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Role of Angiotensin II Type 1A Receptor Phosphorylation, Phospholipase D, and Extracellular Calcium in Isoform-specific Protein Kinase C Membrane Translocation Responses*

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Aleksandra Policha[‡§1], Noriko Daneshtalab[‡], Lina Chen[¶], Lianne B. Dale[‡], Christophe Altier[¶2], Houman Khosravani[¶], Walter G. Thomas[‖], Gerald W. Zamponi[¶3], and Stephen S. G. Ferguson[‡§4]

From the [‡]Cell Biology Research Group, Robarts Research Institute, [§]Department of Physiology and Pharmacology, The University of Western Ontario, 100 Perth Drive, London, Ontario N6A 5K8, Canada, [¶]Department of Physiology, Hotchkiss Brain Institute, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada, and [‖]Baker Heart Research Institute, St. Kilda Road Central, Melbourne 8008, Australia

The angiotensin II type 1A receptor ($AT_{1A}R$) plays an important role in cardiovascular function and as such represents a primary target for therapeutic intervention. The $AT_{1A}R$ is coupled via G_q to the activation of phospholipase C, the hydrolysis of phosphoinositides, release of calcium from intracellular stores, and the activation of protein kinase C (PKC). We show here that PKCβI and PKCβII exhibit different membrane translocation patterns in response to $AT_{1A}R$ agonist activation. Whereas PKCβII translocation to the membrane is transient, PKCβI displays additional translocation responses: persistent membrane localization and oscillations between the membrane and cytosol following agonist removal. The initial translocation of PKCβI requires the release of calcium from intracellular stores and the activation of phospholipase C, but persistent membrane localization is dependent upon extracellular calcium influx. The mutation of any of the three PKC phosphorylation consensus sites (Ser-331, Ser-338, and Ser-348) localized within the $AT_{1A}R$ C-tail significantly increases the probability that persistent increases in diacylglycerol levels and PKCβI translocation responses will be observed. The persistent increase in $AT_{1A}R$-mediated diacylglycerol formation is mediated by the activation of phospholipase D. Although the persistent PKCβI membrane translocation response is absolutely dependent upon the PKC activity-dependent recruitment of an extracellular calcium current, it does not require the activation of phospholipase D. Taken together, we show that the patterning of $AT_{1A}R$ second messenger response patterns is regulated by heterologous desensitization and PKC isoform substrate specificity.

The angiotensin II type 1A receptor ($AT_{1A}R$)[5] is a member of the large superfamily of G protein-coupled receptors. $AT_{1A}Rs$ are coupled via G_q to the stimulation of phospholipase C (PLC), leading to increases in intracellular diacylglycerol (DAG), inositol 1, 4, 5, trisphosphate formation, and the release of calcium from intracellular stores. Calcium and DAG stimulate the subsequent activation of protein kinase C (1). $AT_{1A}Rs$ have been shown to mediate most of the physiological actions of angiotensin II (Ang II), and this receptor subtype plays a principal role in the control of Ang II-induced vascular functions, including cell growth, vascular contraction, and inflammatory responses as well as salt and water retention (2).

In addition to receptor-G protein coupling, agonist binding to $AT_{1A}Rs$ initiates a series of regulatory processes that contribute to receptor desensitization. $AT_{1A}R$ desensitization is an adaptive process whereby the cellular response to receptor activation diminishes following prolonged agonist exposure (3, 4). There are several mechanisms by which $AT_{1A}R$ desensitization is achieved, but phosphorylation of the $AT_{1A}R$ is the most rapid means by which this receptor is desensitized. $AT_{1A}R$ desensitization is mediated by translocation of both protein kinase C (PKC) and G protein-coupled receptor kinases to the activated receptor, which mediates phosphorylation of serine and threonine residues localized to the carboxyl-terminal tail of the receptor (3–8). The rat $AT_{1A}R$ C-tail contains three consensus sites for PKC phosphorylation. Mutational analysis indicates that endogenous PKC will phosphorylate the $AT_{1A}R$ carboxyl-terminal tail at either Ser-331, Ser-338, or Ser-348 in response to either Ang II or phorbol ester treatment (9).

The release of calcium from intracellular stores in response to G protein-coupled receptor stimulation represents a fundamental mechanism of cell signaling. The patterning of the calcium signal and the activation of calcium-regulated effector enzymes is critically important for the phosphorylation of cytoplasmic targets and the activation of different transcription factors leading to differential gene transcription in developing

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[1] Recipient of an Ontario graduate studentship.

[2] Recipient of fellowships from the Alberta Heritage Foundation for Medical Research (AHFMR) and the Heart and Stroke Foundation of Canada.

[3] An AHFMR Senior Scholar. Holds a Canada Research Chair in molecular neurobiology.

[4] Holds a Canada Research Chair in molecular neurobiology and is a Career Investigator of the Heart and Stroke Foundation of Ontario. To whom correspondence should be addressed: Robarts Research Inst., 100 Perth Dr., P. O. Box 5015, London, Ontario N6A 5K8, Canada. Tel.: 519-663-3825; Fax: 519-663-3314; E-mail: ferguson@robarts.ca.

[5] The abbreviations used are: $AT_{1A}R$, angiotensin II type 1A receptor; Ang II, angiotensin II; DAG, diacylglycerol; EGFP, enhanced green fluorescent protein; mGluR, metabotropic glutamate receptor; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; TRPC, transient receptor potential channel; HEK, human embryonic kidney; YFP, yellow fluorescent protein; siRNA, small interference RNA; HA, hemagglutinin.

systems (10, 11). Calcium signaling via G protein-coupled receptors is complex, involves multiple pathways, and leads to transient, oscillatory, and persistent PKC activation patterns (12–17). Moreover, similar PKC isozymes, such as PKCβI and PKCβII, exhibit different response patterns to metabotropic glutamate receptor 1a (mGluR1a)-stimulated second messenger responses even when expressed in the same cell (17).

Angiotensin II-stimulated calcium signaling is typically characterized by a biphasic response that is comprised of a rapid release of calcium from intracellular stores followed by a slower influx of extracellular calcium (1). The mechanism(s) by which this biphasic response pattern is elicited is unclear, but DAG formation also exhibits biphasic and sustained responses that are essential for the regulation of vascular smooth muscle function.

In the present study, we explored the possibility that differences in the activation patterns for PKCβI and PKCβII might be associated with the biphasic and sustained second messenger responses observed in response to $AT_{1A}R$ activation. We found that mutation of any of the three consensus sites for PKC-mediated phosphorylation in the $AT_{1A}R$ carboxyl-terminal tail results in an increase in the probability that the activation of the $AT_{1A}R$ will result in the recruitment of an extracellular calcium influx, persistent DAG formation in response to phospholipase D (PLD) activation, and the persistent localization of PKCβI to the plasma membrane. The extracellular calcium influx, but not DAG formation, is essential for sustained PKCβI membrane localization. Thus, the ability of the $AT_{1A}R$ to escape heterologous desensitization in PKCβI-expressing cells allows the receptor to recruit additional second messenger responses leading to sustained alterations in activity.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney cells (HEK 293) were from American Tissue Culture Collection (Manassas, VA). Fetal bovine serum, gentamicin, minimal essential medium, and 0.05% trypsin containing 0.5 mM EDTA were purchased from Invitrogen. BAPTA-AM was from Invitrogen Molecular Probes (Burlington, ON). All other biochemical reagents were purchased from Sigma, Fisher Scientific (Ottawa, ON), and VWR (Mississauga, ON).

Cell Culture and Transfection—HEK 293 cells were maintained in minimal essential medium supplemented with 10% (v/v) fetal bovine serum and 50 μg/ml gentamicin at 37 °C in a humidified atmosphere containing 5% CO_2. The cells used in each experiment were transiently transfected using a modified calcium phosphate method (18). Cells were transfected with 5 μg of receptor ($AT_{1A}R$ or $AT_{1A}R$ PKC phosphorylation site mutants), 2 μg of either EGFP-PKCβI, EGFP-PKCβII, or EGFP-PKCδ, and 3 μg of the fluorescent DAG indicator EYFP-PKCδ C1 (19) unless otherwise indicated in the figure legends. Following an 18-h incubation period with the calcium phosphate transfection reagent at 37 °C, the cells were washed with Hanks' balanced salt solution (1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM HEPES, 11 mM glucose, 116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4) and then fed with 10 ml of supplemented minimal essential medium (see above). Five hours later, cells were plated on 15-mm collagen-coated glass coverslips designed for use in a perfusion system (Warner Instrument Corp.) or on collagen-coated 35-mm glass-bottomed culture dishes (MatTek) for confocal live cell imaging studies.

Synthesis of PLD1/2 siRNAs and Transfections—siRNA duplexes corresponding to the human PLD1 (AAGTTAAGAGGAAATTCAAGC) and PLD2 cDNA (GACACAAAGTCTTGATGAG) sequences were purchased from Dharmacon Research (Lafayette, CO), and siCONTROL™ Non-Targeting siRNA 1 was utilized as a control. A full characterization of these siRNA sequences for the knock down of PLD1 and PLD2 protein expression were published previously (20, 21). 100 pM siRNA duplex was transiently co-transfected into HEK 293 cells with cDNA constructs described in the figure legends using Lipofectamine 2000. Cells were utilized 48 h following transfection, and PLD protein knock down was confirmed by immunoblot.

Confocal Microscopy—Confocal microscopy was performed on a Zeiss LSM-510 META laser scanning microscope using Zeiss 63 × 1.4 numerical aperture oil immersion lens. EGFP and YFP fluorescence was visualized with excitation at 488 nm and 515–540-nm emission filter set. Fluorescent signals were collected sequentially every 6.3 s using the Zeiss LSM software time scan function. The cellular distribution and intracellular trafficking of each of the EGFP-conjugated PKCs in cells co-expressing FLAG-$AT_{1A}R$ or HA-$AT_{1A}R$ phosphorylation site mutants were observed in transiently transfected HEK 293 cells plated on glass coverslips. Coverslips were attached to an RC-25F perfusion chamber (Warner Instruments Corp.) with vacuum grease, and the chamber was mounted onto a PH4 platform (Warner Instruments). Coverslips were perfused with solutions from perfusion channels (Model VC-6 apparatus; Warner Instruments) connected via tubing with the perfusion chamber. The perfusing solutions were maintained at 37 °C with the TC-344B Dual Channel Heater Controller (Warner Instruments). The rate at which the perfusing solutions flow through the perfusion chamber is 67 μl/s, and it takes ~30 s for the perfusion chamber to fill with solution. Cells were initially perfused with Hanks' balanced salt solution for 500 s to establish base-line PKC distribution and to test for agonist-independent responses. Next, 100 nM Ang II in Hanks' balanced salt solution was applied for 500 s or the times indicated in the figures, and PKC responses were examined and classified as transient (translocates only once from cytosol to plasma membrane and then back to cytosol in the presence of agonist), persistent (translocates to plasma membrane and remains localized to membrane throughout agonist stimulation), or repetitive (repeated translocation between cytosol and plasma membrane; clear reappearance of PKC at the plasma membrane and loss of cytosolic PKC). Lastly, Hanks' balanced salt solution was again applied for 500 s to wash off agonist. YFP-PKCδ C1 is a DAG indicator that translocates from the cytosol to the plasma membrane in response to increases in membrane DAG concentration (17, 19). The cellular distribution and intracellular trafficking of YFP-PKCδ C1 in HEK 293 cells co-expressing FLAG-$AT_{1A}R$ or HA-$AT_{1A}R$ S331/338/348A were visualized as described above.

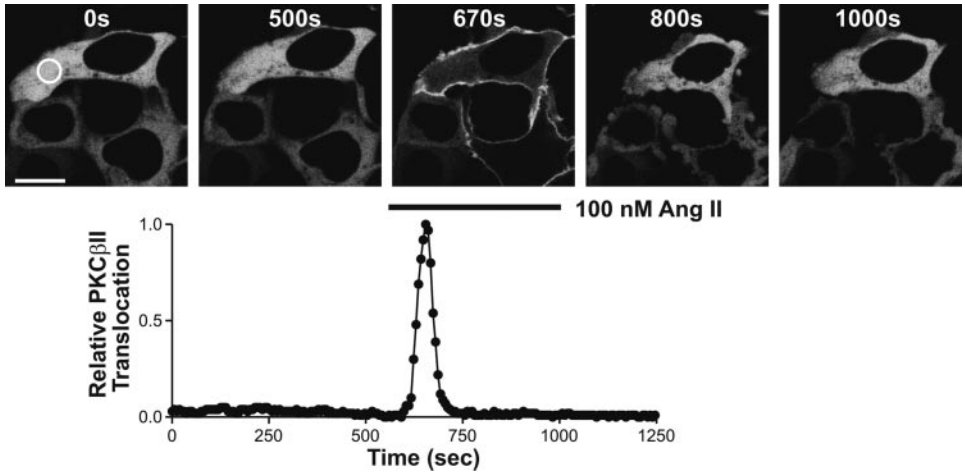

FIGURE 1. **PKCβII translocation response to $AT_{1A}R$ activation.** HEK 293 cells were transfected with cDNA plasmids encoding EGFP-PKCβII (2 μg) and HA-$AT_{1A}R$ (10 μg) and treated with 100 nM Ang II. Shown are representative images selected from a time series of 200–400 laser scanning confocal microscopic images collected at 6.8–12.5-s intervals. The images demonstrate the transient translocation of GFP-PKCβII in 47/47 cells tested. The data are plotted below as the relative change in EGFP fluorescence intensity in the circular (*white*) region of interest with maximal cytosolic fluorescence normalized to zero and minimum cytoplasmic fluorescence normalized to one. The data are collected from six independent experiments. The *white bar* represents 10 μm. The *black bar* represents the time of agonist treatment.

Electrophysiology—Patch clamp recordings on isolated transfected cells were conduced with an Axopatch 200 B amplifier connected to a personal computer via a Digidate 1320 interface. pCLAMP 9 software was used for data acquisition. Borosilicate patch pipettes were pulled with a Sutter microelectrode puller and had typical resistances of 2–4 MΩ when filled with an internal recording solution containing 118 mM Cesium methanesulfonate, 4 mM $MgCl_2$, 0.5 mM EGTA, 9 mM HEPES, pH 7.4. The bath solution contained 116 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM glucose, 10 mM HEPES, pH 7.4. Ang II was applied via a gravity-driven microperfusion system.

Currents were elicited by voltage ramps from −60 to +40 mV from a holding potential of −20 mV. Whole cell conductance was determined from a regression to the current-voltage data at negative voltages (−30 to −60 mV), and in each case, Ang II effects were leak subtracted by subtracting the whole cell conductance determined immediately before Ang II application from that obtained in the presence of Ang II. Data were analyzed in Clampfit and plotted in Sigmaplot. Statistical analysis was conducted in Sigmastat, with statistical significance set at the 0.05 level.

Data Analysis—PKC translocation and DAG responses were recorded as time series of 250–500 confocal images for each experiment. Image analysis was performed using the Zeiss LSM-510 physiology analysis software. The relative change in cytoplasmic fluorescence intensity over time was examined in a 5-μm-diameter region of interest. Data were then normalized between 0 and 1 to show the relative membrane localization of PKC. 0 indicated PKC predominantly in the cytosol, and 1 indicated loss of cytoplasmic PKC, which was taken to indicate that PKC translocated to the plasma membrane. The relative membrane localization was calculated using mean region of interest values obtained for each fluorescent signal collected sequentially during every 6.3-s scan, according to the equation $(max - x)/(max\text{-}min)$, where *max* is the maximum mean region of interest for a given time series, *min* is the minimum value, and x is the value at a given 6.3-s scan. All time course data were plotted using GraphPad Prism software. For each experiment, the number of cells in which the pertinent PKC construct showed transient, persistent, and repetitive responses were counted and expressed as a proportion of total number of responding cells. Data were analyzed for statistical significance with chi-square tests using Excel. Because multiple comparisons were made between control and treatment groups, α values were adjusted with the Bonferroni correction factor (0.05/number of comparison groups). Error bars in the figures indicate two standard deviations (95% confidence intervals). Standard deviations (*S.D.*), were calculated according to the following equation: $S.D. = \mathrm{sqrt}(p(1-p)/n)*100$ where p is the proportion of cells showing a transient or persistent or repetitive response for a given PKC construct and n is the total number of responding cells expressing the given construct.

RESULTS

PKCβ Splice Variant-specific Plasma Membrane Translocation Responses—Because we previously observed differences in EGFP-PKCβI and EGFP-PKCβII plasma membrane translocation responses to mGluR1a activation (17), we tested whether differences in EGFP-PKCβI and EGFP-PKCβII plasma membrane translocation responses might be observed following $AT_{1A}R$ activation with 100 nM Ang II. In HEK 293 cells expressing both HA-$AT_{1A}R$ and EGFP-PKCβII, EGFP-PKCβII translocation to the plasma membrane was observed in 95% of EGFP-PKCβII-transfected cells (Fig. 1). In all responding cells a transient translocation response was the only response pattern observed for this PKC variant. In contrast, HA-$AT_{1A}R$ activation stimulated three different EGFP-PKCβI translocation response patterns: transient (87% of transfected cells), persistent (9% of transfected cells), and repetitive (4% of transfected cells) (Fig. 2A). In cells exhibiting persistent EGFP-PKCβI membrane localization to the plasma membrane, EGFP-PKCβI remained localized to the plasma membrane even after agonist wash out (Fig. 2*B*). In cells exhibiting repetitive EGFP-PKCβI translocation patterns, agonist treatment first resulted in the transient translocation of EGFP-PKCβI to the plasma membrane, which upon the removal of agonist by perfusion was replaced by EGFP-PKCβI oscillations (Fig. 2*C*). To ensure that PKC membrane translocation was $AT_{1A}R$ dependent, control experiments were conducted with cells transfected solely with EGFP-PKCβI or EGFP-PKCβII. No membrane translocation was observed for either isoform in the absence of the $AT_{1A}R$ transfection (data not shown). Taken together these observations suggested that PKCβI and PKCβII exhibit different patterns of activation in response to $AT_{1A}R$ activation.

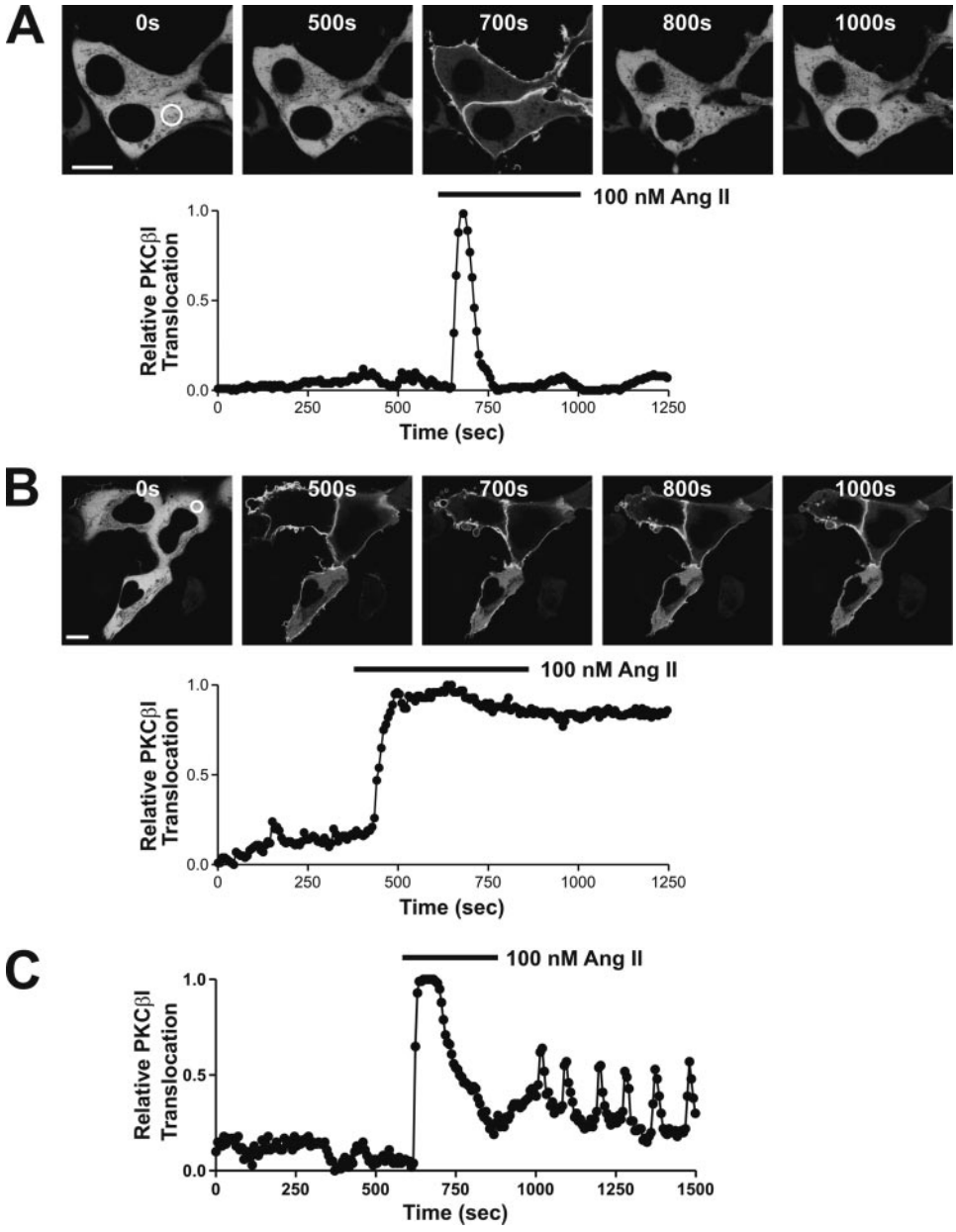

FIGURE 2. **PKCβI translocation response to $AT_{1A}R$ activation.** HEK 293 cells were transfected with cDNA plasmids encoding EGFP-PKCβI (2 μg) and HA-$AT_{1A}R$ (10 μg) and treated with 100 nM Ang II. Shown are representative EGFP-PKCβI response patterns. These responses include transient EGFP-PKCβI translocation (41/47 cells) (*A*), persistent EGFP-PKCβI translocation (4/47 cells) (*B*), and oscillatory EGFP-PKCβI translocation following agonist washout (2/47 cells) (*C*). The data are plotted below as the relative change in EGFP fluorescence intensity in the circular (*white*) region of interest with maximal cytosolic fluorescence normalized to zero and minimum cytoplasmic fluorescence normalized to one. The data are collected from six independent experiments. The *white bars* represent 10 μm. The *black bar* represents the duration of agonist treatment.

PKCβI-dependent Alterations in $AT_{1A}R$-stimulated DAG Formation—Previously, we observed that the co-expression of PKCβI, but not PKCβII, with mGluR1a led to persistent increases in intracellular DAG levels in response to receptor activation (15). Therefore, we examined whether PKCβI expression altered DAG responses in HEK 293 cells transfected with HA-$AT_{1A}R$ utilizing a YFP-PKCδ C1 domain construct that translocates from the cytosol to the plasma membrane in response to increases in intracellular DAG concentrations (19). We found that in cells expressing the $AT_{1A}R$ alone, agonist-stimulated DAG responses were predominantly transient (72% of cells) versus persistent (28%) and in cells expressing both PKCβII and the $AT_{1A}R$ a similar result was obtained (Fig. 3). However, in cells expressing PKCβI, $AT_{1A}R$-stimulated DAG responses were predominantly persistent (60% of cells) (Fig. 3). Taken together, our data suggested that DAG signaling in response to $AT_{1A}R$ was altered by the expression of PKCβI.

Role of $AT_{1A}R$ Phosphorylation in PKC Splice Variant-specific Translocation Responses—Because PKCβI expression altered $AT_{1A}R$-stimulated DAG formation from a predominantly transient to persistent response pattern, we tested whether PKC isoform-specific phosphorylation at three PKC consensus sites (Ser-331, Ser-338, and Ser-348) (9) in the $AT_{1A}R$ C-tail might contribute to differences in PKCβI and PKCβII translocation patterns and DAG responses. The mutation of all three C-tail $AT_{1A}R$ PKC consensus sites had no effect on the transient nature of EGFP-PKCβII translocation in response to agonist treatment (Fig. 4). However, the mutation of any one of the PKC phosphorylation consensus sites in $AT_{1A}R$, either individually or in combination, significantly increased the number of cells exhibiting either persistent or repetitive EGFP-PKCβI translocation responses (Fig. 4). Therefore, we examined whether agonist-stimulated DAG responses were also altered by $AT_{1A}R$-S331/338/348A-expressing cells using the YFP-PKCδ C1 domain DAG reporter construct. We found that the activation of the $AT_{1A}R$-S331/338/348A resulted in persistent DAG formation in 98% of cells (Fig. 5). Thus, similar to the overexpression of PKCβI, the mutation of PKC consensus sites in the $AT_{1A}R$ C-tail also resulted in alterations in coupling of the $AT_{1A}R$ to DAG formation.

Role of PLDs in $AT_{1A}R$-stimulated Persistent DAG Formation and PKC Translocation—Not only was the $AT_{1A}R$ reported to bind directly to PLD and regulate the activity of the enzyme (22), but PLD activation via the metabolism of phosphatidic acid provides an alternative cellular mechanism for the formation of DAG (23). Therefore, we tested whether persistent DAG formation and PKCβI translocation was PLD-mediated by co-transfecting cells with siRNAs for PLD1 and PLD2 that were previously reported to effectively knock down PLD protein

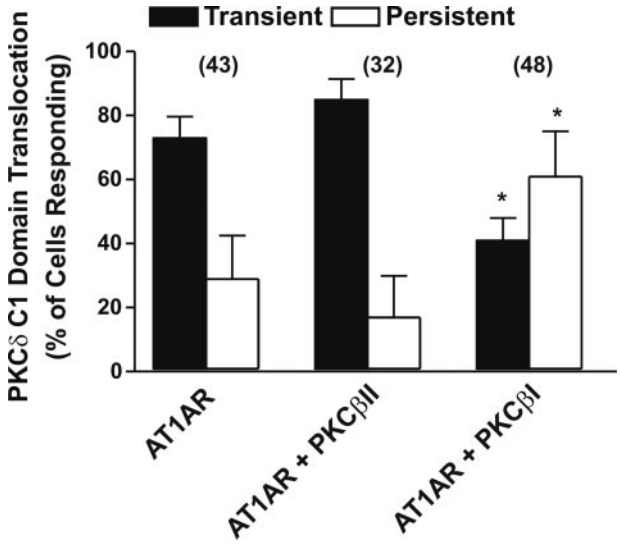

FIGURE 3. **$AT_{1A}R$-stimulated DAG formation in PKCβI- and PKCβII-expressing cells.** HEK 293 cells were transfected with cDNA plasmids encoding HA-PKCβI (2 μg) or HA-PKCβII (2 μg) along with YFP-PKCδ C1 domain (1 μg) and HA-$AT_{1A}R$ (10 μg) and treated with 100 nM Ang II. Changes in intracellular DAG concentrations were assessed by the cytosol to plasma membrane translocation of a YFP-PKCδ C1 domain reporter construct. Cells were scored as either exhibiting transient or persistent YFP-PKCδ C1 domain translocation responses. The *numbers in parentheses* represent the total number of cells counted from six different experiments. *Error bars* indicate 2 S.D. (95% confidence intervals). *, $p < 0.05$ compared with cells expressing PKCδ C1 and $AT_{1A}R$.

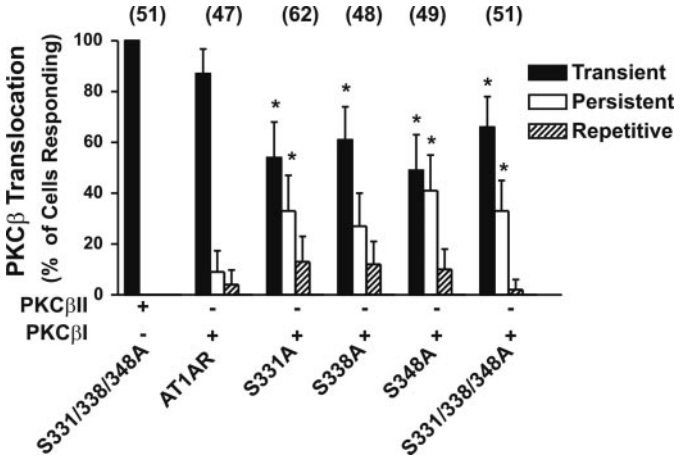

FIGURE 4. **PKCβ translocation responses in cells expressing $AT_{1A}R$ PKC consensus site mutants.** HEK 293 cells were transfected with cDNA plasmids encoding EGFP-PKCβI (2 μg) or EGFP-PKCβII (2 μg) along with 10 μg of plasmid cDNA encoding either HA-$AT_{1A}R$, HA-$AT_{1A}R$-S331A, HA-$AT_{1A}R$-S338A, HA-$AT_{1A}R$-S348A, or HA-$AT_{1A}R$-S331/338/348A and treated with 100 nM Ang II. Cells were scored as exhibiting either transient, persistent, or repetitive EGFP-PKCβ translocation responses. The *numbers in parentheses* represent the total number of cells counted from six different experiments. *Error bars* indicate 2 S.D. (95% confidence intervals). *, $p < 0.05$ compared with the percentage of cells in which PKCβI showed the same type of response when co-expressed with wild type $AT_{1A}R$.

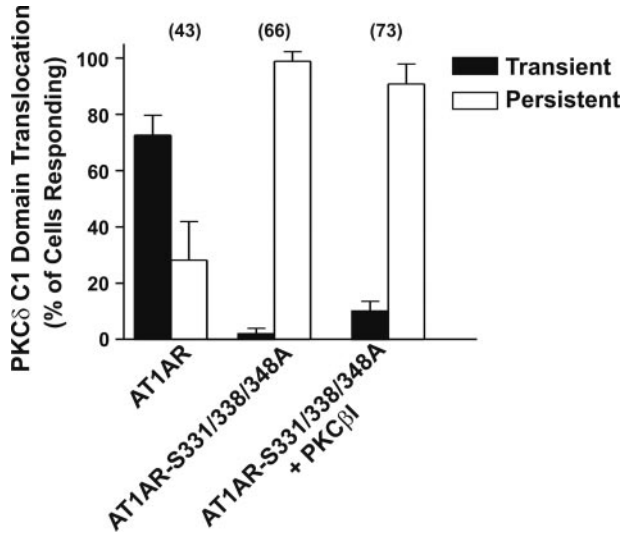

FIGURE 5. **Wild type and mutant $AT_{1A}R$-stimulated DAG formation.** HEK 293 cells were transfected with cDNA plasmids encoding YFP-PKCδ C1 domain (1 μg) along with 10 μg of plasmid cDNA encoding either HA-$AT_{1A}R$, HA-$AT_{1A}R$-S331/338/348A, or HA-$AT_{1A}R$-S331/338/348A cotransfected with HA-PKCβI (2 μg) and treated with 100 nM Ang II. Changes in intracellular DAG concentrations were assessed by the cytosol to plasma membrane translocation of the YFP-PKCδ C1 domain reporter construct. Cells were scored as either exhibiting transient or persistent YFP-PKCδ C1 domain translocation responses. The *numbers in parentheses* represent the total number of cells counted from six different experiments. *Error bars* indicate 2 S.D. (95% confidence intervals). *, $p < 0.05$ compared with cells expressing PKCδ C1 and $AT_{1A}R$.

expression. In cells transfected with scrambled siRNA $AT_{1A}R$-S331/338/348A activation resulted in the persistent plasma membrane localization of the YFP-PKCδ C1 domain DAG reporter construct in all cells tested (Fig. 6*A*). In contrast, in cells transfected with PLD1 and PLD2 siRNAs DAG responses became predominantly transient (76% of cells) (Fig. 6*A*), indicating that PLD activation was involved in persistent DAG formation following $AT_{1A}R$-S331/338/348A activation. Treatment of cells with both PLD1 and PLD2 siRNAs was required to prevent persistent DAG formation (data not shown). However, in cells treated with PLD1/2 siRNA persistent PKCβI translocation responses to $AT_{1A}R$-S331/338/348A-stimulated responses were unaltered when compared with cells transfected with control siRNA (Fig. 6*B*). In contrast, in cells expressing $AT_{1A}R$-S331/338/348A and treated with PLD1/2 siRNA the plasma membrane translocation of EGFP-PKCδ, a novel PKC isozyme that lacks the calcium binding C2 domain, became predominantly transient rather than persistent (Fig. 6*C*). PKCβII translocation was not affected by the PLD1 and PLD2 siRNAs (data not shown). Interestingly, the initiation of persistent DAG formation, as well as persistent PKCβI and PKCδ translocation responses, was dependent upon the activation of PLC as the pretreatment of cells with the PLC inhibitor U73122 prevented the plasma membrane translocation of the YFP-PKCδ C1 domain, EGFP-PKCβI, and EGFP-PKCδ (Fig. 6*D*).

Role of Calcium in $AT_{1A}R$-stimulated PKCβI and PKCβII Membrane Translocation—Because persistent PKCβI translocation responses were not attenuated by PLD siRNA treatment, we investigated the role of both intracellular and extracellular calcium pools in regulating the plasma membrane translocation of GFP-PKCβI and GFP-PKCβII. To examine the role of intracellular calcium stores, cells were pretreated for 30 min with either 50 μM calcium chelator BAPTA-AM or 1 μM thapsigargin to deplete intracellular calcium stores prior to agonist stimulation of the $AT_{1A}R$-S331/338/348A mutant. Treatment of cells with either BAPTA-AM or thapsigargin prevented AngII-stimulated translocation of both EGFP-PKCβI and EGFP-PKCβII in all

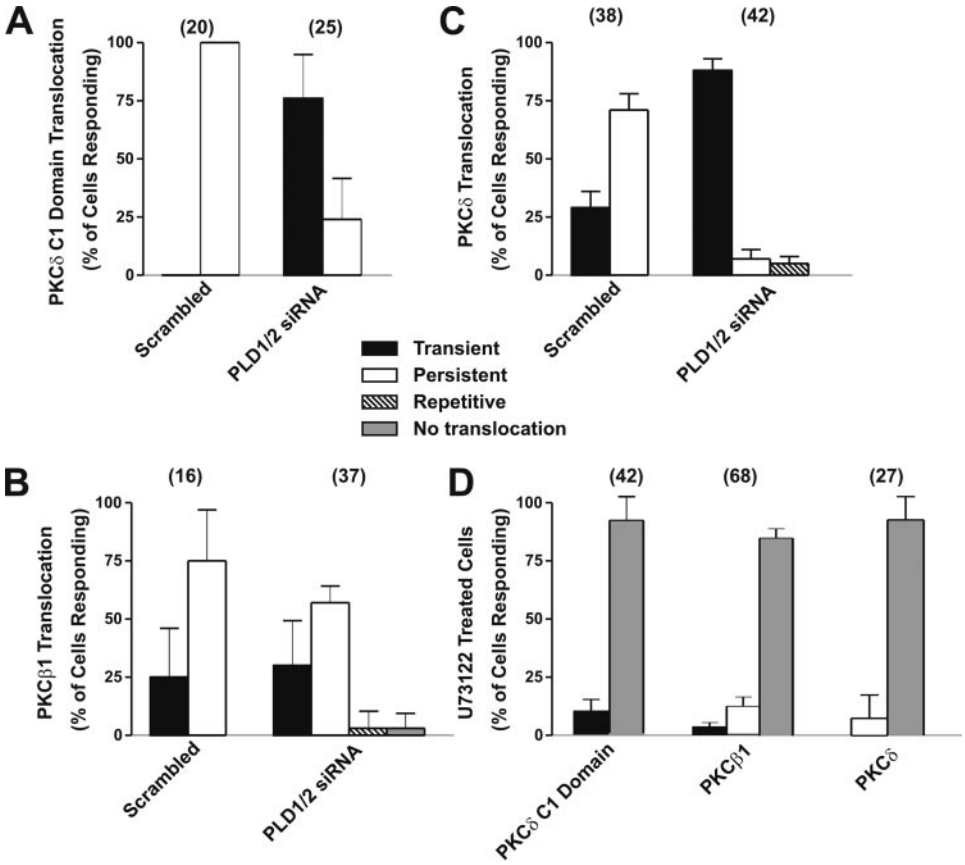

FIGURE 6. **Effect of PLD1/2 siRNA on persistent DAG formation as well as persistent PKCβI and PKCδ translocation responses in cells expressing AT_{1A}R-S331/338/348A.** HEK 293 cells were transfected with cDNA plasmids encoding HA-AT_{1A}R-S331/338/348A (1 μg) and either the YFP-PKCδ C1 domain reporter construct (*A*), EGFP-PKCβI (*B*), or EGFP-PKCδ (*C*) along with either scrambled (100 pM) or both PLD1 (100 pM) and PLD2 (100 pM) siRNA duplex and stimulated with 100 nM AngII. *D*, HEK 293 cells transfected with cDNA plasmids encoding HA-AT_{1A}R-S331/338/348A along with either the YFP-PKCδ C1 domain reporter construct, EGFP-PKCβI, or EGFP-PKCδ were pretreated with 10 μM U73122 for 15 min prior to receptor stimulation with 100 nM AngII. Shown are the percentage of HEK 293 cells that exhibited either transient, persistent, repetitive, or no translocation responses for each of the fluorescent reporter constructs. The *numbers in parentheses* represent the total number of cells counted from six different experiments. *Error bars* indicate 2 S.D. (95% confidence intervals).

cells tested (data not shown). In contrast, preincubation of cells with 2.5 mM EDTA did not prevent the transient translocation of either GFP-PKCβI or GFP-PKCβII in response to AT_{1A}R-S331/338/348A activation (Fig. 7). However, persistent PKCβI translocation responses were not observed in AT_{1A}R-S331/338/348A-activated cells treated with EDTA. Taken together, these data suggested that the release of calcium from intracellular stores was essential for the initiation of PKCβI and PKCβII translocation responses and that the influx of extracellular calcium must also be required for the persistent localization of PKCβI to the plasma membrane.

To further evaluate the role of extracellular calcium in the activation of PKCβI by AT_{1A}R, we tested the effect of perfusing cells with 2.5 mM EGTA subsequent to the induction of a persistent EGFP-PKCβI membrane translocation response. Agonist activation of the AT_{1A}R-S331/338/348A mutant resulted in persistent EGFP-PKCβI plasma membrane translocation in 23/47 cells tested, and when the bath medium was switched by perfusion to include EGTA, EGFP-PKCβI translocation was terminated in all 23 cells (Fig. 8). However, when both Ang II and EGTA were washed out by perfusion, PKCβI translocated back to the plasma membrane in 21/23 cells. These results suggested that persistent PKCβI membrane localization was also dependent upon the recruitment of an extracellular calcium influx that remained engaged in the absence of agonist stimulation.

Electrophysiological Characterization of the Extracellular Calcium Current—To determine whether the AT_{1A}R-mediated increase in extracellular calcium entry could be resolved via electrophysiology, we carried out whole cell patch clamp recordings from HEK 293 cells transfected with either wild type AT_{1A}R or AT_{1A}R-S331/338/348A along with either PKCβI or PKCβII. Cells were held at a potential of −20 mV before application of a voltage ramp from −60 to +40 mV. In the absence of Ang II treatment, only negligible current activity could be observed (Fig. 9, *A* and *B*, *0s*). Application of 100 nM AngII to a subset of cells expressing mutant AT_{1A}R-S331/338/348A along with EGFP-PKCβI resulted in the appearance of substantial current activity that reversed at about −15 mV (Fig. 8*A*, *600s*), which is consistent with a non-selective cation channel. In contrast, Ang II tended to evoke much smaller currents in cells expressing AT_{1A}R-S331/338/348A and EGFP-PKCβII (Fig. 9*B*, *600s*). Fig. 9*C* summarizes the effects of Ang II treatment across a total of 91 cells examined. In cells expressing EGFP-PKCβI, ~70% of the cells failed to respond to Ang II application with a significant (*i.e.* <0.05 nS conductance) inward current, whereas the remaining 30% responded with an average whole cell conductance of ~2 nS, consistent with what we observed for persistent PKCβI translocation responses. When recording from cells expressing the AT_{1A}R-S331/338/348A mutant, the fraction of responding cells increased to ~55%, with an average conductance of responding cells of just greater than 2 nS. Treatment of the cells with 1 μM PKC inhibitor chelerythrine reduced the inward current in responding cells that expressed the AT_{1A}R-S331/338/348A mutant and PKCβI (Fig. 9*C*). Hence, cells expressing PKCβII tended to show only a small degree of current activity, whereas cells expressing PKCβI were able to support larger inward currents. Moreover, in the PKCβI-expressing cells, the AT_{1A}R-S331/338/348A mutant was more effective in triggering current activity than wild type AT_{1A}R, and this was dependent upon PKCβI activity. These results were consistent with the observation that persistent PKCβI translocation

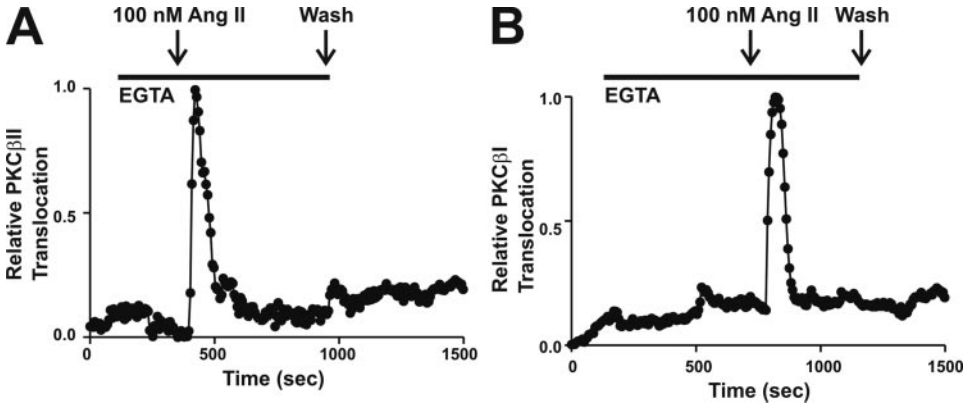

FIGURE 7. **Independence of transient PKCβ translocation on extracellular calcium.** HEK 293 cells were transfected with cDNA plasmids encoding HA-AT_{1A}R-S331/338/348A (10 μg) and either EGFP-PKCβII (2 μg) (*A*) or EGFP-PKCβI (2 μg) (*B*) and were pretreated with 2.5 mM EGTA prior to being exposed to 100 nM Ang II in the presence of 2.5 mM EGTA. *Black bars* shows the duration of EGTA treatment. *Arrows* indicate the times of agonist infusion and washout. The data are plotted as the relative change in GFP fluorescence intensity with maximal cytosolic fluorescence normalized to zero and minimum cytoplasmic fluorescence normalized to one. The data are representative of six independent experiments.

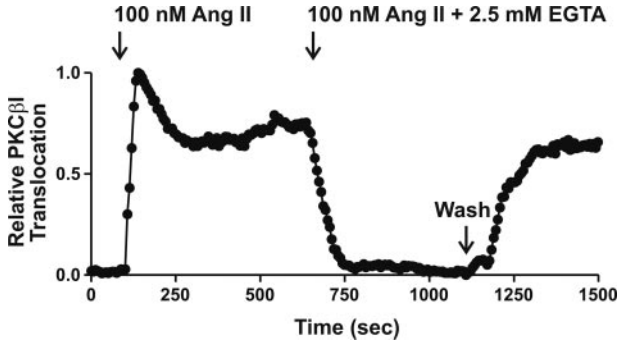

FIGURE 8. **Role of extracellular calcium in persistent PKCβI translocation responses.** HEK 293 cells were transfected with cDNA plasmids encoding HA-AT_{1A}R-S331/338/348A (10 μg) and EGFP-PKCβI (2 μg) and were treated with 100 nM Ang II prior to being exposed to 2.5 mM EGTA in the presence of 100 nM Ang II. *Arrows* indicate the times at which cells were treated with agonist, treated with agonist in the presence of 2.5 mM EGTA, and washed with agonist- and EGTA-free medium. The data are plotted as the relative change in EGFP fluorescence intensity with maximal cytosolic fluorescence normalized to zero and minimum cytoplasmic fluorescence normalized to one. The data are representative of 21/23 cells showing persistent translocation responses from six independent experiments.

responses were more reliably observed in AT_{1A}R-S331/338/348A mutant-expressing cells.

Fig. 10, *A* and *B*, displays the time courses for the Ang II-stimulated effects on whole cell conductance for the wild type (Fig. 10*A*) and S331/338/348A mutant (Fig. 10*B*) AT_{1A}R for cells exhibiting current increases. For both wild type and S331/338/348A mutant AT_{1A}R, the effects of Ang II occur with an approximate 3-min delay after agonist application. Moreover, in particular for cells expressing the AT_{1A}R-S331/338/348A mutant, a separation in current responses between cells expressing PKCβI and PKCβII is clearly evident (Fig. 10*B*), again consistent with the observed translocation responses for EGFP-PKCβI and EGFP-PKCβII (Fig. 4).

DISCUSSION

In the present study we have shown that the alternative splice variants for PKCβ, PKCβI and PKCβII, exhibit distinct patterns of membrane translocation in response to AT_{1A}R activation. We find that, whereas PKCβII transiently translocates to the plasma membrane in response to AT_{1A}R agonist activation, PKCβI displays two additional response patterns: 1) persistent plasma membrane translocation and 2) repetitive localization between the cytosol and plasma membrane following the removal of agonist. The observed difference in AT_{1A}R-stimulated PKCβI and PKCβII responses appears to be regulated, at least in part, by PKC phosphorylation at three consensus sites localized to the AT_{1A}R carboxyl-terminal tail. Mutation of any individual PKC consensus site: 1) increases the probability that PKCβI will persistently translocate to the plasma membrane, 2) results in sustained DAG responses, 3) leads to the recruitment of an extracellular calcium current and 4) does not alter PKCβII responses. Taken together, these results suggest that PKC-mediated phosphorylation may play an important role in regulating AT_{1A}R coupling to persistent DAG formation and the recruitment of an extracellular calcium current.

Angiotensin II-stimulated calcium and DAG signaling are typically characterized by both biphasic and sustained responses (1). Therefore, we propose the following model for the mechanism underlying the observed switch from transient to sustained calcium and DAG responses following AT_{1A}R activation (Fig. 11). First, stimulation of the AT_{1A}R results in transient increases in intracellular calcium, DAG, and inositol 1,4,5 trisphosphate levels, resulting in the release of intracellular calcium stores and the transient activation of both PKCβI and PKCβII. Second, the release of calcium from intracellular stores is essential for the initiation of all alternative PKC translocation responses. Third, we propose that under some conditions the AT_{1A}R escapes phosphorylation at each of three PKC phosphorylation consensus sites (see following sections for discussion), resulting in sustained PLD-mediated DAG formation via the metabolism of phosphatidic acid. Fourth, AT_{1A}R coupling to PLD-mediated DAG formation is also increased by the expression of PKCβI, but not PKCβII, suggesting that PKC isoform-specific receptor phosphorylation may be involved in regulating this process. One possible explanation for this observation may be that PKCβII, but not PKCβI, recognizes the AT_{1A}R as a substrate. Fifth, we also observed that in PKCβI-expressing cells the AT_{1A}R exhibits the capacity to recruit an extracellular calcium current and the probability that this will occur is significantly enhanced by the mutation of PKC consensus sites in the AT_{1A}R C-tail. Sixth, although both calcium and DAG are known to contribute to the membrane localization of conventional PKC isozymes, the PLD-mediated source of DAG is not required for the sustained localization of PKCβI at the membrane. However, it is essential for the persistent translocation of the novel PKCδ isoform. Finally, the sustained localization of PKCβI at the plasma membrane is absolutely dependent upon AT_{1A}R-stimulated influx of extracellular calcium and PKCβI activity, as the establishment of this current is only observed in

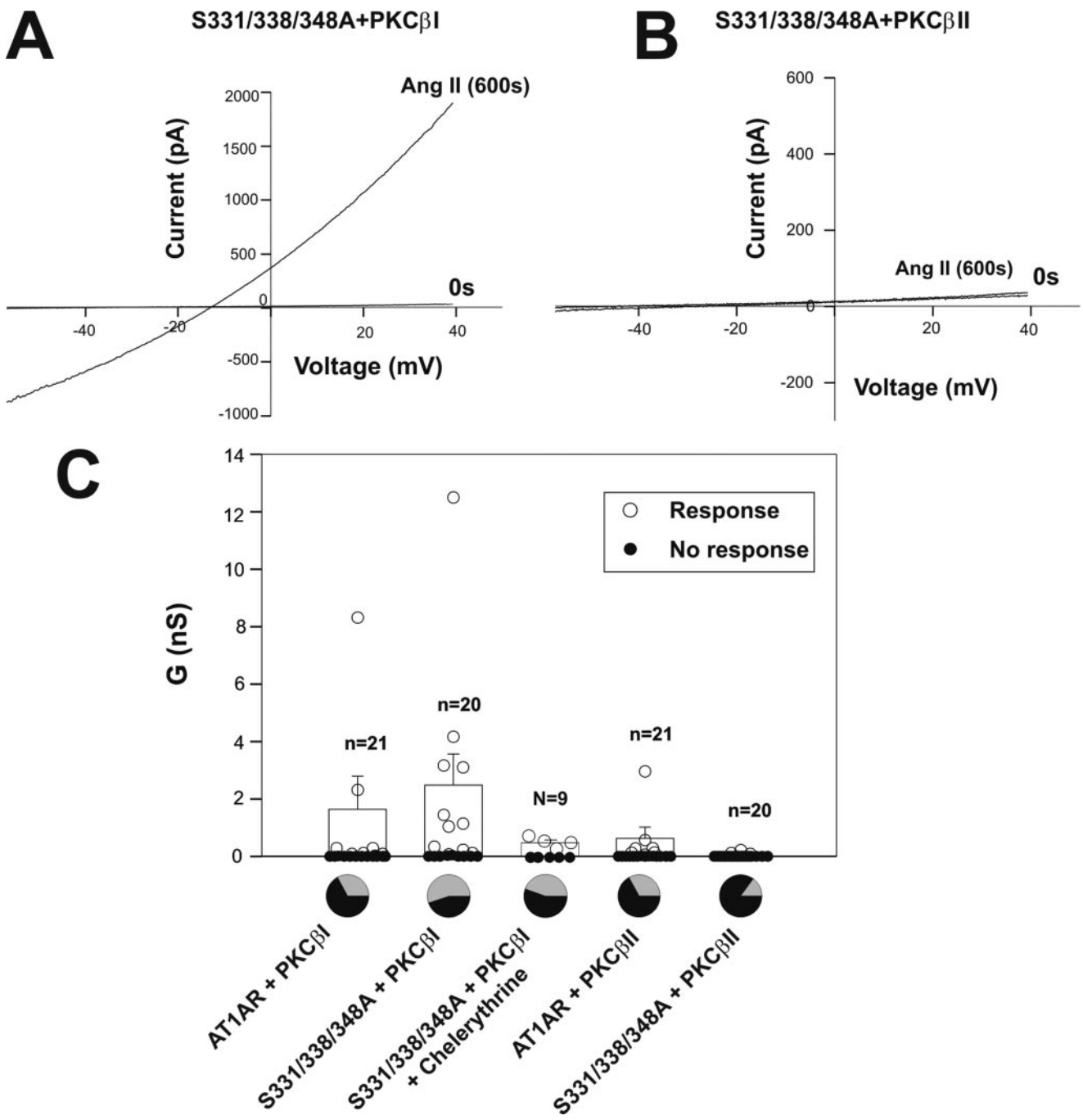

FIGURE 9. **Whole cell patch clamp recordings from HEK 293 cells.** HEK 293 cells were transfected with cDNA plasmids encoding either HA-AT_{1A}R (10 μg) or HA-AT_{1A}R-S331/338/348A (10 μg) along with either GFP-PKCβI (2 μg) or GFP-PKCβI (2 μg). Shown are representative current traces reflecting whole cell current activity in tsA-201 cells expressing the HA-AT_{1A}R-S331/338/348A with either PKCβI (*A*) or PKCβII (*B*) before and after stimulation with 100 nM Ang II. Currents were elicited by application of a voltage ramp (between −60 mV and +40 mV) from a holding potential of −20 mV. *C*, shown is a summary of Ang II-mediated whole cell current activity. Currents were elicited as outlined under "Experimental Procedures", and whole cell conductance values were determined by liner fits to the inward current data between −60 and −30 mV. In each case, conductance values obtained in the absence of Ang II were subtracted from those observed after Ang II application. *Hollow* symbols reflect Ang II-mediated conductances >0.05 nS (*i.e.* responding cells); *filled symbols* reflect cells where Ang II-mediated effects smaller than 0.05 nS. The *bars* reflect mean conductance values obtained from only the responding cells. The *black* portions of the pie charts indicate the percentage of cells that responded to Ang II application. *Error bars* denote S.E.

PKCβI-expressing cells and can be prevented by a PKC inhibitor. This suggests that PKCβI, but not PKCβII, activity is required for the recruitment of the current. The repetitive translocation of PKCβI in some cells may involve repetitive activation of PLC similar to what we have previously reported for mGluR1 (17). Overall, PKC isoform-specific activity contributes to the regulation of AT1AR-stimulated patterns of second messenger activation.

There are a number of potential mechanisms by which PKCβ splice variants may respond differently to alterations in second messenger levels. One potential explanation for PKC subtype-specific response patterns is related to the differential sensitiv-

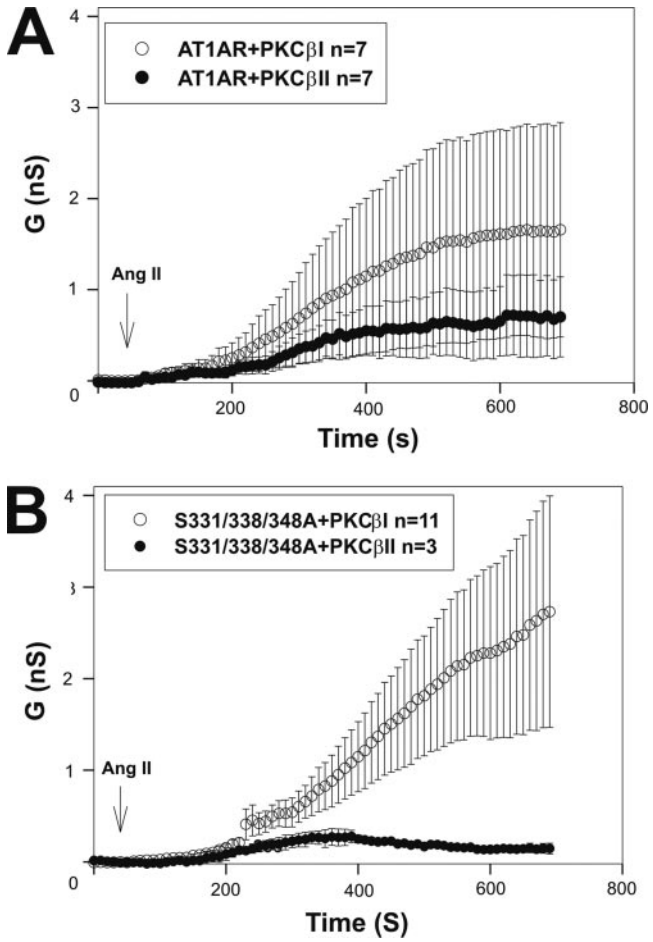

FIGURE 10. **Time course of Ang II-stimulated currents in HEK 293 cells.** *A*, time course for AngII-stimulated currents in HEK 293 cells expressing HA-$AT_{1A}R$ with and without EGFP-PKCβI and EGFP-PKCβII. *B*, time course for AngII-stimulated currents in tsA-201 cells expressing HA-$AT_{1A}R$-S331/338/348A with and without EGFP-PKCβI and EGFP-PKCβII. Only cells with Ang II-induced effects on whole cell conductance >0.05 nS are included in the figure. *Error bars* denote S.E.

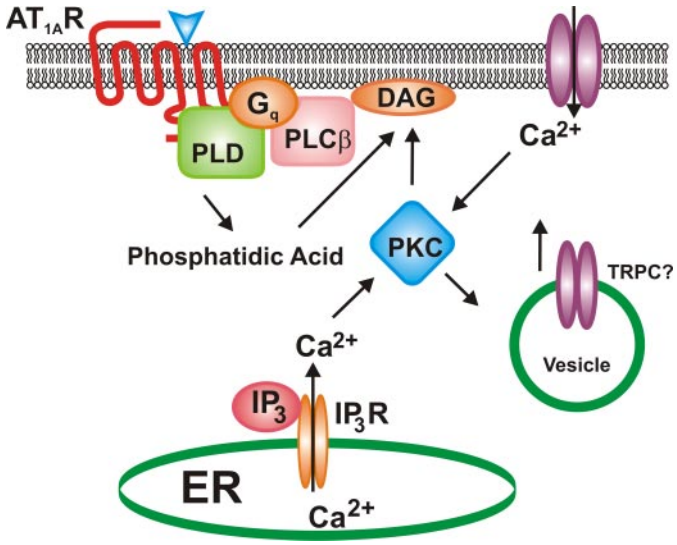

FIGURE 11. **Schematic representation of the proposed model for $AT_{1A}R$-stimulated PKC isoform-specific translocation responses.** A detailed description of the figure can be found under "Discussion." *Ca^{2+}*, calcium; *ER*, endoplasmic reticulum; *IP_3*, inositol 1,4,5 trisphosphate; *IP_3R*, IP_3 receptor; *PKC*, protein kinase C; *PLCβ*, phospholipase Cβ; *PLD*, phospholipase D; *TRPC*, transient receptor potential channel.

ity of PKCβI and PKCβII to increases in intracellular calcium and DAG levels. Keranen and Newton (24) have demonstrated that the PKCβI and PKCβII V5 domains regulate differences in the calcium-dependent affinity of the enzymes for acidic membranes. Consistent with this observation, we showed that PKCβI and PKCβII exhibit distinct translocation patterns in response to mGluR1a activation, even when expressed in the same cell (17). PKCβII activation appears to be primarily regulated by alterations in intracellular calcium released from intracellular stores and is relatively insensitive to changes to elevated DAG, whereas PKCβI activity is sensitive to both calcium and DAG. We previously identified two residues in the PKCβII V5 domain that suppress PKCβI-like responses for PKCβII.

The initiation of both transient and persistent PKCβI responses by $AT_{1A}R$ requires the release of calcium from intracellular stores. However, only the persistent responses required the influx of extracellular calcium. This influx of extracellular calcium is unlikely to involve $AT_{1A}R$ coupling to calcium-specific voltage-activated plasma membrane channels, because HEK 293 cells do not express these channels (25). Rather the dependence on extracellular calcium for the persistent PKCβI plasma membrane translocation is likely due to the recruitment of nonspecific cation channels, such as transient receptor potential channels (TRPCs) (Fig. 11). Cayouette *et al.* (26) have recently shown that activation of muscarinic receptors in HEK 293 cells or depletion of intracellular calcium increases the levels of TRPC6 at the plasma membrane. Similarly, other groups have shown that TRPC can be activated by stimulation of G_q-coupled receptors (27, 28). Therefore, one potential mechanism by which the calcium-dependent persistent membrane localization of PKCβI can be mediated is through activation of $AT_{1A}R$-stimulated membrane targeting and activation of TRPCs. The activation of the TRPC current by the $AT_{1A}R$ appears to be dependent upon PKCβI activity. However, the relevance and/or identity of the specific TRPC mediating the influx of extracellular calcium in our model remain to be determined.

Unlike PKCβII, PKCβI exhibits the capacity to be persistently localized at the plasma membrane in response to $AT_{1A}R$ activation; this response persists even after the cells are perfused with agonist-free medium. This differs from what we previously observed for mGluR1a, where the perfusion of cells with agonist-free medium led to the cessation of PKCβI activation and localization at the plasma membrane (17). Moreover, PKCβI localization at the plasma membrane in mGluR1a-expressing cells was solely dependent upon persistent changes in DAG formation without concomitant alterations in intracellular calcium concentrations. This suggests that in response to $AT_{1A}R$, but not mGluR1a activation, there is a PKCβ splice variant-specific alteration in HEK 293 calcium homeostasis that becomes independent of receptor activation. Of particular interest is the possibility that PKCβI contributes directly to the $AT_{1A}R$-dependent recruitment of a TRPC-mediated influx of extracellular calcium. Several studies have suggested that TRPC activity is activated by increases in intracellular DAG levels (27, 28). However, in our study, $AT_{1A}R$ coupling to extra-

cellular calcium is only observed in PKCβI-expressing cells, whereas sustained levels of DAG signaling are observed in both PKCβI- and PKCβII-expressing cells. An alternative mechanism by which TRPC activity is recruited is through the receptor-regulated exocytotic insertion of an intracellular vesicular pool of TRP channels into the plasma membrane (26). Interestingly, we often observe the translocation of PKCβI, but not PKCβII, to intracellular vesicles in response to $AT_{1A}R$ activation (data not shown). Thus, it is possible that PKCβI activation may directly modulate TRPC exocytosis. However, extensive additional experimentation will be required to test this hypothesis.

We observed that the mutation of any individual PKC phosphorylation consensus site in the $AT_{1A}R$ carboxyl-terminal tail results in the persistent activation of DAG formation and increases the probability that PKCβI will persistently localize to the plasma membrane in response to $AT_{1A}R$ activation. In contrast, PKCβII response patterns remain unchanged in cells expressing the $AT_{1A}R$-S314/338/348A mutant. This observation suggests that feedback phosphorylation and heterologous $AT_{1A}R$ desensitization by PKCβI and PKCβII may play an important role in regulating PKCβI membrane translocation patterns. The probability that PKCβI will exhibit persistent plasma membrane localization is increased following the mutation of any of the three PKC consensus sites localized to the $AT_{1A}R$ carboxyl-terminal tail. This suggests that phosphorylation at all three sites may be required to attenuate PKCβI translocation. However, the alterations in DAG signaling by PKC phosphorylation site $AT_{1A}R$ mutants does not appear to require the overexpression of PKCβI, because persistent activation of DAG signaling is observed in 98% of cells expressing the $AT_{1A}R$-S314/338/348A mutant alone. Thus, endogenously expressed PKC isozymes are likely sufficient to mediate heterologous $AT_{1A}R$ desensitization and prevent $AT_{1A}R$ coupling to a PLC-independent source of DAG.

In summary, we have uncovered an intricate and complicated mechanism by which the activity of PKCβI and PKCβII is differentially regulated in response to $AT_{1A}R$ activation. It appears that persistent membrane translocation of PKCβI is dependent upon both the release of calcium from intracellular stores and the recruitment of an extracellular calcium source. We hypothesize that this source of calcium might involve the PKCβI-specific recruitment of a TRP channel. Overall, we find that the patterning of $AT_{1A}R$ second messenger response patterns is regulated by PKC isoform-specific sensitivity to alterations in intracellular calcium and DAG levels, heterologous desensitization, and PKC isoform substrate specificity.

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Role of Angiotensin II Type 1A Receptor Phosphorylation, Phospholipase D, and Extracellular Calcium in Isoform-specific Protein Kinase C Membrane Translocation Responses

Aleksandra Policha, Noriko Daneshtalab, Lina Chen, Lianne B. Dale, Christophe Altier, Houman Khosravani, Walter G. Thomas, Gerald W. Zamponi and Stephen S. G. Ferguson

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