Early Endosomal Antigen 1 (EEA1) Is an Obligate Scaffold for Angiotensin II-induced, PKC-α-dependent Akt Activation in Endosomes

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Akt/protein kinase B (PKB) activation/phosphorylation by angiotensin II (Ang II) is a critical signaling event in hypertrophy of vascular smooth muscle cells (VSMCs). Conventional wisdom asserts that Akt activation occurs mainly in plasma membrane domains. Recent evidence that Akt activation may take place within intracellular compartments challenges this dogma. The spatial identity and mechanistic features of these putative signaling domains have not been defined. Using cell fractionation and fluorescence methods, we demonstrate that the early endosomal antigen-1 (EEA1)-positive endosomes are a major site of Ang II-induced Akt activation. Akt moves to and is activated in EEA1 endosomes. The expression of EEA1 is required for phosphorylation of Akt at both Thr-308 and Ser-473 as well as for phosphorylation of its downstream targets mTOR and S6 kinase, but not for Erk1/2 activation. Both Akt and phosphorylated Akt (p-Akt) interact with EEA1. We also found that PKC-α is required for organizing Ang II-induced, EEA1-dependent Akt signaling in early endosomes. EEA1 expression enables PKC-α phosphorylation, which in turn regulates Akt upstream signaling kinases, PDK1 and p38 MAPK. Our results indicate that PKC-α is a necessary regulator of EEA1-dependent Akt signaling in early endosomes. Finally, EEA1 down-regulation or expression of a dominant negative mutant of PKC-α blunts Ang II-induced leucine incorporation in VSMCs. Thus, EEA1 serves a novel function as an obligate scaffold for Ang II-induced Akt activation in early endosomes.

Angiotensin II (Ang II) is a pluripotent hormone in VSMCs that stimulates contraction, inflammation, and senescence, as well as growth responses resulting in VSMC hypertrophy (1–3). These effects of Ang II are mediated primarily through the G protein-coupled Ang II type 1 receptor (AT1R) (4). AT1R, once activated, moves into caveolin-enriched plasma membrane lipid rafts where it facilitates EGF receptor (EGFR) transactivation (5). EGFR-dependent outputs emanating from this domain activate at least two discrete signaling axes (6). One is represented by Erk1/2 and its downstream targets and another involves activation of p38 MAPK, PDK1, Akt, and p70S6K (7, 8). This complexity of the AT1R signaling repertoire was anticipated by early findings in which we showed that Ang II stimulation of phospholipase-mediated generation of diacylglycerol is biphasic in VSMCs. Strategies that slowed or prevented internalization mechanisms inhibited the second, sustained phase but not the transient first phase of signaling (9). We thus posited the existence of at least two discrete Ang II signaling domains in VSMCs at the cell membrane and putatively in an intracellular compartment (10). Subsequently, it was found that many well described membrane receptor signaling pathways, such as those for EGF and insulin as well as the β2-adrenergic receptor, also generate signals from various endosomal compartments, now known collectively as “signaling endosomes” (11–13). Multiple intracellular compartments provide platforms for signal generation by recruiting and co-localizing unique combinations of signaling molecules. These posited signaling loci include the following: early endosomes; APPL1 (adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper containing 1) endosomes; multivesicular bodies; and late endosomes (14–17). Thus, endosomal signals may be functionally, mechanistically, and temporally distinct from those generated at the cell surface (18, 19).

Ang II signaling events at the plasma membrane are relatively well understood, especially in the context of the canonical model involving heterotrimeric G protein-coupled receptors. AT1Rs, for example, activate phospholipases that generate second messengers such as calcium, phospholipids, and diacylglycerol, which (20) activate classical PKC isoforms (21). PKCs are a class of membrane-bound Ser/Thr kinases that influence the organization of signaling scaffold complexes and the spatial localization of the resulting output signals (22). The function of PKC, in turn, can be influenced by the scaffold without being an integral component of the complex (22). PKCs have been increasingly implicated in the spatial organization of signal propagation in disparate compartments in multiple cell types (30).
Although Akt/PKB is a central node in initiating major pathophysiological outcomes, including Ang II-induced hypertrophy in blood vessels, the mechanisms of its activation are not fully understood. In the conventional model, Akt activation mainly occurs at the plasma membrane in lipid rafts and nonlipid raft microdomains (23, 24). We and others (15, 25–28) reported that general inhibitors of endocytosis and/or intracellular membrane trafficking modulate signaling events initiated at the plasma membrane. For example, a dynamin dominant negative mutant, concanavalin A and methyl-β-cyclodextrin prevent Akt activation in several cell types (27, 29). More specifically, methyl-cyclodextrin prevent Akt activation in several cell types (27, 29). However, the mechanism by which methyl-cyclodextrin inhibits Ang II-dependent activation of Akt in VSMCs (29). Recent experiments infer mechanistic insights. The APPL1 containing endosome is a transitional structure leading to the PI3K-dependent formation of EEA1-positive early endosomes (16). Down-regulation of APPL1 by siRNA inhibits Akt activation/phosphorylation. Akt but not p-Akt interacts with APPL1 through its PTB domain (30, 31). p-Akt was not detected in APPL1 endosomes, which raises the question of whether p-Akt is formed in the downstream EEA1-positive endosomes. Definitive understanding of the mechanisms by which Ang II-induced signals activate Akt in VSMCs requires definition of the subcellular compartment(s) in which these events occur.

EEA1 endosomes were recognized originally as organelles contributing to the trafficking and degradation of plasma membrane components and inactivation of signaling molecules (32). EEA1 is a membrane bound, endosome fusion-promoting protein and an effector of the small GTPase Rab5 that regulates early endocytosis events (33, 34). Together, these proteins enable early endosome formation. Because EEA1 is not found in other endosomal compartments, it has become a de facto marker of the early endosome. Recent evidence infers that EEA1 endosomes may organize and compartmentalize signaling events. In neutrophils stimulated by platelet-activating factor, EEA1 protein binds to the NADPH oxidase p40phox-p67phox subunit complex (35). Platelet-activating factor also stimulates Akt1 association with both p40- and p67-phox. Association of Akt directly with EEA1 was not demonstrated and the phosphorylation of Akt was alluded to but not directly addressed (35). Thus, EEA1 may play a functional role in organizing signaling events.

We posited that Ang II activation/phosphorylation of Akt in VSMCs occurs in EEA1-containing early endosomes. Here, we show that EEA1 is required for activation of PKC-α, which is upstream of Akt phosphorylation. Our study provides compelling evidence for a novel physiological role of EEA1 as a signal organizer/scaffold for PKC-α-dependent Akt activation in early endosomes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies to EEA1 and dynamin were purchased from BD Biosciences. Rabbit polyclonal antibodies to Akt1, APPL1, EGFR, and PKC-α were from Santa Cruz Biotechnology (San Diego, CA). Antibodies to p-Akt (Ser-473, Thr-308), p-Erk1/2 (Thr-202/Tyr-204), Erk1/2 MAP kinase (9102), p-mTOR (Ser-2448), p-PDK1 (Ser-241) were from Cell Signaling Technology, Inc. (Danvers, MA). All other chemicals and reagents, including DMEM, were from Sigma.

**Cell Culture, Adenovirus Transduction, and Ang II Treatments**—VSMCs were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion. Cells were grown in DMEM with 25 mM HEPES and 4.5 g/liter glucose supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For adenovirus-induced protein expression, cells at 40–50% confluence were incubated with adenoviruses for 30 min in serum-free medium. Cells were then washed and grown for 48 h in serum-supplemented medium. Expression of proteins was tested by Western blot using specific antibodies. For Ang II treatments, cells were grown in serum-free DMEM overnight and incubated with 100 nM Ang II at 37 °C.

**Western Blot and Immunoprecipitation**—VSMCs were collected and lysed in lysis buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, and 1 mM sodium orthovanadate). Lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with specific antibodies. For immunoprecipitation, cell lysates were clarified by centrifugation at 16,000 × g for 10 min, and the supernatant was incubated with specific antibodies or IgG control. Protein-antibody complexes were then pulled down with protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). After five washes, samples were separated by SDS-PAGE, transferred to membranes, and analyzed by Western blot with specific antibodies.

**Lipid Raft Flotation**—Caveolae-enriched lipid raft fractions were isolated by sucrose gradient flotation as described previously (24). VSMCs were lysed in lysis buffer containing 1% Triton X-100 during 30 min at 4 °C, and sucrose was added to reach 1.5 M. Samples were loaded on the bottom and overlaid with 1.2 M sucrose followed by 0.15 M sucrose prepared in the same buffer without Triton X-100. After centrifugation at 38,000 rpm (Beckman L8 ultracentrifuge) for 18 h at 4 °C, fractions were collected from top to bottom and analyzed by Western blot.

**Sucrose Gradient Fractionation**—VSMCs were grown on 100-mm dishes for 72 h. After overnight serum starvation, cells were stimulated with Ang II. After stimulation, all procedures were carried out at 4 °C. Cells were collected, washed, and resuspended in ice-cold homogenization buffer (50 mM HEPES, pH 7.4, 0.25 mM sucrose, complete mixture of protease inhibitors). Cell suspensions were homogenized using 30 strokes with a glass dounce homogenizer. Post-nuclear fraction was loaded on top of a 10–50% sucrose multistep gradient and sedimented at 36,000 rpm for 16 h (Beckman L8 ultracentrifuge). Fractions were collected from the top and analyzed by SDS-PAGE. Distribution of specific markers was monitored by Western blot.

**Immunofluorescence**—VSMCs were incubated with anti-Akt, anti-APPL1, or anti-EEA1 for 1 h at room temperature and then incubated in either FITC-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA) or Rhodamine Red X-conjugated secondary antibodies for 1 h at room temperature. Cells on coverslips were mounted in Vectashield.
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(Vector Laboratories, Burlingame, CA) and examined using the 488- and 543-nm lines of the argon ion and green HeNe lasers with 515/30-nm band pass and 585-nm-long pass filters, respectively, in a confocal imaging system (Zeiss LSM 510 META). For double-labeling experiments, FITC and Rhodamine Red X images were scanned with the multi-tracking mode on a Zeiss LSM 510 META confocal microscope. Controls with no primary antibody showed no fluorescence labeling, and single-label controls were performed in double-labeling experiments. To distinguish random color overlap from co-localization due to either co-compartmentalization or interaction of proteins, Imaris Coloc software was utilized (36). Imaris software enables nonbiased co-localization analysis by providing automatic threshold selection. All images were quantified using the automatic mode with no manual adjustment of the thresholds.

Proximity Ligation Assay (PLA)—VSMCs were grown on cover glasses; paraformaldehyde fixed and labeled with EEA1 or APPL1, Akt, and p-Akt antibodies. Next, we followed the PLA protocol (Olink Bioscience, Uppsala, Sweden) in which short DNA strands are bound to antibodies of interest. In this system, antibodies against a protein pair are attached to short chains of complementary DNA oligonucleotides, which hybridize when in close proximity. Enzymatic ligation of oligonucleotides generates a circularized DNA strand that serves as a template. The amplification reaction product that remains attached to the antibody-protein complex is visualized through the hybridization of fluorescently labeled oligonucleotides (37, 38). A fluorescence signal occurs only when two proteins are in close proximity (<40 nm) (38). Signals of fluorescent PLA probes indicating co-localization/co-compartmentalization of two proteins (38) were acquired with a confocal imaging system (Zeiss LSM 510 META).

siRNA Transfection—siRNA for EEA1 (3’- AAGTTTCA-GATTCTTTACAAA) or scrambled siRNA as a control (Ambion) were transfected into VSMCs using the Basic Nucleofector® kit for primary smooth muscle cells (Amaxa Biosystem, Gaithersburg, MD). The efficiency and specificity of siRNA depletion were verified by Western blot using specific antibodies.

[^3H]Leucine Incorporation—VSMCs were grown in DMEM containing 0.1% serum for 48 h. Next, cells were incubated with [^3H]Leucine (1 μCi/ml) in the presence or absence of 100 nm Ang II for 48 h. Before harvesting, cells were washed with phosphate-buffered saline, incubated with 5% trichloroacetic acid for 5 min on ice, and lysed with 0.4 M NaOH. Radioactivity of cell-incorporated [^3H]Leucine was analyzed using a liquid scintillation counter.

Statistical Analysis—All values were expressed as mean ± S.E. and analyzed using analysis of variance of the repeated experiments, followed by the Tukey’s post hoc test. Statistical significance was accepted at p < 0.05.

RESULTS

Ang II Stimulation of Akt in VSMCs Requires Internalization and Does Not Occur in Caveolae/Lipid Raft Fractions—It has been reported that growth factor-mediated Akt signaling in endothelial cells is organized in caveolae/lipid rafts (24) and, as noted earlier, Ang II-mediated signaling events in VSMCs require the transactivation of the EGFR tyrosine kinase in a caveolae/lipid raft-dependent manner (29). Thus, we first examined whether Ang II-stimulated Akt phosphorylation occurs in lipid rafts in VSMCs. Ang II activates Akt with a peak between 3 and 5 min. Using flotation sucrose sedimentation, we isolated caveolin-enriched lipid rafts as indicated by the presence of caveolin 1 and Rac-1 (Fig. 1A). Caveolin 1, as expected, was also detected in noncaveolae fractions due to its location in multiple compartments including the Golgi and endoplasmic reticulum (39, 40). Akt was not recruited into caveolin-enriched lipid rafts after 1 or 3 min of stimulation with Ang II (Fig. 1A, lane 2). Phosphorylated Akt (p-Akt) localized exclusively in heavy, nonlipid raft fractions, thus providing evidence that Akt activation by Ang II occurs in nonlipid raft cellular compartments (Fig. 1A, lane 6).

Signaling of G protein-coupled receptors (including AT1R) is initiated at the plasma membrane; however, activation of the entire Ang II signaling repertoire requires internalization (9, 41). To confirm the importance of internalization for Akt activation in VSMCs, we overexpressed the dominant negative K44A dynamin mutant (DynK44A) to prevent internalization. Overexpression of DynK44A prevented Akt phosphorylation at Ser-473 and Thr-308 after 3 and 5 min of Ang II stimulation (Fig. 1B). For control, we tested Erk1/2 activation that was significantly but not completely inhibited under these conditions indicating intact signaling from AT1R. These findings are consistent with the notion that internalization to intracellular compartments is required for Ang II-induced Akt phosphorylation at both of its activation sites.

Akt Is Activated in EEA1 Early Endosomes—To investigate the nature of Akt activation site(s), we performed subcellular fractionation of cells in basal and Ang II-stimulated conditions. Basally, Akt localized primarily in the cytosol (Fig. 2A, fractions 1 and 2). After Ang II stimulation, both Akt and pAkt co-migrated with the early endosomal markers EEA1 and Rab5 (Fig. 2A, fractions 3 and 4). Migration of markers to detect cytosol (GAPDH), plasma membrane (EGFR and Rac1), early endosomes (EEA1 and Rab5), late endosomes (Rab7), and caveolin-enriched fractions (Fig. 2B) were unaffected by 1- or 3-min incubation with Ang II (data not shown). As shown recently, Akt associates with APPL1 protein in HeLa cells in response to insulin-like growth factor and in rat adipocytes in response to insulin (15, 42), raising the possibility that Ang II activation of Akt could occur in APPL1 endosomes in VSMCs. The notion that this compartment is the precursor of EEA1 endosomes (16) and that both compartments contain Rab5 led us to investigate whether Akt is phosphorylated in an early endosomal compartment containing EEA1, APPL1, or both. To that end, we assessed the co-localization of Akt with both markers by immunofluorescence. After 1 and 3 min of stimulation, Ang II robustly increased Akt co-localization with EEA1 (Fig. 3, A and C). There was a modest basal interaction between APPL1 and Akt, which was not enhanced by Ang II treatment (Fig. 3, B and C). Furthermore, we used Imaris software to visualize the extent of co-localization of EEA1 and Akt, based on three-dimensional images of z-stack sections. As shown in
supplemental Fig. 1, EEA1 signals (presented on images with surface generated by Imaris software) associate in endosomal-like structures that do not co-localize under basal conditions with the Akt signal. Ang II stimulation changes the Akt-staining pattern and brings Akt into EEA1-positive structures. Moreover, immunoprecipitation experiments showed that EEA1 and Akt interacted in response to Ang II treatment (Fig. 3D). To confirm this interaction further, we used PLA, a technology that allows the detection of in vivo protein-protein interactions (38). As shown in Fig. 3F, incubation with Akt and EEA1 antibodies generates a vesicle-like staining in basal conditions that increased after Ang II incubations, indicating that EEA1 and Akt interact. These results support the hypothesis that phosphorylation of Akt occurs in EEA1 rather than APPL1 endosomes and that EEA1 may facilitate Akt activation. Using PLA, we found that stimulation of VSMCs with Ang II led to a robust EEA1 and p-Akt interaction, as reflected by the increase in fluorescence signal with a vesicular-like distribution (Fig. 4A). There was a basal background APPL1/p-Akt interaction that was not up-regulated with Ang II stimulation. (Fig. 4B). These results are consistent with the results from the immunofluorescence (Fig. 3A) and subcellular fractionation experiments (Fig. 2), showing direct evidence that Akt is activated in EEA1 early endosomes.

EEA1 Down-regulation Prevents Akt Activation—The activation of Akt in EEA1 early endosomes and its interaction with EEA1 protein support the hypothesis that EEA1 organizes signaling events required for Akt activation. To investigate this possibility further, we knocked down EEA1 expression with siRNA and assessed Akt activation. The data in Fig. 5A demonstrate that silencing of EEA1 abolished Ang II-induced Akt phosphorylation at both Thr-308 and Ser-473 sites without affecting the expression level of Akt. To gain insights regarding the specificity of EEA1 knockdown in Akt signaling, we examined activation of Erk1/2, which functions in a distinctly different signaling pathway (43, 44). In contrast to the inhibition of Akt activation, Erk1/2 phosphorylation was unaffected (Fig. 5A), indicating that EEA1 has a specific role in Akt activation by Ang II requires internalization. A, VSMCs were stimulated with 100 nM Ang II (AngII) for 1 or 3 min. Caveolin (cav)-enriched lipid raft fractions (lanes 2 and 3) containing caveolin 1 (Cav-1) and Rac-1 were separated from non-caveolae (non-cav) fractions (lanes 5 and 6) using sucrose flotation gradients. Total and phosphorylated Akt are detectable only in non-caveolae fractions, indicating Akt is not activated in caveolin-enriched lipid rafts. B, dynamin (Dyn) K44A dominant negative mutant (lanes 4 – 6), expressed in VSMCs known to prevent endocytosis, inhibits activation of Akt, suggesting that Akt activation may take place within internal compartments. Overexpression of dynamin was confirmed by probing with dynamin-specific antibody. Bar graphs represent averaged data (mean ± S.E.) expressed as fold of change over control LacZ adenovirus infected cells (Ctr) (n = 3). *, p < 0.05 significantly different from control conditions.

Akt Activation in Early Endosomes Requires EEA1

FIGURE 1. Akt activation by Ang II requires internalization. A, VSMCs were stimulated with 100 nM Ang II (AngII) for 1 or 3 min. Caveolin (cav)-enriched lipid raft fractions (lanes 2 and 3) containing caveolin 1 (Cav-1) and Rac-1 were separated from non-caveolae (non-cav) fractions (lanes 5 and 6) using sucrose flotation gradients. Total and phosphorylated Akt are detectable only in non-caveolae fractions, indicating Akt is not activated in caveolin-enriched lipid rafts. B, dynamin (Dyn) K44A dominant negative mutant (lanes 4 – 6), expressed in VSMCs known to prevent endocytosis, inhibits activation of Akt, suggesting that Akt activation may take place within internal compartments. Overexpression of dynamin was confirmed by probing with dynamin-specific antibody. Bar graphs represent averaged data (mean ± S.E.) expressed as fold of change over control LacZ adenovirus infected cells (Ctr) (n = 3). *, p < 0.05 significantly different from control conditions.

FIGURE 2. Akt is recruited to early endosomes by Ang II. Postnuclear supernatants of VSMCs stimulated with Ang II (100 nM for 3 min) or nonstimulated were separated in a 10–50% sucrose gradient. Endosomal localization of total and phosphorylated Akt was tested using Western blot with specific antibodies. Major fraction of phosphorylated Akt co-migrates with EEA1 indicating Akt is activated within early endosomes (A). To characterize the gradient, Western blots were performed with antibodies against EEA1 and Rab5 as markers of early endosomes (Early endos.; lanes 3 and 4), GAPDH for cytoplasmic fractions (Cyto; lanes 1 and 2), Rab7 for late endosomes (lane 8), and EGFR and Rac-1 for plasma membrane fractions (PM; lanes 6 –8). Representative of three independent experiments. Bar graph (B) represents averaged data of A (mean ± S.E.) expressed as fold of change over basal. *, p < 0.05 significantly different from basal conditions. Cav-1, caveolin 1.
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A

AngII 0 min

AngII 1 min

AngII 3 min

B

AngII 0 min

AngII 1 min

AngII 3 min

C

Relative changes in co-localization

AngII (min)

0 1 3 0 1 3 NC

* * *

D

AngII (min)

0 1 3 6 10 NC

E

Relative changes in Akt

AngII (min)

0 1 3 6 10

F

AngII 0 min

AngII 1 min

AngII 3 min

EEA1/Akt
EEA1 Is Required for Activation of Akt Upstream Kinases —
To investigate the mechanism by which EEA1 regulates Akt activation, we explored whether EEA1 modulates Akt upstream kinases. In many cells, mTOR complex 2 (mTORC2) has been implicated in Akt activation; however, such a role in VSMCs has not been established. Prolonged treatment with rapamycin inhibits, phosphorylation at Ser-2448 and Ser-2481, markers of activation of mTORC1 and mTORC2, respectively (46, 47). As shown in supplemental Fig. S3 prolonged rapamycin treatment of VSMCs inhibited Ang II-induced activation of both complexes as indicated by lack of phosphorylation of the specific activation sites. Akt phosphorylation, however, was not affected. These data indicate that in contrast to other model systems (24, 46, 47), mTORC2 may not directly participate in Akt activation in VSMCs, at least in response to Ang II.

Next, we examined the effects of EEA1 silencing upon the following: activation of PDK1, a well established kinase for Akt Thr-308 phosphorylation (48–50); p38 MAPK, which was reported as a kinase upstream of Akt Ser-473 (51); and PKC-α, which was recently established as an Akt regulator (24, 52). We first examined whether these kinases are recruited to EEA1 endosomes in response to Ang II. Similar to Akt, PKC-α, PDK1, and p38 MAPK move from the cytosol to EEA1 containing fractions after 3 min of Ang II stimulation (Fig. 6A). Consistent with observations in other cell types, we observed a transient translocation of PKC-α and PDK1 to plasma membranes after 1 min of Ang II stimulation. Next, we tested the role of EEA1 in Akt upstream signaling kinase activation. We found that down-regulation of EEA1 inhibited the Akt signaling axis. Furthermore, transactivation of EGFR, an early event in Ang II signaling that occurs in the plasma membrane, was also unaffected in EEA1-deficient cells compared with control scrambled siRNA transfected cells (data not shown). We also examined the effects of EEA1 down-regulation on Akt downstream signaling. Silencing of EEA1 expression prevented phosphorylation of mTOR at Ser-2448 and S6 kinase at Ser-389 without changes in expression of these downstream proteins (Fig. 5B). As a control, we tested whether other functions of early endosomes independent of Ang II signaling or internalization events were affected by EEA1 down-regulation. In particular, we examined EGF-induced EGFR degradation that depends on internalization and trafficking through early endosomes (45). There were no major differences in EGF-induced EGFR degradation between control and EEA1-deficient cells (supplemental Fig. S2). These findings indicate that Ang II-induced Akt phosphorylation in early endosomes and activation of its downstream targets requires the presence of EEA1. Moreover, these effects exhibit specificity for the Ang II-initiated activation of Akt and thus are not due to a broad inhibition of endocytosis-dependent events.

FIGURE 4. Akt is activated in EEA1 early endosomes. Phosphorylated Akt localize in close proximity (<40 nm) to EEA1 (A), but no increase in signal was detected for APPL1 (B) as indicated by the red fluorescent signal generated by PLA in VSMCs stimulated with Ang II (100 nm for 1 or 3 min). The blue signal indicates nuclei stained with DAPI.

FIGURE 3. Akt co-localizes with EEA1. A and B, VSMCs incubated with or without (100 nm Ang II for 1 or 3 min) were fixed with 4% paraformaldehyde and processed for immunofluorescence as described under “Experimental Procedures.” Confocal images were acquired in samples co-stained with antibodies against EEA1 (red) or APPL1 (red) and Akt (green). Nuclei were localized by DAPI (blue). The colocalization channel (Colo) generated by overlapping pixels of EEA1 or APPL1 and Akt immunofluorescent signals using Imaris software shows that Akt co-localize with EEA1 in response to Ang II, but no significant changes were observed for APPL1 endosomes. C, bar graph represents averaged data of A and B (mean ± S.E.) expressed as fold of change over basal. *, p < 0.05 significantly different from basal conditions. NC represents negative control in which the primary anti EEA1 antibody was omitted. D, EEA1 was immunoprecipitated (IP) from VSMCs. Ang II (100 nm) stimulates association of Akt and EEA1. E, bar graph represents quantified data of D expressed as fold of change over basal. F, interaction of EEA1 and Akt after Ang II (100 nm) visualized as a red fluorescent signal detected by PLA (as described under “Experimental Procedures”). Blue signal of DAPI indicates nuclei.
Ang II-induced PDK1, p38 MAPK, and PKC-α phosphorylation (Fig. 6B). To test whether PKC-α is upstream of Akt kinases in VSMCs, we overexpressed a PKC-α dominant negative mutant (DN-PKC). We observed that the DN-PKC reduced Akt, p38 MAPK, and PDK1 phosphorylation in response to Ang II (Fig. 6C). Thus, PKC-α acts as an important regulator of Ang II-Akt signaling pathway by modulating the activation of both Akt Ser-473 and Thr-308 upstream kinases in early endosomes.

**EEA1 and PKC-α Are Required for Ang II-induced [3H]Leucine Incorporation in VSMCs**—Akt has an established role in Ang II-induced [3H]leucine incorporation implicated in VSMC hypertrophy (51). Thus, we tested whether downregulation of EEA1 expression or overexpression of DN-PKC-α prevents Ang II-induced [3H]leucine incorporation. As shown in Fig. 7, A and B, both interventions significantly inhibited Ang II-induced [3H]leucine incorporation, confirming the physiological relevance of EEA1 and PKC-α for Ang II-induced Akt activation in early endosomes.

**DISCUSSION**

Previously, the primary functions associated with endosomes were trafficking, sorting, and recycling between plasma membrane and internal compartments. Reports in various systems support the concept that the internalization and endocytotic machineries are involved directly in signal transduction (14, 17, 18). Our previous work showed that a major component of Ang II signaling, including Akt activation in VSMCs, depends on internalization (9). Thus, endosomes may serve as platforms for generating discrete activation of downstream pathways that refine the original agonist-mediated membrane signal into one with enhanced target specificity. Akt is centrally involved in Ang II-induced pathological hypertrophy of VSMCs, but despite its importance, the mechanisms regulating its activation by Ang II are incompletely understood. In this study, we report that Ang II recruits Akt into early endosomes in VSMCs, where it is activated and initiates downstream signaling. Moreover, we provide evidence that EEA1, a structural component of early endosomes, facilitates activation of Akt upstream kinases and Akt itself. We also identify PKC-α as a key regulator of the upstream Akt kinases p38 MAPK and PDK1 and show that EEA1 and PKC-α are required for Ang II-induced [3H]leucine incorporation, a process implicated in hypertrophic growth of VSMCs. Our data are consistent with a model (Fig. 8) in which Ang II induces the recruitment of PKC-α, Akt, and its upstream kinases to early endosomal compartments where Akt becomes activated. EEA1 thus functions as a scaffold enabling Akt phosphorylation in early endosomes in VSMCs.

**FIGURE 5. Down-regulation of EEA1 prevents Akt activation.** A, in VSMCs, down-regulation of EEA1 by siRNA (lanes 4–6) compared with scramble siRNA (lanes 1–3) inhibits Ang II induced Akt phosphorylation at Ser-473 and Thr-308 but did not prevent Erk1/2 phosphorylation. B, down-regulation of EEA1 (lanes 4–6) blunts phosphorylation of Akt downstream signaling molecules, S6 kinase, and mTOR compared with scramble siRNA (lanes 1–3). Quantifications of three independent experiments are depicted in the right panel. *, p < 0.05.
cytotic machinery is crucial for β1-adrenergic receptor-induced Akt phosphorylation in neonatal rat cardiac myocytes (27). Recently, it was shown that in 3T3-L1 adipocytes, Akt interacts with endosomes containing the phosphatidylinositol 3-phosphate binding, FYVE (Fab-1, YGL023, Vps27, and EEA1) domain-containing protein WDFY2 in an isoform-specific manner. Depletion of WDFY2 leads to impaired phosphorylation of Akt2 and its substrates, but not Akt1, indicating that these endosomal compartments may facilitate growth hormone signal specification generally (54). Furthermore, Rab5, a GTPase regulator of endocytosis and an EEA1 binding partner, is required for EGF-induced Akt phosphorylation in cervical cancer cells (55). Early and recycling endosomes also modulate lysophosphatidic acid-induced PI3K/Akt signaling in human embryonic kidney cells (26). Although it seems clear that Akt can be activated within internal compartments, the mechanisms and location are incompletely defined.

We found that Ang II did not recruit to or activate Akt in caveolin-enriched lipid rafts or APPL1 endosomes. Instead, we show that Ang II-induced Akt activation is dependent upon endocytosis and that Ang II stimulation recruits a major fraction of Akt into early endosomes, peaking at 3 min (Fig. 2A), where it co-migrates with EEA1 (Fig. 2). Strikingly, phosphorylated Akt correlates with total Akt localization and is detected predominantly within the early endosomal fraction, further inferring that phosphorylation occurs in internalized vesicles.

p-Akt and EEA1 interaction was evaluated by PLA that informs the propinquity of two proteins but does not distinguish whether they are interacting directly or are merely co-localized in the same compartment (38). A positive signal in response to Ang II was observed for EEA1, but insignificant
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FIGURE 8. EEA1 is a scaffold for Akt activation in early endosomes. Ang II induces the recruitment of Akt, PDK1, and p38 MAPK and the Akt regulator PKC-α to EEA1 endosomes to facilitate Akt activation in this compartment. Ang II-induced signaling events mediated by EEA1 and PKC-α are required for the activation of S6 kinase and mTOR and subsequent hypertrophy of VSMCs.

changes were found for APPL1 (Fig. 4, A and B). This result raised the possibility that EEA1 itself may play a role in Akt activation. Moreover, EEA1 co-immunoprecipitates with Akt after Ang II, providing additional evidence that EEA1 serves as an organizer of Akt signal generation. EEA1 siRNA not only inhibited Akt activation but also blocked activation of critical Akt upstream kinases, PKC-α, p38 MAPK, and PDK1 (Fig. 6B). This enabling function of EEA1 appears to be specific for the Akt signaling axis in VSMCs, because EGFR transactivation, which is localized in lipid rafts (56), and Erk1/2 activation were not affected. These observations indicate that in VSMCs Ang II-initiated Akt phosphorylation occurs in early endosomes and is EEA1-dependent.

PKC-α has been implicated in Akt activation in other cell types (24, 57). For example, PKC-α is a critical organizer of insulin-mediated activation of Akt within lipid rafts of endothelial cells, as noted (24). Loss of PKC-α activity led to mislocalization of signaling complex components and inhibition of Akt phosphorylation. Similarly, our results show that PKC-α also coordinates the activation of Akt in Ang II-stimulated VSMCs, at least in part, by regulating its upstream signaling kinases, p38 MAPK, and PDK1 (50, 51). These results reveal PKC-α as a key regulator of the Ang II signaling leading to Akt activation in VSMCs and, together with previous studies, indicate that PKC-α is a common regulator of Akt signaling from both receptor tyrosine kinases (24) and G protein-coupled receptors (AT1R), regardless of the cellular compartment in which Akt phosphorylation takes place.

Our previous studies established a key role of the Ang II-Akt signaling axis in hypertrophy of VSMCs, a hallmark of hypertensive cardiovascular disease (51). Consistent with this observation, we found that inhibition of the Akt pathway by either EEA1 siRNA knockdown or expression of DN-PKC-α significantly prevents Ang II-induced [3H]leucine incorporation that has been associated with hypertrophic growth in VSMCs. Our study infers physiological importance of the early endosomal compartment in Ang II-induced Akt activation and may inform more generally a novel role of EEA1 in intracellular signal organization.

Thus, we show that EEA1 per se is critical for Ang II-induced Akt activation/phosphorylation and provide compelling evidence that it acts as a scaffold for assembling protein signaling complexes in early endosomes. Our data reveal a previously unrecognized role of EEA1 and early endosomes in Ang II signaling.

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Supplemental Fig. S1. **3-D visualization of Akt and EEA1 fluorescent signals.** VSMCs were immunostained for Akt and EEA1. High quality Z-sections of confocal images of VSMCs under basal conditions (AngII 0) or after AngII stimulation (100nM; AngII 3 min) taken with Zeiss LSM 510 META. Images were analyzed by Imaris software. Akt (green) and EEA1 (red) signals have been visualized as 3D images.
Supplemental Fig. S 2. **EGFR degradation is not affected by EEA1 down-regulation.** EGFR degradation requires internalization and trafficking through early endosomes. Scramble or EEA1 siRNA transfected VSMCs were stimulated with 100 ng of EGF for 30 and 60 min. EGFR degradation induced by EGF evaluated by Western blot using EGFR antibody. Down-regulation of EEA1 does not have broad inhibitory effect on endocytosis-dependent events. Membrane was blotted for tubulin to confirm equal loading. Representative of n=3 experiments.
Supplemental Fig. S3. AngII-induced Akt activation does not involve mTORC1 and mTORC2. Prolonged rapamycin treatment (24h) that prevents mTOR complex 2 and 1 phosphorylation/activation as indicated by mTOR phosphorylation at Ser2481 and Ser2448, respectively shows no effect on Ang II-induced Akt activation. Additionally, LC3 expression was tested to ensure efficiency of rapamycin treatment. Representative of n=3 experiments.
Early Endosomal Antigen 1 (EEA1) Is an Obligate Scaffold for Angiotensin II-induced, PKC-α-dependent Akt Activation in Endosomes
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