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Novel Role of Protein Kinase C Delta Tyr^{311} Phosphorylation in Vascular Smooth Muscle Cell Hypertrophy by Angiotensin II

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Abstract

We have previously shown that activation of protein kinase C δ (PKCδ) is required for angiotensin II (AngII)-induced migration of vascular smooth muscle cells (VSMCs). Here, we have hypothesized that PKCδ phosphorylation at Tyr^{311} plays a critical role in VSMC hypertrophy induced by AngII. Immunoblotting was used to monitor PKCδ phosphorylation at Tyr^{311}, and cell size and protein measurements were used to detect hypertrophy in VSMCs. PKCδ was rapidly (0.5–10 min) phosphorylated at Tyr^{311} by AngII. This phosphorylation was markedly blocked by a Src family kinase inhibitor and dominant-negative Src, but not by an epidermal growth factor receptor kinase inhibitor. AngII-induced Akt phosphorylation and hypertrophic responses were significantly enhanced in VSMCs expressing PKCδ wild type compared with VSMCs expressing control vector, whereas the enhancements were markedly diminished in VSMCs expressing PKCδ Y311F mutant. Also, these responses were significantly inhibited in VSMCs expressing kinase-inactive PKCδ K376A compared with VSMCs expressing control vector. From these data, we conclude that not only PKCδ kinase activation but also the Src-dependent Tyr^{311} phosphorylation contributes to Akt activation and subsequent VSMC hypertrophy induced by AngII, thus signifying a novel molecular mechanism for enhancement of cardiovascular diseases induced by AngII.

Keywords

angiotensin II; AT_1 receptor; signal transduction; protein kinase C δ; Src; hypertrophy; vascular smooth muscle cells

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Disclosures
None.

Introduction

Angiotensin II (AngII) plays a major role in vascular remodeling outside of its hemodynamic effects. In cultured vascular smooth muscle cells (VSMCs), cardiac myocytes, and cardiac fibroblasts, AngII has been shown to promote hypertrophy and/or hyperplasia. There are two subtypes of AngII receptors, AT_1 and AT_2, although the major physiological and pathophysiological actions of AngII are facilitated through the AT_1 receptor. In VSMCs, activation of the AT_1 receptor coupled to G_q increases intracellular Ca^{2+} and activates protein kinase C (PKC) [1,2]. In this regard, several PKC isoforms including PKCδ are believed to be activated by AngII in VSMCs [3–5]. In addition, various tyrosine kinases and serine/threonine kinases are rapidly activated by AngII and likely play important roles in mediating vascular remodeling induced by AngII [6,7]. However, the detailed role of each PKC isoform in mediating AngII-induced vascular remodeling as well as the possible signal crosstalk with other kinases has been insufficiently characterized.

Increasing evidence suggest that PKCδ is involved in many mechanisms promoting VSMC remodeling and dysfunction [8–11]. It was reported that PKCδ is activated by mechanical stress and VSMCs from PKCδ–null mice migrate slower than control VSMCs [12]. Previously, we have shown that PKCδ kinase activity is required for activation of several tyrosine kinases by AngII or reactive oxygen species in VSMCs [4,13,14]. Moreover we have recently reported that PKCδ is required for activation of Rho, Rho-kinase and c-Jun NH_2-terminal kinase, and subsequent migration of VSMCs by using kinase-inactive PKCδ over expression [15]. These data suggest an important role of PKCδ in mediating vascular remodeling induced by AngII.

PKCδ is also phosphorylated on tyrosine residues in many cells including VSMCs and cardiac myocytes [13,16–18]. Although there are multiple tyrosine phosphorylation sites on PKCδ, Tyr^{311} located between the regulatory and catalytic domains is of particular interest. This is because the Tyr^{311} phosphorylation has been linked to increased kinase activity in cells treated with H_2O_2 19. PKCδ phosphorylation at Tyr^{311} may also affect the selectivity of substrates [17]. Taken together with the above information, we have tested the hypothesis that PKCδ Tyr^{311} phosphorylation plays a major role in AngII-induced vascular hypertrophy. We found that PKCδ phosphorylation at Tyr^{311} was induced by AngII through a Src family kinase and that this phosphorylation was involved in Akt activation and subsequent VSMC hypertrophy.

Methods

An expanded Methods section describing reagents, primary antibodies, cell culture and statistical analysis is available at http://hyper.ahajournals.org.

Retrovirus infection

Wild type or Y311F PKCδ containing enhanced green fluorescent protein (GFP) at the C-terminus [20] was cloned into the pBM-IRES-PURO vector and high titer retroviral supernatants were generated [21]. VSMCs were infected with retrovirus and the infected VSMCs were selected as previously described [22,23]. To assess complete viral transformation after an antibiotic selection, in addition to the detection of the over-expression by immunoblotting, we routinely confirmed more than 99% infection efficiency of our retrovirus vectors by the GFP tagged to the mutants and detected under a fluorescent microscope.

Adenovirus infection

The generation of adenovirus encoding wild type and a kinase-inactive K376A PKCδ mutant construct and dominant negative K295M+Y527F Src was described previously [24,25]. The titer (pfu/ml) of adenovirus was determined by Adeno-XTM Rapid Titer Kit (BD Biosciences, Palo

Alto, CA). VSMCs were infected with adenovirus for 2 days as previously described [14]. To assess complete viral transformation, we routinely confirmed more than 99% infection efficiency of our adenovirus vectors by GFP encoded by these vectors separately and detected under a fluorescent microscope.

Immunoblotting

Cell lysates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane as previously described [26]. The membranes were then exposed to primary antibodies overnight at 4 °C. After incubation with the peroxidase-linked secondary antibody (Amersham Biosciences, Piscataway, NJ) with dilution between 1:1,000 – 1:10,000 (depending on the primary antibody) for 1 hour at room temperature, the immunoreactive proteins were visualized by a chemiluminescence reaction kit (Pierce, Rockford, IL).

Protein Assay

VSMCs on 12 well culture plates were incubated with serum-free DMEM for 3 days in retrovirus infected VSMCs. For adenovirus infection, VSMCs were incubated with serum-free DMEM for 1 day and infected with adenovirus at 100 multiplicity of infection in serum-free DMEM for 2 days. The cells were further incubated with or without 100 nmol/L AngII for 3 days. After aspiration of the medium, cells were washed twice with ice-cold Hanks balanced salt solution, and the total amount of cellular protein was measured as previously described [27].

Cell Volume Assay

After the pretreatments described in the protein assay, VSMCs were washed with Hanks balanced salt solution and trypsinized. The cells were then suspended in PBS and the cell volume was measured by Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Fullerton, CA) [27].

Proliferation Assay

After the pretreatments described in the protein assay, cell proliferation was measured using a CellTiter 96 Aqueous cell proliferation assay kit (Promega, Madison, WI) following the manufactures' protocol as previously described [27]. Basically, this assay measures cell viabilities upon the PKCδ manipulation with or without AngII stimulation for 3 days.

BrdU assay

After the adenovirus infection as described for the protein assay, the cells were pretreated with or without 100 nmol/L AngII for 24 hours, and BrdU incorporation was determined for an additional 24 hours by a BrdU incorporation kit (Calbiochem, La Jolla, CA) according to the manufactures' protocol.

Results

Phosphorylation of PKCδ at Tyr^{311} by AngII through the G_q-coupled AT_1 receptor

In 3 day serum-starved rat aortic VSMCs, AngII (100 nmol/L) stimulated phosphorylation of PKCδ at Tyr^{311} in a rapid (within 0.5 min) and transient manner with a peak of 2 to 5 min (Figure 1). The phosphorylation returned to the baseline level at 40 min (Figure S1A, please see http://hyper.ahajournals.org.). Thus, in subsequent experiments, unless otherwise stated, VSMCs were stimulated with AngII for 2 min for evaluation of the PKCδ phosphorylation. Pretreatment with an AT_1 receptor antagonist, RNH6270, totally blocked PKCδ phosphorylation by AngII (Figure S1B, please see http://hyper.ahajournals.org.). The AT_1 receptor is mainly coupled to the heterotrimeric G-protein G_q, whereas G protein-independent

signal transduction by the AT_1 has been reported [2]. Thus, we determined whether G_q contributed to the AngII-induced PKCδ phosphorylation. Pretreatment with a selective G_q inhibitor, YM-254890 [22,28], completely blocked PKCδ phosphorylation at Tyr^{311} by AngII (Figure S1C, please see http://hyper.ahajournals.org.), indicating that AngII-induced phosphorylation of PKCδ at Tyr^{311} was mediated through G_q activation.

Involvement of Src in PKCδ Tyr^{311} phosphorylation by AngII

Activation of the AT_1 receptor by AngII leads to rapid transactivation of the epidermal growth factor (EGF) receptor which appears to mediate many key components of downstream signal transduction in VSMCs [29], whereas a Src family kinase has been implicated as a PKCδ Tyr^{311} kinase [17]. To clarify the involvement of Src family kinase and/or EGF receptor transactivation in PKCδ phosphorylation, we pretreated VSMCs with PP2, a Src family kinase inhibitor, or AG1478, an EGF receptor family kinase inhibitor. Interestingly, AngII-induced PKCδ Tyr^{311} phosphorylation was markedly blocked by PP2 (5 μmol/L), whereas AG1478 (1 μmol/L) had no inhibitory effect. As expected, AG1478, but not PP2, inhibited AngII-induced EGF receptor transactivation as detected by its autophosphorylation at Tyr^{1068} (Figure 2A). Also, PP3 (5 μmol/L), the inactive control chemical for PP2, had no inhibitory effect on AngII-induced PKCδ Tyr^{311} phosphorylation (Figure S1D, please see http://hyper.ahajournals.org.). To support these pharmacological experiments, the effect of dominant negative Src was examined. Infection of adenovirus encoding dominant negative Src, but not control vector, markedly inhibited PKCδ Tyr^{311} phosphorylation induced by AngII, whereas neither virus affected the EGF receptor transactivation (Figure 2B). Sufficient over expression of the dominant negative Src mutant as compared to endogenous Src was confirmed (Figure 2B). These data suggest that Src but not EGF receptor mediates AngII-induced PKCδ Tyr^{311} phosphorylation.

Involvement of PKCδ Tyr^{311} phosphorylation and PKCδ kinase activity in AngII-induced VSMC hypertrophy

To verify the functional significance of the Tyr^{311} phosphorylation, we established VSMCs which over-express wild type PKCδ or a PKCδ Y311F mutant containing GFP at the C terminus using retrovirus infection (Figure 3A). In wild type PKCδ expressing VSMCs, the AngII-induced increase in cellular protein was significantly enhanced compared to the control VSMCs. However, the enhancement was much less in Y311F expressing VSMCs (Figure 3B). Moreover, in wild type PKCδ VSMCs, AngII significantly increased the cell volume, whereas no enhancement was observed in Y311F mutant expressing cells (Figure S2A, please see http://hyper.ahajournals.org.). There was no significant change in cell number among these VSMCs stimulated by AngII (Figure S2B, please see http://hyper.ahajournals.org.). The confluency state of these VSMCs at the time of the measurements was less than 90% because without a mitogen the VSMC did not significantly proliferate after serum-starvation. Also, BrdU incorporation was not significantly changed by AngII regardless of wild type PKCδ over expression in VSMCs (Figure S2C, please see http://hyper.ahajournals.org.). In addition, there was no enhancement of an apoptotic marker, cleaved caspase-3, detected in both control and PKCδ over expressing VSMCs with 4 h AngII stimulation (Figure S3, please see http://hyper.ahajournals.org.).

To investigate whether the kinase activity of PKCδ is also required for AngII-induced protein synthesis in VSMCs, we infected VSMCs with an adenovirus encoding a kinase-inactive PKCδ mutant (K376A). In VSMCs expressing K376A, both AngII-induced protein synthesis (Figure 4) as well as the increase in cell volume (Figure S4A, please see http://hyper.ahajournals.org.) was significantly inhibited compared with control VSMCs. Again, there was no significant change in cell number among these VSMCs stimulated by AngII (Figure S4B, please see http://hyper.ahajournals.org.). These data suggest that PKCδ

Tyr^{311} phosphorylation and PKCδ kinase activity are both required for AngII-induced hypertrophy in VSMCs.

It has been demonstrated that both Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) are involved in AngII-induced VSMC hypertrophy [30–32]. To assess the functional role of PKCδ Tyr^{311} phosphorylation and kinase activity in AngII-induced hypertrophic signaling, we examined Akt and ERK1/2 activation in the above treated cells. In wild-type PKCδ expressing VSMCs, AngII-induced Akt phosphorylation was markedly enhanced, whereas no enhancement of Akt phosphorylation by AngII was seen in Y311F expressing VSMCs (Figure 5A). In contrast, neither wild-type or Y311F expression altered AngII-induced ERK phosphorylation in VSMCs. Also, PKCδ Tyr^{311} phosphorylation by AngII precedes the Akt phosphorylation. Previously, we have demonstrated that K376A PKCδ had no inhibitory effect on ERK1/2 phosphorylation induced by AngII in VSMCs [15]. In contrast, Akt phosphorylation induced by AngII was markedly inhibited in K376A expressing VSMCs (Figure 5B). These data suggest that AngII-induced VSMC hypertrophy is positively regulated by PKCδ kinase activation and Tyr^{311} phosphorylation through their involvement with Akt activation but not ERK activation.

Discussion

The major novel findings of this study revealed that PKCδ activation associated with Tyr^{311} phosphorylation mediates AngII-induced VSMC hypertrophy through a mechanism involving Akt. Also, AngII appears to induce PKCδ Tyr^{311} phosphorylation through the G_q-coupled AT_1 receptor via Src. These findings provide a new signaling mechanism by which the AT_1 receptor activation leads to PKCδ-mediated vascular remodeling and may serve as a potential therapeutic target toward cardiovascular diseases.

AngII-induced rapid PKCδ Tyr^{311} phosphorylation has been reported [18] with a slightly more sustained time course which may be due to a shorter serum starvation than the present study. However, the upstream regulators of PKCδ Tyr^{311} phosphorylation have not yet been identified. The present study using a selective G_q inhibitor indicates that the phosphorylation is mediated through G_q activation. This is in agreement with our recent observation suggesting that AngII-induced PKCδ Tyr^{311} phosphorylation in VSMCs requires intracellular Ca^{2+} elevation [33]. Since the AT_1 receptor is the dominant receptor expressed in our cultured VSMCs [34], we have not evaluated the possible confounding of these signal transductions by the AT_2 receptor. Increasing evidence suggest the counter regulatory functions of the AT_2 receptors toward the AT_1 receptor-dependent functions including vascular hypertrophy as well as hyperplasia in vivo [35,36]. Therefore, it will be interesting to further characterize a possible signal crosstalk of the PKCδ regulation between these subtype receptors in vivo.

Here, we report that PKCδ phosphorylation at Tyr^{311} by AngII is at least in part Src-dependent in VSMCs. Supporting this finding is the fact that several others have reported that PKCδ Tyr^{311} phosphorylation in select cell types was dependent on Src family kinases when stimulated with various non-GPCR agonists [19,37,38]. Also, Src family kinases have been shown to be complexed with PKCδ in several cell types including VSMCs [13,38–40]. However, possible contribution of other Src family kinases (Fyn and yes) expressed in VSMCs [41] in AngII-induced PKCδ Tyr^{311} phosphorylation remains to be determined. Although, we have not studied AngII-induced Src phosphorylation, such as at the positive regulatory Tyr^{416} residue, the Src inhibitor PP2 used in this study has been shown to block this phosphorylation effectively in VSMCs [42,43]. In addition, our data suggest that the involvement of EGF receptor transactivation in the PKCδ phosphorylation by AngII is unlikely. However, the EGFR kinase inhibitor AG1478 if used at 10 times more concentration than in the present study, partially attenuated c-Src phosphorylation at Tyr^{416} induced by AngII in VSMCs [42]. Thus, further careful evaluation

may be necessary regarding the possible partial but minor involvement of the EGF receptor transactivation in this PKCδ cascade.

We have previously utilized a PKCδ inhibitor, rottlerin, to elucidate the role of this PKC isoform in signal transductions of the AT_1 receptor in VSMCs [4,15]. However, we have not utilized this inhibitor in the present study because of the reported off target effects [44], which would be inappropriate for our long term hypertrophic experiments. In PKCδ-deficient VSMCs, cytoskeletal signaling, reorganization and subsequent migration in response to mechanical stress was diminished [12]. Also, an over-expression study using the wild type PKCδ suggested that PKCδ mediates p38 mitogen activated protein kinase (p38MAPK) activation induced by high glucose in VSMCs [45]. However, by using the K376A mutant as well as rottlerin, our previous studies have shown that PKCδ kinase activity is essential for AngII-induced activation of a select set of protein kinases, which include JAK2, Rho-kinase, p21-activated kinase and c-Jun NH_2-terminal kinase, but not ERK or p38MAPK [14,15,33]. Thus, involvement of PKCδ in p38MAPK activation may be agonist-dependent.

It has been reported that PKCδ deficient mouse VSMCs are resistant to apoptotic responses compared to control VSMCs [46]. Over expression of PKCδ in VSMC cell lines also results in G1 arrest and apoptosis [10,47]. These apoptotic or necrotic changes if they occur could be associated with enlargement of cell volume [48]. However, this scenario is quite unlikely in our present study because there was no difference in caspase-3 cleavage or cell viability with PKCδ over expression regardless of AngII stimulation as shown in the supplemental figures.

Here, we further revealed Akt as a PKCδ-dependent kinase in VSMCs stimulated by AngII which plays a significant role in VSMC hypertrophy [31]. To support our notion, a similar link between PKCδ and Akt was observed in thrombin-induced NF-κB activation in endothelial cells [49]. In addition, other mechanisms may coordinately regulate VSMC hypertrophy upon PKCδ activation by AngII such as expression of Smad3 and transforming growth factor β [11], and the Tyr^{311} phosphorylation of PKCδ.

Interestingly, our data suggest that AngII-induced PKCδ Tyr^{311} phosphorylation is also required for enhanced Akt activation and VSMC hypertrophy observed in VSMCs over-expressing wild-type PKCδ. However, the PKCδ Y311F mutant did not show a dominant-negative effect to inhibit Akt activation below the vector-transfected cells and one of the hypertrophic responses was still slightly greater than the control cells, demonstrating distinct characteristics of the PKCδ mutants. The kinase-inactive mutant, K376A, not only loses its wild-type ability to positively regulate Akt activation and subsequent hypertrophy, but also competes with endogenous PKCδ and thus acts as a dominant-negative PKCδ inhibitor. Y311F mutant also loses most of its own hypertrophic characteristics, whereas it is unable to interfere with the endogenous wild-type PKCδ. Although PKCδ Tyr^{311} phosphorylation has been proposed to enhance kinase activity, recent findings suggest additional roles of the Tyr^{311} phosphorylation in mediating unique functions of this PKC isoform [17,50]. The Tyr^{311} phosphorylation may be the additional component required for the complex formation between PKCδ, Akt, and its upstream kinase, 3-phosphoinositide dependent kinase 1/PDK1, and subsequent Akt activation which appears to require the PKCδ kinase activity [51]. Taken together, it is attractive to speculate that the PKCδ phosphorylation may contribute to Akt activation and subsequent hypertrophy independent from the kinase activity. To support this notion, we observed a comparable autophosphorylation of Y311F PKCδ at $Ser^{643/676}$ phosphorylation to that of wild-type in AngII-stimulated VSMCs (unpublished observation), thus reflecting the kinase activity remains intact in Y311F mutant.

In the present study, we have not employed a standard protein synthesis assay measuring a radio-labeled leucine incorporation. However, we believe that our two distinct methods utilized

here measure the hypertrophic effects of AngII just as sufficiently and perhaps more directly. Our data demonstrating hypertrophic responses by AngII stimulation in VSMCs are consistent with highly sited past articles using intact aortic segments [52] and cultured aortic VSMCs [53]. Moreover, no significant enhancement of DNA synthesis was observed in AngII stimulated VSMCs regardless of PKCδ over expression. However, since our data rely on over expression strategies, a future study should be conducted by using specific RNA silencing to evaluate the overall roles of PKCδ in mediating VSMC hypertrophy induced by AngII. In addition, slight distinctions in control cell responses between Figure 3B and 4A may be caused by distinct control vectors utilized as well as by a selection of the permanently infected cells in the retroviral experiment. It is also unlikely that PKCδ Y311F mutant affects other PKC isoforms expressed in VSMCs non-specifically, because this residue is unique to PKCδ. Beside the data shown in Figure 3A, we and others have previously demonstrated the specificities of the PKCδ mutants utilized in the present study [14,54–57].

A recent study utilizing proteomic analysis of PKCδ-deficient VSMCs revealed that more than 30 proteins are altered including enzymes related to glucose and lipid metabolism, thus highlighting the critical role of PKCδ in the development of cardiovascular diseases [9]. PKCδactivation increases O_2 derived free radical generation from mitochondria and thereby promotes a pro-oxidant state [58]. Therefore, it will be interesting to expand the present findings by evaluating the regulation of proteins such as pyruvate dehydrogenase and heat shock proteins which are likely involved in atherosclerosis as well as metabolic disorders [59,60].

Perspectives

We found that in addition to PKCδ kinase activity, PKCδ phosphorylation at Tyr^{311} appears to be required for Akt activation and subsequent VSMC hypertrophy induced by AngII, which is considered a potential mechanism of atherosclerosis and restenosis after vascular injury. However, our findings are limited within cell culture experiments. Therefore, further clarification of the signal transduction in vivo could contribute to a better understanding of the molecular mechanism of cardiovascular diseases as well as to the development of better strategies for their treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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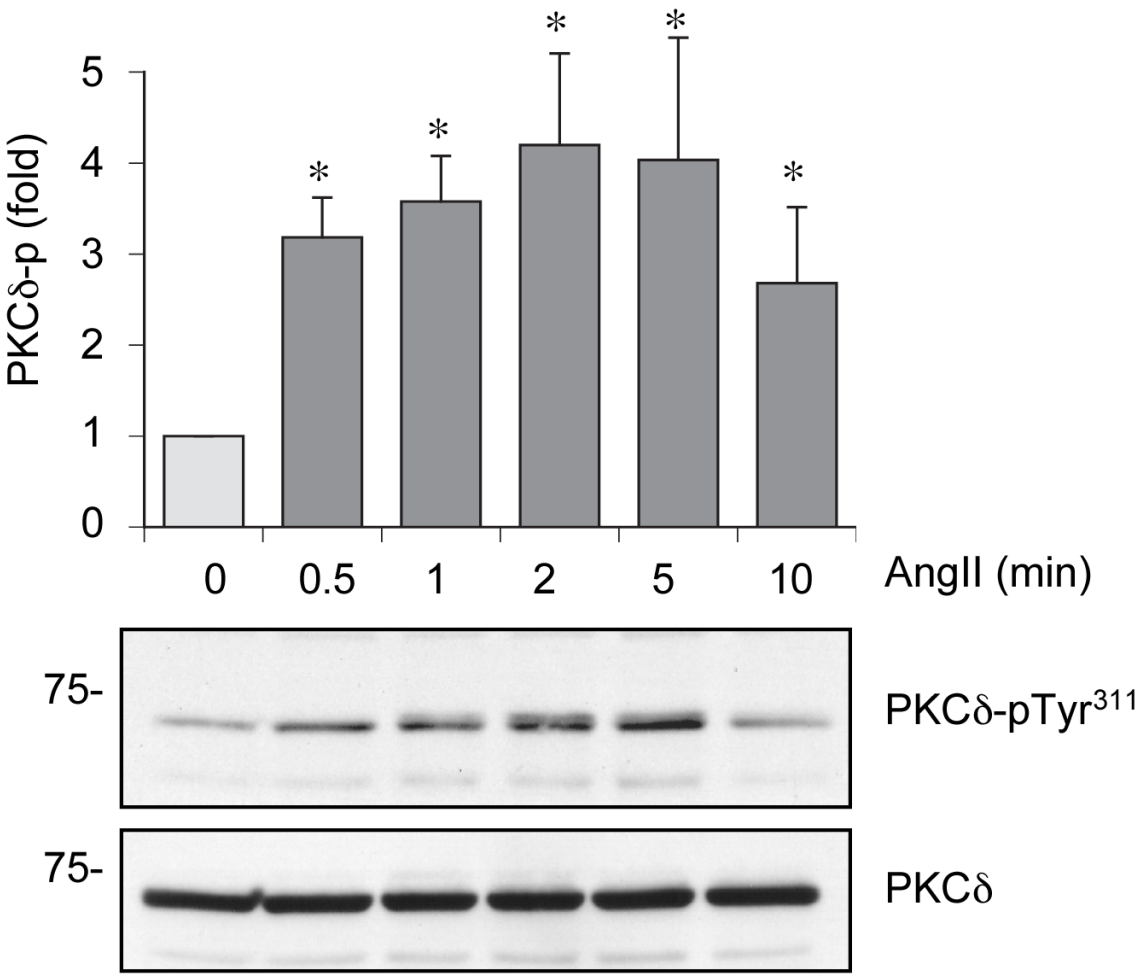

Figure 1.

Phosphorylation of PKCδ at Tyr^{311} by AngII. VSMCs were stimulated with 100 nmol/L AngII for the indicated time periods. The cell lysates were immunoblotted with phospho-selective antibody which detects PKCδ Tyr^{311} phosphorylation and anti-PKCδ antibody. The PKCδ phosphorylation at Tyr^{311} was measured by densitometry, normalized to total PKCδ and shown as mean ± SEM (n=3). *$P < 0.05$ compared to the basal control.

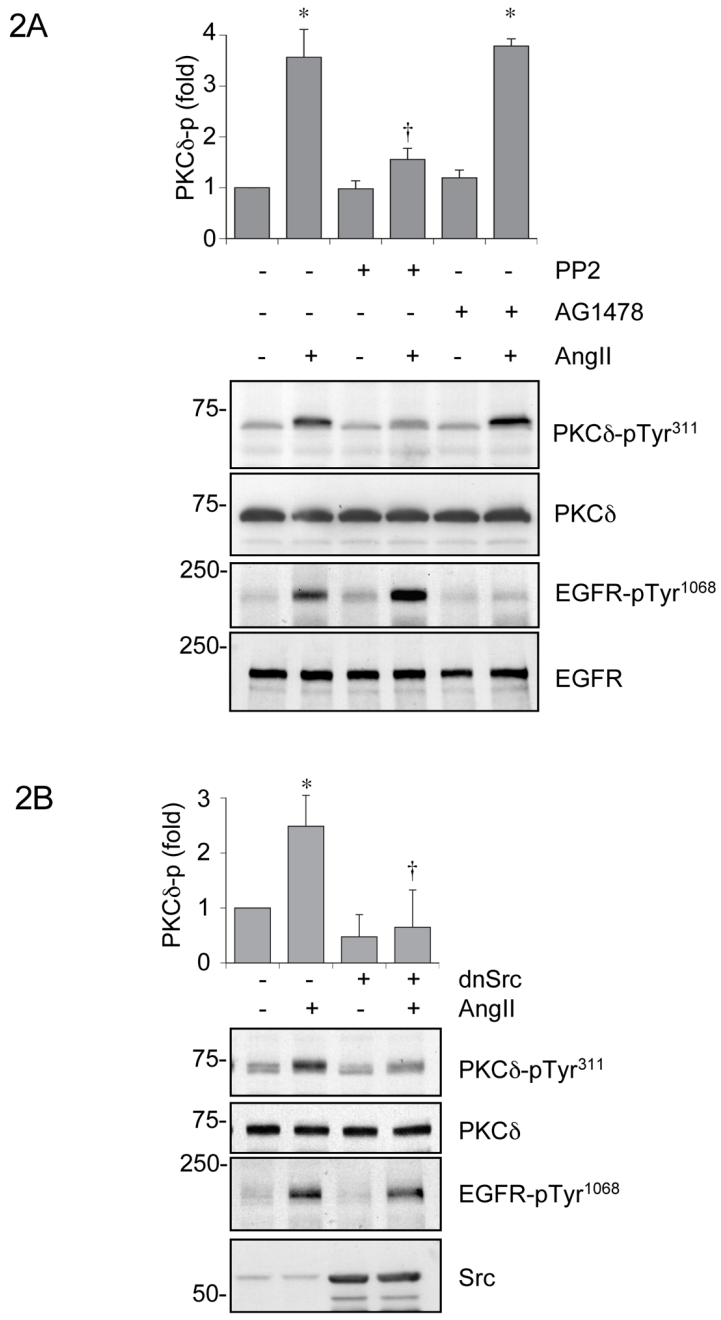

Figure 2.

Involvement of Src in PKCδ Tyr^{311} phosphorylation induced by AngII. **A**, VSMCs were pretreated with a Src family kinase inhibitor, PP2 (5 μmol/L) or an EGF receptor kinase inhibitor, AG1478 (1 μmol/L) for 30 min and stimulated with 100 nmol/L AngII for 2 min. **B**, VSMCs were infected with adenovirus encoding dominant negative (dn) Src or control vector, and stimulated with 100 nmol/L AngII for 2 min. A and B, the cell lysates were immunoblotted with phospho-specific antibodies which detect PKCδ Tyr^{311} phosphorylation or EGF receptor autophosphorylation at Tyr^{1068} and with anti-PKCδ, anti-EGF receptor and anti-Src antibodies as indicated. The PKCδ phosphorylation at Tyr^{311} was measured by

densitometry and shown as mean ± SEM (n=3). *$P < 0.05$ compared to the basal control. †$P < 0.05$ compared to the stimulated control.

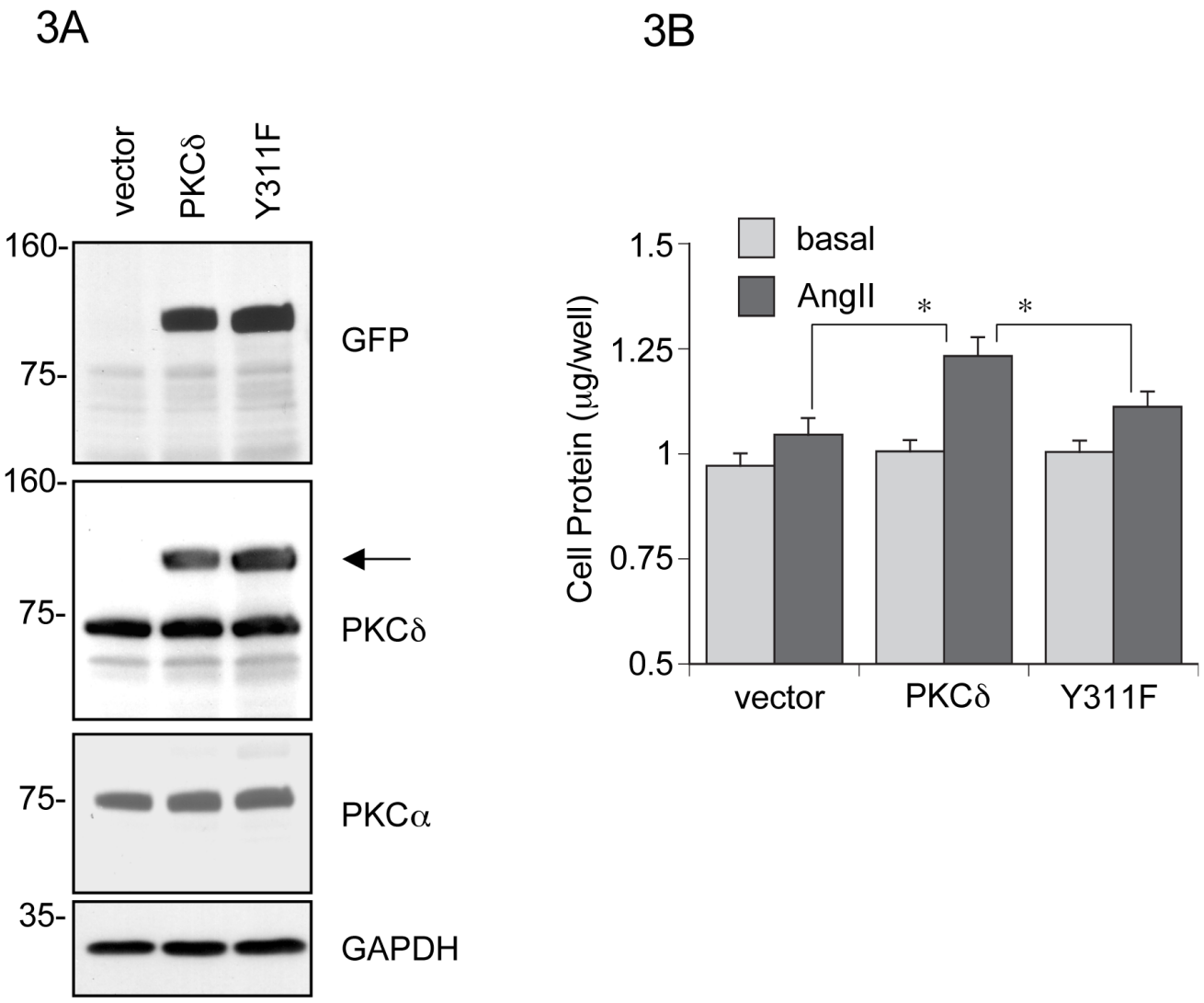

Figure 3.

PKCδ phosphorylation at Tyr^{311} contributes to VSMC hypertrophy induced by AngII. **A**, VSMCs were infected with retrovirus encoding control vector, wild type PKCδ tagged with GFP, or PKCδ Y311F mutant tagged with GFP. The cell lysates were immunoblotted with antibodies as indicated. An arrow denotes exogenously introduced GFP-tagged PKCδ. **B**, VSMCs infected with the above retrovirus were stimulated with 100 nmol/L AngII for 3 days. Afterwards, cellular protein levels were measured by a protein assay kit. The data was presented as fold basal (mean ± SEM, n=3). *$P < 0.05$.

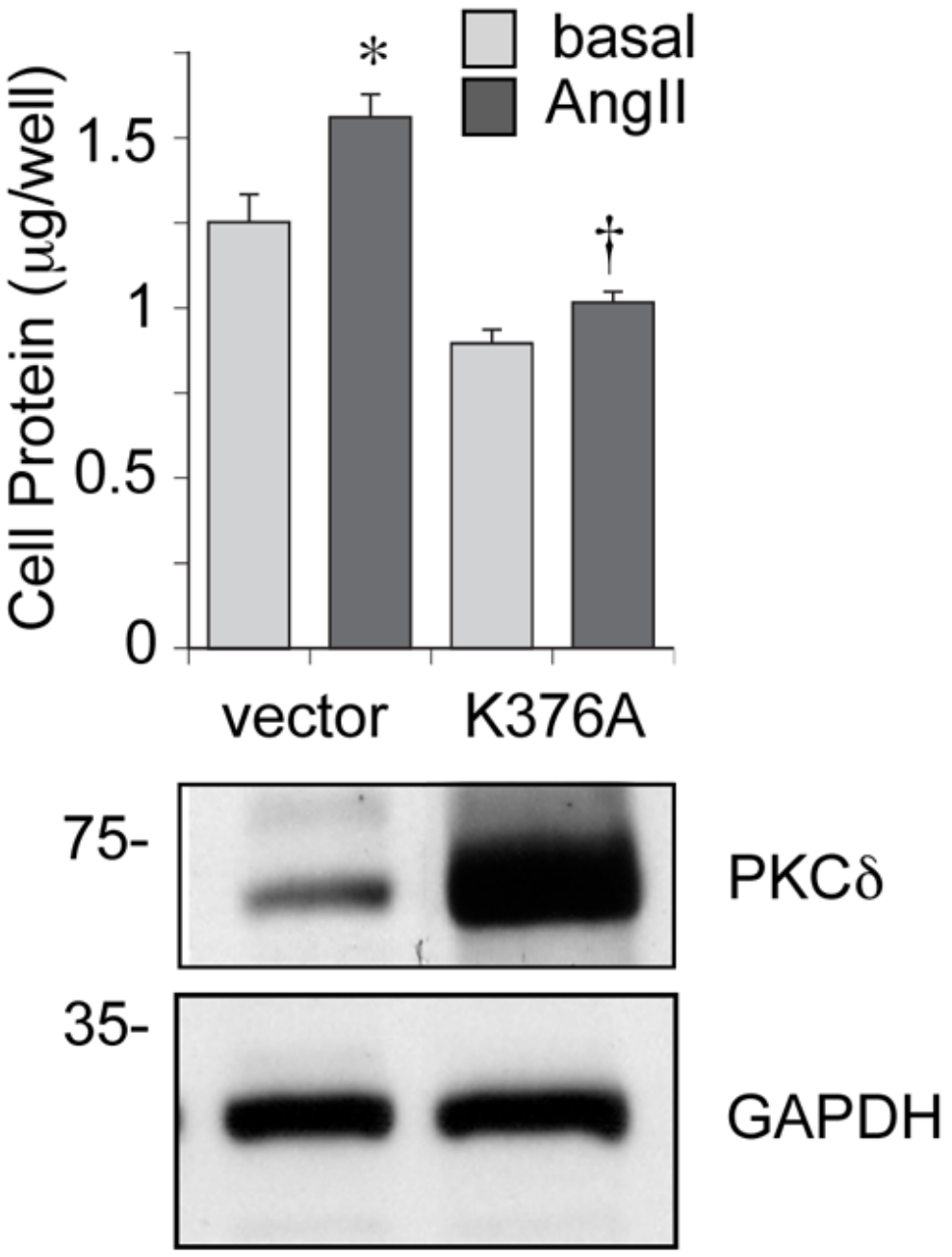

Figure 4.

Kinase activity of PKCδ is required for AngII-induced protein synthesis in VSMCs. VSMCs were infected with adenovirus encoding a kinase-inactive PKCδ mutant (K376A) or control empty vector, and stimulated with 100 nmol/L AngII for 3 days. Afterwards, cellular protein levels were measured by a protein assay kit. The data was presented as fold basal (mean ± SEM, n=3). **P* < 0.05 compared to the basal control. †*P* < 0.05 compared to the stimulated control. Also, immunoblotting of PKCδ and GAPDH to confirm K376A over-expression was performed.

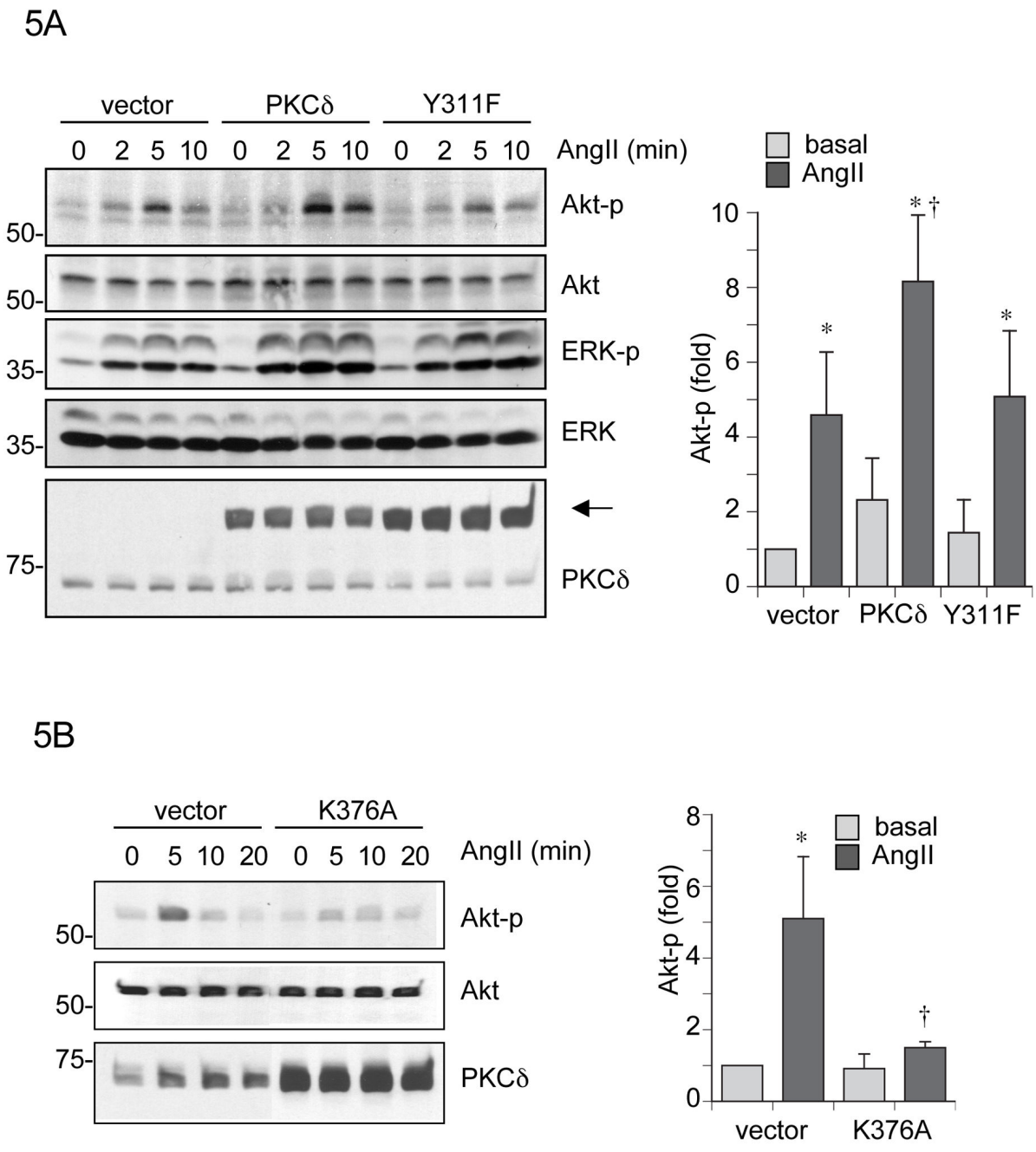

Figure 5.

PKCδ kinase activity and Tyr^{311} phosphorylation are required for AngII-induced Akt activation of VSMCs expressing PKCδ mutants. **A**, the retrovirus-infected VSMCs (vector, wild type PKCδ, or Y311F mutant) were stimulated with 100 nmol/L AngII for the indicated time periods. An arrow indicates GFP-tagged PKCδ position. **B**, the adenovirus-infected VSMCs (vector or K376A mutant) were stimulated with 100 nmol/L AngII for the indicated time periods. A and B, cell lysates were immunoblotted with antibodies as indicated. The Akt Ser^{473} phosphorylation signal was measured by densitometry and shown as mean ± SEM (n=3). **P* < 0.05 compared to the basal control. †*P* < 0.05 compared to the stimulated control.