Phosphoinositide Kinases Play Key Roles in Norepinephrine- and Angiotensin II-induced Increase in Phosphatidylinositol-4,5-Bisphosphate and Modulation of Cardiac Function

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Running title: NE and Ang II increase activity of PtdIns kinases and cardiac PIP2 level

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Background: The mechanism and significance of phosphoinositide metabolism during heart stress stimulations are not clear.

Results: Norepinephrine and angiotensin II increase cardiac phosphatidylinositol-4,5-bisphosphate levels via an enhanced interaction between phosphatidylinositol 4-kinase IIIβ and PKC, which correlate with a maintained systolic function.

Conclusion: Cardiac phosphoinositides turnover is enhanced.

Significance: A novel mechanism of phosphoinositide metabolism is described for modulation of cardiac function.

In this study, we studied cardiac phosphoinositide levels in both cells and whole animals under the stimulation of norepinephrine (NE), angiotensin II (Ang II), and other physiologically relevant interventions. The results demonstrated that activation of membrane receptors related to NE or Ang II caused an initial increase and a later fall in phosphatidylinositol-4,5-bisphosphate (PIP2) levels in the primary cultured cardiomyocytes from adult rats. The possible mechanism underlying this increase in PIP2 was found to be through an enhanced activity of phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ), which was mediated by an upregulated interaction between PI4KIIIβ and PKC; the increased activity of phosphatidylinositol 4-phosphate 5-kinase γ was also involved for NE-induced increase of PIP2. When the systolic
functions of the NE/Ang II-treated cells were measured, a maintained or failed contractility was found to be correlated with a rise or fall in corresponding PIP2 levels. In two animal models of cardiac hypertrophy, PIP2 levels were significantly reduced in hypertrophic hearts induced by isoprenaline but not in those induced by swimming exercise. This study describes a novel mechanism for phosphoinositide metabolism and modulation of cardiac function.

Phosphoinositides are important membrane phospholipids that serve important cellular functions. Among them, phosphatidylinositol-4,5-bisphosphate (PIP2) is a minor but central phosphoinositide that anchors and modulates numerous important signaling and cytoskeletal proteins (1). PIP2 regulates diverse cellular activities, including endocytosis, exocytosis, cell migration, focal adhesion formation, angiogenesis, transcriptional regulation, and mRNA processing (2, 3). Furthermore, the activities of numerous membrane proteins including ion channels and transporters are also regulated by PIP2 (4, 5).

Phosphoinositides and PIP2 levels in the cell membrane are dynamically regulated (6, 7). One of the well-established cellular events involved in PIP2 dynamics is mediated by the activation of the Gq protein-coupled receptor (GqPCR): The stimulation of GqPCR activates phospholipase C (PLC), which in turn, hydrolyzes PIP2 into inositol trisphosphate (IP3) and diacylglycerol (DAG) (8). Meanwhile, most PIP2 is synthesized at the membrane by the sequential phosphorylation of phosphatidylinositol PI and phosphatidylinositol phosphate (PIP) (9). However, the seemingly paradoxical Gq agonist-stimulated phosphoinositide production has been known since the early 1950s (10-12). In pancreas and brain cortex slices, the synthesis of phosphoinositides is increased many fold by acetylcholine (10). Furthermore, total PIP levels in guinea pig heart increase with the activation of Gq/PLC-dependent pathways by carbachol and phenylephrine (13). In superior cervical ganglion neurons, enhanced phosphatidylinositol 4-kinase activity is thought to be responsible for compensated membrane PIP2 levels when challenged with bradykinin (14). Furthermore, rather than a global dynamic regulation, PIP2 hydrolysis in the cell membrane might be a localized event. Distinct pools of membrane PIP2 have been suggested early in the development of the concept of phosphoinositide signaling (6), but the exact mechanisms of formation and maintenance of such pools, indeed even their existence, remain controversial. However, such mechanisms are supported by the fact that membrane PIP2 is characterized by low lateral mobility (15), which suggests that a signaling pathway that utilizes PIP2 could use PIP2 turnover machinery locally. It is still an intriguing issue as to how phosphoinositide metabolism is modulated in the event of Gq/PLC pathway activation.

The GqPCR-PLC-PIP2 signaling pathway is arguably the most important cell signaling pathway involved in cardiac hypertrophy and failure (8). It is well established that activation of Gq/PLC signaling via several extracellular signals directly stimulates the hypertrophic response, and blocking the Gq/PLC pathway in vivo can partially attenuate the hypertrophy induced by pressure overload (16). Some important hypertrophic agonists, including norepinephrine (NE) and angiotensin II (Ang II), are well-established GqPCR agonists (17); their involvement in the development of different types of cardiac hypertrophy has been well documented (8, 16, 18, 19).
In this study, we studied the modulation and mechanism of cardiac phosphoinositides by NE and Ang II at both the cellular and whole animal levels. We describe an agonist-induced increase in both PIP and PIP2 levels and present a novel mechanism for these increases to be the result of the enhanced activity of PI4-kinase IIIβ, mediated by an upregulated interaction between PI4KIIIβ and PKC; furthermore, for NE, an increased activity of phosphatidylinositol 4-phosphate 5-kinase γ was also involved. This enhanced PIP2 turnover is correlated with maintained cardiac systolic function in stimulated cardiac hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Cardiomyocyte isolation and stimulation with agonists**—Cardiomyocytes were isolated from male Sprague–Dawley rats (200–250 g). The rats were anesthetized (sodium pentobarbital, 120 mg/kg) and then the hearts were rapidly excised and mounted on a Langendorff apparatus. The hearts were perfused for 1 minute with Ca2+-free Tyrode solution (pH 7.4), containing (in mM) 140 NaCl, 5.4 KCl, 1.0 MgCl2, 10 HEPES, and 10 glucose, gassed with 95% O2–5% CO2, and kept at 37°C. The hearts were then transferred to the perfusion medium containing 0.05% collagenase II, 0.1% BSA, and 50 μM CaCl2. At the end of a 24-minute recirculation period, the ventricle was cut into small pieces to obtain cardiomyocytes that were suspended for 20 minutes each in buffers containing gradually increasing extracellular Ca2+ concentrations (250, 500, and 750 μM) to a final concentration of 1 mM containing 1% BSA. The final pellet was resuspended in medium-199 (M-199, Gibco 12340, Invitrogen, USA) and plated onto a 6-well plate at 4.5 × 105 cells/well in a 5% CO2 humidified incubator at 37°C.

After a 24-hour incubation, cells were subjected to treatments with 5 μM NE or 3 μM Ang II for variable times. Further experiments were carried out in which cardiomyocytes were pretreated for 30 minutes with prazosin (3 μM), losartan (3 μM), propranolol (3 μM), U73122 (0.8 μM), U73343 (0.8 μM), bisindolylmaleimide-1 (Bis-1, 2 μM), H89 (30 μM), wortmannin (10 μM), or cycloheximide (CHX, 10 μM) followed by incubation with NE or Ang II.

**Lipids extraction and membrane PIP2 assay**—Cellular lipids were extracted from cardiomyocytes (4.5 × 105 cells/sample) after different treatments. The cell pellets were first washed with 0.75 mL of 0.5 M TCA and 0.5 mL of 5% TCA/1 mM EDTA, and were then resuspended in 0.325 mL chloroform/methanol/12 N HCl (40:80:1 v/v/v) for 15 minutes at room temperature and vortexed vigorously 4 times during this period. Then, 0.125 mL chloroform and 0.225 mL of 0.1 N HCl were added to the cells, which were then vortexed for 2 minutes and centrifuged (1,500 rpm for 5 minutes). The bottom layer was transferred to another tube for drying in a vacuum dryer. This method of lipid extraction is modified from the protocol described previously (20).

The dried lipid samples were reconstituted with chloroform/methanol/water (5:5:1 v/v/v), vortexed, and sonicated in an ice water bath for 5 minutes. Phospholipids in the samples were then analyzed using TLC (21, 22). One-microliter aliquots of the reconstituted lipid samples were subjected to analysis on TLC plates (GF254). The mobile phase for TLC was chloroform/methanol/4 N NH4OH (45/35/10, v/v/v). Phospholipids were visualized with iodine vapor. PIP and PIP2 were confirmed by mass spectrometry, and the images of the spots were analyzed by densitometry analysis software.

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Lipid-protein overlay assay--For this assay, a PIP2 Mass Strip Kit (Echlon Biosciences, USA) was used. Three microliters of phospholipid from each cardiomyocyte sample (per sample/15 μl in 80:80:1 chloroform/methanol/1 N HCl) was spotted onto a nitrocellulose membrane and dried. Membranes were blocked in PBS containing 3% BSA at room temperature for 1 hour. The procedure followed the manufacturer's protocol. Images of the spots were analyzed by densitometry analysis software.

Co-immunoprecipitation and western blot--Cardiomyocytes (0.9~1 × 10⁶ cells/sample) were harvested in lysis buffer. One hundred microliters of cell lysate were incubated with 0.5 μg antibody and gently rocked for 12 hours at 4 °C. Then 20 μL protein G beads (GE Biotech, USA) were added with gentle rocking for 2 hours at 4°C. After washing five times with washing buffer (PBS buffer, protease inhibitors, 0.5% Nonidet P-40, and 0.1% Triton X-100), the co-immunoprecipitated samples were subjected to SDS-PAGE and immunoblot (IB) analysis using appropriate antibodies. Cell lysates incubated with protein G beads alone were routinely used as a negative control. Studies were repeated at least three times, and the antibodies used for immunoprecipitation and IB were also exchanged.

The proteins was mixed with loading buffer (10% glycerol, 50 mM Tris-HCl, 2% SDS, 5% mercaptoethanol, and 0.02% bromophenol blue), heat-denatured at 95°C for 5 minutes and subjected to SDS-PAGE. The proteins resolved by 10% SDS-PAGE were transferred to nitrocellulose membranes (Millipore, USA) in transfer buffer (20% methanol, 15.6 mM Tris-base, and 120 mM glycine) for 3 hours at 100 V, and were probed with anti-PIP5Kγ or anti-PI4KIIIβ antibodies (1:500 dilution; Upstate, USA) or PKC isoforms antibodies (1:500 dilution; Epitomics, USA), overnight at 4°C. Nonspecific binding was blocked with 1.5% (w/v) evaporated skimmed milk (Diōco, USA) in TBS (154 mM NaCl, 10 mM Tris-base). Anti-rabbit or anti-mouse secondary antibodies conjugated to IRDye700DX (1:5000 dilution; Rockland, USA) were used to probe primary antibodies. Protein bands were detected and quantified on an Odyssey two-color infrared imaging system (LI-COR Biosciences, USA).

Contraction measurements--The cardiomyocytes were transferred to a chamber mounted on the stage of an inverted microscope and superfused with Tyrode solution (1.8 mM Ca²⁺) at room temperature. The cells were field stimulated with a 4-ms bipolar, supramaximal pulse stimulator (IonOptix, Milton, MA, USA), and the frequency of the stimulation was chosen to be 1 Hz (15 V). Prior to recording, the cells were stimulated for 3 minutes at a constant pacing rate to allow them to adapt to the new frequency and reach steady state (23). Sarcomere shortening was recorded using an IonOptix system, and recordings were analyzed using the IonOptix analysis package (IonOptix, Milton, MA, USA).

Rat cardiac hypertrophic models--Swimming exercise was used to establish the physiological hypertrophic model. The rats (180 ± 20 g) underwent adaptive training for the first 3 days, then the training period was increased to 2 hours per day, 6 days per week. The training procedure normally lasted for 8 weeks. To establish the pathological hypertrophic model, the isoproterenol was injected (s.c. 5 mg/kg per day) for 1 week (24-26).

Statistical analysis--All results are reported as the mean ± S.E.M. Comparisons of two groups only were accomplished using
the unpaired Student’s $t$ test. Experiments with more than two groups were compared by one-way analysis of variance. All data were analyzed using Origin7.5, and the figures were plotted using Adobe Illustrator CS4.

RESULTS

NE and Ang II increase PIP$_2$ levels in cardiomyocytes—NE and Ang II are well-known GqPCR agonists, which are expected to induce membrane PIP$_2$ hydrolysis. We first tested the effects of these agonists on total PIP$_2$ abundance in cultured cardiomyocytes from adult rats. It has been shown that incubation of adult cardiomyocytes with NE (5 μM) results in a rapid activation of the Gq/PLC pathway with a maximal effect occurring at 2 hours (27), whereas Ang II needed a longer time (24 hours) but a lower working concentration (28, 29). After incubation with NE (5 μM, 2 hours) or Ang II (3 μM, 24 hours), the total abundance of PIP$_2$ in cardiomyocytes (from $4.5 \times 10^5$ cells), measured using TLC, was found to be significantly increased by 19 ± 6% and 18 ± 5%, respectively (Fig. 1A). We also used a lipid-protein overlay assay (30) (Fig. 1B), which confirmed the TLC results. We tried shorter incubation times with Ang II (2 hours) or NE (20 minutes) treatment but failed to observe any prominent effects on PIP$_2$ levels (see Fig. 5A). A specific antagonist of the α-adrenergic receptor (α1), prazosin, or Ang II receptor (AT1), losartan (3 μM), abolished the effects of NE and Ang II, respectively (Fig. 1C), indicating the involvement of GqPCR pathway.

PLC, PKC, and PI4K are involved in the increase in PIP$_2$ level—It has long been known that Gq agonists can stimulate phosphoinositide production, but the mechanism is not clear (10-13). We made an effort to understand this mechanism.

In parallel with changes in PIP$_2$ levels, PIP levels were also increased after treatment with NE or Ang II (Fig. 2B and D), indicating an increased activity of PI4-kinase (31). PLC and PKC are signaling molecules of GqPCR and PI4-kinase is the key enzyme for PIP$_2$ synthesis. The roles of PLC, PKC, and PI4-kinase were examined for their involvement in the observed effects of NE and Ang II on phosphoinositide production. The PLC inhibitor U73122 (0.8 μM; an inactive analog U73343, 0.8 μM was used as a control), the PKC inhibitor Bis-1 (2 μM), and the PI4-kinase inhibitor wortmannin (10 μM) were used to assess the roles of these molecules. Cardiomyocytes were treated with these inhibitors for 30 minutes before being treatment with NE or Ang II. Figure 2A and B shows the results of NE-treated cells and panels C and D show the results of Ang II-treated cells. Pretreatment of cardiomyocytes with U73122, Bis-1, and wortmannin prevented the NE- or Ang II-induced increase in PIP and PIP$_2$. These results indicate that both the Gq/PLC signaling pathway and PI4-kinase are involved in the NE- and Ang II-induced increase of phosphoinositides in cardiomyocytes.

The interaction between PI4KIIIβ and PKC is responsible for enhanced PIP$_2$ synthesis—Four mammalian PI4-kinases have been cloned and characterized; two isoforms of type III PI4-kinases are sensitive to inhibition by high micromolar concentrations of wortmannin, and two isoforms of type II PI4-kinases are wortmannin-insensitive but can be inhibited by low micromolar concentrations of adenosine (9, 32). We first tested the effect of PIK93, a specific blocker of PI4-kinase IIIβ, which can inhibit the activity of PI4KIIIβ by approximately 90% at a
concentration of 250 nM (33). PIK93 abolished the NE- and Ang II-induced increases in PIP₂ and PIP (Fig. 3A), and reduced the levels of PIP level slightly (Fig. 3A). Similar to wortmannin (13) but contrary to adenosine, PIK93 had no effect on the basal level of PIP₂ (Fig. 3A). We then tested the effect of adenosine. Adenosine (2 μM) greatly reduced basal PIP₂ and PIP levels. Under this influence, total PIP₂ and PIP levels (basal plus NE induced) in the presence of NE and adenosine were not significantly different from control PIP₂ and PIP levels (basal only) in the absence of NE and adenosine. These results suggest that adenosine does not inhibit NE-induced increases in phosphoinositides and may even enhance the effect of NE, considering that the absolute increase in phosphoinositides induced by NE was greater in the presence of adenosine than in the absence of NE (Fig. 3B). A similar conclusion could also be made for the effect of adenosine on Ang II-induced effects on the phosphoinositides (Fig. 3B). These results suggest that PI4KIII is mostly involved in NE- and Ang II-induced increases in phosphoinositide production.

We then further investigated how PI4KIII is involved in phosphoinositide production. Our previous study in Xenopus oocytes demonstrated that activation of PKC increases the activity of PI4KIII through an enhanced interaction between PKC and PI4KIII (21, 34). Since PKC is also involved in the NE- and Ang II-induced increase in phosphoinositides in this study (Fig. 2), we reasoned that a similar mechanism could exist in cardiomyocytes. We thus studied the interaction of PI4-kinase with two PKC isoforms: PKCα and PKCβII. A previous study suggested that both PKCα and PKCβII could interact with PI4KIIIβ in Xenopus oocytes (21, 34). For the cardiomyocytes studied here, co-immunoprecipitation results showed that both PKC isoforms α (Fig. 3D) and βII (Fig. 3E), were able to interact with PI4KIIIβ, but not with PI4KIIIα (Fig. 3C). Furthermore, the interaction between PKC and PI4KIIIβ was enhanced after treatment of cardiomyocytes with NE (5 μM) or Ang II (3 μM) for 2 hours, or PMA (a potent PKC activator, 0.5 μM) for 20 minutes, yet the expression of PI4KIIIβ did not change markedly. Thus although both isoforms (α and β) of PI4KIII are known to supply the PI4P substrate during GqPCR-activated PLC signaling (35), only PI4KIIIβ was able to interact with activated PKC. Furthermore, treatment of cardiomyocytes with isoprenaline (Iso, 0.5 μM, 2 hours) which selectively activates β-adrenergic receptors, did not affect the interaction between PKC and PI4-kinase. These results suggest that activation of the Gq/PLC pathway (and subsequent activation of PKC) could enhance the interaction between PKC and PI4KIIIβ, which contributes to increased cellular PIP₂ levels.

Role of PIP5K in the NE-induced PIP₂ increase—NE, unlike Ang II, apart from activating the Gq/PLC/PKC pathway, also activates the Gs/cAMP/PKA pathway mediated by β-adrenergic receptors (36, 37). This difference may explain the fact that the NE-induced increase of PIP₂ occurs faster than the Ang II-induced does. We thus tested whether the Gs/cAMP/PKA pathway is also involved in the NE-induced PIP₂ increase. For this, we compared the effects of NE and Ang II on the expression of PIP5-kinase protein, a key kinase in PIP₂ synthesis (38). Among the three isoforms (α, β, and γ) of PIP5K, the expression of the γ, but not the α and β isoforms (data not shown), was found in cardiomyocytes to be upregulated after stimulation with NE (5 μM, 2 hours), Iso (0.5 μM, 2 hours), Ang II (3 μM, 2 hours)
and PMA (0.5 μM, 20 minutes). However, the effect of NE and Iso on PIP5Kγ was significantly greater than that of Ang II or PMA (Fig. 4A). Consistent with the involvement of the β receptor/cAMP/PKA pathway, the enhanced expression of PIP5Kγ by NE was abolished by propranolol (a β receptor blocker) and H89 (a PKA blocker), but not by prazosin (an α1 receptor blocker) and Bis-1 (a PKC blocker) (Fig. 4B). Consistent with these results, propranolol abolished the NE-induced increase in PIP2 (Fig 4C), and Iso induced an increase in PIP2 levels, although it was less efficient than NE (11 ± 3% vs. 19 ± 6%, Fig. 4C). These results suggest that activation of β-adrenergic receptors and the activation of the downstream signaling pathway by NE lead to an increased expression of PIP5Kγ, which contributes to the increased synthesis of PIP2.

The above results suggest a relatively rapid turnover of PIP5Kγ. It has been reported that lipid kinases like PIP5-kinase turned over rapidly enough to alter cellular PIP2 levels within 1 hour (39). Nonetheless, we tested the effects of cycloheximide (CHX), a protein synthesis inhibitor. In the presence of CHX, the NE-induced increase in total PIP2 and PIP5Kγ expression in cardiomyocytes was inhibited (Fig. 4D).

**PIP2 level is correlated with the cardiac function**—Finally, we studied whether the NE- and Ang II-induced effects on PIP2 turnover are associated with their effects on the function of cardiomyocytes. Both NE and Ang II have long been known to be associated with cardiac hypertrophy and heart failure, which often manifest an altered cardiac function. We found that a prolonged incubation of cardiomyocytes with NE or Ang II resulted in a pattern of PIP2 changes, which correlated with altered cardiomyocyte systolic function. When cardiomyocytes were incubated with NE (5 μM) or Ang II (3 μM) for up to 72 hours (the maximal incubation time for NE was 48 hours, because many cells died after a 72-hour treatment.), both NE and Ang II induced a gradual increase in PIP2 levels within the first 24 hours, but afterwards, the effects of NE and Ang II began to recede and PIP2 levels fell back to control or even lower levels (Fig. 5A). The systolic function of the cultured cardiomyocytes was assessed by measuring sarcomere shortening induced by an electrical stimulation (for details see EXPERIMENTAL PROCEDURES), which represents the amplitude of cardiomyocyte contraction (Fig. 5B). In our hands, cultured cardiomyocytes can maintain their systolic function up to 48 hours, although the contraction gradually weakened with time. When applied to cultured cardiomyocytes up to 48 hours, NE (5 μM) or Ang II (3 μM) affected the contraction of cardiomyocytes with a pattern and time course similar to the effect of NE and Ang II on PIP2 levels: The contraction gradually increased within a 24-hour incubation with either NE or Ang II, but fell back to control levels after a 48-hour incubation (Fig. 5C). In the cells treated with NE for 48 hours, contraction actually weakened to levels lower than the control.

To further investigate the relationship between PIP2 level and cardiac function, we studied changes in PIP2 level in intact hearts from two rat models of cardiac hypertrophy: swimming-induced compensatory physiological hypertrophy and Iso-induced pathological hypertrophy. The systolic and diastolic functions of these two models were first assessed (data not shown), and the cardiac functions of the swimming rats were better maintained in comparison with those of the Iso-injected rats. As shown in Figure 5D, the PIP2 levels in the Iso-induced hearts were significantly reduced compared with
the time-matched controls; PIP$_2$ levels in swimming-induced hearts were increased compared with control hearts by 23 ± 12%, although the increase did not reach statistical significance. The above results suggest that maintained PIP$_2$ levels in the membrane of cardiomyocytes are important for compensatory cardiac function.

**DISCUSSION**

Activation of GqPCR is expected to hydrolyze PIP$_2$ into IP$_3$ and DAG; thus membrane PIP$_2$ levels would be expected to fall. However, in this study, we demonstrated that activation of $\alpha_1$ receptor (and $\beta_1$ receptor) or AT1 receptor with NE and Ang II, respectively, did not reduce but increased cellular PIP$_2$ levels in primary cultured adult rat cardiomyocytes. These results, combined with earlier findings in pancreas, brain (10-12), and hearts (13), point to a general phosphoinositide metabolism event in the cell: Activation of GqPCR not only initiates the hydrolysis of PIP$_2$ but also simultaneously starts the biochemical mechanisms that favor the increased resynthesis of PIP$_2$.

Importantly, we provided a novel mechanism for this GqPCR-mediated phosphoinositide production: PKC mediates the enhancement of PI4-kinase activity. First, using specific blockers, we found that two downstream signaling molecules, PLC and PKC, were indispensable to the NE/Ang II-induced increase in PIP$_2$ levels. Thus, it seems that the agonist-induced activation of the Gq/PLC pathway and the hydrolysis of PIP$_2$ itself initiate the compensatory mechanism of enhanced PIP$_2$ synthesis, a handy mechanism for maintaining global PIP$_2$ stabilization. We then demonstrated that the key to the coupling of PIP$_2$ hydrolysis and enhanced synthesis was the interaction between PKC, the downstream product of PIP$_2$ hydrolysis, and PI4-kinase; activation of PKC by the Gq-PLC-PIP$_2$ pathway increases its interaction with PI4-kinase, which increases the activity of the latter.

We went further to show that only PI4KIII$\beta$, but not all PI4-kinases, selectively participates in this PKC-mediated event. Mammalian cells express two classes of PI4-kinases, termed types II and III, and each class contains $\alpha$- and $\beta$-isoforms (40). The type-III kinases are inhibited by micromolar concentrations of wortmannin (9). The fact that wortmannin and PIK93, a specific blocker of PI4KIII$\beta$, abolished the NE/Ang II-induced PIP$_2$ increase but did not affect basal PIP$_2$ levels suggests that NE/Ang II selectively activates the type-III kinases, more specifically, PI4KIII$\beta$. On the other hand, adenosine, a proposed type-II kinase inhibitor, reduced basal PIP$_2$ levels but did not affect the NE/Ang II-induced PIP$_2$ increase. These results suggest that PIP$_2$ in the membrane could come from different pools that are synthesized by different PI4-kinases. The type-III PI4-kinases come into play only when phosphoinositides begin to be depleted in the membrane with continued PLC activation (41). As membrane trafficking to the cell membrane is increased during GqPCR activation, membrane insertion during receptor activation likely brings PI4-kinases to the surface membrane, along with other materials required for the synthesis of PIP$_2$.

In our previous study, we reported that an enhanced interaction between PKC and PI4KIII$\beta$ is responsible for the PKC-mediated activation of PIP and PIP$_2$ synthesis in *Xenopus* oocytes (34). In this study, we confirmed that a similar mechanism exists in cardiomyocytes, with an important role linking the Gq/PLC pathway and membrane phosphoinositide
metabolism.

Although NE is more often considered to be an agonist of α-adrenergic receptors, it does activate β-adrenergic receptors (19, 36), which also contribute to phosphoinositide production. However, the β receptor-mediated PIP$_2$ increase was primarily via the increased expression of PIP5Kγ. This result implies that the expression of PIP5Kγ is regulated within minutes of NE action. Although we did not find markedly increased expression of PI4-kinase (only its increased interaction with PKC) in this study, we did find increased expression of PI4-kinase in *Xenopus* oocytes when treated with PMA within tens of minutes (34). These results are not surprising because enzymes of crucial significance in metabolic and growth processes frequently have short half lives as their function is to appear promptly when there is expression of a vital process and to decline rapidly when their role is completed. Involvement of the rapid turnover of PIP5Kγ in NE-induced synthesis in this current study was manifested by the effect of CHX, a protein synthesis inhibitor.

β receptor is a Gs-coupled receptor, the activation of which usually leads to the activation of cAMP/PKA pathway. Not surprisingly, the increased expression of PIP5Kγ induced by NE was blocked by PKA blocker H89. Although Ang II and PMA also increased the expression of PIP5Kγ, their effects were much weaker than that of NE or Iso. The weak effect brought by Ang II and PMA on PIP5Kγ expression is likely the result of another mechanism: There have been reports suggesting that crosstalk between the cAMP/PKA pathway and PKC activation might regulate PIP5-kinase activity (38).

What then is the physiological significance of the NE/Ang II-induced modulation of cardiomyocyte PIP$_2$? As the activities of PLC and PKC are essential to NE/Ang II-induced cardiomyocyte hypertrophy (8, 42), PIP$_2$ may play an important role in the hypertrophic remodeling process through the functional modulation of signaling proteins and ion channels (43). Several transporters and ion channels families in cardiac myocytes, including K$_{ATP}$ channels, and Na$^+$-Ca$^{2+}$ exchangers (NCX) (44), GIRK/K$_{Ach}$ (45), and KCNQ/I$_{Ks}$ (46), need PIP$_2$ for their physiological functions. Importantly, these ion channels are modulated by GqPCR agonists, including phenylephrine and Ang II through modulation of membrane PIP$_2$ (47). It is not clear whether the PIP$_2$-mediated modulation of these ion channels and transporters directly contributes to the modulation of cardiac function in this study. Nonetheless, in this study, the measured systolic function correlated well with the PIP$_2$ levels in cultured cardiomyocytes treated with NE or Ang II. Furthermore, the apparent disparity in cardiac PIP$_2$ level between the two hypertrophic animal models indicates that PIP$_2$ has different metabolic patterns in different functional states of hypertrophic processes. It has already been reported that wortmannin can induce heart failure (48-50). Thus, blocking the resynthesis of PIP$_2$ with wortmannin may accelerate the process of cardiomyocyte hypertrophy. Overall, the results indicate that the increased in cellular PIP$_2$ levels is a possible compensatory mechanism related to maintaining normal cardiomyocyte systolic function. Our present results suggest that membrane PIP$_2$ could be a crucial factor in determining compensatory capability, at least systolic function, in hearts under stress.
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FOOTNOTES

The abbreviations used are: PtdIns (PI), phosphatidylinositol; PIP₂, phosphatidylinositol-4,5-bisphosphate; GqPCR, Gq protein-coupled receptor; PLC, phospholipase C; IP₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PIP, phosphatidylinositol 4-phosphate; PI4K, phosphatidylinositol 4-kinase; NE, norepinephrine; Ang II, angiotensin II; PKC, protein kinase C; TLC, thin layer chromatography; Bis-1, bisindolylmaleimide-1; PMA, phorbol 12-myristate 13-acetate; Iso, isoprenaline; PKA, protein kinase A; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; CHX, cycloheximide

FIGURE LEGENDS

FIGURE 1. The effects of NE and Ang II on PIP₂ levels in cultured adult rat cardiomyocytes. A. Total PIP₂ levels in adult rat cardiomyocytes treated with Ang II (3 μM, 24 hours) or NE (5 μM, 2 hours) measured by TLC. Iodine staining of phospholipids is shown in the left panel. Normalized PIP₂ levels obtained from TLC analysis were shown in the right panel. *P < 0.01 vs. control (n=17 animals). B. Representative results of the lipid-protein overlay assay of PIP₂ levels in adult rat cardiomyocytes. C. The effect of prazosin (3 μM) and losartan (3 μM) on the NE- and Ang II-induced increase in total PIP₂, respectively (n=7 animals).

FIGURE 2. The effects of blockers of PLC, PKC, and PI4K on the NE- and Ang II-induced increase in PIP₂ and PIP levels. A–D. The effects of U73122 (0.8 μM), U73343 (0.8 μM), Bis-1 (2 μM) and wortmannin (wort, 10 μM) on the NE- (A and B) or Ang II- (C and D) induced increase in total PIP₂ and PIP levels in cardiomyocytes. All blockers were added 30 minutes before either NE or Ang II was given. PIP and PIP₂ were extracted after incubation with NE (2 hours) or Ang II (24 hours). These results were confirmed using lipid-protein overlay assay. *P < 0.05 vs. control.

FIGURE 3. NE, Ang II, and PMA enhanced the interaction between PKC and PI4KIIIβ. A and B. The effects of PIK93 (A, 250 nM) and adenosine (B, 2 μM) on basal and the NE- (2 hours) or Ang II (24 hours)-induced increase in PIP and PIP₂ in primary cultured cardiomyocytes (n=5–7 animals): Adenosine (2 μM, 2 hours), but not PIK93 (250 nM, 2 hours), reduced basal PIP₂ levels in primary cultured cardiomyocytes. C. Co-immunoprecipitation of PKC isoforms and PI4KIIIα. D and E. Representative results of co-immunoprecipitation experiments for interactions between PKCα and PI4KIIIβ (E), between PKCβII and PI4KIIIβ (F) are shown in the left panels. Protein lysates from cardiomyocytes, which were treated with NE (5 μM), Ang II (3 μM), or Iso (0.5 μM) for 2 hours, or PMA (0.5 μM) for 20 minutes, were immunoprecipitated and blotted with anti-PI4KIIIβ, anti-PKCα, or anti-PKCβII antibodies as indicated. The right panel shows summary data for the normalized relative intensity of the precipitates (n=7 animals). *P < 0.05 vs. control.

NE and Ang II increase activity of PtdIns kinases and cardiac PIP2 level
FIGURE 4. Activation of the β-adrenergic receptor increases expression of PIP5Kγ and PIP₂ levels. Representative western blots of membrane proteins from cardiomyocytes probed with antibodies against PIP5Kγ and Akt are shown in the left panels of A and B. Normalized data from densitometry analyses are shown in the right panels. *P < 0.05 vs. control. #P < 0.05. The western blot band intensity of each group was first normalized to each corresponding Akt band intensity. The concentrations of the drugs used were as follows: NE (5 μM), Iso (0.5 μM), Ang II (3 μM), PMA (0.5 μM), propranolol (3 μM), H89 (30 μM), prazosin (3 μM) and Bis-1 (2 μM). C. The effect of Iso on PIP₂ levels and the effect of propranolol on the NE-induced increase of PIP₂ levels. D. Left panel: effect of CHX (10 μM) on the NE-induced increase in PIP₂ levels; Right panel: effect of CHX (10 μM) on PIP5Kγ expression. CHX was able to downregulate cardiac PIP5Kγ expression with or without NE. The summary data were normalized to the corresponding control values, which were obtained from cells treated with solvent alone for the same period of time as for CHX treatment (n=5 animals). *P < 0.05, **P < 0.01 vs. control.

FIGURE 5. Correlation of PIP₂ level with cardiomyocyte function and different hypertrophic hearts. A. Time course for the effects of NE and Ang II on PIP₂ level of cardiomyocytes. NE (5 μM, red circles) and Ang II (3 μM, black squares) were administered to cultured rat cardiomyocytes for the periods of time indicated (n = 6–15 animals). B and C. The time course of the effects of NE and Ang II on cardiomyocyte systolic function. B. A recording sample of sarcomere shortening; myocytes were first incubated with either NE (5 μM) or Ang II (3 μM) for the periods of times indicated. C. The time course of the effects of NE and Ang II on cardiomyocyte systolic function. The data show the percentage of cell length shortening. The control values were from the cells treated with solvent for the same periods of time as indicated (n=11–17 animals). D. Total PIP₂ levels in rat hearts from Iso- and swimming-induced cardiac hypertrophy (n=4 animals). *P < 0.05, **P < 0.01 vs. control.
Figure 1

NE and Ang II increase activity of PtdIns kinases and cardiac PIP2 level

A

B

C
Figure 2

*NE and Ang II increase activity of PtdIns kinases and cardiac PIP2 level*
**Figure 3**

*NE and Ang II increase activity of PtdIns kinases and cardiac PIP2 level*

**A**

![Graph A](image_url)

**B**

![Graph B](image_url)

**C**

![IB: PI4KIIIα](image_url)

**D**

![IP: PKCα](image_url)

**E**

![IP: PI4KIIIβ](image_url)
NE and Ang II increase activity of PtdIns kinases and cardiac PIP2 level

A

B

C

D

Figure 4

Normalized PIP5Kγ expression control

NE

Iso

PMA

Ang II

1.0

0.5

0.0

0

1

2

3

4

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Figure 5

NE and Ang II increase activity of PtdIns kinases and cardiac PIP2 level

A

![Graph showing normalized PIP2 level over time with Ang II and NE treatments.]

B

![Images showing tissue samples with control, NE 24 h, and Ang II 24 h treatments.]

C

![Bar graph showing amplitude of contraction (% shortening) at different times with control, NE, and Ang II treatments.]

D

![Bar graph showing normalized PIP2 level with control, Iso, and swim treatments.]

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