RBCK1, a Protein Kinase CβI (PKCβI)-interacting Protein, Regulates PKCβ-dependent Function*

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RBCK1 (RBCC protein interacting with PKC β) has originally been identified as a protein kinase CβI (PKCβI)-binding partner by a two-hybrid screen and as one of the gene transcripts that increases during adult cardiac hypertrophy. To address whether RBCK1 and PKCβI functions are interconnected, we used cultured neonatal myocytes where we previously found that the activity of PKCβI is required for an increase in cell size, also called hypertrophy. In this study, we showed that acute treatment of cardiac myocytes with phenylephrine, a prohypertrophic stimulant, transiently increased the association of RBCK1 with PKCβI within 1 min. A prolonged phenylephrine treatment also resulted in an increase of the interaction of the two proteins. Endogenous RBCK1 protein levels increased upon phenylephrine-induced hypertrophy. Further, adenovirus-based RBCK1 overexpression in the absence of phenylephrine required PKCβ activity, since the increase in cell size was inhibited when the RBCK1-expressing cells were treated with PKCβ-selective antagonists, supporting our previous observation that both PKCβI and PKCβII are required for hypertrophy. Unexpectedly, RBCK1-induced increased cell size was inhibited by phenylephrine. This effect correlated with a decrease in the level of both PKCβ isoforms. Most importantly, RNA interference for RBCK1 significantly inhibited the increase in cell size of cardiac myocytes following phenylephrine treatment. Our results suggest that RBCK1 binds PKCβI and is a key regulator of PKCβI function in cells and that, together with PKCβII, the three proteins are essential for developmental hypertrophy of cardiac myocytes.

Activation of protein kinase C (PKC) isoforms, a family of 10 serine-threonine kinases, is associated with translocation from the soluble to the particulate cell fraction (1). PKC activation depends on phosphatidylycerine and diacylglycerol and to different extents on calcium and other lipid second messengers. Translocation of PKC to the particulate fraction was initially thought to reflect direct association of the enzyme with membrane lipids. However, such association cannot solely reflect the diverse subcellular location of each inactive and active PKC isoform. Data from several laboratories indicate that the functional selectivity of individual PKC isoforms is determined, at least in part, by protein-protein interactions. Different types of PKC-interacting proteins have been described, including several AKAP scaffold proteins (2, 3), STICKs (substrates that interact with C kinase) (4–6), and RACKs (receptors for activated C kinase) (7–9). In neurons, the scaffold protein AKAP79 assembles three enzymes in a complex, including PKC, the cAMP-dependent protein kinase, and calcineurin, a protein phosphatase (2). STICKs, such as MARCKS (myristoylated alanine-rich C kinase substrate) and adducin, are PKC-binding proteins as well as PKC substrates (4, 6). The functional specificity of each PKC isoform is determined, in part, by the differential localization of the C kinase-specific RACKs (7, 9, 10). RACKs anchor activated PKC isoforms in close proximity to their selective substrates (9), but are not PKC substrates. Several of the RACK-binding sites on the classical and novel PKC isoforms have been identified (11–16), and short peptides derived from these binding sites specifically inhibit the function of the corresponding PKC isoforms both in vitro and in vivo (15, 17–20). Like AKAPs, RACKs anchor other proteins in addition to PKC (21) (e.g. in addition to binding to activated PKCβII (7), RACK1 interacts with Src (22), phospholipase C (23), integrins (24), ribosomal RNA (25), and PDE4D5 (26)). Furthermore, upon activation, PKCβII associates with RACK1 prior to their movement to the active site of PKCβII, suggesting a role for RACK1 as a shuttle protein (27). Finally, RCKs (receptors for inactive C kinase) were also postulated to inhibit and sequester PKC isoforms prior to their activation (28).

Here, we describe a PKCβI-interacting protein, RBCK1, and the functional consequences of this interaction in neonatal cardiac myocytes. RBCK1 has previously been identified as a PKC-binding partner by a yeast two-hybrid system using the regulatory domain of PKCβI as bait (29). RBCK1 is a 498-amino acid protein containing two coiled-coil regions, a RING finger, and two SH3 domains, integrating (24), ribosomal RNA (25), and PDE4D5 (26)). Furthermore, upon activation, PKCβII associates with RACK1 prior to their movement to the active site of PKCβII, suggesting a role for RACK1 as a shuttle protein (27). Finally, RCKs (receptors for inactive C kinase) were also postulated to inhibit and sequester PKC isoforms prior to their activation (28).

We set out to characterize the interaction of RBCK1 with PKCβI in primary heart cells in culture. The choice of this culture stems from our earlier finding that PKCβ function is required for the increase in cardiac myocyte cell size, a process
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Isolation and Infection of Rat Neonatal Cardiac Myocytes—Care of rats in this investigation conforms to Ref. 57. Cardiac myocytes were isolated as previously described from 1-day-old Sprague-Dawley rat litters (37) or by using the cardiomyocyte isolation kit from Cellutron. Cardiac myocytes represent 90–95% of total adherent cells. Cells were maintained in Eagle’s minimal essential medium with Earle’s balanced salt solution (containing 50 units/ml penicillin, 80 μM vitamin B₁₂, 0.1 mM bromo-oxyuridine, and 80 μM vitamin C) with 10% serum after plating. For all of the experiments, cells were transferred in serum-free medium (Eagle’s minimal essential medium with Earle’s balanced salt solution containing 10 μg/ml insulin, 10 μg/ml transferrin, 80 μM vitamin C, 50 units/ml penicillin, and 80 μM vitamin B₁₂) on day 3. Infections were performed on day 3 at a multiplicity of infection of 5. Twenty-four hours after the infection, the medium containing the adenovirus was removed and replaced by fresh serum-free medium. Phenylephrine was added at this time for the 48-h prolonged treatment.

RNA Interference Knockdown and Reverse Transcription-PCR—Stealth RNAi molecules were designed using the Invitrogen site on the World Wide Web and transfected into cardiac myocytes with TransIT-TKO transfection reagent following the recommended protocol (Mirus). Stealth RNAi transfection efficiency was determined by visualizing labeled stealth RNAi in cells. Transfected cells with stealth RNAi labeled or not were recovered for 24 h, serum-starved, and treated with the hypertrophic agent phenylephrine. The time course of the knockdown in RBCK1 expression was identified after isolating the RNA and performing reverse transcription-PCR or protein blots after transfections of the stealth RNAi. Glyceroldehyde-3-phosphate dehydrogenase was used as a standard. The primer sequences were as follows: RBCK1, GGAGGCGCTGCGCCAGTATGA (forward) and CAGGGGACAGGAGCGCCCGGA (reverse) to amplify a 310-bp product; glyceraldehyde-3-phosphate dehydrogenase, CCAGTATGATTCTACCCACGGC (forward) and CCAGATGATGACCTTTTGGC (reverse) to amplify a 141-bp product.

Cell Size Determination of Cultured Rat Neonatal Cardiac Myocytes—Myocytes overexpressing GFP or GFP-RBCK1 were photographed at ×63 magnification, and the single cell size was determined using PhotoShop software by outlining the cell periphery and measuring pixel number. For each measurement, all GFP-expressing myocytes in one chamber and an equal amount of myocytes in the GFP-RBCK1-expressing chamber were counted. The effect of RBCK1 knockdown was assessed on cardiac myocytes that were not overexpressing GFP. Phase-contrast pictures of these cells were taken using ×63 magnification, and cell size was determined.

Delivery of Peptide—The PKCBI/V5-3- and PKCBII/V5-3-selective antagonist peptides derived from PKCBI and PKCBII sequences (KLFIMNL and QEVIRNN, respectively) were conjugated to the TAT-(47–57) carrier peptide for transmembrane delivery as previously described (38). The TAT-(47–57) peptide was used as a control. All peptides were delivered three times every 4 h on day 4 (660 nM/each) and day 5 (330 nM). On day 6, the peptides were added 1 h prior to the cell lysis (330 nM).
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**Immunoprecipitation Experiments and Western Blot Analysis**—On day 6, cardiac myocytes infected with adenoviruses encoding for GFP or GFP-RBCK1 constructs were washed with cold phosphate-buffered saline and incubated in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1:300 protease inhibitor mixture, 1:300 phosphatase inhibitor mixture) for 30 min at 4 °C by gentle rocking. Myocytes were then scraped, disrupted by repeated aspiration through a 21-gauge needle, and collected in microcentrifuge tubes. Lysates were incubated with 20 μl of protein G-agarose beads for 30 min at 4 °C by gentle rocking and centrifuged at 10,000 × g for 10 min at 4 °C. Supernatants were collected, mixed with the anti-PKC or anti-GFP antibody (2 μg), and incubated at 4 °C for 1 h. Twenty microliters of protein G-agarose beads were then added, and the samples were incubated at 4 °C for 1 h. The beads were washed three times with 1 ml of lysis buffer. Immunocomplexes were resuspended in 50 μl of Laemmli buffer, loaded on SDS-PAGE, and transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibody followed by visualization by ECL. The phosphorylation of RBCK1 was determined using the mixture of anti-phosphoserine and phosphothreonine antibodies listed under “Materials.” The amount of phosphorylated RBCK1 was corrected to the amount of immunoprecipitated RBCK1.

**Translocation of PKCβI**—After acute treatment with phenylephrine, cardiac myocytes were washed with cold phosphate-buffered saline, scraped in homogenization buffer, and spun at 100,000 × g for 30 min at 4 °C. The supernatants were collected, mixed with the anti-PKC or anti-GFP antibody followed by phosphothreonine antibodies listed under “Materials.” The amount of phospho-PKCβ was determined using the mixture of anti-phosphoserine and phosphothreonine antibodies listed under “Materials.” The amount of phosphorylated PKCβ was corrected to the amount of immunoprecipitated PKCβ.

**RESULTS**

**RBCK1 Binds PKCβI in Rat Neonatal Cardiac Myocytes**—When co-expressed in COS-7 cells, RBCK1 bound preferentially to PKCβI (29). Based on these data, we first set out to determine whether RBCK1 binds PKCβI in neonatal cardiac myocytes and whether PKC activation with phenylephrine regulates their interaction. To this end, GFP-fused RBCK1 was overexpressed using adenovirus-mediated gene transfer. Greater than 95% of cells expressed the full-length RBCK1-GFP within 24 h after infection. The interaction between RBCK1 and PKCβI was assessed by immunoprecipitating endogenous PKCβI using an anti-PKCβI antibody followed by Western blot analysis of the immunoprecipitates with an anti-GFP antibody. Although similar amounts of endogenous PKCβI were immunoprecipitated (Fig. 1A), the amounts of RBCK1-GFP co-immunoprecipitated with PKCβI increased after a 1-min treatment with 5 μM phenylephrine followed by a decline 3 min after stimulation (Fig. 1, A and B). The kinetics of association correlated with that of PKCβI translocation to the cell particulate fraction and PKCβI transiently translocated within 1 min of phenylephrine treatment (Fig. 1C). These results demonstrate that activation of PKCβI with phenylephrine is associated with an increased binding to RBCK1. Note that because endogenous RBCK1 has a molecular weight that is close to that of the IgG, we carried out immunoprecipitation experiments from cross-linked cardiac myocyte lysates. Unfortunately, after this procedure, our antibodies did not recognize PKCβI or RBCK1. We were therefore unable to analyze the binding of PKCβI to endogenous RBCK1.

**Increased Interaction between PKCβI and RBCK1 after Prolonged Phenylephrine Treatment**—Prolonged treatment of cardiac myocytes with phorbol 12-myristate 13-acetate or phenylephrine causes increased cell size, which was shown to be dependent on PKCβ activation (15). We therefore set out to determine whether a prolonged treatment of cultured myocytes with phenylephrine modulates the interaction between endogenous PKCβI and RBCK1. Under basal conditions, a small amount of RBCK1 was seen to co-immunoprecipitate with endogenous PKCβI, and the interaction between both proteins increased after 48-h treatment with phenylephrine (Fig. 2A). An increased amount of PKCβI was co-immunoprecipitated with RBCK1 upon prolonged phenylephrine treatment as well (Fig. 2B). Finally, RBCK1 did not co-immunoprecipitate with endogenous PKCβII (Fig. 2C). A splice variant of PKCβII that differs only in the last 50 amino acids, RBCK1 did not co-immunoprecipitate either with PKCζ (Fig. 2D), an isozyme previously shown to interact with RBCK1 when co-expressed in COS cells (29). When PKCζ was success-
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**FIGURE 2.** Specific interaction between endogenous PKCβI and RBCK1-GFP in primary culture of neonatal cardiac myocytes upon prolonged phenylephrine treatment. A, representative blot of co-immunoprecipitation (IP) with an anti-PKCβI antibody, probed with an anti-PKCβI and then with an anti-GFP antibody. Cells treated with 5 μM PE for 48 h exhibited enhanced PKCβI/RBCK1-GFP protein-protein interaction when compared with untreated cells. The amount of RBCK1 that was co-immunoprecipitated with PKCβI is normalized to the amount of PKCβI that was immunoprecipitated. Quantitation of four independent co-immunoprecipitation experiments (*, p < 0.002 versus no stimulation), B, representative blot of co-immunoprecipitation with an anti-GFP antibody, probed with an anti-PKCβI and then an anti-GFP antibody showing the reverse co-immunoprecipitation from A. C, RBCK1-GFP did not co-immunoprecipitate with PKCβI. Representative blot of co-immunoprecipitation with an anti-PKCβI antibody, probed with an anti-PKCβI antibody and then an anti-GFP antibody. D, RBCK1-GFP did not co-immunoprecipitate with PKCζ. A representative blot is shown of co-immunoprecipitation with an anti-GFP antibody, probed with an anti-PKCζ antibody and then an anti-GFP antibody. E, RBCK1-GFP is more phosphorylated upon PE treatment. Shown is a representative experiment of three independent experiments showing the level of phosphorylation of RBCK1-GFP, immunoprecipitated with an anti-GFP antibody and probed with a mixture of anti-phosphoserine and phosphothreonine antibodies (top blot) or anti-GFP antibody (bottom blot). Cells were treated with or without PE (5 μM PE for 1 min, 3 min, or 48 h). The lower panel shows a 48% increase in RBCK1 phosphorylation after 1 min and a 73% increase after 48 h of PE treatment relative to RBCK1 from nontreated cells.

**FIGURE 3.** Endogenous RBCK1 protein levels are increased in cultured cardiac myocytes in response to prolonged phenylephrine treatment. A, whole lysates from untreated or PE-treated cardiac myocytes (5 μM PE for 48 h) were analyzed by Western blot for RBCK1 expression. A representative blot of endogenous RBCK1 is shown. B, histogram showing a significant increase in RBCK1 protein levels upon phenylephrine-induced hypertrophy. Quantitative analysis from three independent experiments is shown. Western blotting against glyceraldehyde-3-phosphate dehydrogenase of the same membrane was used as an internal control for loading and for normalization. *, p < 0.0001 versus no stimulation.

PKCβI observed following both acute (Fig. 1) and 48-h (Fig. 2A) treatment with phenylephrine is correlated with an increased phosphorylation of RBCK1.

**RBCK1 Protein Levels Increase during Hypertrophy**—Although two gene expression profiling studies have demonstrated changes of RBCK1 mRNA levels in association with cardiac hypertrophy (34, 39), no data were provided on whether the protein levels were also altered. Using RBCK1 antisera, raised against the C-terminal amino acids 420–434, we found that the endogenous RBCK1 protein was present in cultured neonatal myocytes. Most importantly, the level of endogenous RBCK1 increased by 2-fold in the presence of the hypertrophic stimulus (phenylephrine, 5 μM, 48 h) (Fig. 3).

**PKCβ Is Required for RBCK1-induced Increase of Cell Size**—We showed a correlation between an increase of RBCK1 protein levels and myocyte hypertrophy, but evidence of direct causality has not been established. Therefore, we next determined whether increased RBCK1 protein level mediates the development of myocyte hypertrophy or whether it is a consequence of this phenotype. Again, we used RBCK1-GFP overexpression.

One hallmark of hypertrophy is increased cell surface area. The GFP tag enabled us to focus only on live beating cells (i.e. cardiac myocytes that express the transgenes). As shown in Fig. 4A, the quantification of several independent experiments demonstrated a significant increase in cell surface area when RBCK1 was overexpressed (153%; lane 5 versus lane 1). Fig. 4B, which shows representative pictures of control and RBCK1-overexpressing cells, demonstrates the differences described. Overexpression of RBCK1 was therefore sufficient to increase the cell size of cardiac myocytes in the absence of phenylephrine.

The mechanism whereby overexpression of RBCK1 promotes cardiac myocyte hypertrophy is unknown. However, PKCβ was previously reported to mediate cardiac hypertrophy (15, 32, 33, 40, 41), and our results showed that RBCK1 interacts with PKCβ in cardiac myocytes (Figs. 1 and 2). Based on these data, we hypothesized that the RBCK1 pathway may involve PKCβ. Therefore, we set out to measure cell size in cardiac myocytes overexpressing RBCK1 after delivery of either PKCβI- or PKCβII-selective antagonist. The cell surface area of
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**FIGURE 4.** Adenovirus-mediated overexpression of RBCK1 is sufficient to induce cardiac myocyte hypertrophy in the absence of phenylephrine; dependence on PKCβ activation. A, cardiac myocyte cell surface areas were quantified from GFP- or RBCK1-GFP-infected cultures. Beating cells (cardiac myocytes) were imaged, and surface areas were calculated with Photoshop software. Values are relative to GFP-expressing cells that received no other treatment (lane 1). Overexpression of RBCK1-GFP increased the cardiac myocyte cell surface by 50% in the absence of PE treatment (lane 5). Treatment with the specific PKCβ (lanes 3 and 7) or PKCβ antagonist peptide (lanes 4 and 8) prevented the effect of RBCK1-GFP overexpression on the increase in cell surface. Averaged results from four independent experiments are shown, *p < 0.02 versus RBCK1-GFP control; #, p < 0.04 versus GFP control. Tat carrier peptide was used as a control (lanes 2 and 6). B, shown are representative photographs of cardiac myocytes from A.

Heart myocytes overexpressing RBCK1 and treated with the PKCβ- or PKCβII-selective inhibitors was significantly smaller than untreated myocytes overexpressing RBCK1 (Fig. 4A, lanes 7 and 8 versus lane 5) and comparable with the cell surface area of myocytes overexpressing GFP as a control (Fig. 4A, lanes 7 and 8 versus lane 1). Representative images are shown in Fig. 4B. In conclusion, βIV5-3 and βIIIV5-3 peptides inhibited the effects of RBCK1-induced increase in cell size, suggesting that both PKCβ isozymes are critical downstream components of the signaling pathways activated by RBCK1 in neonatal cardiac myocytes. Note that both peptide treatments had no significant effect on the cell size of control cells overexpressing GFP (Fig. 4A, lanes 3 and 4 versus lane 1).

**RBCK1-induced Hypertrophy Inhibition by Phenylephrine Is Correlated to the Reduction of the Level of Endogenous PKCβ**—We next determined whether RBCK1 affects phenylephrine-induced hypertrophy. As previously reported (42, 43), 48-h stimulation with phenylephrine induced an increase in cell size of control myocytes overexpressing GFP alone to 130% (Fig. 5, A (lane 2 versus lane 1) and B). However, the cell size with both phenylephrine and overexpressed RBCK1 was smaller than the cell size in the presence of phenylephrine alone (Fig. 5, lane 7 versus lane 2) or overexpressed RBCK1 alone (Fig. 5, A (lane 7 versus lane 6) and B). Our data demonstrate that, unexpectedly, RBCK1-induced hypertrophy is inhibited by phenylephrine treatment. The expression level of RBCK1 in phenylephrine-treated cells was comparable with untreated cells (data not shown). Therefore, the lack of hypertrophy in RBCK1-overexpressing cells treated with phenylephrine is mediated through an independent mechanism. Moreover, the PKCβ- or PKCβII-selective antagonist peptides prevented phenylephrine-induced hypertrophy of GFP-overexpressing myocytes (Fig. 5, lanes 4 and 5 versus lane 2). On the other hand, PKCβ- or PKCβII-specific inhibition did not induce additional decrease of the cell size of RBCK1-overexpressing myocytes treated with phenylephrine (Fig. 5, lanes 9 and 10 versus lane 7), suggesting that both PKCβ1 and PKCβIII are no longer active compared with phenylephrine-treated control cells.

We were puzzled by the lack of hypertrophic effects following the concomitant RBCK1 overexpression and phenylephrine treatment, since each induce hypertrophy through a PKCβ-dependent pathway. We therefore determined the levels of PKCβ1 and PKCβIII in RBCK1-overexpressing cells. In the absence of phenylephrine, the levels of PKCβ1 were not significantly affected by RBCK1 overexpression (Fig. 6A, left bars). However, phenylephrine-induced increase in expression of PKCβ1 and PKCβIII was completely blocked by the overexpression of RBCK1 (Fig. 6, A and B, right bars). These results demonstrate that RBCK1 overexpression abrogates the phenylephrine-inhibited hypertrophy of myocytes.
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Endogenous RBCK1 modulates phenylephrine-induced hypertrophy. A, the cell surface area of cardiac myocytes treated for a prolonged period of time with phenylephrine was quantified. Values are relative to the untreated control cells. Treatment with 5 μM PE for 48 h induced a 50% increase in cell surface of control cells. The cell surface area of cardiac myocytes transfected with a control siRNA was comparable with control cells. Transfection with RBCK1 siRNA prevented the PE-induced increased cell size of cardiac myocytes. Averaged results from three independent experiments are shown. #, p < 0.005 versus untreated control cells; *, p < 0.008 versus PE-treated control cells. B, RBCK1 mRNA and protein levels after siRNA transfection. Reverse transcription-PCR and Western blot analysis were carried out on control cells (1), cells transfected with the control siRNA (2), and cells transfected with RBCK1 siRNA. On the day of the experiments, RBCK1 levels were significantly reduced.

overexpressed atypical PKCζ was previously described (29). PKCζ, which is not activated by phorbol 12-myristate 13-acetate or phenylephrine and does not play a role in the development of cardiac hypertrophy, did not bind to RBCK1 in our experimental model (Fig. 2D), using the same conditions for co-immunoprecipitation of RBCK1 with PKCβI (Fig. 2A). A possible explanation for this discrepancy is that RBCK1 binding to PKCζ is cell type-specific or does not occur with endogenous levels of PKCζ. Together, our present data strongly suggest that, in rat neonatal cardiac myocytes, RBCK1 preferentially interacts with PKCβI.

Moreover, RBCK1 could be the anchoring protein or RACK (9, 11) for PKCβI. In addition, like the PKCβI-specific RACK, which shuttles that isozyme to its site of activity (27), RBCK1, which shuttles between the cytosol and the nucleus (30), may participate in PKCβI translocation. Supporting this suggestion, we found that the association of PKCβI with RBCK1 has a similar time course to the translocation of PKCβI from the cell soluble to the cell particulate fraction (Fig. 1). Because RBCK1 reversibly binds PKCβI upon phenylephrine-induced translocation (Fig. 1), RBCK1 is probably a RACK or/and a protein that shuttles PKCβI to its site of activity. In our effort to identify the mechanism by which phenylephrine increases the affinity of RBCK1 for PKCβI, we found that RBCK1 phosphorylation is increased following both acute and long term treatment with phenylephrine (Fig. 2E). However, the identification of the kinase responsible for RBCK1 phosphorylation and determination of whether RBCK1 phosphorylation is leading to the increased affinity for PKCβI require further experiments.

The expression of PKCβ is increased during pathological cardiac hypertrophy in adult animals and in failing hearts of humans (44–47), and transgenic mice overexpressing PKCβ in

FIGURE 7. Endogenous RBCK1 modulates phenylephrine-induced hypertrophy. A, the cell surface area of cardiac myocytes treated for a prolonged period of time with phenylephrine was quantified. Values are relative to the untreated control cells. Treatment with 5 μM PE for 48 h induced a 50% increase in cell surface of control cells. The cell surface area of cardiac myocytes transfected with a control siRNA was comparable with control cells. Transfection with RBCK1 siRNA prevented the PE-induced increased cell size of cardiac myocytes. Averaged results from three independent experiments are shown. #, p < 0.005 versus untreated control cells; *, p < 0.008 versus PE-treated control cells. B, RBCK1 mRNA and protein levels after siRNA transfection. Reverse transcription-PCR and Western blot analysis were carried out on control cells (1), cells transfected with the control siRNA (2), and cells transfected with RBCK1 siRNA. On the day of the experiments, RBCK1 levels were significantly reduced.

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The myocardium develop hypertrophy (32, 33). The primary culture of neonatal cardiac myocytes is often referred to as a model representing developmental hypertrophy. In culture, cardiac myocyte cell size increases following prolonged treatment with phenylephrine (42, 43), and we have showed that this mechanism is dependent, at least in part, on the activation of PKCβ (15). Because the PKCβ1-RBCK1 interaction increases during prolonged phenylephrine treatment, we hypothesized that RBCK1 is a potential regulator of PKCβ-mediated hypertrophy. Indeed, with the use of adenovirus, we showed that RBCK1 overexpression increases hypertrophy by a downstream activation of PKCβ (Fig. 4). We previously reported a role for both PKCβ1 and PKCβII in cardiac hypertrophy (15). What we have shown here is that although PKCβ1 and not PKCβII binds to RBCK1, both affect RBCK1-induced hypertrophy (Fig. 4). These data strongly suggest that PKCβII is downstream from RBCK1. Most importantly, we showed that following RBCK1 knockdown, cultured neonatal cardiac myocytes do not undergo phenylephrine-induced hypertrophy. To our knowledge, this is the first evidence that RBCK1 plays a role in regulating the cell growth of cardiac myocytes.

Our study suggests that phenylephrine-induced hypertrophy may involve the same signal transduction mechanisms induced by RBCK1 overexpression. βI and βII PKC-selective antagonists blocked both phenylephrine-induced hypertrophy and RBCK1-induced hypertrophy (Fig. 3). Therefore, endogenous RBCK1 may contribute to the development of cardiac hypertrophy. However, the exact pathways by which endogenous RBCK1 modulates PKCβ are not yet known.

Unexpectedly, phenylephrine prevented RBCK1-induced hypertrophy in a process that is independent of PKCβ activation, but may be due to PKCβ down-regulation. Given that phenylephrine that activates PKCβ induces a hypertrophic response and that the hypertrophic responses to RBCK1 involve PKCβ, we hypothesized that PKCβ may be inactivated in the presence of both hypertrophic stimulations over a prolonged time course. Supporting our hypothesis is the finding that, in contrast to control cells, those overexpressing RBCK1 do not show increases in the level of PKCβ with phenylephrine stimulation. It is possible that PKCβ degradation is increased. Indeed, one established way to inactivate PKC upon sustained activation is a degradation pathway known as down-regulation (48). In that context, it is interesting to note that RBCK1 has a potential E3 ubiquitin ligase activity (30). It has been shown that HOIL-1, the human splice variant of RBCK1, has a ubiquitin ligase activity for IRP2 (heme-oxidized iron-regulatory protein-2) (49). Once ubiquitinated, proteins are targeted to proteasomal degradation (50, 51), and importantly, several studies have shown ubiquitination-mediated degradation of PKC (52–56). Therefore, one explanation could be that during phenylephrine treatment, RBCK1-induced ubiquitination of PKCβ leads to the degradation of PKCβ by the proteasome. However, we were unable to detect any difference in PKCβ protein steady-state levels or accumulation of multiubiquitinated forms of PKCβ in the presence of MG132, a proteasome inhibitor (10 μM, 6 h, data not shown). Therefore, the reduced PKCβ level in phenylephrine-treated cells concomitant with the overexpression of RBCK1 may be mediated independently of a ubiquitin ligase activity. Since RBCK1 is also a transcription factor (29–31), it is more likely that RBCK1 modulates the mRNA levels of PKCβI and PKCβII. Supporting this explanation is our observation that the phenylephrine-induced increase in protein levels of PKCβII, which does not interact with RBCK1, is also prevented upon RBCK1 overexpression.

In summary, a major finding of this study is the interaction of PKCβI with RBCK1 in neonatal cardiac myocytes and the potential function of this association in the modulation of cardiac cell size during developmental hypertrophy, a novel role for RBCK1 in the heart. Our data also indicate that PKCβ may serve as a key signaling mechanism for the manifestation of RBCK1-induced hypertrophy. Finally, the selective interaction of PKCβI with RBCK1 suggests that this protein serves as a RACK or a scaffold protein for PKCβII. It remains to be determined whether RBCK1 and its interaction with PKCβII are altered in cardiac disease and whether the PKCβ-RBCK1 interaction would serve as a potential therapeutic target to treat pathological hypertrophy.

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