An Essential Role for Autophosphorylation in the Dissociation of Activated Protein Kinase C from the Plasma Membrane*

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The cellular localization of protein kinase C (PKC) is intimately associated with the regulation of its biological activity. Previously we have demonstrated that the redistribution of PKC to the plasma membrane in response to physiological stimuli is followed by a rapid return of PKC back to the cytoplasm (Feng, X., Zhang, J., Barak, L. S., Meyer, T., Caron, M. G., and Hannun, Y. A. (1998) J. Biol. Chem. 273, 10755–10762). Although the process of PKC membrane targeting has been extensively studied, the molecular mechanism underlying the dissociation of membrane-bound PKC remains unclear. In the present study, by examining the dynamic distribution of wild-type PKC βII and its kinase-deficient mutant (K371R), we demonstrate that kinase activity is required for PKC membrane dissociation. Moreover, the inability of PKC βII(K371R) to dissociate from the plasma membrane in cells overexpressing wild-type PKC βII suggests that autophosphorylation activity of the kinase might be essential for its membrane dissociation. This was further supported by mutational analysis of two in vivo autophosphorylation sites on PKC βII. The replacement of Ser660 or Thr641 by alanine (S660A or T641A) was found to synergistically reduce the reversal of PKC βII membrane translocation, whereas the replacement of the same amino acids by glutamic acid (S660E or T641E), an amino acid commonly used to mimic phosphate, results in mutants behaving similar to wild-type PKC βII. These findings point to an essential role for autophosphorylation in the dissociation of activated PKC from the plasma membrane and suggest that, like PKC membrane translocation, the returning of PKC to the cytoplasm after its activation is also delicately regulated.

Protein kinase Cs (PKCs) comprise a family of phospholipid-dependent serine/threonine kinases that play critical roles in many cellular signaling events by catalyzing specific substrate phosphorylation (1, 2). Activation of PKC can be triggered by stimulating a wide variety of cell surface receptors including those for hormones, neurotransmitters, and growth factors. These receptors transduce a diversity of extracellular signals to either phospholipase C or phospholipase D, which when activated results in the generation of membrane lipid mediator diacylglycerol (DAG) and/or mobilization of intracellular Ca²⁺ (3). The activation of PKC by DAG, Ca²⁺, and many other lipid mediators has been intimately associated with many important biological processes such as cell proliferation, differentiation, and gene expression (1, 2).

Previous biochemical and immunocytochemical studies have indicated that the biological activity of PKCs is closely regulated by their subcellular localization (1, 4–6). For instance, upon activation of cell surface receptors, PKCs were found to translocate from the cytoplasm to the plasma membrane and subsequently activated (7, 8). It is believed that the changes in the cellular levels and intracellular localization of Ca²⁺ and DAG result in the redistribution of PKC to the plasma membrane, which is enriched with phosphatidylinositol, a lipid cofactor for PKC. This process may also involve PKC binding proteins (3). The regions on classic PKCs (i.e. PKC α, β, γ) that bind various regulatory factors are located within the amino-terminal regulatory domain (Fig. 1), which contains an autoinhibitory sequence (i.e. the pseudosubstrate in V1 region) and two membrane-targeting protein modules, namely C1 and C2 regions. The C1 region contains the binding site for DAG, whereas the C2 region binds phospholipids whose binding is allosterically facilitated by the association of Ca²⁺ to the same region. The interaction of PKCs with DAG and phospholipids results in the removal of pseudosubstrate from the catalytic domain and the subsequent mobilization of PKCs to the plasma membrane (3). Recently, with the development of green fluorescent protein (GFP)-conjugated PKCs, this PKC membrane trafficking process has been further studied in live cells through real time visualization (9, 10). Interestingly, it was observed that shortly after their membrane targeting, PKCs dissociate from the plasma membrane and rapidly return to the cytoplasm (9). However, the molecular mechanisms underlying this reversal of PKC translocation have remained unclear.

The maturation of PKCs during their biosynthesis involves phosphorylation of the enzymes at multiple serine/threonine sites (11, 12). In the case of PKC βII, three major in vivo phosphorylation sites have recently been identified (13, 14). Briefly, phosphorylation of PKC βII occurs initially at Thr⁵⁰⁰ located within the activation loop at the entrance of PKC catalytic site (15). This is followed by sequential autophosphorylation of the enzyme at Thr⁶⁴₁ and Ser⁶⁶⁰ (14). The inability of PKC βII itself to cause phosphorylation at Thr⁵⁰⁰ has suggested that the phosphorylation of this residue involves a distinct protein kinase (15). It is now widely believed that the phosphorylation of these serine/threonine sites on PKC is critical for the regulation of PKC enzymatic activity and subcellular localization (16–20). Interestingly, it was recently discovered that under the condition of oxidative stress, PKC βII is also modified by phosphorylation at tyrosine residues, and the tyrosine phosphorylation directly activates PKCs even in the absence of lipid metabolites and second messengers including

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The abbreviations used are: PKC, protein kinase C; MHC-PKC, myosin II heavy chain-specific protein kinase C; GFP, green fluorescent protein; AT₁AR, angiotensin II type 1A receptor; PMA, phorbol 12-myristoyl 13-acetate; DAG, diacylglycerol.
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DAG and Ca\(^{2+}\) (21). Therefore, like DAG, Ca\(^{2+}\), and many other lipid mediators, in vivo phosphorylation of PKCs provides an additional way for effectively regulating PKC functions but at the level of enzymes themselves. Although there are lines of evidence suggesting that the phosphorylation status of PKCs may contribute to their enzymatic activity and localization in cells, the exact role for phosphorylation in PKC dynamic trafficking between the plasma membrane and cytoplasm has not been established.

In a previous study, we reported the development of a GFP-conjugated PKC \(\beta\) (GFP-PKC \(\beta\)) for studying PKC cellular distribution in response to G protein-coupled receptor activation and desensitization (9). GFP-PKC \(\beta\) is fully functional in terms of its phospholipid-dependent kinase activity and its ability to translocate from cytoplasm to the plasma membrane in response to phorbol ester stimulation. The visualization of GFP-PKC \(\beta\) distribution has revealed a dynamic and reversible nature of PKC trafficking between the plasma membrane and cytoplasm in response to physiological stimuli (9). In the present study, using GFP-PKC \(\beta\) and several of its mutants, we further examine the molecular events underlying the reversal of PKC membrane translocation. Our results reveal an essential role for PKC kinase activity in the reversible translocation of PKC \(\beta\) in response to \(G_{q}\)-coupled angiotensin II type 1A receptor (\(AT_{1a}\)) activation. More specifically, we demonstrate that autophosphorylation activity of the enzyme is essential for its membrane dissociation. These findings suggest a molecular mechanism for regulating PKC inactivation and indicate that, like PKC membrane targeting, the process of PKC dissociation from the plasma membrane is also exquisitely regulated.

**EXPERIMENTAL PROCEDURES**

**Materials**—Eagle’s minimum essential medium, phosphate-buffered saline and HEPES buffer were from Life Technologies, Inc. Human embryonic kidney cells (HEK 293) were provided by the American Type Culture Collection. [\(\gamma\)-\(^{32}\)P]ATP was purchased from NEN Life Science Products. Purified human PKC \(\beta\) was from Pan Vera. Monoclonal antibody against GFP was from CLONTECH. Monoclonal antibody against amino terminus of PKC \(\beta\) was from Transduction Laboratories. Fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG was from Jackson Immunoresearch Laboratories, Inc. Restriction enzymes were from Promega or New England Biolabs. Ampli-Taq DNA polymerase was obtained from Perkin-Elmer. Protein A-Sepharose CL-4B was from Amersham Pharmacia Biotech. Phosphatidylinerine and sphingosylglycerol were purchased from Avanti Polar Lipids Inc. All other chemicals were from Sigma.

**Plasmid Constructs**—All recombinant DNA procedures were carried out following standard protocols. pBK-CMV-GFP-PKC \(\beta\) was constructed as described previously (9). The kinase-deficient mutant (K371R) was generated by polymerase chain reaction to mutate codon AAG (Lys\(^{175}\)) in the coding sequence to CCG (Arg) according to a previous study on PKC \(\alpha\) (22). Similarly, mutations of single autophosphorylation site (Thr\(^{641}\) or Ser\(^{660}\)) were generated by polymerase chain reaction to mutate ACA (Thr\(^{641}\)) to GCA (Ala) or GAA (Glu) and to mutate TAA (Ser\(^{660}\)) to GCC (Ala) or GAA (Glu). The mutation of both autophosphorylation sites, designated GFP-DA, was generated by mutating both Thr\(^{641}\) and Ser\(^{660}\) to alanine simultaneously (Fig. 1). The sequences of the constructs were confirmed by DNA sequencing.

**Cell Culture and Transfection**—HEK 293 cells were maintained in Eagle's minimum essential medium supplemented with 10% (v/v) fetal bovine serum in a 5% CO\(_{2}\) incubator at 37 °C. Cells were seeded at a density of 2.0 × 10\(^5\) cells/100-mm dish and transfected using a modified calcium phosphate method with 1–15 μg of plasmids (23).

**Protein Kinase C Assay**—For assaying PKC activity in cells transfected with wild-type or mutant GFP-PKC \(\beta\), the cell lysates (200 μg) were first immunoprecipitated with 5 μl of monoclonal GFP antibody. The immunoprecipitates were washed 48 h after transfection as described previously (9). The PKC autophosphorylation activity and PKC kinase activity toward substrate histone II\(S\) were assessed using the vesicle assay for PKC as described previously (9). The phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

**Immunoblot**—Cell lysates from HEK 293 cells transfected with GFP-PKC \(\beta\) or GFP-K371R or cell lysates from stable HEK 293 cell lines overexpressing PKC \(\beta\) I were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The membranes were probed with monoclonal anti-PKC \(\beta\) antibody (1:200) or rabbit anti-PKC \(\beta\) antibody (1:2000) and exposed using the ECL Western blotting detection system (Amersham Pharmacia Biotech).

**Indirect Immunofluorescence**—HEK 293 cells stably overexpressing wild-type PKC \(\beta\) I were seeded on glass coverslips placed in six-well dishes at a density of 5 × 10\(^5\) cells/well. The cells were fixed in 3.7% paraformaldehyde for 10 min and permeabilized in phosphate-buffered saline containing 0.2% Triton X-100 for another 10 min. After 1 h of incubation with rabbit anti-PKC \(\beta\) antibody (1:100), the cells were washed and incubated with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:100 dilution) for 1 h. The coverslips were mounted onto the slides, and PKC \(\beta\) immunofluorescence was observed with a Zeiss LSM-410 laser scanning microscope at 488-nm excitation. The differential interference contrast images were recorded at the same time.

**Confocal Microscopy**—HEK 293 cells were transfected with GFP-PKC \(\beta\) or one of its mutants together with \(\Delta T_{1a}\). 24 h after transfection, the cells were plated onto 35-mm glass bottom culture dishes (MatTek) at a density of 4 × 10\(^3\) and incubated for another 24 h to allow the cells to attach to glass. The cells expressing GFP-PKC \(\beta\) or its mutants were observed with a Zeiss LSM-410 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 scannings. Angiotensin II, staurosporine, and FMA were applied to the cells during the scannings. Quantitative analysis of relative fluorescence intensity was performed on a Macintosh computer using the public domain NIH Image program. 2 The relative change of plasma membrane fluorescent intensity was calculated according to a previously reported method (24). Briefly, from a series of images recorded before and after angiotensin II stimulation, line intensity profiles across each cell were determined. The relative fluorescence intensity on the plasma membrane was calculated by the formula \(I_{\text{mb,40}} - I_{\text{mb,180}}\), \(I_{\text{mb}}\) represents the amplitude of fluorescence signal on plasma membrane, and \(I_{\text{cyt}}\) is the average cytosolic fluorescent intensity.

**Quantitation of the Reversal of PKC Translocation**—Sequential confocal images of a population of transfected cells before and after various treatments were recorded as described above. The numbers of cells in which GFP-PKC mobilizes to the plasma membrane within 40 s (\(C_{40}\)) and in which GFP-PKC returns to the cytoplasm within 180 s (\(C_{180}\)) were counted, respectively. The percentage of the reversal of PKC translocation is calculated as \(100 - C_{180}/C_{40}\). For each experiment, \(C_{40}\) was more than 50.

**Data Analysis**—The mean and S.E. are expressed for values obtained from the number of separate experiments indicated. Statistical significance was determined by an unpaired two-tailed t test.

**RESULTS**

The visualization of GFP-PKC \(\beta\) trafficking in live HEK 293 cells has demonstrated that membrane translocation of PKC \(\beta\) I in response to \(AT_{1a}\) activation is followed immediately by the returning of the enzyme back to the cytoplasm (9). To understand the molecular events underlying the dynamic nature of PKC redistribution, initial experiments examined the role of phosphorylation in the reversal of PKC membrane translocation using a kinase-deficient GFP-PKC \(\beta\) mutant K371R (Fig. 1). As a conserved lysine at the ATP-binding site of the enzyme was replaced by an arginine, GFP-K371R completely lost its ability to auto-phosphorylate and to phosphorylate other substrates such as histone II\(S\) (Fig. 2A). When tested in HEK 293 cells, GFP-K371R was observed to undergo a rapid translocation to the plasma membrane within 40 s in response to \(AT_{1a}\) activation, similar to that observed for wild-type GFP-PKC \(\beta\) I. However, although the membrane localization of GFP-PKC \(\beta\) I was transient and mem-

2 The NIH Image program was developed at the National Institutes of Health and is available on the Internet at http://rsb.info.nih.gov/nih-image/.
PKC fluorescence remained on the plasma membrane even after 300 s after angiotensin II stimulation, whereas over 90% GFP-K371R of wild-type GFP-PKC association was further supported by the diminution of reversal membrane.

The requirement of kinase activity for PKC membrane dissociation was further supported by the diminution of reversible wild-type GFP-PKC βII membrane translocation in the presence of staurosporine, a potent protein kinase inhibitor that reduces PKC activities (25). When HEK 293 cells co-transfected with GFP-PKC βII and AT₁R were stimulated by angiotensin II, the percentage of cells in which GFP-PKC βII had the ability to undergo membrane dissociation was reduced markedly in a staurosporine concentration-dependent manner (Fig. 3). At a staurosporine concentration of 500 nM, GFP-PKC βII completely lost its ability to return to the cytoplasm in almost all transfected cells. However, staurosporine alone was not found to affect GFP-PKC βII distribution (data not shown). Taken together, these results indicate that PKC kinase activity is essential for its dissociation from the plasma membrane and returning to the cytoplasm following activation of cell surface receptors in HEK 293 cells.

In live cells, PKCs have the ability to mediate intramolecular phosphorylation (autophosphorylation) and intermolecular phosphorylation (transphosphorylation) including phosphorylation of various protein substrates. Unless autophosphorylation is involved, the lack of membrane dissociation of the kinase-deficient PKC βII should be resuscitable by expressing wild-type PKC βII in the same cells. For examining this possibility, stable cell lines were generated that overexpress PKC βII at a level of about 1 µg/mg whole cell protein as estimated using purified PKC βII as the control (Fig. 4A). Indirect immunofluorescence microscopy further confirmed that all cells of the stable cell lines evenly expressed PKC βII (Fig. 4B). When GFP-K371R and AT₁R were co-transfected into such a PKC βII stable cell line, similar to what was observed in native HEK 293 cells, GFP-K371R distributed evenly in the cytoplasm in unstimulated cells but underwent an irreversible membrane translocation in response to AT₁R activation (Fig. 4C, top panel). The irreversibility of GFP-K371R redistribution was apparent because no additional increase of membrane fluorescence was observed when cells were exposed to a second pulse of phorbol ester PMA at 13 min after the initial stimulation by angiotensin II (Fig. 4C, top panel). As an additional control, the distribution of wild-type GFP-PKC βII was also examined in...
the PKC βII stable cell line (Fig. 4C, bottom panel). Similar to what was observed in native HEK 293 cells, GFP-PKC βII underwent a rapid reversible membrane translocation following activation of AT₁A R, demonstrating that overexpression of PKC βII does not affect the ability of GFP-PKC βII to dissociate from the membrane (Fig. 4C, bottom panel). Therefore, the inability of wild-type PKC βII to rescue the kinase deficiency of GFP-K371R suggests that autophosphorylation, rather than transphosphorylation, of the enzyme may be important for its membrane dissociation.

Two major in vivo autophosphorylation sites, i.e., Ser⁶⁶⁰ and Thr⁶⁴¹, have been identified at the carboxyl terminus of PKC βII by biochemical studies (13, 14). Therefore, mutational analysis of the two sites was performed to further study the role of autophosphorylation in PKC βII dynamic trafficking. In these experiments, GFP-PKC βII, GFP-K371R, and various GFP-PKC βII autophosphorylation mutants were transfected into HEK 293 cells together with the AT₁A R, and the percentage of cells in which GFP-PKC molecules underwent reversible membrane translocation were quantitated as described under “Experimental Procedures.” Upon stimulation of the AT₁A R, 96.4% of cells transfected with wild-type GFP-PKC βII exhibited reversible PKC membrane translocation, whereas PKC membrane dissociation was observed in only 9.5% of cells transfected with GFP-K371R (Fig. 5). Interestingly, the replacement of Ser⁶⁶⁰ or Thr⁶⁴¹ by alanine (S660A or T641A) to eliminate autophosphorylation of these residues was found to reduce the reversal of PKC βII membrane translocation by as much as 37.5 and 22.2%, respectively, whereas double replacement of both Ser⁶⁶⁰ and Thr⁶⁴¹ by alanine (S660A/T641A) further diminished PKC membrane dissociation by as much as 66.4%. In contrast, the replacement of Ser⁶⁶⁰ or Thr⁶⁴¹ by glutamic acid (S660E or T641E), an amino acid commonly used to mimic phosphate, had no detectable effect on the reversal of PKC βII membrane translocation. Both mutants behaved like wild-type PKC βII. These results provide strong evidence supporting an essential role of autophosphorylation in PKC membrane dissociation and indicate that the failure of membrane dissociation of PKC kinase-deficient mutant is the consequence of its lack of autophosphorylation.

**DISCUSSION**

Our findings provide the first evidence for the involvement of PKC enzymatic activity in the dynamic trafficking of the enzyme in response to physiological stimuli and indicate that the kinase activity of PKC is essential for the returning of PKC to the cytoplasm following its membrane translocation. Moreover, the inability of wild-type PKC βII to rescue the lack of membrane dissociation of the kinase-deficient PKC βII mutant suggests that autophosphorylation, rather than transphosphorylation, of PKC βII is crucial for its membrane dissociation. This idea is further corroborated by the observation that the replacement of two major autophosphorylation sites on PKC βII with alanine, but not glutamic acid, markedly reduces the reversibility of PKC membrane translocation. The exact cellular mechanism by which activated PKC dissociates from the plasma membrane remains undetermined, but our results implicate autophosphorylation in PKC inactivation and therefore provide a start point from which to begin to dissect the processes regulating the association of PKC membrane targeting activities.

**FIG. 3.** Dose-dependent inhibition by staurosporine of GFP-PKC βII membrane dissociation following AT₁A R activation-mediated PKC translocation. HEK 293 cells were transfected transiently with 1 µg of pBK-GFP-PKC βII and 10 µg of pcDNA I/amp-AT₁A R. The cells were stimulated with 0.5 µM angiotensin II in the presence of various concentrations of staurosporine. The reversal of PKC βII translocation was quantitated as described under “Experimental Procedures.” The shown result was a representative of three separate experiments.

**FIG. 4.** Effect of overexpressing wild-type PKC βII on the membrane dissociation of the kinase-deficient PKC βII in response to AT₁A R activation. pBK-CMV-PKC βII was transfected into HEK 293 cells for constructing stably cell lines overexpressing PKC βII (HEK 293/PKC βII). The expression levels of PKC βII in HEK 293/PKC βII cell lines were determined by immunoblot analysis in comparison with purified baculovirus-expressed human PKC βII (A). The purity of the cell lines and distribution of PKC βII in the HEK 293/PKC βII cells were examined by differential interference contrast and indirect immunofluorescence microscopy with PKC βII-specific antibody (B). In C, the HEK 293/PKC βII cells were transfected with 2 µg of pBK-CMV-GFP-PKC K371R (top panels) or 1 µg of pBK-CMV-GFP-PKC βII (bottom panels) together with 10 µg of pcDNA I/amp-AT₁A R. The cells were stimulated with 0.5 µM angiotensin II. 13 min after the first stimulation, the GFP-K371R-transfected cells were exposed to 1 µM PMA. Indicated in the micrographs are the time points following the first angiotensin stimulation. The experiments were performed independently on five different occasions with two different stable HEK 293/PKC βII cell lines, and each time 10–20 cells from independent stimulations were recorded. The confocal micrographs are representative of more than 75% of the cells observed.
modules (i.e. C1 and C2 regions) with plasma membrane lipids and proteins.

Associated with its biological activity, PKC cellular localization is regulated in various PKC-mediated signal transduction events (1, 2, 5, 6). Although cellular distribution of PKC has been extensively studied biochemically and immunocytochemically, the present study represents the first investigation of mechanisms regulating PKC trafficking in live cells. The visualization of GFP-PKC fluorescence provides a direct and sensitive means for assessing real time PKC cellular localization and therefore is uniquely suited for following cellular events regulating the transient and reversible trafficking of PKCs in response to extracellular signals (9, 10). In particular, we have examined the dynamic redistribution of several GFP-conjugated PKC βII mutants either deficient in ATP binding and catalysis (K317R) or unable to be autophosphorylated (T641A and/or S660A) in response to activation of G protein-coupled AT1AR. Although these mutants underwent membrane translocation upon receptor activation with time profiles and extents similar to GFP-PKC βII, unlike their wild-type counterpart, which immediately returned to the cytoplasm, the mutants remained stably associated with the plasma membrane. These results provide direct visual evidence supporting a novel role of PKC βII kinase activity and autophosphorylation in the membrane dissociation of the enzyme.

Previous biochemical studies have suggested that the phosphorylation status of PKCs is associated with their subcellular partition (3). Studies on the Dictyostelium homolog of classic PKC, myosin II heavy chain-specific protein kinase C (MHC-PKC), have revealed that deletion of the carboxyl-terminal autophosphorylation sites results in a mutant (MHC-PKCIαST) irreversible to the chemoattractant cAMP and persistently localized on the plasma membrane (28). Like mammalian PKC βII, MHC-PKC normally resides in the cytoplasm and undergoes reversible membrane translocation in response to cAMP stimulation. Thus, the membrane localization of MHC-PKC is consistent with our findings that PKC autophosphorylation is required for its membrane dissociation. Interestingly, unlike PKC βII whose autophosphorylation mutants are mainly distributed in the cytoplasm, MHC-PKCIαST was found predominantly on the plasma membrane under nonstimulated conditions (26). Considering the diverse variety of different PKC activators, binding proteins as well as a large number of PKC isoenzymes, it is conceivable that the cellular distribution of PKCs in response to extracellular signals is not only a function of cellular environment (i.e. protein, lipid, ion composition, etc.) but also an intrinsic property governed by the biochemical structures of distinct PKC isoenzymes. Moreover, because in both PKC βII and MHC-PKC, autophosphorylation sites reside at the carboxyl termini and are not within the identified membrane targeting modules (i.e. C1 and C2 region), it is likely that autophosphorylation of PKCs confers conformational or electrostatic changes that modulate PKC association with membrane lipids and proteins via their regulatory domains (18, 27).

PKC Autophosphorylation of PKCβII has been known for years and has been associated with some changes in the kinetic behavior of the enzyme in vitro (17). However, the present study defines an important physiological function for PKC autophosphorylation by demonstrating its critical role in the reversible PKC βII membrane trafficking in response to cell surface receptor activation. Moreover, the requirement for kinase activity and autophosphorylation in PKC βII dissociation from the plasma membrane following translocation reveals a novel regulatory mechanism modulating PKC activity. Thus, membrane dissociation should no longer be considered as a default step following PKC translocation, but rather as a regulated step requiring kinase autophosphorylation. Failure of this regulation may result in “permanently” membrane-associated proteins with subsequent alterations in cellular signaling and functions. In conclusion, our results demonstrate an exquisite control of the subtle interaction of PKC with the plasma membrane, and have revealed the dynamic and regulated nature of this process. Furthermore, by implicating the involvement of autophosphorylation in turning off PKCs, these findings also suggest that autophosphorylation of PKCs may be important in preventing abnormal cell proliferation, differentiation, and gene expression.

REFERENCES

1. Nishizuka, Y. (1992) Science 258, 607–614
2. Blobe, G. C., Stirling, S., Obeid, L. M., and Hannun, Y. A. (1996) Cancer Surv. 27, 213–248
3. Newton, A. C. (1997) Curr. Opin. Cell Biol. 9, 161–167
4. Nishizuka, Y. (1995) FASEB J. 9, 484–496
5. Mochly-Rosen, D. (1996) Science 268, 247–251
6. Mochly-Rosen, D., and Gordon, A. S. (1998) FASEB J. 12, 35–42
7. Farrar, W. L., Thomas, T. P., and Anderson, W. B. (1985) Nature 315, 235–237
8. Farrar, W. L., and Anderson, W. B. (1985) Nature 315, 233–235
9. Feng, X., Zhang, J., Barak, L. S., Meyer, T., Caron, M. G., and Hannun, Y. A. (1998) J. Biol. Chem. 273, 10755–10762
10. Sakai, N., Sasaki, K., Igekagi, N., Shirai, Y., Ono, Y., and Saito, N. (1997) J. Cell Biol. 139, 1465–1476
11. Bornner, C., Filipuzzi, U., Wartmann, M., Eppenberger, U., and Fabbro, D. (1989) J. Biol. Chem. 264, 13992–13999
12. Flint, A. J., Paladini, R. D., and Koshland, D. E., Jr. (1990) Science 249, 408–411
13. Tokunaga, S. E., Medzhibozsky, K. F., Flint, A. J., Burlingame, A. L., and Koshland, D. E., Jr. (1995) J. Biol. Chem. 270, 26807–26812
14. Keranen, L. M., Dutli, E. M., and Newton, A. C. (1995) Curr. Biol. 5, 1394–1403
15. Orr, J. W., and Newton, A. C. (1994) J. Biol. Chem. 269, 37715–37718
16. Dutli, E. M., Keranen, L. M., DePaoli-Roach, A. A., and Newton, A. C. (1994) J. Biol. Chem. 269, 29359–29362
17. Huang, K. P., Chan, K. F., Singh, T. J., Nakabayashi, H., and Huang, F. L. (1986) J. Biol. Chem. 261, 12314–12319
18. Edwards, A. S., and Newton, A. C. (1997) J. Biol. Chem. 272, 13832–13890
19. Lee, J. Y., Hannun, Y. A., and Obeid, L. M. (1996) J. Biol. Chem. 271, 13169–13174
20. Bornancin, F., and Parker, P. J. (1996) Curr. Biol. 6, 1114–1123
21. Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikukawa, U., and Nishizuka, Y. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11233–11237
22. Ohno, S., Ronno, Y., Akita, Y., Yano, A., and Suzuki, R. (1999) J. Biol. Chem. 265, 6296–6300
23. Cullen, B. R. (1987) Methods Enzymol. 152, 684–704
24. Ono, E., Teruel, M. N., Quest, A. F., and Meyer, T. (1998) J. Cell Biol. 140, 485–498
25. Ward, N. E., and O’Brien, C. A. (1992) Mol. Pharmacol. 41, 387–392
26. Dembinsky, A., Rubin, H., and Ravid, S. (1997) J. Biol. Chem. 272, 828–834
27. Hunter, T. (1995) Cell 83, 1–4

FIG. 5. The requirement of autophosphorylation in the membrane dissociation of PKC βII. 1–2 μg of GFP-conjugated PKC βII, PKC βII(K371R) and five PKC βII autophosphorylation mutants were transfected into HEK 293 cells together with 10 μg of pcDNA I/amp-AT1AR. The reversal of PKC translocation was quantitated as described under “Experimental Procedures.” The data represent the means ± S.E. of three separate experiments. ***, p < 0.001 compared with the wild-type values.