Functional proteomic analysis of a three-tier PKCε-Akt-eNOS signaling module in cardiac protection

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Am J Physiol Heart Circ Physiol 288: H954–H961, 2005. First published November 4, 2004; doi:10.1152/ajpheart.00756.2004.—Cardiac protective signaling networks have been shown to involve PKCε. However, the molecular mechanisms by which PKCε interacts with other members of these networks to form task-specific modules remain unknown. Among 93 different PKCε-associated proteins that have been identified, Akt and endothelial nitric oxide (NO) synthase (eNOS) are of importance because of their independent abilities to promote cell survival and prevent cell death. The simultaneous association of PKCε, Akt, and eNOS has not been examined, and, in particular, the formation of a module containing these three proteins and the role of such a module in the regulation of NO production and cardiac protection are unknown. The present study was undertaken to determine whether these molecules form a signaling module and, thereby, play a collective role in cardiac signaling. Using recombinant proteins in vitro and PKCε transgenic mouse hearts, we demonstrate the following: 1) PKCε, Akt, and eNOS interact and form signaling modules in vitro and in the mouse heart. Activation of either PKCε or Akt enhances the formation of PKCε-Akt-eNOS signaling modules. 2) PKCε directly phosphorylates and enhances activation of Akt in vitro, and PKCε activation increases phosphorylation and activation of Akt in PKCε transgenic mouse hearts. 3) PKCε directly phosphorylates eNOS in vitro, and this phosphorylation enhances eNOS activity. Activation of PKCε in vitro increased phosphorylation of eNOS at Ser1177, indicating eNOS activation. This study characterizes, for the first time, the physical, as well as functional, coupling of PKCε, Akt, and eNOS in the heart and implicates these PKCε-Akt-eNOS signaling modules as critical signaling elements during PKCε-induced cardiac protection.

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INTRACELLULAR SIGNALING NETWORKS are composed of protein modules specified to execute distinct tasks (5, 17, 35). In the heart, complex cellular phenotypes, such as resistance to ischemic cell death, involve activation of various signaling molecules. Despite this information, the manner in which the actions of these individual proteins are integrated into functional modules has only begun to be understood. Numerous studies have demonstrated modules composed of two molecules; however, the next level of hierarchical structure, i.e., a three-tier module, remains scarcely defined. Although “three-tier” modules have been described in certain canonical signaling responses (e.g., MAPKs), there is a paucity of investigations demonstrating simultaneous association of three molecules as a generalized organizational pattern to recruit proteins with disparate functions together for shared signaling actions. The present study was designed to address this limitation of our understanding.

Protein kinase Cε (PKCε) has been well documented to play an important role in the genesis of cardioprotection (11, 22). In particular, previous studies from our laboratory have shown that activation of PKCε in the heart is sufficient to significantly reduce myocardial infarction due to coronary artery occlusion (26, 27). Moreover, Akt/protein kinase B (PKB) (14, 15, 32) and endothelial nitric oxide (NO) synthase (eNOS) (31) have been independently implicated as protective molecules in the setting of oxidative stress and ischemic injury to the myocardium. As described above, however, the information gained about these molecules through previous investigations was acquired in isolation [e.g., transgenic activation of PKCε is sufficient to reduce infarct size (26)] or in a binary sense [e.g., regulation of NO production through eNOS by Akt (3, 15, 34)]. To our knowledge, no previous investigations examined the possibility that these three molecules, PKCε, Akt, and eNOS, together constitute a module, the assembly of which is a critical mechanism of protective signaling in the heart. In the present study, we took a combined proteomic and biochemical approach to characterize native protein complexes containing PKCε, Akt, and eNOS. We examined PKCε-Akt-eNOS signaling modules in vitro and in the mouse heart in terms of molecular architecture (i.e., protein-protein interactions) and signal transduction (i.e., posttranslational modification and alteration of enzymatic activity). Our findings indicate formation of a three-tier module in the heart and suggest that such signaling units, similar to that formed by PKCε, Akt, and eNOS, may represent a mechanism utilized to manipulate multipurpose signaling proteins to carry out distinct tasks in the myocyte.

MATERIALS AND METHODS

All procedures were performed in accordance with the Animal Research Committee guidelines at the University of California, Los Angeles, and the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Materials. Recombinant active Akt-1, nonactive Akt-1, and pleckstrin homology (PH) domain of Akt-1, and PH domain-deleted Akt-1 fusion proteins corresponding to human Akt-1/PKB-α were purchased...
from Upstate Biotechnology (Lake Placid, NY). Recombinant human PKCε and bovine eNOS were purchased from Biomol (Plymouth Meeting, PA) and Calbiochem (San Diego, CA), respectively. Anti-Akt-1 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PKCε and anti-eNOS monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA). Anti-phospho-Akt (Thr308 and Ser27) , anti-phospho-eNOS (Ser1177) , and anti-phosphoglycogen synthase kinase (GSK)-3β (Ser21/9) monoclonal antibodies and Akt kinase assay kit were obtained from Cell Signaling Technology (Beverly, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

PKCε transgenic mice. The transgenic mice with cardiac-specific activation of PKCε used in this study exhibited ~6.2-fold overexpression of PKCε and have been previously described (26). Transgenic mice and their nontransgenic littermates were used at 9–12 wk of age.

Immunoprecipitation. Immunoprecipitation was performed as previously described (2, 12, 26–28, 33, 36). Briefly, covalently cross-linked PKCε monoclonal antibodies were incubated with protein samples overnight at 4°C. Immunocomplexes were then washed three times with buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 10 mM EDTA, 1% (vol/vol) Nonidet P-40 (NP-40), 1 mM Na3VO4, and a protease inhibitor cocktail (Roche, Indianapolis, IN). After the final wash, the protein sample was eluted from the beads by resuspension in Laemmli buffer, boiled, and then subjected to SDS-PAGE.

Immunoblotting. Standard protocols were applied for immunoblotting (2, 26–28, 36). Briefly, after SDS-PAGE separation, proteins were transferred to nitrocellulose membranes and blotted in 5% milk or 5% bovine serum albumin (BSA) for phosphospecific antibodies in Tris-buffered saline supplemented with 0.03 mg/ml L-ε-phosphatidyl-1-serine (PS), 2.5 μg/ml phorbol 12-myristate 13-acetate (PMA), 3.5 mM dithiothreitol, 100 μM ATP, 6.5 mM MgCl2, 50 mM Tris-HCl, pH 7.5, and 0.2 μCi of [γ-32P]ATP (for in vitro phosphorylation assay) at 30°C for 30 min. The resulting nitrate was converted to nitrite with nitrate reductase treatment. Total nitrite was measured by the Griess method and quantified by a Wallace 1420 multilabel counter (16).

Statistical analysis. Values are means ± SE. Differences among the experimental groups were analyzed using one-way ANOVA. If the ANOVA showed an overall significance, post hoc contrasts were performed with Student’s t-test (27, 37). P < 0.05 was considered significant.

RESULTS

Formation of PKCε-Akt-eNOS modules in vitro. Formation of this three-tier module was initially examined using recombinant proteins in vitro.

We first determined the ability of these three proteins to directly interact in a binary fashion. GST-PKCε fusion proteins were incubated with recombinant Akt or eNOS, GST pull-down was performed, and the products were separated by SDS-PAGE and subjected to Western blotting for Akt or eNOS. Figure 1 demonstrates direct interaction between PKCε and Akt in vitro. Two further observations regarding this interaction warrant remark. I) GST-PKCε preferentially interacted with recombinant active Akt (130.0 ± 2.3% of nonactive Akt) vs. nonactive Akt with no treatment (Fig. 1A). 2) Recombinant PH-deleted Akt and Akt PH domains were used to demonstrate that the interaction between PKCε and Akt occurred via the PH domain of Akt (Fig. 1B). Interaction of Akt and eNOS is well established and was also observed in this study (data not shown).

Next, we investigated the formation of this module when all three components were present. Akt and eNOS were incubated in the presence or absence of PKCε, and the sample was subjected to immunoprecipitation for eNOS and immunoblotting for Akt. The data indicate that addition of PKCε increases the interaction between eNOS and Akt (Fig. 2A). To determine the role of PKCε activation in its ability to interact with Akt and eNOS, these respective proteins were incubated with PKCε in the presence or absence of the potent PKC ε activators PMA and PS, and the samples were subjected to immunoprecipitation for PKCε and immunoblotting for Akt or eNOS. Figure 2B shows that activation of PKCε with PMA and PS enhances its interaction with Akt and eNOS. Similarly, to examine the effect of Akt’s activation on its interaction with PKCε and eNOS, these respective proteins were incubated with active or nonactive Akt recombinant protein and then subjected to immunoprecipitation and immunoblotting. Interestingly, Akt activation not only enhanced its interaction with PKCε (Fig. 2C, bottom), but, also, activation of Akt enhanced the interaction between PKCε and eNOS (Fig. 2C, top). These
data suggest that activation of Akt is a key event to facilitate assembly of PKCε-Akt-eNOS modules.

Formation of PKCε-Akt-eNOS modules in the mouse heart.

Having established formation of functional PKCε-Akt-eNOS modules in vitro, we next wanted to explore the assembly of, and signal transduction by, these modules in the heart. Previous studies showed that Akt and eNOS are present in cardiac PKCε signaling complexes (28). Despite this information, nothing was known regarding the nature of interactions between PKCε and Akt or eNOS (i.e., whether they modulate each other’s activity or posttranslational modification state), the architecture of the signaling complexes formed by these molecules, and whether the three localize in the same signaling unit in the heart.

To assess native, i.e., intact, complexes that have not been disrupted by harsh detergents or heating and, thus, maintain their endogenous interactions, mouse hearts were homogenized and separated via gel filtration chromatography. With this method, intact myocardial protein complexes are separated on the basis of their physical size. Individual gel filtration fractions were immunoprecipitated with PKCε antibodies to isolate the native complexes containing PKCε from the given elution fraction (representative of a given molecular size). This last step is essential, because although some native complexes of a

Fig. 1. PKCε directly interacts with Akt and endothelial nitric oxide (eNOS) in vitro. Glutathione S-transferase (GST)-PKCε was incubated with recombinant Akt or eNOS, subjected to SDS-PAGE, and immunoblotted for Akt or eNOS. A: GST-PKCε favors interaction with active (lanes 1 and 2), compared with nonactive (lanes 3 and 4), Akt. GST without insert proteins (GST-null) exhibited minimal interactions with active or nonactive Akt (lanes 6 and 7, respectively). B: GST-PKCε favors interaction with the Akt pleckstrin homology (PH) domain (lanes 3 and 4) compared with PH domain-deleted Akt (AktΔPH; lanes 1 and 2). Lanes 6 and 7, positive controls (direct loading of recombinant proteins) for AktΔPH and Akt PH domain, respectively. C: GST-PKCε directly interacts with eNOS (lanes 1 and 2). GST without insert proteins exhibited minimal interactions with eNOS (lane 4). Results are representative of ≥3 independent experiments. +, Presence of a protein; −, absence of protein.

Fig. 2. Activation of PKCε or Akt enhances PKCε-Akt-eNOS module formation in vitro. A: recombinant Akt and eNOS were incubated in the absence (lane 1) or presence (lane 2) of PKCε and then subjected to immunoprecipitation (IP) for eNOS and immunoblotting (IB) for PKCε or Akt. PKCε is sufficient to increase interactions between eNOS and Akt. B: recombinant PKCε was incubated with Akt and eNOS in the absence (lane 1) or presence (lane 2) of the PKCε activators phorbol 12-myristate 13-acetate (PMA) and phosphatidyl-L-serine (PS) and then subjected to immunoprecipitation for PKCε and immunoblotting for eNOS or Akt. Activation of PKCε with PMA and PS increases its affinity for Akt and eNOS. C: active (lane 2) or nonactive (lane 3) Akt recombinant proteins were incubated with PKCε and eNOS and then subjected to immunoprecipitation for PKCε and immunoblotting for Akt or eNOS. Activation of Akt increases its interaction with PKCε and increases interaction of PKCε with eNOS. Results are representative of ≥3 independent experiments, and IgG and beads-alone controls demonstrated negligible signal (data not shown). +, Presence of a protein or lipids; −, absence of protein or lipids.
given size may contain PKCe, other complexes of identical or similar sizes most certainly exist that do not contain PKCe but may coelute from the column nonetheless. The isolated PKCe immunocomplexes were then separated by denaturing SDS-PAGE and immunoblotted for PKCe, Akt, and eNOS. Figure 3A shows the Western immunoblot analysis of the chromatographic elution fractions after immunoprecipitation for PKCe in the wild-type nontransgenic mouse heart. The data indicate that Akt and eNOS associate with PKCe in multiple fractions, suggesting that these three molecules interact with each other in a variety of different-sized multiprotein complexes. Next, the identical analysis was performed using hearts from PKCe transgenic mice (Fig. 3B), which are inherently resistant to ischemic injury (26). We observed a shift toward a higher molecular weight (i.e., a lower elution fraction) in expression patterns of PKCe-Akt-eNOS signaling modules from PKCe transgenic mice compared with those from nontransgenic mouse hearts, indicating that, during protection, PKCe-Akt-eNOS signaling modules are assembled within native complexes of greater molecular size.

Signal transduction through a three-tier PKCe-Akt-eNOS module. To investigate signal transduction by PKCe-Akt-eNOS modules, the effect of these proteins to posttranslationally modify each other and to influence each other’s enzymatic activity was determined.

Recombinant Akt or eNOS was incubated with recombinant PKCe in the presence of the PKC activators PMA and PS and [γ-32P]ATP. PKCe was found to directly phosphorylate Akt and eNOS (Fig. 4A). Next, the effect of these PKCe-induced modifications on the activation status of Akt and eNOS was examined. Akt phosphorylation activity directed at the well-known substrate GSK-3β was significantly enhanced in the presence of PKCe (Fig. 4B), suggesting that the PKCe-dependent phosphorylation of Akt (Fig. 4A) leads to increased Akt kinase activity. Similarly, addition of PKCe significantly enhanced eNOS activity, as measured by NO2/NO3 production (178.8 ± 11%, P < 0.05 vs. eNOS alone) compared with eNOS alone (Fig. 4B). These data suggest that the PKCe-dependent phosphorylation of eNOS (Fig. 4A) triggers the increase in eNOS activity.

Akt is known to be activated by phosphorylation of Thr308 within its activation loop, a modification that stabilizes the active conformation of the molecule. Subsequently, phosphorylation of Ser1173 at the carboxyl terminal is essential for full activation of Akt. Although it is well known that phosphoinositide-dependent kinase (PDK)-1 is responsible for Thr308 phosphorylation, the upstream kinase(s) that catalyzes the phosphorylation of Ser1173 has not been defined. In this study, we tested whether PKCe could be a putative “candidate PDK-2” that targets Ser1173 for phosphorylation and completes the activation process of Akt. Indeed, addition of PKCe in vitro resulted in a 58.3 ± 2.8% increase in Akt phosphorylation at Ser1173 above that seen in the absence of PKCe (using a site-specific antibody to phospho-Ser1173; Fig. 5), suggesting that PKCe may be a kinase responsible for PDK-2 activity directed at Akt. This possibility was further supported by the data described below from PKCe transgenic mice.

Role of PKCe-Akt-eNOS modules in PKCe-induced cardiac protection. To confirm the functional importance of these posttranslational modifications observed in vitro, Akt and eNOS phosphorylation were also examined in the mouse heart. First, PKCe cardiac-protected transgenic mice hearts were used to test the effect of activation of PKCe on Akt by examining the two conserved phosphorylation sites of Akt. Using the

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recombinant Akt (ε) or eNOS (H9252) was incubated with recombinant PKC ε\(^{2}\) in the presence of PKC activators PMA and PS and \([γ-32P]ATP\). After SDS-PAGE separation, phosphorylation was visualized by autoradiography. Lanes 1 and 3 demonstrate minimal background phosphorylation signal. B: after incubation with PKCε, Akt (top; lanes 2 and 3) and eNOS (bottom) activities were assessed by the ability of Akt to phosphorylate glycogen synthase kinase (GSK)-3β or eNOS to produce NO\(_2/\text{NO}_3\). In the absence of PKCε, nonactive Akt does not phosphorylate GSK-3β (lane 1). Results are representative of ≥3 independent experiments. +, Presence of a protein; −, absence of a protein.

Fig. 4. PKCε phosphorylates and enhances activity of Akt and eNOS. A: recombinant Akt (A) or eNOS (B) was incubated with recombinant PKCε (lane 2) in the presence of PKC activators PMA and PS and \([γ-32P]ATP\). After SDS-PAGE separation, phosphorylation was visualized by autoradiography. Lanes 1 and 3 demonstrate minimal background phosphorylation signal. B: after incubation with PKCε, Akt (top; lanes 2 and 3) and eNOS (bottom) activities were assessed by the ability of Akt to phosphorylate glycogen synthase kinase (GSK)-3β or eNOS to produce NO\(_2/\text{NO}_3\). In the absence of PKCε, nonactive Akt does not phosphorylate GSK-3β (lane 1). Results are representative of ≥3 independent experiments. +, Presence of a protein; −, absence of a protein.

Fig. 5. PKCε phosphorylates Akt on Ser\(^{473}\) in vitro. PKCε was incubated with nonactive Akt (lanes 3 and 4), and Ser\(^{473}\) phosphorylation was detected by site-specific antibody-based immunoblotting (lanes 1 and 2, nonactive Akt negative control). In the absence of PKCε (lanes 5 and 6), Akt is not phosphorylated at Ser\(^{473}\). Results are representative of ≥3 independent experiments. +, Presence of a protein; −, absence of a protein.

DISCUSSION

Many subcellular functions, such as signal transduction, are accomplished by multiprotein complexes. These complexes are differentially assembled within cells in response to given stimuli, and they coordinate modules of proteins (≥2 interacting molecules) that are targeted to carry out specific tasks. The functions of these modules, therefore, are engendered by the properties of the molecules within them and the interactions among these individual components. Several recent studies have emphasized the role of modules containing two proteins (KA) as effectors of signaling tasks in the setting of cardiac protection. However, the next level of hierarchical structure, i.e., integration of a third molecule into these modules, is poorly understood. On the basis of findings from other laboratories suggesting the potential for a three-tier module containing PKCε, Akt, and eNOS, we designed the present study to functionally characterize this signaling unit containing two kinases and an NO-generating enzyme.

The molecular architecture of, and signal transduction by, PKCε-Akt-eNOS signaling modules was first examined in the in vitro setting. The role of Akt to activate eNOS has been established (3, 15, 25, 38), and several investigations have suggested that PKC signaling can directly influence Akt activity (12, 20, 21, 23). However, the role of the ε isoform of PKC in modulation of Akt is completely unknown, and formation of a module containing PKCε, Akt, and eNOS has never been studied. In the present study, all three molecules were found to form protein-protein interactions with each other in a pairwise fashion, but, importantly, the interaction was significantly potentiated when all three molecules were present (Fig. 2). We
found that PKCe preferentially interacts with the PH domain of Akt, congruent with previous studies in noncardiac cells, indicating that this domain of Akt is critical for its activation (4). Moreover, we found that the PH domain of Akt appears not to be necessary for binding of eNOS to Akt in vitro (data not shown), suggesting that the interactions of PKCe and eNOS with Akt are noncompeting; i.e., they occur through different domains. These data suggest that the module concept, whereby two or more molecules are recruited into close apposition for signal transduction, is tenable, because a linear, or “pathway,” model may not involve simultaneous interaction of more than two molecules.

To determine whether these interactions lead to signal transduction, we examined the ability of PKCe to posttranslationally modify Akt and eNOS and to regulate their activity. The findings demonstrate that PKCe phosphorylates Akt and leads to increased Akt activity (as evidenced by GSK-3β phosphorylation; Fig. 4). Similarly, PKCe phosphorylates and activates eNOS, leading to increased NO2/NO3 production. Thus PKCe is physically and functionally coupled to Akt and eNOS in vitro, providing the impetus to examine this module in vivo.

Accordingly, we next examined PKCe-Akt-eNOS modules in a line of PKCe transgenic mice with cardiac-specific overexpression of an active mutant of PKCe that display a powerful inherent resistance to ischemia-reperfusion injury (26). We wanted to examine the presence and characteristics of PKCe-Akt-eNOS modules in these PKCe mice, with the goal to understand the role of these modules in a cardiac-protective phenotype.

Indeed, our in vitro findings were substantiated by the studies in murine hearts: activation of PKCe (PKCe transgenic mice) was associated with increased formation of functional PKCe-Akt-eNOS modules. PKCe mice demonstrated enhanced posttranslational modification of Akt and eNOS, as well as enhanced activation of these molecules (analogous to the in vitro experiments in which PKCe was activated with PMA and PS). The phosphorylation and inactivation of GSK-3β observed in this study is in agreement with that documented during cardiac protection by other investigators (34). Recombinant PKCe alone could increase the phosphorylation of GSK-3β (Fig. 4B). However, this was considerably less than when Akt was also present, suggesting that the preferred mechanism of activation of GSK-3β by PKCe in this module is indirect, i.e., through Akt. Therefore, although we cannot rule out that the increase in phosphorylation of GSK-3β in the PKCe transgenic mice is at least partly due to a direct action by PKCe, we believe that this is more likely achieved through Akt activation. Additional investigations are necessary to elucidate the functional importance of the direct effects of PKCe on GSK-3β phosphorylation during ischemic injury and protection.

Using nondenaturing gel filtration liquid chromatography (to separate protein complexes) followed by immunoprecipitation for PKCe (to isolate PKCe-containing native complexes), we were able to examine intact protein complexes containing...
PKCε. To determine which of these complexes contained Akt and eNOS, we separated the complexes immunoprecipitated from the gel filtration fractions by SDS-PAGE and subjected them to Western blotting for Akt and eNOS (Fig. 3). The data indicate that PKCε, Akt, and eNOS reside together in multiple native complexes in the mouse heart and that these complexes display a large molecular weight range. Furthermore, PKCε-induced cardiac protection (i.e., PKCε transgenic mice) was associated with increased localization of these PKCε-Akt-eNOS modules to higher-molecular-weight complexes. To our knowledge, these findings are the first to demonstrate formation of native complexes in the myocardium that are modulated with regard to their molecular components (here, PKCε-Akt-eNOS modules) concomitant with changes in the phenotype of the organ. These findings are of salient interest, because they suggest that native protein complexes, such as PKCε complexes containing modules such as the PKCε-Akt-eNOS module described here, are mechanisms of signal transduction and are not solely artifacts of biochemical analyses. Taken with the striking data described above regarding posttranslational modifications and enhanced enzymatic activity engendered by assembly of this signaling unit, these findings support PKCε-Akt-eNOS modules as critical components of PKCε protective signal transduction. Because each component within the complex contributes to the complex formation, it is very likely that expression changes in Akt and/or eNOS may also influence assembly of this module. Consequently, future studies are required to investigate Akt- and eNOS-induced changes in PKCε-Akt-eNOS module formation, potentially with use of transgenic mouse models with cardiac-specific overexpression of Akt or eNOS (6, 9).

In contrast to our findings, other investigators reported that in A549 and HEK293 cells the phosphatidylinositol 3-kinase-Akt signaling pathway was regulated by PKC in a negative manner (10, 39). PMA-induced apoptosis was accompanied by an inhibition of Akt activity (29). Similarly, PKC activation inhibited eNOS activity by attenuating eNOS phosphorylation on Ser1177 and increasing phosphorylation of Thr298 in cardiovascular endothelial cells (24, 25). The present findings suggest that cardiac PKCε serves as a positive regulator of the formation and regulation of PKCε-Akt-eNOS signaling modules in mouse hearts. Possible reasons for these differences include isoform-specific functions of PKC and/or distinct cell types. These findings highlight the versatility of the enzymes to participate in a host of distinct cellular processes and emphasize the importance of studying modules of proteins (and correlating these findings with a phenotype) that are regulated multiplicatively in the setting of opioid-induced protection (13). Whether other isoforms of PKC also form a similar signaling module with Akt and eNOS in these species remains to be determined.

Akt has two conserved phosphorylation sites, Thr308 and Ser473, which must be modified to induce full activation. The upstream kinase that phosphorylates Thr308 is PDK-1. However, the protein kinase responsible for Ser473 phosphorylation is unknown and has been tentatively named “PDK-2” (7). Several candidates have been proposed, including atypical PKC (40), lipid raft-associated activity (18), or simply the autophosphorylation processes that are triggered by PDK-1 activity (1). Because of the data in the present study indicating a link between PKCε and Akt, we examined the ability of PKCε to phosphorylate Akt at Ser473, thereby behaving as the aforementioned PDK-2. Interestingly, we found that PKCε can, in fact, phosphorylate Akt at Ser473 in vitro (Fig. 5). Taken with the data demonstrating increased Ser473 phosphorylation of Akt in PKCε transgenic mice (Fig. 6), these findings strongly suggest that PKCε is a candidate for the in vivo “PDK-2 activity” associated with the cardiac-protected phenotype displayed by these PKCε mice. Moreover, these findings have important implications for regulation of the PKCε-Akt-eNOS module. Specifically, once Akt has been primed by PDK-1, integration into the PKCε-Akt-eNOS module would theoretically allow for full activation of Akt on the basis of the PDK-2 activity ascribed here to PKCε. Further experimentation is required to fully establish or refute this concept.

In summary, we have demonstrated assembly of PKCε-Akt-eNOS signaling modules in vitro and in the mouse heart. These modules appear to be involved in cardiac protection and provide a mechanistic link between these three proteins previously associated with such protection. The concept of modules of proteins is not new; indeed, the field of MAPK signaling, for instance, has long recognized the importance of conserved mechanisms of activation. A departure from previous investigations that is offered by these studies is the principle that multipurpose signaling proteins, themselves not constrained to mandatory activation pathways, can be manipulated by the cell to form distinct signaling modules. In other words, module formation may be an organizational tool of the intracellular signaling network to elicit specialized responses.

GRANTS

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