Signal transduction via protein kinase C (PKC) is closely regulated by its subcellular localization. In response to activation of cell-surface receptors, PKC is directed to the plasma membrane by two membrane-targeting domains, namely the C1 and C2 regions. This is followed by the return of the enzyme to the cytoplasm, a process shown recently to require PKC autophosphorylation (Feng, X., and Hannun, Y. A. (1998) J. Biol. Chem. 273, 26870–26874). In the present study, we examined mechanisms of translocation and reverse translocation and the role of autophosphorylation in these processes. By visualizing the trafficking of wild-type as well as mutant PKC/II in live cells, we demonstrated that in response to cell-surface receptor activation, the function of the C1 region is required but not sufficient for recruitment of the enzyme to the plasma membrane. The C2 region is also critical in anchoring the enzyme to the plasma membrane. Furthermore, the inability of a kinase-deficient PKC to undergo reverse translocation was restored by the addition of intracellular calcium chelators, suggesting a role for the C2 region in the persistent phase of translocation. On the other hand, the inability of a C2 deletion mutant (C1 region intact) to translocate in response to agonist was reversed in mutants lacking kinase activity or by mutation of the Ser660 autophosphorylation site to alanine, suggesting that autophosphorylation of this site is required for opposing the action of the C2 region. Therefore, the membrane-targeting function of the C1 region is facilitated by the C2 region and appears to be opposed by autophosphorylation. Taken together, these findings provide novel evidence of the functional regulation of reversible PKC membrane localization by autophosphorylation, and they show that the dynamic translocation of PKC in response to agonists is tightly regulated in a collaborative fashion by the C1 and C2 regions in balance with the effects of autophosphorylation.

Protein kinase C (PKC) represents a family of serine/thre-
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for many years that PKC is phosphorylated at multiple serine/threonine sites during its biogenesis (13, 14). In the case of PKCβII, three major in vivo phosphorylation sites, Thr500, Thr641, and Ser660, have been identified (15–17). The inability of PKCβII itself to phosphorylate at Thr500 has suggested that the phosphorylation of this residue involves a protein kinase distinct from PKC (18). In contrast, Thr641 and Ser660 are identified as the residues that are sequentially autophosphorylated following phosphorylation of Thr500 during PKC maturation (16). Autophosphorylation of PKC has been shown to regulate the enzyme regulatory and catalytic domains by enhancing their binding affinity for Ca2+ and ATP, as well as peptide substrates (19). In addition, it has also been shown to be important for PKC dynamic cellular trafficking, probably by enhancing reverse translocation (20). Therefore, like DAG, Ca2+, and many other lipid mediators, autophosphorylation provides another effective way to regulate the activity and localization of PKC, but at the level of the enzyme itself.

In previous studies, by visualizing real time cellular trafficking of PKCβII conjugated to green fluorescent protein (GFP) in live cells, we showed that PKC undergoes a dynamic and reversible redistribution between the plasma membrane and cytoplasm in response to physiological stimuli such as activation of cell-surface G protein-coupled receptors (GPCRs) (21). Shortly after its membrane translocation, PKC dissociates from the plasma membrane and rapidly returns to the cytoplasm. This is different from the effects of phorbol esters such as PMA or Ca2+ ionsophores such as A23187, which promote a stable association of PKC with the membrane (21–23). In addition, we have demonstrated that the autophosphorylation activity of PKC is essential for its membrane dissociation and therefore plays a key role in the reversible translocation of PKC in response to GPCR activation (20). In the present study, we address how PKC autophosphorylation interacts with membrane targeting through the C1 and C2 regions to achieve a balanced and dynamic movement of PKC in live cells upon activation of the Gα-coupled angiotensin II type 1A receptor (AT1AR). Our results support that the GPCR-promoted PKC membrane trafficking consists of at least three distinct steps, i.e. membrane recruitment, membrane anchorage, and membrane dissociation. The C1 and probably the C2 regions are required for recruiting PKC to the plasma membrane upon receptor stimulation. The C2 region then persistently anchors the enzyme to the membrane. When extracellular signals are turned off, the enzyme returns to the cytoplasm, a process dependent on the autophosphorylation status of PKC. Therefore, although each plays a specific role, the functions of the C1 and C2 regions and autophosphorylation are cooperative and interactive, thus ensuring appropriate timing, duration, and magnitude of dynamic PKC trafficking.

EXPERIMENTAL PROCEDURES

Materials—Eagle’s minimal essential medium and HEPES were from Life Technologies, Inc. Human embryonic kidney (HEK) 293 cells were provided by American Type Culture Collection. Rabbit polyclonal antibody against PKCβII was prepared and extensively characterized as described previously (24). |γ-32P|ATP was purchased from NEN Life Science Products. Restriction enzymes were from Promega or New England Biolabs Inc. Vent DNA polymerase and rabbit anti-phospho-PKC/βSer660 polyclonal antibody were purchased from New England Biolabs Inc. Protein A-Sepharose CL-4B was from Amersham Pharmacia Biotech. Phosphatidylserine and sn-dioctanoylglycerol were purchased from Avanti Polar Lipids, Inc. A23187 was purchased from Calbiochem. BAPTA-AM was from Molecular Probes, Inc. All other chemicals were from Sigma.

Plasmid Constructs—All recombinant DNA procedures were carried out following standard protocols. pBK-CMV-GFP-PKCβII, pBK-CMV-GFP-S660A, and pBK-CMV-GFP-K371R were constructed as described previously (20, 21). pBK-CMV-GFP-mC1 and pBK-CMV-GFP-KR/mC1 were generated from pBK-CMV-YPK-PKCβII and pBK-CMV-GFP-K371R, respectively, by polymerase chain reaction to mutate both codons TGC (Cys5) and TGT (Cys125) in the coding sequence to AGC (Ser) and AGT (Ser) according to a previous study on PKγ (25). pBK-CMV-GFP-ΔC2 and pBK-CMV-GFP-ΔC2/ΔC2 were constructed from pBK-CMV-GFP-PKCβII and pBK-CMV-GFP-K371R, respectively, by polymerase chain reaction to remove 80 amino acids from Lys312 to Arg322. The autophosphorylation mutants (pBK-CMV-ΔC2/SA and pBK-CMV-ΔC2/SE) were generated from pBK-CMV-ΔC2 by polymerase chain reaction to mutate TAA (Ser660) to either GCC (Ala) or GAA (Glu) (see Fig. 1). The DNA sequences of the constructs were confirmed by DNA sequencing.

Cell Culture and Transfection—HEK 293 cells were maintained in Eagle’s minimal essential medium supplemented with 10% (v/v) fetal bovine serum in a 5% CO2 incubator at 37 °C. Cells were seeded at a density of 2.0 × 105 cells/100-mm dish and transfected using a modified calcium phosphate method with 1–5 μg of plasmids. The expression of the hemagglutinin-tagged AT1AR in the presence of wild-type or various mutant GFP-PKCβII constructs was assessed by flow cytometry following antibody staining. The levels of the AT1AR in cells transfected with various mutant GFP-PKCβII constructs were either equivalent to or higher than that in the presence of wild-type GFP-PKCβII.

Protein Kinase C Assay—For assaying PKC activity in cells transfected with wild-type or mutant GFP-PKCβII, the cell lysates (200 μg) were first immunoprecipitated with 5 μg of rabbit anti-PKCβII antibody. The immunoprecipitation was performed 48 h after transfection as described previously (21). The PKC autophosphorylation activity was assessed using the vesicle assay for PKC as described previously (26).

Immunoblotting—Cell lysates from HEK 293 cells transfected with GFP-PKCβII and its mutants were separated by SDS-polyacrylamide gel electrophoresis, followed by autoradiography.

Immunofluorescence—HEK 293 cells transfected with GFP-PKCβII or one of its mutants together with the AT1AR. Twenty-four hours after transfection, the cells were plated onto 35-mm glass-bottom culture dishes (Mattek Corp.) at a density of 4 × 104 and incubated for another 24 h for the cells to attach to glass. The cells expressing GFP-PKCβII or its mutants were observed by confocal microscopy, which was performed on a Zeiss LSM 410 confocal laser scanning microscope using a Zeiss 40× 1.2 NA water immersion lens. The cells were at room temperature in culture medium containing 20 mM HEPES. GFP fluorescent signals were collected sequentially using the Zeiss LSM software time series function with single line excitation (488 nm) with a time interval of 20 or 60 s between two scans. Angiotensin II, A23187, PMA, and EGTA/BAPTA-AM were applied to the cells during the scans. Quantitative analysis of relative fluorescence intensity was performed on a Macintosh computer using the public domain NIH Image program. The relative change in plasma membrane fluorescence intensity was calculated according to a previously reported method (27).

RESULTS

Recruitment of Cytoplasmic PKC to the Plasma Membrane by the C1 Region in Response to Receptor Activation—Previously, we showed that GFP-PKCβII is fully functional in terms of its kinase activity and its ability to undergo membrane translocation (21). In addition, the inherent fluorescence of GFP allowed us to visualize the trafficking of GFP-PKCβII in live HEK 293 cells and to demonstrate that autophosphorylation is essential for the reversibility of PKC membrane translocation in response to GPCR activation (20). To further understand the role of autophosphorylation in association with the membrane-targeting function of the C1 and C2 regions in the dynamic translocation of PKC, a series of GFP-PKCβII mutants were con-
constructed (Fig. 1), and their trafficking properties were studied in live HEK 293 cells.

Initial experiments examined the effects of the C1 region mutation on PKC membrane translocation by altering two essential Cys residues in the two cysteine-rich motifs of the C1 region to Ser (Fig. 1). This C1 region mutant (i.e. GFP-mC1), similar to wild-type GFP-PKCβII, was evenly distributed in the cytoplasm under normal unstimulated conditions (Fig. 2A). Consistent with the role of the cysteine-rich motifs of the C1 region in DAG binding, the GFP-mC1 mutant lost the ability to translocate to the plasma membrane upon stimulation by 1 µM PMA, a phorbol ester commonly used to mimic the action of DAG in targeting PKC to the membrane, but with higher affinity and longer effects (Fig. 2A). In contrast, GFP-mC1 underwent substantial membrane translocation in response to 10 µM A23187, a Ca²⁺ ionophore that causes a maximal increase in intracellular concentrations of Ca²⁺, indicating that the C2 region remained functional in its ability to associate with the membrane (Fig. 2A). To further explore the function of the C1 region in the reversible membrane trafficking of PKC in response to physiological stimuli, GFP-mC1 localization was examined in HEK 293 cells in response to activation of cotransfected AT1AR by its physiological agonist angiotensin II. GFP-mC1 was found to lack the ability to undergo membrane redistribution in response to angiotensin II (Fig. 2B), whereas wild-type GFP-PKCβII underwent a rapid and reversible mem-
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Regulation of Persistent PKC Anchorage to the Plasma Membrane by the C2 Region and the Opposing Role of Kinase Activity—To investigate the possible function of the C2 region in the transient PKC membrane redistribution stimulated by receptor activation, two C2 deletion mutants (GFP-ΔC2 and GFP-KR/ΔC2) were generated by removing 80 amino acids from the C2 region of wild-type GFP-PKCβII and the kinase-deficient mutant GFP-K371R, respectively. In the unstimulated HEK 293 cells, similar to wild-type GFP-PKCβII, GFP-C2 and GFP-KR/ΔC2 were distributed mainly in the cytoplasm (Fig. 3). Stimulation with 1 μM PMA induced a membrane redistribution of both GFP-ΔC2 and GFP-KR/ΔC2, whereas 10 μM A23187 failed to trigger redistribution of the two mutants (Fig. 3). This indicates that the C1 regions, but not the C2 regions, in GFP-mC1 and GFP-KR/mC1 to redistribute to the membrane is due to the inability of their C1 regions to respond to DAG. The inability of these mutants to trigger PKC membrane localization indicates that the recruitment of PKC to the plasma membrane in response to receptor activation requires the function of the C1 region.

Consistent with a role of the C2 region in membrane anchoring, upon deletion of the C2 region from wild-type GFP-PKCβII, the resulting mutant (GFP-ΔC2) demonstrated a dramatic decrease in its ability to undergo membrane translocation. When quantitated, >90% of GFP fluorescence from wild-type GFP-PKCβII was translocated to the plasma membrane and subsequently returned to the cytoplasm within 100 s of agonist stimulation. In contrast, there was no significant agonist-dependent increase in membrane fluorescence intensity in cells expressing GFP-mC1 (Fig. 2C). As the C2 region of GFP-mC1 was functional (Fig. 2A), the inability of GFP-mC1 to traffic to the plasma membrane suggests that the C1 region is essential for PKC targeting to the plasma membrane following receptor activation and that the C2 region is not sufficient for membrane translocation in response to physiological agonist.

To exclude the possibility that the inability to observe GFP-mC1 mobilization is due to the masking of membrane translocation by very rapid membrane dissociation, we examined the distribution of a kinase-deficient GFP-mC1 mutant (namely GFP-KR/mC1) because loss of kinase activity prevents membrane dissociation in the wild-type enzyme and therefore would serve to trap any translocated enzyme at the plasma membrane (20). GFP-KR/mC1 was generated from the previously described GFP-K371R (20). Converting the conserved Lys at the enzyme ATP-binding site to Arg completely abolishes the ability of PKC to autophosphorylate. Similar to GFP-mC1, when transfected in HEK 293 cells, GFP-KR/mC1 was found mainly in the cytoplasm, and its redistribution to the membrane was induced only by the Ca2+ ionophore A23187, but not by PMA (Fig. 2A). Furthermore, when examined in HEK 293 cells cotransfected with the AT1AR and stimulated with angiotensin II, like GFP-mC1, no significant membrane redistribution of GFP-KR/mC1 was observed (Fig. 2, B and C). These results further confirm that the failure of GFP-mC1 and GFP-KR/mC1 to redistribute to the membrane is due to the inability of their C1 regions to respond to DAG. The inability of these mutants to trigger PKC membrane localization indicates that the recruitment of PKC to the plasma membrane in response to receptor activation requires the function of the C1 region.

**Fig. 2** Effects of the C1 region function and PKC kinase activity on PKCβII membrane trafficking. A, shown are confocal micrographs of HEK 293 cells transfected with 1 μg of pBK-CMV-GFP-PKCβII (upper panels), pBK-CMV-GFP-mC1 (middle panels), or pBK-CMV-GFP-KR/mC1 (lower panels) before (Control) and after stimulation with 1 μM PMA or 0.5 μM A23187 for the indicated time periods. The experiments were performed independently on three different occasions; and each time, 5–10 cells from independent stimulations were recorded. The micrographs are representative of >90% of the cells observed. B, shown are confocal micrographs of HEK 293 cells cotransfected with 5 μg of pcDNA Vamp-AT1R together with 1 μg of pBK-CMV-GFP-PKCβII (upper panels), pBK-CMV-GFP-mC1 (middle panels), and pBK-CMV-GFP-KR/mC1 (lower panels) before (Control) and after stimulation with 0.5 μM angiotensin II (AgII) for the indicated time periods. The experiments were performed independently on three different occasions; and each time, 10–20 cells from independent stimulations were recorded. The micrographs are representative of >75% of the cells observed. C, shown are the time courses of the relative fluorescence average changes in GFP-PKCβII, GFP-mC1, and GFP-KR/mC1 on the plasma membrane following angiotensin II stimulation. Twelve cells overexpressing GFP-PKCβII, 12 cells overexpressing GFP-mC1, or 12 cells overexpressing GFP-KR/mC1 from three individual experiments were used to determine the relative membrane fluorescence intensity as described under “Experimental Procedures.”
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GFP-K371R proteins (Fig. 5Bb), a PKC for phosphorylated Ser660, a conserved PKC autophosphorylation (Fig. 4A). However, unlike GFP-K371R, C2 was observed to translocate rapidly to the plasma membrane within 40 s (Fig. 4A). Therefore, similar to the C1 region, the C2 region is necessary but not sufficient for translocation of PKC in response to agonists.

A major difference between the C1 and C2 regions became apparent when the interaction of these regions with the kinase activity of the enzyme was investigated. Upon agonist stimulation of HEK 293 cells cotransfected with the AT1AR, GFP-KR/mC1 mutant deficient in autophosphorylation (17, 20). Moreover, as expected from the interaction between the C2 region and the kinase activity of PKC, the C2 region does not interfere with the ability of the enzyme to autophosphorylate. Given that GFP-KR/ΔC2 behaves, more or less, similar to the wild-type enzyme, the deletion of the C2 region therefore appears to negate the effects of lack of kinase activity on reversal of translocation, strongly suggesting opposing roles for the C2 region and kinase activity in the regulation of translocation.

It is important to note that the kinase-deficient C1 mutant did not show this behavior (Fig. 2). Therefore, this additional role for the C2 region in the regulation of membrane association was only revealed by comparing the different abilities of GFP-K371R, GFP-KR/mC1, and GFP-KR/ΔC2 to be retained on the plasma membrane following AT1AR-mediated membrane translocation. Given the role of the kinase activity in reversal of membrane translocation and the above results on the opposing effects of the C2 region and the kinase activity, these results suggest a role for the C2 region in anchoring translocated PKC to the plasma membrane, resulting in the persistent membrane localization of PKC in the absence of autophosphorylation.

To test this hypothesis, the function of the C2 region was further evaluated by adding the Ca2+ chelators EGTA and BAPTA-AM, as the binding of the C2 region to plasma membrane lipids is dependent on Ca2+. EGTA and BAPTA-AM have been demonstrated as effective Ca2+ chelators that block A23187- and fatty acid-induced PKCγ membrane translocation in live COS-7 cells and CHO-K1 cells respectively (23, 28). The specificity of their effect was further shown by their inability to inhibit fatty acid-induced membrane translocation of GFP-conjugated PKCε, one of the PKC isoenzymes not regulated by Ca2+ (28). Therefore, the effects of EGTA/BAPTA-AM on the membrane association of GFP-K371R were examined in HEK 293 cells cotransfected with the AT1αR. As shown previously (20), stimulation of the cells by angiotensin II induced a rapid mobilization of GFP-K371R from the cytoplasm to the plasma membrane, and GFP-K371R remained persistently on the membrane (Fig. 6A). However, when the same cells were exposed to 2.5 mM EGTA and 15 μM BAPTA, a rapid return of GFP-K371R to the cytoplasm was observed within 1 min (Fig. 6A). The relative membrane fluorescence intensity was quantitated as shown in Fig. 6B. The addition of EGTA/BAPTA-AM triggered the return of >90% GFP-K371R fluorescence back to the cytoplasm, whereas >90% of GFP-K371R fluorescence continued to be present on the plasma membrane in control cells not exposed to the Ca2+ chelators (Fig. 6B). This requirement for Ca2+ in the persistent membrane association of GFP-K371R further demonstrates that the Ca2+-regulated C2 region plays a critical role in the continuous membrane anchoring of PKCβII once the enzyme is recruited to the plasma membrane.

Functional Cross-talk of Autophosphorylation with the C1 and C2 Regions in Receptor-induced PKC Membrane Trafficking—The above results showed an important and specific interaction between the C2 region and the kinase activity of PKC. To address the role of autophosphorylation per se in this interaction, we used GFP-S660A, which is mutated in one of two major in vivo autophosphorylation sites at the carboxyl terminus of PKCβII. Fig. 7 compares the time course of receptor-mediated membrane trafficking for wild-type GFP-PKCβII and GFP-S660A. Similar to GFP-PKCβII, the mutant GFP-S660A was able to localize to the plasma membrane rapidly in response to angiotensin II stimulation. However, it was apparent that the return of the mutant GFP-S660A from the membrane to the cytoplasm was impaired, suggesting a specific role for this autophosphorylation site in the regulation of reverse translocation.

An additional role for autophosphorylation in regulating PKC membrane targeting was investigated by studying the location in response to angiotensin II compared with the wild-type enzyme (Fig. 4A). Only very weak membrane lightnings were observed in some cells (Fig. 4A, arrows). These results are quantitated in Fig. 4B. Therefore, similar to the C1 region, the C2 region is necessary but not sufficient for translocation of PKC in response to agonists.

These results suggested an important role for the kinase activity of the ΔC2 mutant. Therefore, it became important to demonstrate that PKCβII mutants lacking the C2 region were capable of undergoing autophosphorylation. GFP-PKCβII and GFP-ΔC2 were immunoprecipitated with anti-PKCβII polyclonal antibody from HEK 293 cells overexpressing these two proteins, and their autophosphorylation activities were assessed. Using an in vitro protein kinase assay, it was found that similar to the wild-type enzyme, GFP-ΔC2 was capable of autophosphorylation (Fig. 5A). More important, when the in vivo autophosphorylation status of the kinase was assessed using anti-phospho-PKCβ(Thr660) antibody, which is specific for phosphorylated Ser660, a conserved PKC autophosphorylation site (15–17), both wild-type GFP-PKCβII and GFP-ΔC2 were found to be phosphorylated to a similar extent at Ser660 (Fig. 5B), further indicating that GFP-ΔC2 retains the ability to autophosphorylate in cells. On the other hand, only little Ser660 phosphorylation was detected in cells overexpressing GFP-K371R proteins (Fig. 5B), a PKCβII mutant deficient in autophosphorylation (17, 20). Moreover, as expected from the nature of the antibody, anti-phospho-PKCβ(Thr660) antibody also failed to detect the mutant GFP-S660A, in which Ser660 was converted to Ala (data not shown). Therefore, the deletion of the C2 region does not interfere with the ability of the enzyme to autophosphorylate. Given that GFP-KR/ΔC2
translocation of the C2 deletion mutant and the combined C2 deletion/S660A mutant. If the lack of GFP-ΔC2 membrane translocation was indeed related to its ability to autophosphorylate, mutation of Ser660 to alanine in GFP-ΔC2 (i.e., GFP-ΔC2/SA) should result in an enhanced membrane translocation. If not, the results would point to a role for phosphorylation of different substrates rather than autophosphorylation. In HEK 293 cells cotransfected to express the AT1AR, GFP-ΔC2/SA was observed to undergo a reversible membrane translocation, unlike GFP-ΔC2, whose distribution was unchanged by AT1AR activation (Fig. 8A). The time frame for the return of GFP-ΔC2/SA appeared to be longer than that of the wild-type enzyme as shown in Fig. 8B, consistent with the finding that GFP-S660A is defective in membrane dissociation (Fig. 7). As a control, mutation of Ser660 to Glu, which mimics phosphorylated Ser, resulted in a mutant (GFP-ΔC2/SE) that underwent only a very weak membrane redistribution (Fig. 8A), and the phenotype exhibited by GFP-ΔC2/SE was closer to that of GFP-ΔC2 than to that of GFP-ΔC2/SA (Fig. 8B). At the peak of membrane translocation (t = 60 s) in response to AT1AR activation, the average relative membrane fluorescence intensity for GFP-ΔC2/SA was much higher than those for GFP-ΔC2 and GFP-ΔC2/SE.

Taken together, these results demonstrate that the absence of GFP-ΔC2 membrane trafficking results from the counteraction between the function of the C1 region and autophosphorylation; and, as a consequence, the removal of an autophosphorylation site to reduce membrane dissociation rescues the ability of GFP-ΔC2/SA to translocate to the membrane. The inhibitory effect of autophosphorylation on PKC membrane association is only obvious in the absence of the C2 region.

**DISCUSSION**

The real time visualization of GFP-PKCβII localization in response to AT1AR activation in live HEK 293 cells has demonstrated that receptor-mediated PKC membrane trafficking is
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PKC immunoblot
PKCβII Ab

PKC autophosphorylation

FIG. 5. Characterization of GFP-ΔC2 autophosphorylation in vitro and in cells. A, HEK 293 cells were transfected with 5 μg of pBK-CMV-GFP-PKCβII or pBK-CMV-GFP-ΔC2. Twenty micrograms of cell homogenates were loaded in each lane, separated on 8% SDS-polyacrylamide gel, and probed with rabbit polyclonal antibody (Ab) against PKCβII (upper panel). The above homogenates (200 μg) were immunoprecipitated with rabbit anti-PKCβII polyclonal antibody, and the immunocomplexes were assayed for their kinase activity under standard conditions as described previously (26) (lower panel). B, HEK 293 cells were transfected with 5 μg of pBK-CMV (Vector), pBK-CMV-GFP-PKCβII, pBK-CMV-GFP-K371R, or pBK-CMV-GFP-ΔC2. Twenty micrograms of cell homogenates were loaded in each lane, separated on 8% SDS-polyacrylamide gel, and probed with rabbit polyclonal antibody against phospho-PKCβ(Ser660) (upper panel) and then stripped and reprobed with polyclonal rabbit antibody against PKCβII (lower panel) sequentially. Shown are representative results from two independent experiments.

a rapid and reversible process (21). The present study provides new insights into the molecular mechanisms underlying the dynamic nature of PKC redistribution. Our results indicate that the functions of the C1 and C2 regions and autophosphorylation are all essential and collaborate/interact to achieve the dynamic trafficking of PKC. Specifically, the C1 region is required for recruiting the enzyme to the plasma membrane from the cytoplasm in response to activation of cell-surface receptors, but it is not sufficient. The Ca$^{2+}$-regulated C2 region then serves to further anchor PKC persistently on the plasma membrane. The dissociation of PKC from the membrane is dependent on autophosphorylation. In addition, autophosphorylation exerts an inhibitory effect on translocation that is most predominant when the C2 region is not functional, suggesting a role for the C2 region in overcoming the inhibitory effects of autophosphorylation on membrane targeting.

PKC membrane translocation has been extensively studied in cells using nonphysiological reagents (11). Phorbol esters such as PMA are commonly used to mimic the action of DAG in inducing PKC translocation from the cytosol to the plasma membrane. Similarly, Ca$^{2+}$ ionophores such as A23187 have been used to raise intracellular Ca$^{2+}$ concentrations. Studies using these reagents have suggested that both the C1 and C2 regions independently target the enzyme to the membrane (11, 29). However, the high affinity PKC membrane association mediated by PMA and the high Ca$^{2+}$ concentrations induced by ionophore do not precisely reflect the PKC membrane trafficking properties stimulated by physiological stimuli such as activation of cell-surface receptors. For instance, the reversibility of receptor-induced PKC membrane trafficking cannot be mimicked by PMA or A23187 (21, 23).

By visualizing the trafficking of wild-type and mutant GFP-PKCβII in live HEK 293 cells, we found that although PKC mutants deficient in the C1 region function still responded to Ca$^{2+}$ ionophore, they lack the ability to undergo membrane redistribution in response to GPCR activation (Table I and Fig. 2). This not only further indicates that PKC membrane translocation induced by receptor activation is different from that induced by PMA or A23187, but also suggests an important physiological role for the C1 region in receptor-mediated targeting of the enzyme to the membrane. It is likely that the targeting of PKC to the plasma membrane by the C1 region is mediated by DAG (in a phosphatidylinerse-dependent manner) (30, 31); and therefore, these data provide very strong support for a direct signaling effect for DAG on PKC.

On the other hand, the C2 deletion mutant, which has an intact phorbol ester-responsive C1 region, also fails to translocate, thus showing that the C1 region is not sufficient for PKC translocation. Therefore, the C1 region is necessary but not sufficient for membrane translocation in response to agonists. The C2 region is commonly believed to be another important membrane-targeting module that interacts with multiple intracellular targets, including phospholipids and PKC-binding proteins (32, 33). The functional involvement of the C2 region in PKC membrane translocation has been demonstrated in cells by C2 region-derived peptides that effectively inhibit both hormone-induced translocation of PKCβII and its signaling functions (34). Structural analysis has revealed that two Ca$^{2+}$ molecules are coordinated by five aspartate residues in the C2 region of PKCβII (35, 36). This may account for the Ca$^{2+}$-dependent regulation of the C2 region. In addition, mutational analysis has identified in the C2 region of PKCα two basic residues and two hydrophobic residues that may provide electrostatic and hydrophobic interactions with membrane components, specifically anionic phospholipids (37). Interestingly, the residues involved in binding to Ca$^{2+}$ and phospholipids are localized to the loops that connect the β-sheet sandwich structure of the C2 region. However, despite the increasing structural information on the interaction of the C2 region with its targets, the physiological roles of the C2 region in receptor-induced PKC activation have not been fully understood.

In the present study, we also observed that deletion of the C2 region abrogates agonist-induced translocation. Therefore, the C2 region is necessary for translocation. On the other hand, the C1 deletion mutant, which retains an intact ionophore-responsive C2 region, also fails to translocate, showing that the C2 region is not sufficient for agonist-induced membrane translocation.

The more significant and unexpected result came from studies with the deletion of the C2 region from GFP-K371R. The GFP-K371R mutant of PKCβII loses the ability to dissociate from the membrane. The deletion of the C2 region from this mutant restored the ability of the enzyme to undergo membrane dissociation (Table I and Fig. 4). This indicates that the persistent membrane association of GFP-K371R is a consequence of the C2 region function in the absence of kinase...
activity (probably through autophosphorylation; see below). This was further supported with studies using intracellular calcium chelators that were able to undo the defect of the K371R mutant in reverse translocation, again showing that the persistence of the enzyme on the membrane is due to calcium effects that are mediated through the C2 region. Therefore, the C2 region serves an additional function to anchor PKC to the membrane until autophosphorylation-dependent membrane dissociation occurs.

The role for autophosphorylation per se in receptor-mediated PKC trafficking was explored in the present study in association with the functions of the C1 and C2 region. Interestingly, we found that the membrane targeting and association properties mediated by the C1 and C2 regions are differentially modulated by the ability of the enzyme to autophosphorylate. It was initially surprising to observe that the C1 region of GFP-KR/D2 was able to target the enzyme to the plasma membrane whereas GFP-D2, also with a functional C1 region, lost the ability to traffic. As the replacement of Lys371 with Arg at the ATP-binding site abolishes the ability of GFP-KR/D2 to undergo phosphorylation and autophosphorylation, it is possible that the lack of GFP-ΔC2 translocation results from an inhibitory effect of autophosphorylation on the C1 region function. The linkage between autophosphorylation and the C1

**FIG. 6.** Membrane dissociation of GFP-K371R promoted by Ca\(^{2+}\) chelators following AT\(_{1}\)R activation-induced PKC membrane translocation. A, HEK 293 cells were transfected transiently with 1 µg of pBK-GFP-K371R and 5 µg of pcdNA I/amp-AT\(_{1}\)R. The cells were stimulated with 0.5 µM angiotensin II (AgII) to induce GFP-K371R membrane translocation and were then exposed to 2.5 mM EGTA in the presence of 15 µM BAPTA 180 s after the first stimulation. Indicated in the confocal micrographs are the time points following the first angiotensin II stimulation. The experiments were performed independently on three different occasions; and each time, 10–20 cells from independent stimulations were recorded. The confocal micrographs are representative of >75% of the cells observed. B, shown are the time courses of the relative fluorescence changes in GFP-K371R on the plasma membrane following stimulation by 0.5 µM angiotensin II (AgII), 2.5 mM EGTA and 15 µM BAPTA ( ), or 2.5 mM EGTA and 15 µM BAPTA 180 s after 0.5 µM angiotensin II stimulation ( ). The representative image series including the cells shown in A was used to determine the relative membrane fluorescence intensity as described under “Experimental Procedures.”

**FIG. 7.** Effects of the S660A mutation on the reversible membrane translocation of PKC\(\beta\)II in response to AT\(_{1}\)R activation. Shown are representative confocal micrographs of HEK 293 cells cotransfected with 5 µg of pcdNA I/amp-AT\(_{1}\)R together with 1 µg of pBK-CMV-GFP-PKC\(\beta\)II (upper panels) or pBK-CMV-GFP-S660A (lower panels) before (Control) and after stimulation with 0.5 µM angiotensin II (AgII) for the indicated time periods. The experiments were performed independently on three different occasions; and each time, 10–20 cells from independent stimulations were recorded. The micrographs are representative of >75% of the cells observed.
region function was further confirmed by the fact that the replacement of an autophosphorylation residue (Ser660) with Ala (but not Glu) rescued the ability of GFP-DCC2 to undergo membrane redistribution. Furthermore, the functions of autophosphorylation and the C2 region also are counteractive. The persistent membrane association of PKC regulated by the C2 region was most dominant in the absence of autophosphorylation (i.e. GFP-K371R), and the enzyme membrane dissociation mediated by autophosphorylation was most predominant when the C2 region was not functional (i.e. GFP-ΔC2). Taken together, these results provide direct visual evidence supporting the hypothesis that the receptor-mediated dynamic trafficking of PKC requires the functional collaboration of PKC autophosphorylation with the C1 and C2 regions.

A role for autophosphorylation in regulating PKC membrane association is also in agreement with a recent functional study on autophosphorylation of the calcium-activated PKC AplI from *Aplysia* using an anti-phospho-PKC antibody specific for

![Fig. 8. Effects of autophosphorylation on the membrane translocation of PKCβII with the C2 deletion. A, shown are representative confocal micrographs of HEK 293 cells cotransfected with 5 μg of pcDNA l/amp-AT1R together with 1 μg of pBK-CMV-GFP-ΔC2/SA (upper panels), pBK-CMV-GFP-ΔC2/SE (middle panels), or pBK-CMV-GFP-ΔC2 (lower panels) before (Control) and after stimulation with 0.5 μM angiotensin II (AgII) for the indicated time periods. The experiments were performed independently on three different occasions; and each time, 20–50 cells from independent stimulations were recorded. The micrographs are representative of >75% of the cells observed. B, shown are the time courses of the relative fluorescence changes in GFP-ΔC2/SA, GFP-ΔC2/SE, and GFP-ΔC2 on the plasma membrane following angiotensin II stimulation. The relative membrane fluorescence intensity was determined as described under “Experimental Procedures.” The data represent the means ± S.E. summarized from 20 cells overexpressing GFP-ΔC2, 20 cells overexpressing GFP-ΔC2/SA, and 19 cells overexpressing GFP-ΔC2/SE from three independent experiments.

**Table I**

| GFP-tagged PKC protein | Translocation stimuli | PMA | A23187 | Angiotensin II |
|------------------------|-----------------------|-----|--------|---------------|
| GFP-PKCβII             | Yes (irreversible)    | Yes (irreversible) | Yes (reversible) |
| GFP-K371R              | Yes (irreversible)    | —   | Yes (irreversible) |
| GFP-mC1                | No                    | Yes (irreversible) | No |
| GFP-KR/mC1             | No                    | Yes (irreversible) | No |
| GFP-ΔC2                | Yes (irreversible)    | No  | Yes (reversible) |
| GFP-KR/ΔC2             | —                     | —   | —      |
| GFP-ΔC2/SA             | —                     | —   | —      |
| GFP-ΔC2/SE             | —                     | —   | —      |

*—, membrane translocation under this condition was not determined.

Due to a very weak membrane redistribution, the reversibility of membrane translocation could not be assessed.
step III, and membrane dissociation (step III), involving the C1 and C2 region functions and PKC autophosphorylation, respectively. See "Discussion" for details. P, phosphate on autophosphorylation residues; kinase, the PKC catalytic domain.

Thr$^{613}$, one of the conserved autophosphorylation residues among members of the PKC family (38, 39). Autophosphorylation of AplI was found to remove the enzyme from the plasma membrane by decreasing its affinity for calcium. There are two autophosphorylation sites identified in PKCβII that are well conserved among the different isoenzymes in the PKC family (15, 16). These residues are surrounded by several hydrophobic residues to form an autophosphorylation motif (FXxF(S/T)(F/Y)), one of which has recently been confirmed using anti-phospho-PKCβ(Ser$^{660}$) antibody (17). Also, it has been suggested that the autophosphorylated serine or threonine residues together with the surrounding hydrophobic residues modulate the membrane affinity of PKC (19, 40). In addition, consistent with our observation that the functions of the C1 and C2 regions are regulated by autophosphorylation, there is evidence indicating that autophosphorylated residues at the carboxyl terminus of PKC may be folded very close to the C1 and C2 regions, as the binding affinity of the enzyme for Ca$^{2+}$ was altered upon replacement of autophosphorylation residues by alanine (19). The potential mechanisms underlying the functions of the autophosphorylated carboxyl terminus are anticipated as either indirect modulation of the conformation of membrane-targeting protein modules or direct involvement as part of the interface between the membrane and the enzyme (19, 34). The molecular details associated with the functions of autophosphorylation await further exploration.

Combining our findings with the current knowledge of PKC trafficking, we propose the following model (10, 11, 30). Briefly, the dynamic membrane trafficking of PKC in response to cell-surface receptor activation occurs in three major steps, namely membrane recruitment, persistent membrane anchorage, and membrane dissociation (Fig. 9). In unstimulated cells, PKC is mainly distributed in the cytoplasm with the pseudosubstrate blocking the catalytic site to prevent the enzyme from substrate binding and catalysis. Upon stimulation by extracellular signals such as the binding of seven-transmembrane G$\alpha$-coupled receptors with their physiological agonists, the cellular levels of DAG and Ca$^{2+}$ are increased as a result of activation of phospholipase C-mediated signaling pathway by GPCRs. Consequently, PKC is recruited to the plasma membrane by the C1 region via its binding to DAG and the C2 region through binding to calcium. Following the recruitment of PKC to the membrane, the high affinity membrane anchorage of the recruited enzyme involves the calcium-dependent function of the C2 region or is achieved by the collaboration of the C1 and C2 regions. One important intrinsic property of the G protein-coupled receptor is that for most GPCRs, agonist activation is followed immediately by desensitization of the receptors through phosphorylation by either G protein-coupled receptor kinases or second messenger-activated kinases (41). This inactivation of the receptors results in the subsequent turning off of phospholipase C and a decrease in the cellular DAG and Ca$^{2+}$ levels. The decrease in these intracellular PKC activators undermines the function of the C1 and C2 regions; and subsequently, the enzyme is removed from the membrane, which is enhanced by autophosphorylation.

An important issue that arises is whether autophosphorylation is regulated or not. Although it cannot be inferred from our study when autophosphorylation of the enzyme occurs, it is believed that PKCs are autophosphorylated during their maturation, independent of any activators (16). If autophosphorylation is stoichiometric in the resting state or under normal growing conditions, then one hypothesis would be that autophosphorylation of PKC keeps the enzyme from associating with the plasma membrane and thus retains the enzyme in the cytoplasm. This is supported by the studies on the Aplysia PKC (39). On the other hand, we found in preliminary studies that autophosphorylation at Ser$^{660}$ was enhanced in response to angiotensin II and phorbol esters. This raises the possibility that initial translocation and activation of PKC result in further autophosphorylation, which then provides a further force for reverse translocation and signal termination.

Many important cellular processes, including cell proliferation and differentiation, involve the transduction of extracellular signals to specific intracellular sites. During these processes, signaling molecules, including protein kinases and phosphatases, need to traffic to specific cellular locations for effective action on their downstream targets (42). Due to the dynamic nature of cellular signal transduction, the trafficking of these signaling molecules has been difficult to follow. In the present and previous studies (20), we have utilized GFP con-

FIG. 9. Schematic representation of the regulation of receptor-induced PKC membrane trafficking. Briefly, the rapid reversible PKC membrane translocation in response to receptor activation is proposed to consist of at least three steps: membrane recruitment (step I), persistent membrane anchorage (step II), and membrane dissociation (step III), involving the C1 and C2 region functions and PKC autophosphorylation, respectively. See "Discussion" for details. P, phosphate on autophosphorylation residues; kinase, the PKC catalytic domain.

2 K. P. Becker and Y. A. Hannun, unpublished observations.
jugates to study the regulatory mechanisms for PKC trafficking and signal transduction. In particular, the real time visualization of GFP-PKC fluorescence provides a direct and sensitive means for assessing the dynamic PKC trafficking between the plasma membrane and cytoplasm. Trafficking to intracellular locations other than the plasma membrane and cytoplasm.

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