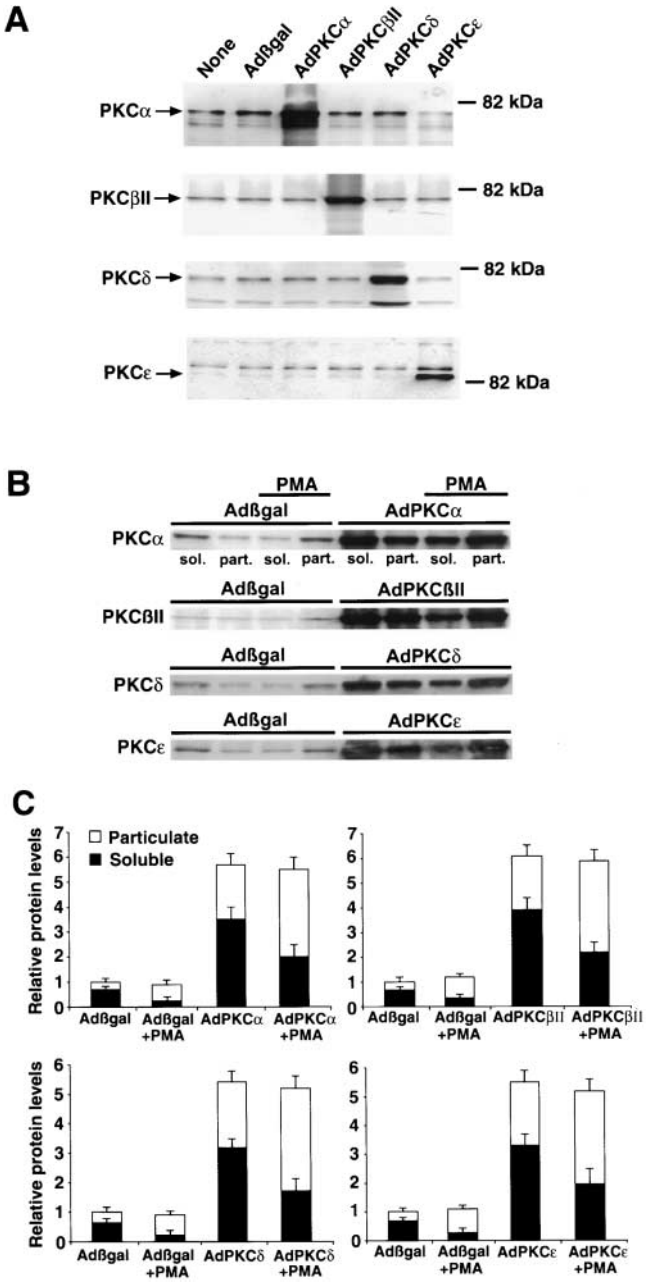

Figure 1. **Adenoviral-mediated gene transfer of wild-type PKCα, βII, δ, and ε in cardiomyocytes.** (A) Western blot analysis for antibody cross-reactivity from PKCα, βII, δ, and ε from AdPKCα, AdPKCβII, AdPKCδ, AdPKCε, or control Adβgal-infected neonatal cardiomyocytes. (B) Western blot analysis of cytosolic and particulate subcellular protein distribution of each PKC isoform at baseline or after stimulation with PMA. Distribution of both endogenous and overexpressed protein are shown. (C) Histogram representation of PKC particulate (open area) and soluble (dark area) protein levels demonstrates a 5–6-fold increase in protein expression ($P < 0.05$). Results were averaged from three independent experiments.

*Abbreviations used in this paper: ANF, atrial natriuretic factor; ERK1/2, extracellular signal–regulated kinase1/2; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; moi, multiplicity of infection; PE, phenylephrine; pfu, plaque forming units; RACK, receptor of activated C kinases.

induced cardiomyocyte hypertrophy. PKCα-dependent regulation of cardiomyocyte hypertrophy was shown to require extracellular signal–regulated kinase1/2 (ERK1/2) activation, suggesting a downstream mechanism of action. Collectively, these results implicate PKCα as a critical regulator of cardiomyocyte hypertrophic growth.

Results

Characterization of PKC isozyme subcellular localization

Adenoviral-mediated gene transfer into cultured neonatal cardiomyocytes was employed to evaluate the necessary and sufficient functions of selected PKC isozymes in regulating hypertrophic growth. Western blotting was performed from AdPKCα-, AdPKCβII-, AdPKCδ-, or AdPKCε-infected cardiomyocytes to verify the integrity of each PKC isozyme–encoding adenovirus, to quantify overexpression levels, and to evaluate antibody specificity (multiplicity of infection [moi] of 100 plaque forming units [pfu]/ml, resulting in >95% infectivity). The data showed no cross-reactivity between any of the four isozyme-specific antibodies used, although nonspecific bands were observed with PKCδ and PKCε antibody (Fig. 1 A). Western blot quantitation revealed a 5–7-fold increase in protein expression relative to endogenous levels (Fig. 1, B and C). To verify that adenoviral-mediated overexpression did not compromise PKC regulation or the ability to translocate in cardiomyocytes, membrane (particulate), and cytosolic (soluble) protein extracts were generated and subjected to Western blot analysis. The data demonstrate that PMA (200 nM) induced a similar profile of cytosolic and membrane-associated protein redistribution between endogenous and overexpressed PKC isozymes (Fig. 1, B and C). These results indicate that adenoviral-mediated PKC overexpression gives the same ratio of membrane and cytosolic localization as compared with their endogenous isozyme. However, wild-type PKC isozyme overexpression also increased the total PKC localization to the membrane protein fraction at baseline, suggesting increased activity (Fig. 1, B and C).

Characterization of PKCα, βII, δ, and ε subcellular localization is currently an area of ongoing investigation, which has been complicated by inadequate antibodies, epitope masking, and by low levels of endogenous protein in cardiomyocytes. To further evaluate the subcellular localization and redistribution profile of PKCα, βII, δ, and ε, immunocytochemistry was performed in cardiomyocytes infected with each PKC-expressing adenovirus. However, the specificity of each PKC isozyme antibody was first evaluated by immunocytochemistry in AdPKCα-, AdPKCβII-, AdPKCδ-, and AdPKCε-infected cardiomyocytes, which revealed no cross-reactivity and further validated the integrity of each antibody (Fig. 2 A). At baseline, PKCα, βII, and ε were each broadly distributed in a diffuse pattern in unstimulated cardiomyocytes, consistent with their predominant localization to the cytosolic protein fraction (Fig. 2 B). PKCδ also demonstrated a diffuse pattern of localization at baseline, although a significant concentration was observed surrounding the nucleus (Fig. 2 B). After 30 min of PMA or phenylephrine (PE) stimulation, each PKC isozyme demonstrated a distinct redistribution pattern

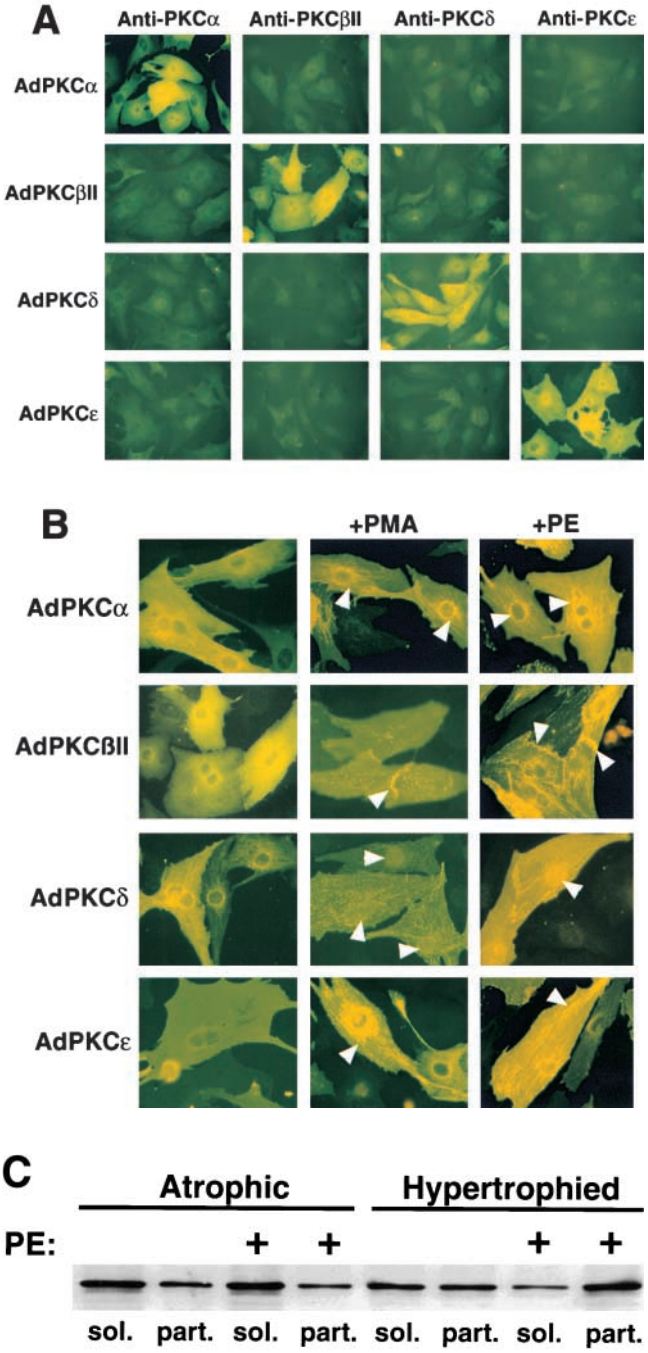

Figure 2. **Adenoviral-mediated gene transfer of PKCα, βII, δ, and ε in neonatal cardiomyocytes demonstrates distinct subcellular localization and translocation after agonist treatment.** (A) Immunocytochemical analysis of PKC antibody specificity from AdPKCα-, AdPKCβII-, AdPKCδ-, and AdPKCε-infected cardiomyocytes. Antibodies are shown on the top while viral infection is shown on the left. (B) PKC isozyme distribution was characterized before and after PMA or PE treatment. Similar results were obtained in three independent experiments. The arrows show areas of significant redistribution after agonist stimulation. (C) PKCα shows efficient translocation to the particulate fraction only in previously hypertrophied cardiomyocytes.

(Fig. 2 B). Specifically, PKCα protein became localized in a perinuclear pattern and with a microtubule-like network (see below). PKCβII protein translocated to a punctate pattern throughout the cell with focal concentrations at cell–cell con-

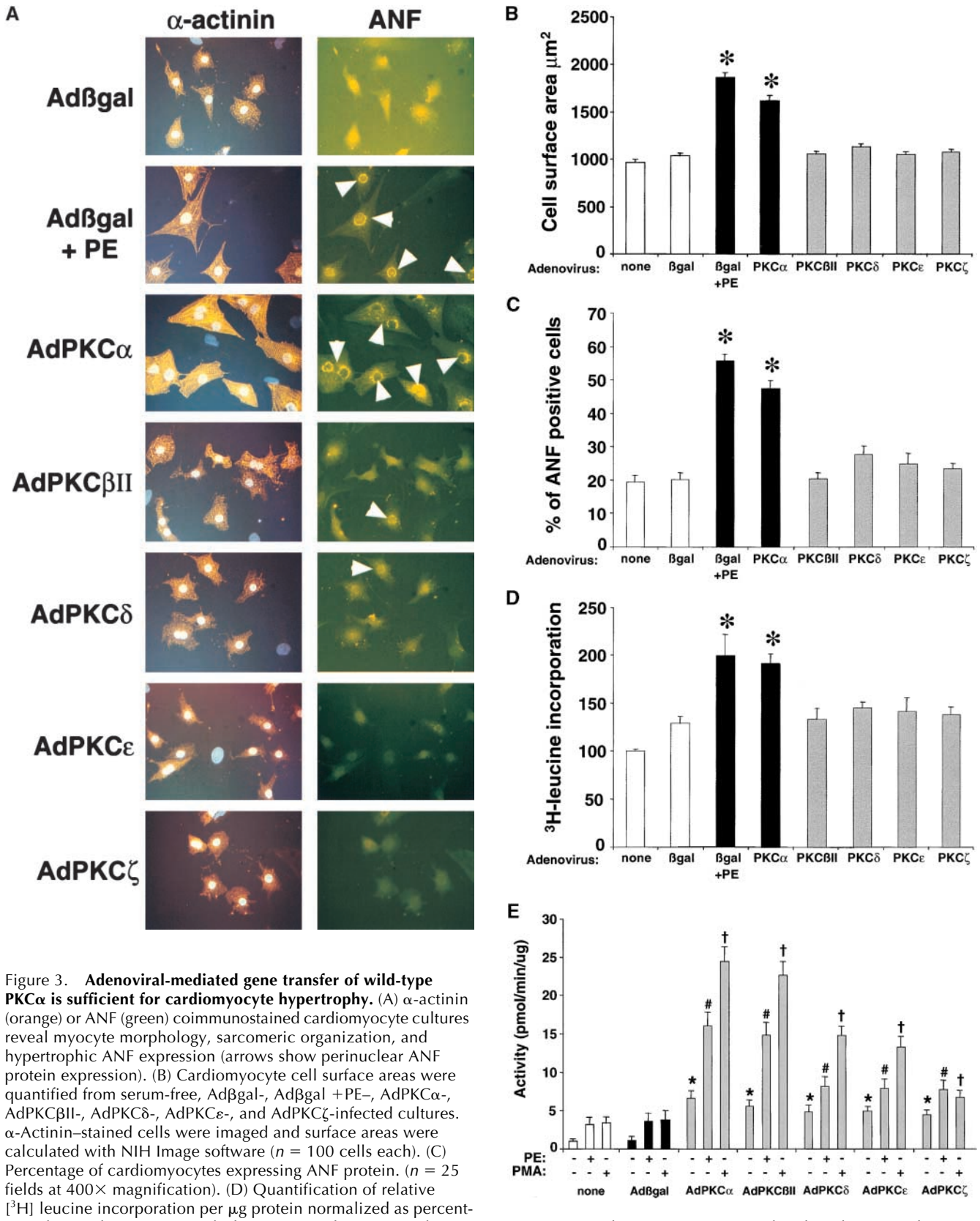

Figure 3. **Adenoviral-mediated gene transfer of wild-type PKCα is sufficient for cardiomyocyte hypertrophy.** (A) α-actinin (orange) or ANF (green) coimmunostained cardiomyocyte cultures reveal myocyte morphology, sarcomeric organization, and hypertrophic ANF expression (arrows show perinuclear ANF protein expression). (B) Cardiomyocyte cell surface areas were quantified from serum-free, Adβgal-, Adβgal +PE–, AdPKCα-, AdPKCβII-, AdPKCδ-, AdPKCε-, and AdPKCζ-infected cultures. α-Actinin–stained cells were imaged and surface areas were calculated with NIH Image software ($n = 100$ cells each). (C) Percentage of cardiomyocytes expressing ANF protein. ($n = 25$ fields at 400× magnification). (D) Quantification of relative [^{3}H] leucine incorporation per μg protein normalized as percentage of control. (E) PKC-specific kinase assay shows a significant increase in activity from overexpression of each isoform. Results were averaged from three independent experiments for A–D, and a representative experiment is shown for E. $^*P < 0.05$ vs. Adβgal, $^{\#}P < 0.05$ vs. Adβgal + PE, $^{\dagger}P < 0.05$ vs. Adβgal + PMA.

tacts. PKCδ demonstrated a significant redistribution of protein to within the nucleus, whereas PKCε showed an increased association with the sarcomeres (Fig. 2 B). Collectively, the unique patterns of PKCα, βII, δ, and ε subcellular redistribution in neonatal cardiomyocytes suggests different regulatory roles for each isozyme.

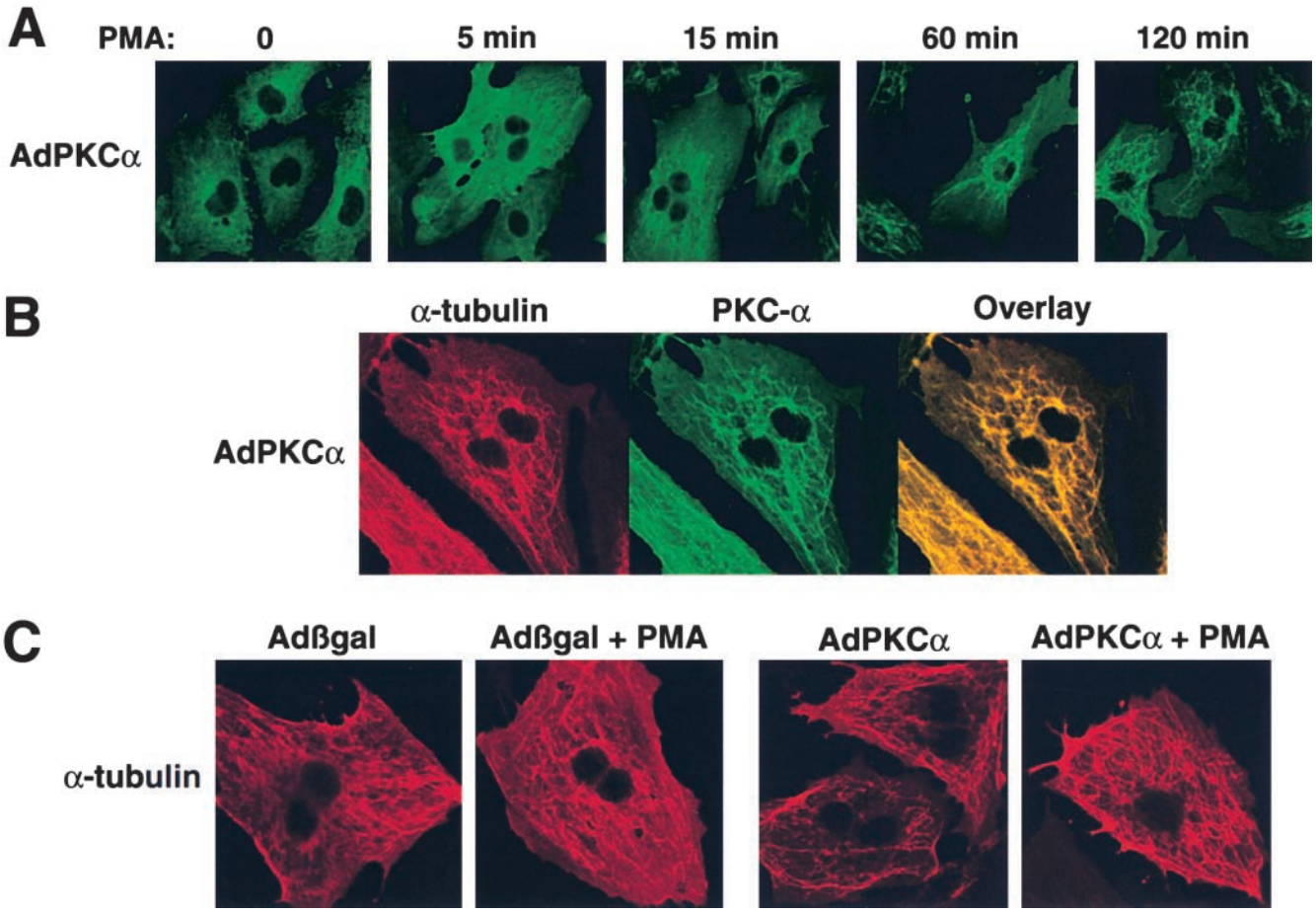

Figure 4. **Adenoviral-mediated gene transfer of PKCα in rat neonatal cardiomyocytes colocalizes with α-tubulin.** (A) Immunocytochemical analysis of PKCα translocation at progressive times after PMA stimulation. (B) Costaining for PKCα (green) and α-tubulin (red) demonstrates coincident immunoreactivity. (C) As a control, neither AdPKCα, Adβgal, nor PMA altered the normal pattern of α-tubulin localization. Similar results were observed in three independent experiments.

We also attempted to examine the subcellular localization and redistribution pattern of endogenous PKCα, βII, δ, and ε in cardiomyocytes. However, only endogenous PKCδ and PKCα were reasonably detectable using standard immunocytochemical techniques and the specific antibodies used here (Fig. 2 A). Although somewhat faint, PKCδ and PKCα antibody reactivity was observed in a nuclear and perinuclear pattern, respectively, consistent with their localization pattern observed by overexpression (Fig. 2 A). Endogenous PKCβII and PKCε were not readily detected in neonatal cardiomyocytes using the antibodies described here (see Discussion).

The observation that PE induced PKCα translocation by immunocytochemistry is in contrast to four previous reports that failed to identify significant PKCα translocation to the particulate fraction after either PE or endothelin-1 stimulation of cardiomyocytes (Clerk et al., 1994, 1996; Deng et al., 1998; Hayasaki-Kajiwara et al., 1999). However, our analysis of translocation was performed in previously hypertrophied cardiomyocytes (stimulated with 2% fetal bovine serum for 24 h), which act in a more physiologic manner compared with smaller atrophic myocytes. Indeed, PE stimulation did not promote translocation of PKCα in atrophic myocytes, but significant translocation was readily observed in previously hypertrophied myocytes (Fig. 2 C, last lane) (see Discussion).

PKCα uniquely induces hypertrophic growth

Although a large number of studies have demonstrated activation of PKC isozymes in association with cardiac hypertrophic growth, evidence of direct causality has not been established. To this end, each PKC isozyme was overexpressed in neonatal cardiomyocytes by adenoviral infection to evaluate their ability to induce hypertrophic growth. In serum-free medium, neonatal cardiomyocytes infected with a control β-galactosidase–expressing adenovirus (Adβgal) showed an atrophic phenotype without significant ANF protein expression (Fig. 3 A). However, α-adrenergic agonist (PE) stimulation invoked sarcomeric organization, an increase in cell size, and ANF protein expression (perinuclear) (Fig. 3 A). Interestingly, overexpression of PKCβII, δ, ε, and ζ did not stimulate hypertrophic growth or ANF expression after 48 h (Fig. 3 A). In contrast, overexpression of wild-type PKCα promoted significant sarcomeric organization, increased cell size, and increased ANF protein expression (Fig. 3 A). Quantitation of three independent experiments demonstrated significantly greater cell surface area, percentage of cells expressing ANF, and [^{3}H]-leucine incorporation in AdPKCα, but not AdPKCβII-, AdPKCδ-, AdPKCε-, or AdPKCζ-infected neonatal cardiomyocytes (Fig. 3, B–D) ($P < 0.05$). Al-

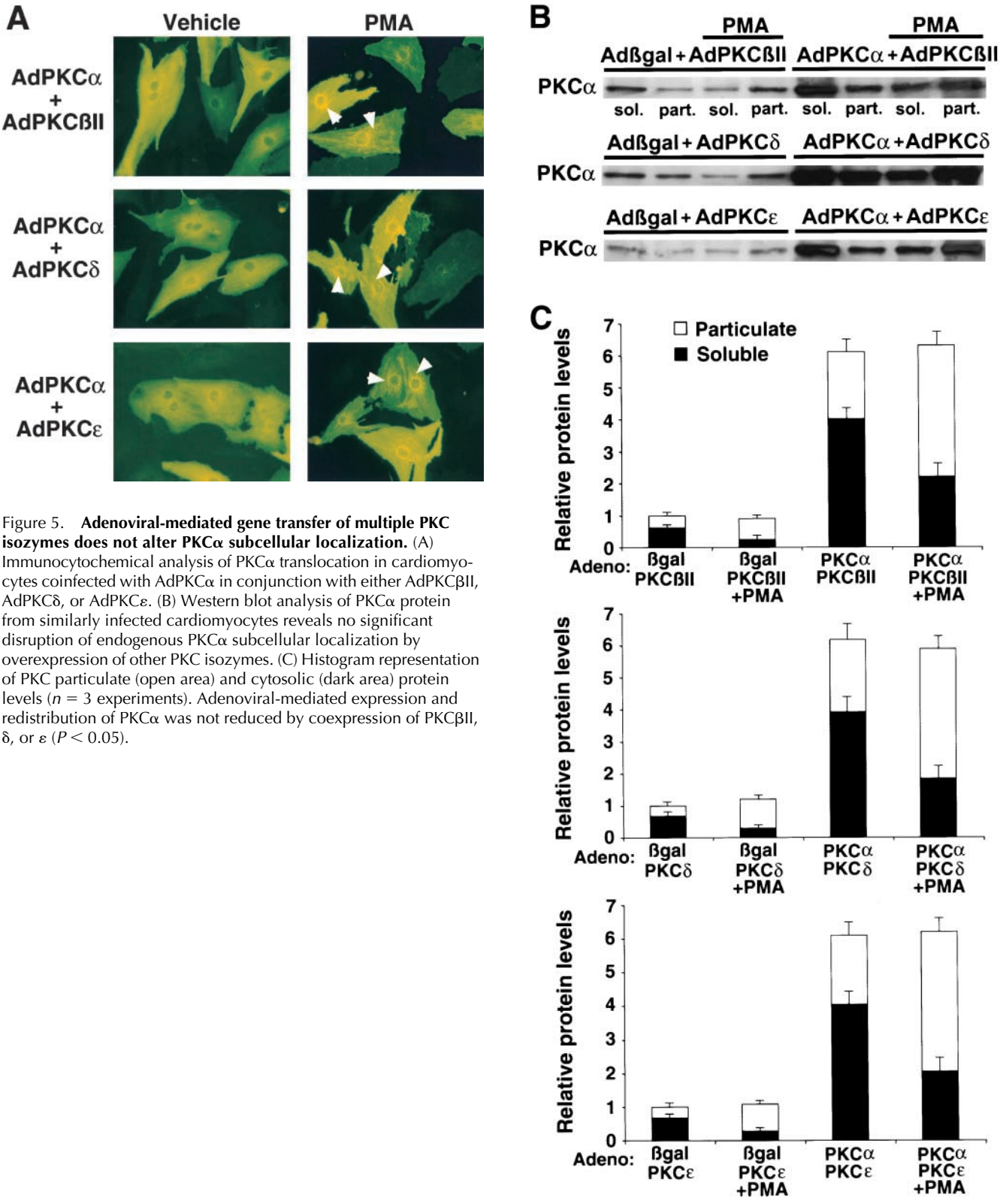

Figure 5. **Adenoviral-mediated gene transfer of multiple PKC isozymes does not alter PKCα subcellular localization.** (A) Immunocytochemical analysis of PKCα translocation in cardiomyocytes coinfected with AdPKCα in conjunction with either AdPKCβII, AdPKCδ, or AdPKCε. (B) Western blot analysis of PKCα protein from similarly infected cardiomyocytes reveals no significant disruption of endogenous PKCα subcellular localization by overexpression of other PKC isozymes. (C) Histogram representation of PKC particulate (open area) and cytosolic (dark area) protein levels ($n = 3$ experiments). Adenoviral-mediated expression and redistribution of PKCα was not reduced by coexpression of PKCβII, δ, or ε ($P < 0.05$).

though these results suggest that PKCα is a unique inducer of cardiomyocyte growth, it was also important to verify the integrity of each adenoviral-expressed isozyme. To this end, PKC-specific enzymatic assays were performed from AdPKCα-, AdPKCβII-, AdPKCδ-, AdPKCε-, and AdPKCζ-infected cardiomyocytes, which each demonstrated an ~5-fold increase in kinase activity compared with no infection or Adβgal infection ($P < 0.05$) (Fig. 3 E). In addition, control and AdPKC-infected cardiomyocytes were also stimulated with PE or PMA for 30 min to evaluate induction of kinase activity. PMA induced a further ~7-fold increase in kinase activity in AdPKCα and AdPKCβII infected cardiomyocytes, while AdPKCδ and AdPKCε infected cells showed an ~3-fold increase in kinase activity

($P < 0.05$) (Fig. 3 E). A similar profile, albeit less robust, was observed after PE stimulation (Fig. 3 E).

Activated PKCα colocalizes with α-tubulin in neonatal cardiomyocytes

Since PKCα appeared to uniquely induce cardiomyocyte hypertrophy in culture, it was of interest to more carefully examine its subcellular localization. A time course of PMA-induced redistribution of PKCα was first performed, which demonstrated detectable movement by 15 min of stimulation that reached a maximum by 60 min (Fig. 4 A). The observed localization of activated PKCα suggested an association with a filamentous or tubular network. Indeed, activated PKCα localization was coincident with α-tubulin as assessed by confocal microscopy with individual antibodies (PKCα is green and α-tubulin is red) (Fig. 4 B). As an important control, neither Adβgal infection, AdPKCα infection, nor PMA stimulation altered the endogenous pattern of α-tubulin localization, suggesting that the observed colocalization between activated PKCα and α-tubulin does not result from alterations in α-tubulin localization (Fig. 4 C).

PKCα localization is not affected by PKCβII, δ, or ε overexpression

Overexpression of PKCα, βII, δ, and ε revealed distinct subcellular localizations in cardiomyocytes, suggesting unique docking complexes between each of these PKC isozymes. However, it was of concern that overexpression of one PKC isozyme might influence the docking and subcellular distribution of other isozymes. To control for secondary effects associated with isozyme-specific overexpression, AdPKCα was coinfected with either AdPKCβII, AdPKCδ, or AdPKCε and the ability of PKCα to undergo the proper redistribution was analyzed by immunocytochemistry. Overexpression of PKCβII, δ, and ε did not affect PKCα localization in unstimulated cardiomyocytes, nor did it affect PMA-induced PKCα redistribution to the tubulin-associated network (Fig. 5 A) (moi 100 pfu/ml).

To further assess potential nonspecific effects associated with overexpression, Western blotting was performed using coinfected cardiomyocytes at baseline or after PMA stimulation. Such an analysis was performed for both endogenous and overexpressed PKCα in the presence of PKCβII, δ, and ε overexpression (Fig. 5 B). Specifically, the translocation of endogenous PKCα in response to PMA was not affected by overexpression of PKCβII, δ, and ε (Fig. 5 B). Furthermore, overexpressed PKCα also demonstrated a similar profile of PMA-induced redistribution, even in the presence of PKCβII, δ, and ε overexpression (Fig. 5 B). Identical results were observed and quantified in three independent experiments (Fig. 5 C). Collectively, these results indicate that specific PKC isozyme overexpression does not significantly alter the activity or other PKC isozymes. These results further suggest that the hypertrophic response induced by PKCα overexpression is likely an intrinsic function of this isozyme and not the result of indirect effects on other PKC isozymes.

PKCα is uniquely required for cardiomyocyte hypertrophy

It was of interest to examine the requirement of PKC isozyme–specific activities as necessary mediators of agonist-induced cardiomyocyte hypertrophic growth. Accordingly, adenoviral vectors expressing dominant negative mutants of PKCα, βII, δ, and ε were used in cardiomyocytes. Each dominant negative mutant encodes the full-length protein, but contains a single amino substitution in a critical ATP binding residue, rendering the kinase inactive (Ohba et al., 1998; Matsumoto et al., 2001). Previous work has demonstrated that such mutations in PKC generate effective dominant negative proteins (Li et al., 1996; Hong et al., 1999; Pass et al., 2001; Strait et al., 2001). As assessed by Western blotting from three independent experiments, adenoviral infection at an moi of 100 pfu/ml resulted in ~7–9-fold higher levels of each dominant negative isozyme 48 h postinfection compared with the wild-type isozymes (Fig. 6, A and B).

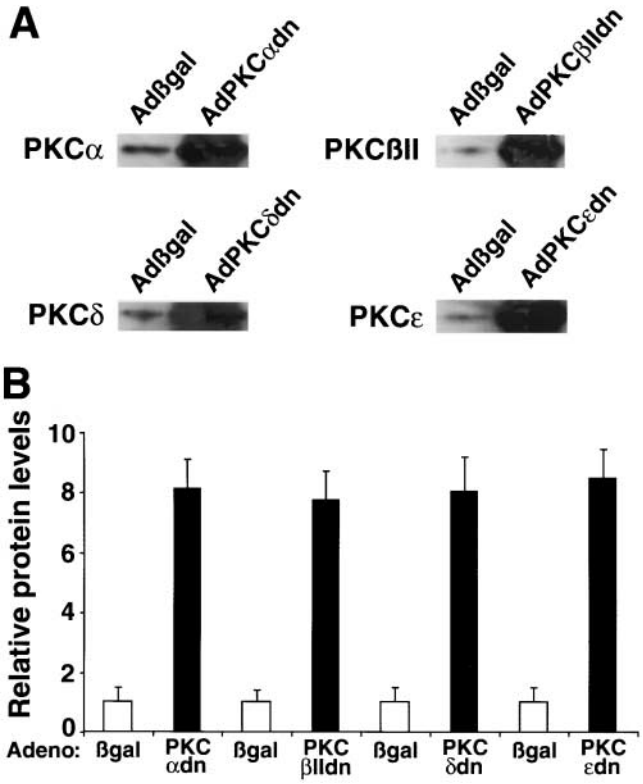

Figure 6. **Adenoviral-mediated gene transfer of dominant negative PKCα, βII, δ, and ε in neonatal cardiomyocytes.** (A) Western blot analysis of protein expression levels from AdPKCαdn-, AdPKCβIIdn-, AdPKCδdn-, or AdPKCεdn-infected cardiomyocytes. (B) Histogram representation of total PKC isozyme protein levels demonstrates a 7–9-fold increase in protein after adenoviral infection compared with endogenous wild-type protein levels ($n = 3$).

To examine the necessity of PKC isozyme activation in regulating hypertrophic growth, cardiomyocytes overexpressing each dominant negative isozyme were stimulated with the α-adrenergic agonist PE. As a control, a mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1)-expressing adenovirus was included as an established inhibitor of agonist-induced hypertrophy through MAPK blockade (Bueno et al., 2001). The data demonstrate that AdPKCαdn and AdPKCεdn each attenuated or blocked PE-induced sarcomeric organization, increased surface area, ANF protein expression, and [^{3}H]-

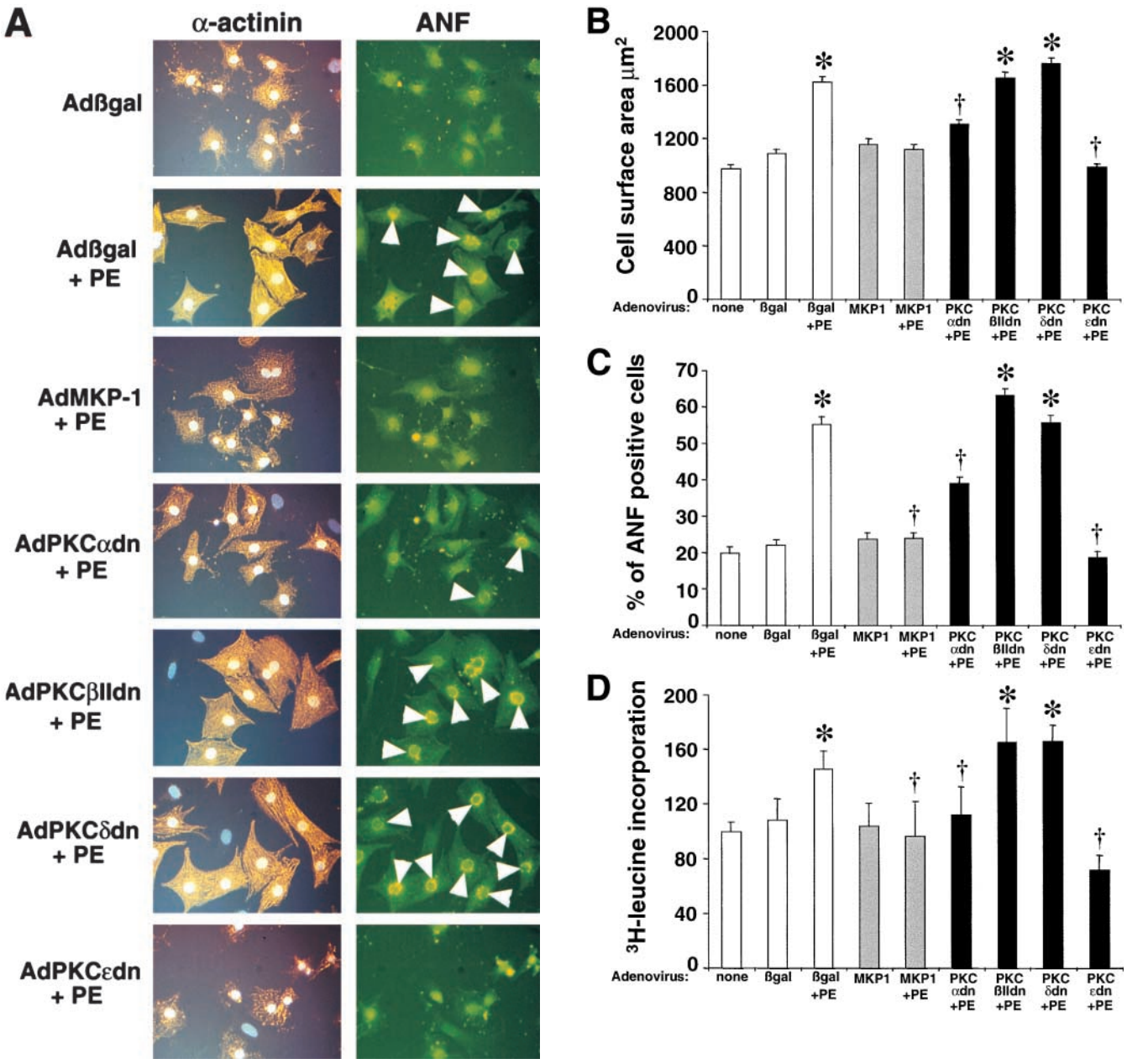

Figure 7. **Assessment of hypertrophy in cardiomyocytes expressing dominant negative PKC isozymes.** (A) α-Actinin (orange) and ANF (green) coimmunostained cardiomyocyte cultures. (B) Cardiomyocyte cell surface area was quantified from serum-free, Adβgal-, Adβgal + PE–, AdMKP-1–, AdMKP-1 + PE–, AdPKCαdn + PE–, AdPKCβIIdn + PE–, AdPKCδdn + PE–, and AdPKCεdn + PE–infected cultures. α-Actinin–stained cells were imaged with confocal microscopy and digitized, and surface areas were calculated with NIH Image software ($n = 100$ cells each). (C) Percentage of cardiomyocytes expressing ANF protein ($n = 25$ fields at 400× magnification). (D) Quantification of [^{3}H] leucine incorporation per μg protein. All results were obtained in three independent experiments. *$P < 0.05$ vs. Adβgal. †$P < 0.05$ vs. Adβgal + PE.

leucine incorporation (Fig. 7, A–D). In contrast, AdPKCβIIdn and AdPKCδdn infection did not attenuate any parameter of PE-induced cardiomyocyte hypertrophy (Fig. 7, A–D). Although dominant negative PKCε overexpression inhibited hypertrophy, a significant loss of infected myocytes was observed, suggesting a primary effect on cell viability, resulting in a secondary effect on hypertrophy. Indeed, AdPKCεdn infection enhanced cardiomyocyte apoptosis as assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) and poly(ADP-ribose) polymerase (PARP) protein cleavage, suggesting that PKCε activity is normally required to maintain cardiomyocyte viability in culture (unpublished data). However, an important observation is that PKCα acts as a necessary mediator of cardiomyocyte hypertrophic growth in culture without negatively affecting cell viability.

The mechanism of PKCα-induced hypertrophy utilizes ERK1/2 MAPK

The mechanism whereby overexpression of PKCα promotes cardiomyocyte hypertrophy is uncertain. However, recent

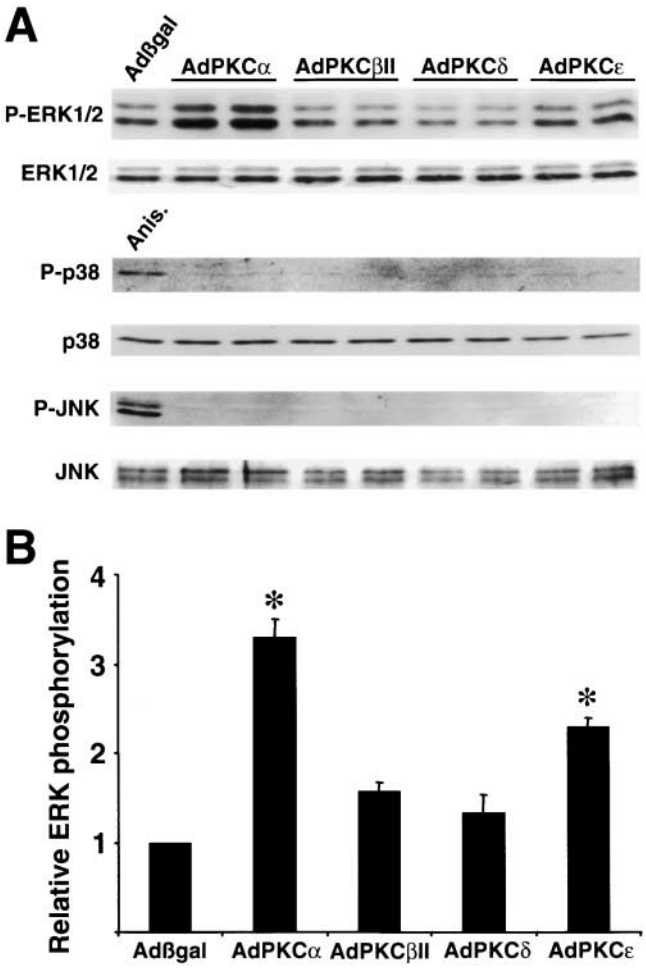

Figure 8. **Overexpression of PKCα and ε induces ERK1/2 phosphorylation.** (A) Western blot analysis for ERK1/2, p38, and JNK wild-type and phosphorylated forms in AdPKC-infected cardiomyocytes. Anisomycin was used as a control for JNK and p38 activation only. (B) Quantitation from three independent experiments demonstrates significant ERK1/2 activation induced by AdPKCα and ε infection compared with Adβgal infection. $^*P < 0.05$ vs. Adβgal.

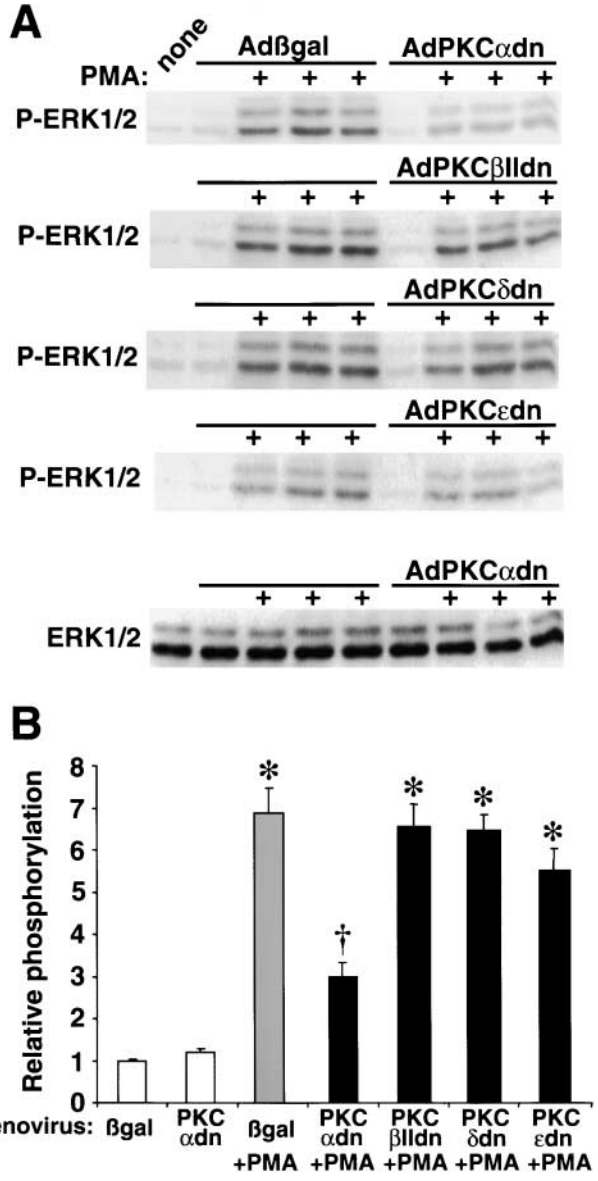

Figure 9. **AdPKCαdn inhibits PMA-induced ERK1/2 phosphorylation in neonatal cardiomyocytes.** (A) Western blot analysis of phosphorylated ERK1/2 from AdPKCαdn-, AdPKCβIIdn-, AdPKCδdn-, and AdPKCεdn-infected cardiomyocytes before and after PMA treatment. The amount of total ERK1/2 did not vary. (B) Histogram showing a significant decrease in phosphorylated ERK1/2 only with AdPKCαdn infection ($n = 3$). $^*P < 0.05$ vs. Adβgal; $^\dagger P < 0.05$ vs. Adβgal + PMA.

investigation in multiple cell types has demonstrated cross-talk between PKC and MAPK-signaling pathways (Kolch et al., 1993; Clerk et al., 1994; Schonwasser et al., 1998; Ping et al., 1999; Rohde et al., 2000; Strait et al., 2001). Accordingly, the activation status of ERK1/2, p38, and c-Jun NH_2-terminal kinase (JNK) were analyzed in neonatal cardiomyocytes overexpressing each wild-type PKC isozyme. Western blot analyses showed a 3.3-fold increase in ERK1/2 phosphorylation in PKCα overexpressing cardiomyocytes and a mild increase associated with PKCε overexpression (Fig. 8, A and B). In contrast, PKCβII and δ overexpression did not significantly induce ERK1/2 phosphorylation in neonatal cardiomyocytes (Fig. 8, A and B). Analysis of p38 and JNK phosphorylation demonstrated no activation associated with any PKC isozyme overexpression in cardiomyocytes, although control experiments with anisomycin-treated, Adβgal-infected myocytes showed activation (Fig. 8 A). Collectively, these results indicate that PKCα and ε each uniquely cross-talk with the ERK1/2 MAPK signaling pathway in cardiomyocytes. The prominent activation of ERK1/2 associated with PKCα overexpression also suggests a mechanism of hypertrophic growth regulation (see below).

The interconnectivity between PKC isozymes and MAPK was further evaluated in PMA-stimulated cardiomyocytes infected with AdPKCαdn, AdPKCβIIdn, AdPKCδdn, or AdPKCεdn. Interestingly, immunoblotting with phosphorylation-specific antibodies demonstrated a partial inhibition of ERK1/2 activation in dominant negative PKCα-expressing cardiomyocytes (Fig. 9 A). In contrast, overexpression of dominant negative PKCβII, δ, and ε did not significantly attenuate ERK1/2 activation (Fig. 9 A). Results from three independent experiments demonstrated a 2.5-fold reduction in ERK1/2 activation in AdPKCαdn-infected cardio-

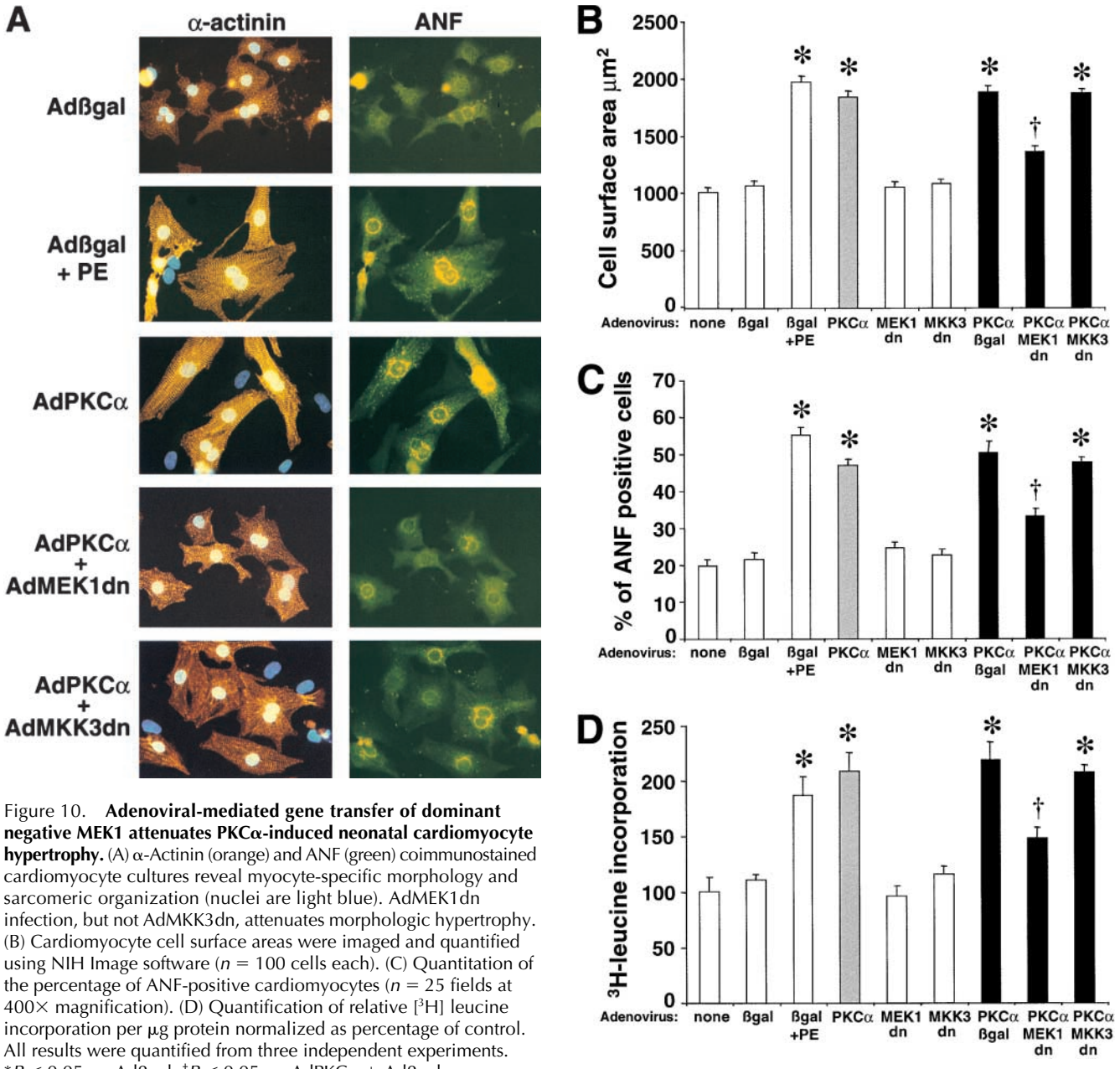

Figure 10. **Adenoviral-mediated gene transfer of dominant negative MEK1 attenuates PKCα-induced neonatal cardiomyocyte hypertrophy.** (A) α-Actinin (orange) and ANF (green) coimmunostained cardiomyocyte cultures reveal myocyte-specific morphology and sarcomeric organization (nuclei are light blue). AdMEK1dn infection, but not AdMKK3dn, attenuates morphologic hypertrophy. (B) Cardiomyocyte cell surface areas were imaged and quantified using NIH Image software ($n = 100$ cells each). (C) Quantitation of the percentage of ANF-positive cardiomyocytes ($n = 25$ fields at 400× magnification). (D) Quantification of relative [^{3}H] leucine incorporation per μg protein normalized as percentage of control. All results were quantified from three independent experiments. *$P < 0.05$ vs. Adβgal; †$P < 0.05$ vs. AdPKCα + Adβgal.

myocytes, but no significant inhibition by AdPKCβIIdn, AdPKCδdn, or AdPKCεdn (Fig. 9 B). p38 and JNK activation were not affected by any of the four dominant negative PKC isozymes (unpublished data). Together, these results further suggest cross-talk between PKCα and ERK1/2 as part of the mechanism responsible for cardiomyocyte hypertrophic growth.

To examine the functional consequence of PKCα and ERK1/2 cross-talk in regulating cardiomyocyte hypertrophic growth, AdPKCα was coinfected with an adenovirus expressing a dominant negative mutant of MEK1 (AdMEK1dn). An identical dominant negative MEK1-encoding adenovirus was shown previously to inhibit ERK1/2 activity and to block agonist-induced neonatal cardiomyocyte hypertrophy in culture (Ueyama et al., 2000). Remarkably, AdMEK1dn attenuated AdPKCα-mediated cardiomyocyte hypertrophy as assessed by gross morphology, cell surface area, ANF protein expression, and [^{3}H]-leucine expression (Fig. 10, A–D). In contrast, a dominant negative MKK3-expressing adenovirus, which blocks p38 activation (Li et al., 2001), did not reduce AdPKCα-induced cardiomyocyte hypertrophy (Fig. 10, A–D). These results indicated that PKCα regulates cardiomyocyte hypertrophic growth, in part through ERK1/2 MAPK activation.

Discussion

PKC isozymes demonstrate unique subcellular localizations

Previous analyses of PKCα, βII, δ, and ε subcellular localization in neonatal cardiomyocytes have shown both similar and

dissimilar patterns of localization to the data shown in this report. Mochly-Rosen and colleagues demonstrated endogenous PKCα localization to the perinuclear region of neonatal cardiomyocytes, although an association with microtubules was not reported (Disatnik et al., 1994; Johnson et al., 1996a). Such a pattern of redistribution is interesting given the known role of microtubules in trafficking intracellular organelles, vesicles, and large protein complexes between the nucleus and cell membrane. An association with assembled α-tubulin might suggest a role for PKCα in either regulating intracellular trafficking during the hypertrophic response, or that PKCα interacts with other regulatory factors that also associate with microtubules. That activated PKCα prominently redistributes to the perinuclear region (continuous with the endoplasmic reticular membrane) also suggests a potential regulatory role given the number of important signaling factors that have been localized to the perinuclear region (Cdc42, Pyk2, and cytosolic phospholipase A2) (Hirabayashi et al., 1999; Klingbeil et al., 2001; Murphy et al., 2001). More significantly, the perinuclear region is an important site of reactive calcium regulation through the inositol triphosphate receptor (Guihard et al., 1997). Collectively, these observations suggest that PKCα redistribution is associated with sites of reactive signaling and altered calcium handling.

Mochly-Rosen and colleagues also showed PKCδ localization to the nucleus and perinucleus, whereas PKCε was localized in a cross-striated pattern in neonatal cardiomyocytes (Johnson et al., 1996a; Dorn et al., 1999). Although we identified a similar pattern of PKCε localization and redistribution, the pattern of PKCδ translocation was somewhat different. Here it was shown that PKCδ normally resides in the perinuclear membrane in unstimulated neonatal cardiac myocytes, but stimulation with PMA promoted movement out of the perinuclear region and into the nucleus itself (Fig. 2). PKCβII was shown previously to redistribute to the perinuclear area and to filamentous structures at the periphery of the neonatal cardiomyocyte (Disatnik et al., 1994). We also observed redistribution to the perinuclear region and the periphery of the cell, but a significant concentrations of PKCβII was also observed at cell-to-cell contacts upon stimulation with PMA in neonatal cardiomyocytes (Fig. 2). Although all four isozyme-specific antibodies readily detected their respective isozyme when overexpressed without loss of specificity, endogenous PKCβII and ε were not readily detected despite positive accounts in the literature, suggesting either low protein abundance, partial epitope masking, or that previous descriptions may have employed antibodies with different specificity.

That PKCα, βII, δ, and ε each demonstrate unique subcellular localizations and redistribution patterns upon activation suggests regulation by isozyme-specific docking complexes. Indeed, PKCα, βII, δ, and ε have each been shown to contain unique interacting domains in their NH_2 terminus that provide specificity for anchoring proteins (RACKs), which regulate the specificity of substrate phosphorylation (for review see Mackay and Mochly-Rosen, 2001). A similar paradigm of isozyme-specific actions was also observed in cultured rat pituitary cells in which overexpression of PKCα, βII, δ, and ε revealed a unique function for PKCε in regulating prolactin secretion (Akita et al., 1994). Collectively, numerous studies suggest that overexpression approaches can be employed to dissect isozyme-specific functions of PKC factors given the high degree of fidelity by which docking and substrate recognition are controlled in vivo.

PKCα uniquely induces neonatal cardiomyocyte hypertrophic growth

Data implicating PKC isozymes as a regulators of cardiomyocyte hypertrophy have largely been derived by association. Specifically, agonist-induced cardiomyocyte hypertrophy has been shown to activate a diverse array of intracellular signaling factors, including PKC (for review see Molkentin and Dorn, 2001). To date, direct evidence implicating a specific PKC isozyme as a dominant regulator of myocyte hypertrophic growth is lacking. In culture, permeabilized neonatal cardiomyocytes treated with a generalized PKC pseudo-substrate peptide demonstrated reduced [^{14}C]phenylalanine incorporation, suggesting a necessary role of PKC signaling in general (Johnson et al., 1996b). More recently, adenoviral-mediated overexpression of a constitutively active mutant of PKCε was reported to enhance hypertrophic marker gene expression and to increase cell length, but interestingly, overexpression did not increase cell surface area or protein-to-DNA ratio (Strait et al., 2001). In this report, PKCα, but not PKCβII, δ, ε, or ζ, induced cardiomyocyte hypertrophic growth characterized by increased cell surface area, [^{3}H]leucine incorporation and ANF expression.

It was of concern whether simple overexpression of a wild-type PKC isozyme would function similar to an activation event of that particular endogenous isozyme. Overexpression studies of other wild-type signaling factors have demonstrated efficacy through a net increase in both the activated and inactivated forms of the given factor, such that the inactivated state is innocuous while the activated state is functional. For example, overexpression of wild-type Gαq or Gαs in the mouse heart was sufficient to activate downstream targets (Iwase et al., 1996; D'Angelo et al., 1997). Consistent with these reports, overexpression of each wild-type PKC isozyme by adenoviral gene transfer produced a 5–7-fold increase in particulate association, suggesting greater activation by mass action.

Additional data examining PKC isozyme functions in regulating myocyte growth came from the use of transgenic mice expressing PKCβ or PKCε in the heart. Overexpression of wild-type or an activated deletion mutant of PKCβ in the mouse heart was initially reported to induce a hypertrophic response (Bowman et al., 1997; Wakasaki et al., 1997). However, more recent investigation of PKCβ transgenic mice suggests that physiologic activation of this isozyme in the adult heart does not promote cardiomyopathy and actually benefits cardiac contractility and ischemic recovery (Tian et al., 1999; Huang et al., 2001). Indeed, targeted disruption of the PKCβ gene in the mouse did not affect the ability of these hearts to undergo hypertrophic growth (Roman et al., 2001). Similar disparity of interpretation surrounds the effect of PKCε in the hearts of transgenic mice. Expression of a PKCε activating peptide in the mouse heart, which promoted a physiologic activation of PKCε, did not result in cellular hypertrophy (Mochly-Rosen et al., 2000). In contrast, a second group reported that overexpres-

sion of an activated PKCε mutant protein in the mouse heart promoted cardiac hypertrophy (Takeishi et al., 2000). However, a third group demonstrated that high levels of PKCε overexpression promoted hypertrophy and cardiomyopathy, while more physiologic levels of expression were beneficial to the heart and rendered it resistant to myocardial ischemia (Pass et al., 2001). In general, these previous studies suggest a level of uncertainty as to whether PKCβ or PKCε regulates the hypertrophic growth of cardiac myocytes.

PKCα is required for neonatal myocyte growth

Overexpression of dominant negative mutants of PKCα, βII, δ, and ε in growth-stimulated neonatal cardiomyocytes suggested a necessary role for PKCα and PKCε. Such data implicate critical roles for these two latter isozymes as regulators of myocyte hypertrophy. However, AdPKCεdn infection promoted significant terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) and poly(ADP-ribose) polymerase (PARP) protein cleavage, suggesting induction of apoptosis (unpublished data). Consistent with this interpretation, high levels of PKCε inhibitory peptide expression in the mouse heart by transgenesis induced a lethal cardiomyopathy, suggesting a critical role for basal signaling through PKCε in maintaining cellular homeostasis (Mochly-Rosen et al., 2000). Such results suggest that the inhibition of cardiomyocyte hypertrophic growth observed with AdPKCεdn is secondary to the loss of cell viability. In contrast, overexpression of dominant negative PKCα did not affect cardiomyocyte viability, suggesting that inhibition of hypertrophic growth is a direct mechanism and not due to the health of the cells. These results are also consistent with the unique ability of PKCα to induce myocyte hypertrophic growth.

The dominant negative PKC mutations consist of a lysine to arginine substitution in the ATP binding domain that render the kinases inactive but still able to interact with endogenous RACKs. Indeed, an identical PKCε dominant negative mutation (K to R) was recently employed in cardiac-specific transgenic mice, which demonstrated competition with wild-type PKCε for RACK2 binding sites in vivo (Pass et al., 2001). Overexpression of dominant negative PKCε was also shown to only influence the translocation and activity of endogenous PKCε, and not other PKC isozymes (Strait et al., 2001). In other cell types, similar ATP binding site mutations produced potent and isozyme-specific dominant negative regulatory effects (Li et al., 1996; Hong et al., 1999; Pass et al., 2001; Strait et al., 2001). The above studies suggest that overexpression of kinase dead, dominant negative mutants of each PKC isozyme specifically antagonizes the activity of its cognate wild-type isozyme in vivo.

PKCα regulates myocyte growth, in part, through ERK MAPK

The interconnectivity between PKC isozymes and MAPK signaling branches has been previously reported in many cell-types. In cardiac myocytes, dominant negative PKCε was shown to down-regulate endothelin-1–induced ERK1/2 activation (Strait et al., 2001), and in another study, agonist-induced ERK1/2 MAPK activation was correlated with PKCε activation (Jiang et al., 1996). In adult rabbit cardiomyocytes, adenoviral-mediated overexpression of PKCε was reported to activate ERK1/2 MAPK, yet a dominant negative PKCε mutant was reported to have no effect on basal ERK1/2 activation (Ping et al., 1999). More recently, PKCδ and PKCε were also shown to be associated with ERK1/2 activation in cardiac myocytes, suggesting the involvement of other isozymes (Rohde et al., 2000).

In this report, we showed that overexpression of both PKCα and PKCε induced significant ERK1/2 activation, but not p38 or JNK. These results are largely consistent with the reports discussed above. However, we also observed that dominant negative PKCα antagonized PMA-induced ERK1/2 activation, whereas dominant negative PKCε did not. At the functional level, dominant negative MEK1 (blocks ERK1/2 activation) inhibited wild-type PKCα-induced cardiomyocyte hypertrophy, suggesting a critical role for PKCα and ERK1/2 communication in regulating cardiomyocyte hypertrophic growth. Previous reports also support an interconnection between PKCα and ERK1/2 MAPK signaling, although the level at which PKCα interacts with the MAPK cascade is disputed (Kolch et al., 1993; Schonwasser et al., 1998).

Expression of dominant negative PKCβII, δ, and ε did not significantly attenuate PMA-induced ERK1/2 activation in cardiomyocytes. PMA was employed since it acts as a more specific agonist of cPKC and nPKC activation, whereas hypertrophic agonists such as PE, endothelin-1, and angiotensin II stimulate diverse signaling pathways that could indirectly promote activation of selected PKC isozymes. Indeed, the studies discussed above, which demonstrated a role for PKCε and PKCδ in regulating ERK1/2 activity, did not employ a direct PKC agonist such as PMA. In this manner, agonists that function through G-protein–coupled receptors or receptor tyrosine kinases likely utilize additional PKC isozymes, suggesting a complex relationship between PKC and ERK1/2 signaling pathways in cardiomyocytes.

The observation that dominant negative PKCα attenuated PE-induced cardiomyocyte hypertrophy, coupled with the observation that PE augmented PKCα kinase activity and translocation to discrete intracellular locations by immunocytochemistry, suggests that Gαq-coupled receptor signaling activates PKCα. This assertion is in contrast to four previous reports that failed to identify significant PKCα translocation to the particulate fraction after either PE or endothelin-1 stimulation of cardiomyocytes (Clerk et al., 1994, 1996; Deng et al., 1998; Hayasaki-Kajiwara et al., 1999). Two explanations may account for these differing results. First, it is largely assumed that PKC activation is synonymous with the ability to isolate PKC isozymes from a membrane-enriched or particulate protein fraction. This assumption may not be valid in every cell type or may vary depending on the stimulus and the specific isozyme analyzed. Second, our analysis of PE-induced PKCα translocation by immunocytochemistry and kinase activity was performed with hypertrophied neonatal cardiomyocytes (previously serum-stimulated), which are significantly larger and contain more organized sarcomeric structures. Cardiomyocytes in this condition may contain more organized docking units, which might be more amenable to detection of translocation. Alternatively, hypertrophied cardiomyocytes might have more complex membrane–associated signaling com-

plexes, which could respond differently to PE stimulation. Indeed, atrophic cardiomyocytes were largely refractory to PE-induced PKCα translocation (Fig. 2 C). In any event, the conditions used here demonstrated PE-induced PKCα translocation and increased kinase activity in cultured cardiomyocytes that were in a more physiologic state (hypertrophied). Future analysis of PKCα signaling effects in animal models should shed additional light on the physiologic role of PKCα as a regulator of cardiac hypertrophy.

Materials and methods

Primary cardiomyocyte cell culture

Primary cultures of neonatal rat cardiomyocytes were obtained by enzymatic dissociation of 1–2-d-old Sprague-Dawley rat neonates as described previously (De Windt et al., 2000). Cardiomyocytes were cultured under serum-free conditions in M199 media (GIBCO BRL) supplemented with penicillin/streptomycin (100 U/ml) and L-glutamine (2 mmol/L). For analysis of PKC isozyme translocation, cultures were first stimulated with 2% FBS for 24 h to induce sarcomeric organization.

Replication-deficient adenovirus generation

The generation and characterization of adenovirus-encoding wild-type or dominant negative mutants of PKCα, βII, δ, ε, and ζ were described previously (Ohba et al., 1998; Matsumoto et al., 2001) (gift from Dr. Motoi Ohba, Tokyo, Japan). The dominant negative PKCα, βII, δ, and ε cDNAs consisted of a lysine to arginine mutation in the ATP binding domain at amino acid positions 368, 371, 376, and 436, respectively. Each recombinant adenovirus was plaque purified, expanded, and titered in HEK293 cells using the agarose gel overlay method (Mittereder et al., 1996). Typical experiments involved infection of neonatal rat cardiomyocytes at a moi of 100 pfu for 2 h at 37°C in a humidified, 6% CO_2 incubator. Subsequently, the cells were cultured in serum-free M199 media for an additional 24 h before treatments or analysis. Under these conditions ~95% of the cells showed expression of the recombinant protein. Selected cultures were infected at a lower moi of 25 pfu/ml for immunocytochemical analysis so that nonexpressing cells could also be observed and compared with adenoviral infected cells.

Immunocytochemistry

Cardiomyocytes were prepared for immunocytochemistry as described previously (Taigen et al., 2000). To assess sarcomeric organization and cardiomyocyte hypertrophy antibody against α-actinin (EA-53; Sigma-Aldrich) and ANF (Peninsula Laboratories) were used at a dilution of 1:500. Secondary antibodies included anti–mouse TRITC-conjugated antibody (Sigma-Aldrich) and anti–rabbit FITC-conjugated antibody (Sigma-Aldrich) at a dilution of 1:400. Quantitation of cardiomyocyte cell surface area was performed on α-actinin–stained cardiomyocytes using confocal laser microscopy and NIH Image software on a Sun system workstation. Characterization of PKCα, βII, δ, and ε isozyme distribution also used confocal microscopy in conjunction with polyclonal antiserum purchased from Santa Cruz Biotechnology, Inc. (each used at 1:400). α-Tubulin antiserum (1:400) was purchased from Sigma-Aldrich.

Western blot analysis

Protein extracts were generated from cultured cardiomyocytes as described previously (Taigen et al., 2000). Protein samples were subjected to SDS-PAGE (8% gels), transferred to Hybond-P membrane (Amersham Pharmacia Biotech), blocked in 7% milk, and incubated with primary antibodies overnight in 3% milk at 4°C. Secondary antibodies IgG (alkaline phosphatase–conjugated anti–mouse, –rabbit, or –goat) were incubated for 1 h at room temperature in 3% milk (Santa Cruz Biotechnology, Inc.). Chemifluorescent detection was directly performed with the Vistra ECF reagent (RPN 5785; Amersham Pharmacia Biotech) and scanned using a Storm 860 (Molecular Dynamics). Other antibodies included phospho-p38, p38, phospho-JNK, JNK wild-type, phospho-ERK1/2, and ERK1/2 (Cell Signaling).

PKC translocation assay tissue preparation and enzymatic assay

Neonatal cardiomyocyte cultures were prepared in homogenization buffer (25 mM Tris-Cl, pH 7.5, 4 mM EGTA, 2 mM EDTA, 5 mM DTT, 1 mM PMSF, and 1 ug/ml leupeptin) on ice and subsequently spun at 100,000 *g* for 30 min at 4°C. The supernatant product was saved as the cytosolic fraction while the remaining pellet was resuspended in homogenization buffer with the addition of 1% Triton X-100. The sample was then rehomogenized and incubated on ice for 30 min and spun again at 100,000 *g* for 30 min at 4°C, and the remaining supernatant fraction was saved as the particulate sample.

Total PKC activity was determined using a radioactive enzymatic assay (SignaTECT PKC assay system; Promega) in which cardiomyocyte supernatants are passed over a DEAE cellulose column to purify PKC proteins. PKC activity assays were performed in the presence of phospholipids (phosphatidylserine) and a PKC-biotinylated peptide substrate. All reactions were incubated at 30°C for 5 min, and [^{32}P] incorporation was measured by transferring the completed reactions onto membranes (Promega).

Protein synthesis measurements

Rates of protein synthesis in cultured cardiomyocytes were determined by [^{3}H]leucine incorporation. Cardiomyocytes were infected with adenovirus overnight, preincubated with leucine-free RPMI medium for 1 h, and then incubated with 2.5 μCi/ml [^{3}H]leucine for 6 h. PE (20 μM) or vehicle was added to the cultures together with leucine-free medium and [^{3}H]leucine incorporation was then quantified as described previously (Sadoshima et al., 1992).

Statistical analysis

Differences between data groups were evaluated for significance using a Student's *t* test of unpaired data or one-way analysis of variance and Bonferroni's post-test (± standard error of the mean).

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