Visualization of Dynamic Trafficking of a Protein Kinase C \( \beta \mathit{II} \)/Green Fluorescent Protein Conjugate Reveals Differences in G Protein-coupled Receptor Activation and Desensitization*

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Protein kinase C (PKC) links various extracellular signals to intracellular responses and is activated by diverse intracellular factors including diacylglycerol, Ca\(^{2+}\), and arachidonic acid. In this study, using a fully functional green fluorescent protein conjugated PKC\(\beta\mathit{II}\) (GFP-PKC\(\beta\mathit{II}\)), we demonstrate a novel approach to study the dynamic redistribution of PKC in live cells in response to G protein-coupled receptor activation. Agonist-induced PKC translocation was rapid, transient, and selectively mediated by the activation of G\(\alpha\)- but not G\(\alpha\)-coupled receptors. Interestingly, although the stimuli were continuously present, only one brief peak of PKC membrane translocation was observed, consistent with rapid desensitization of the signaling pathway. Moreover, when GFP-PKC\(\beta\mathit{II}\) was used to examine cross-talk between two G\(\alpha\)-coupled receptors, angiotensin II type 1A receptor (AT\(1\)A\R R) and endothelin A receptor (ET\(A\)\R R), activation of ET\(A\)\R R resulted in a subsequent loss of AT\(1\)A\R responsive responsiveness, whereas stimulation of AT\(1\)A\R did not cause desensitization of the ET\(A\)\R signaling. The development of GFP-PKC\(\beta\mathit{II}\) has allowed not only the real-time visualization of the dynamic PKC trafficking in live cells in response to physiological stimuli but has also provided a direct and sensitive means in the assessment of activation and desensitization of receptors implicated in the phospholipase C signaling pathway.

The protein kinase C (PKC)

The abbreviations used are: PKC, protein kinase C; PKA, cAMP-dependent protein kinase; GFP, green fluorescent protein; EGFP, enhanced GFP; AT\(1\)A\R, angiotensin II type 1A receptor; ET\(A\)\R, endothelin A receptor; \(\beta\)AR, \(\beta\)-adrenergic receptor; D\(2\)R, dopamine D\(2\) receptor; HER293 cells, human embryonic kidney 293 cells; PMA, phorbol 12-myristoyl 13-acetate; IP\(_3\), inositol 1,4,5-triphosphate; DAG, diacylglycerol; PBS, phosphate-buffered saline; CMV, cytomegalovirus; GPCR, G protein-coupled receptor.

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muscle contraction (1, 2). Phorbolesters mimic the action of DAG, but they are more potent, and their effects last longer in cells due to their persistence in the cell membrane (1). This has made feasible the study of PKC subcellular localizations before and after activation by phorbolesters by immunofluorescent microscopy in fixed cells (19, 20). However, the same method is not applicable in following the more physiologically relevant redistribution of PKC in response to receptor activation in live cells. Furthermore, it is not clear whether the short term of PKC activation in response to receptor activation is related to the signaling desensitization at the level of receptors.

In the present study, we report the development of a green fluorescent protein conjugated PKCβII (GFP-PKCβII) to study PKC mobilization in response to agonist stimulation of G protein-coupled receptors and to assess the activation and desensitization of these receptors. GFP, originally identified in the jellyfish Aequorea victoria, displays an inherent green bioluminescence and has been used as a fluorescent reporter molecule in the localization of membrane receptors, cytoplasmic proteins, and secretory proteins (21–28). When fused to the amino terminus of PKCβII, the resulting fusion protein, GFP-PKCβII, was found to be fully functional in terms of its phospholipid-dependent kinase activity and its ability to translocate from cytoplasm to the plasma membrane in response to phorbolester (PMA) stimulation, similar to that reported for a PKCβ/GFP conjugate (28). Interestingly, while in PMA-treated cells GFP-PKCβII remained on the plasma membrane, in cells stimulated with physiological signals that activate Gα-coupled receptors, the translocation of GFP-PKCβII to the plasma membrane was found to be transient, reaching a peak and being reversed within minutes. This provided a real-time visual demonstration of the cellular trafficking of a PKC isoenzyme in live cells, revealing a dynamic nature of the interaction of PKCβII with lipid and/or protein molecules on the plasma membrane. Furthermore, since GFP-PKCβII selectively responded to only signals activating Gα, but not Gβ- and Gα-coupled receptors and such responses were transitory, our results also demonstrate an important analytical role of GFP-PKCβII as a reporter in the study of Gα-coupled receptor activation and desensitization.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP was purchased from NEN Life Science Products. Monoclonal antibody against GFP was from CLONTECH. Polyclonal rabbit antibody against PKCβII was prepared and extensively characterized as described previously (29). Mammalian expression vector pBk-CMV and GFP plasmid pEGFP-N1 was from Stratagene and CLONTECH, respectively. 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. Phosphatidyserine and sn-dioctanoyl-glycerol were purchased from Avanti Polar Lipids Inc. Eagle’s minimum essential medium, phosphate-buffered saline (PBS), and 1 ml HEPES buffer were from Life Technologies, Inc. Fetal bovine serum was obtained from Summit Biotechnology Inc. Fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG was from Jackson Immunoresearch Laboratories, Inc. All other chemicals were from Sigma.

Plasmid Constructs—The human PKCβII cDNA was inserted between BamHI and KpnI sites of pBk-CMV. A unique BssHII restriction site was located three base pairs prior to the start codon (ATG) of PKCβII cDNA. To construct GFP-PKCβII, EGFP cDNA without the stop codon was first amplified from plasmid pEGFP-N1 by polymerase chain reaction using 5′-oligonucleotide primer 5′-GTGAGCCGTCGAATGCGTAC-3′ (based on the sequence of pEGFP-N1 from 575 to 595) and 3′-primer 5′-CCATCTTGGGCGCGGCTAGC-3′-CATGC-3′ (with the native sequence of pEGFP-N1 from 1376 to 1396 underlined). The polymerase chain reaction fragment containing EGFP cassette was gel-purified, digested with BamHI and BssHII, and directly inserted between the BamHI and BssHII sites of plasmid pBk-CMV-PKCβII prior to the 5′-end of the PKCβII cDNA (Fig. 1). The sequence of the construct was confirmed by DNA sequencing. The cDNAs of AT1R, ET2R, βAR, and D2R were subcloned in pcDNA I or pcDNA I/amp mammalian expression vectors (Invitrogen).

Cell Culture and Transfection—HEK 293 cells from the American Type Culture Collection (ATCC) were maintained in Eagle’s minimum 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 × 105 cells/100-mm dish and transfected using a modified calcium phosphate method with 1–10 μg of plasmid (30).

Immunoprecipitation—HEK 293 cells were transfected with EGFPPKCβII, or GFP-PKCβII. Immunoprecipitation was performed 48 h after transfection as follows. The cells were washed with cold PBS and solubilized with buffer with protease inhibitors. 150 μM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin/chymotrypsin inhibitor, 5 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride) for 1 h, and the lysates of EGFP, PKCβIII, or GFP-PKCβII transfected cells were immunoprecipitated with PKCβII antibody. Protein A-Sepharose beads were used to absorb immunoprecipitates and were then washed four times with lysis buffer followed by one wash with kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM NaF, 1 mM Na3VO4, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM MgCl2). The kinase activity of immunoprecipitated proteins was analyzed by protein kinase C assay (see below).

Immunoblot—Cells lysates from HEK 293 cells transfected with EGFPPKCβII, or GFP-PKCβII were prepared by SDS-polyacrylamide gel electrophoresis, and electrothermally transferred into nitrocellulose membranes (31). The membranes were blocked in PBS with 0.1% Tween 20 and 5% dried milk, probed with anti-GFP antibody (1:2500 dilution) or rabbit anti-PKCβII antibody (1:2000 dilution), and exposed using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

Protein Kinase C Assay—PKC activity was measured using the vesicle assay for PKC as described previously (32). Standard assay conditions were as follows: 20 mM Tris-HCl, pH 7.4, 100 μM MgCl2, 1 mM CaCl2, 10 μM ATP, 10–15 μC/mg [γ-32P]ATP, 40 μg/ml phosphatidylserine/sn-dioctanoyl-glycerol vesicles, and 200 μg/ml histone HIIS as substrate in a final volume of 250 μl at 30 °C for 10 min. 10 mM EGTA was used in determining basal kinase activity. The phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Indirect Immunofluorescence—Transfected cells were seeded on glass coverslips placed in six-well culture dishes at a density of 5 × 105 cells/well. For experiments involving phorbolester treatment, cells were treated with 100 nM PMA for 5 min. The cells were then rinsed briefly with PBS and fixed in 3.7% paraformaldehyde for 10 min. The fixed cells were permeabilized in PBS containing 0.2% Triton X-100 for 10 min and blocked in PBS containing 0.2% bovine serum albumin for 10 min. After 1 h of incubation with anti-PKCβII polyclonal antibody (1:100 dilution), 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 PKCβII immunofluorescence was determined in living cells. The phosphorylated proteins were analyzed by confocal microscopy.

Confocal Microscopy—HEK 293 cells were transfected with GFP-PKCβII and one or two of the G protein-coupled receptors as described in figure legends. 24 h after transfection, the cells were plated onto 35-mm glass-bottomed culture dishes (MatTek) at a density of 1 × 105 and incubated for another 24 h for the cells to attach to glass. The cells expressing GFP-PKCβII (25–40% of the total cell population) were observed under confocal microscopy. Confocal microscopy was performed on a Zeiss LSM-410 laser scanning microscope at 488-nm excitation.

Comparison of Wild-type PKCβII and GFP-PKCβII—Due to its inherent fluorescence and unique compact structure, GFP has been reported in many studies to serve as a valuable reporter molecule in the localization of various proteins without interfering with their biological activity (21, 33). GFP-PKCβII was constructed by fusing EGFP to the 5′-end of PKCβII (Fig. 1). When examined using SDS-polyacrylamide gel electrophoresis followed by immunoblotting, the GFP-PKCβII was observed to be approximately 6 kDa larger than PKCβII. This difference in size was accounted for by the extra 248 amino acids present in the GFP-PKCβII.
electrophoresis followed by immunoblotting, GFP-PKCβII was found to express to the same extent in HEK 293 cells as wild-type PKCβII but migrated more slowly due to the added mass of GFP (Fig. 2A). To compare the biological activity of GFP-PKCβII with that of wild-type PKCβII, both proteins were immunoprecipitated from HEK 293 cells transiently expressing GFP-PKCβII or PKCβII with an antibody against PKCβII and analyzed for their relative kinase activity using a recently developed PKC assay with histone IIIS as the substrate (32). As shown in Fig. 2B, the cells transfected with EGFP contained very low kinase activity, but transfection of either GFP-PKCβII or PKCβII significantly increased the phosphorylation of histone IIIS. The ability of GFP-PKCβII to phosphorylate histone IIIS in a Ca2+- and phospholipid-dependent manner was comparable to that of wild-type PKCβII, indicating that the fusion of the GFP molecule to the N terminus of PKCβII has no significant effect on PKC activity.

Phorbol esters such as PMA are well known to mimic the action of diacylglycerol in inducing PKC to translocate from cytosol to the plasma membrane (1, 34). Thus, the ability of GFP-PKCβII to respond to PMA was also examined (Fig. 2C, top). Under normal unstimulated conditions, GFP-PKCβII was evenly distributed in the cytoplasm and excluded from the nuclei, a distribution exactly reflecting the cellular localization of wild-type PKCβII detected with indirect immunofluorescence microscopy (Fig. 2C, middle). Stimulation with 1 μM PMA triggered redistribution of GFP-PKCβII to the plasma membrane. This membrane translocation was completed within 5 min, and about 90% of GFP-PKCβII fluorescence was mobilized to the plasma membrane, similar to that observed with transfected wild-type PKCβII in fixed HEK 293 cells (Fig. 2C, top and middle). The distribution of wild-type PKCβII and GFP-PKCβII observed in HEK 293 cells corresponded to the reported distribution of endogenous PKCβII in U937, a cell line expressing endogenous PKCβII (35). In contrast to GFP-PKCβII, unconjugated GFP molecules were distributed throughout the cell body, and their distribution was not affected by PMA (Fig. 2C, bottom). These results indicate that GFP-PKCβII fusion protein retains the biological activity of wild-type PKCβII with respect to kinase activity as well as cellular localization.

Dynamic and Selective Trafficking of GFP-PKCβII in Response to G Protein-coupled Receptor Activation—Although the redistribution of PKC upon phorbol ester stimulation has been extensively studied with indirect immunofluorescence microscopy (19, 20), the kinetics of PKC membrane translocation in response to physiological signals has not been well characterized. One such physiological signal that activates PKC is through activation of G protein-coupled receptors. Therefore, initial studies using confocal microscopy examined the real time cellular distribution of GFP-PKCβII in response to the activation of the Gαi-coupled AT1AR by its physiological ligand angiotensin II (Fig. 3A). This was done at 30 °C in live HEK 293 cells, which had been transiently transfect to overexpress the AT1AR and GFP-PKCβII. In the absence of receptor activation, confocal microscopy revealed that GFP-PKCβII was evenly distributed throughout the cytoplasm. However, upon agonist activation of the AT1AR, a redistribution of GFP-PKCβII to the plasma membrane and clearance of cytosolic fluorescence occurred and peaked within 40 s. This angiotensin II-induced GFP-PKCβII membrane trafficking was rapid compared with the translocation caused by PMA stimulation, which did not peak until after 2–5 min. More interestingly, unlike PMA-induced translocation in which PKC remained persistently localized on the plasma membrane, the mobilization of GFP-PKCβII to the plasma membrane following AT1AR activation was transient, and the redistributed GFP-PKCβII rapidly returned to the cytoplasm within 1 min after translocation. The time frame for GFP-PKCβII recovery in the cytoplasm in the majority of the cells observed ranged from 20 s to 1 min after GFP-PKCβII membrane translocation. Moreover, even in the continuous presence of angiotensin II for 30 min, only an initial brief peak of PKC membrane translocation was observed (data not shown). These visual results are consistent with biochemical studies indicating a dynamic nature of PKCβII in interacting with molecules on the plasma membrane (1, 34).

Among major groups of G protein-coupled receptors, those coupled to Gαi are directly associated with the activation of phospholipase C, resulting in the generation of diacylglycerol and a rise of intracellular Ca2+, whereas the effector for receptors coupled to Gαs and Gαo is mainly adenylyl cyclase associated with the production of cyclic AMP. To examine the specificity and selectivity of agonist-induced PKC trafficking, three additional G protein-coupled receptors were tested for their ability to trigger GFP-PKCβII translocation, including ETaR (coupled to Gαo), β2AR (coupled to Gαo), and D2R (coupled to Gαo). In HEK 293 cells, transiently transfected with GFP-PKCβII and ETaR, a rapid PKC membrane translocation was observed in response to the ETaR endogenous agonist endothelin (Fig. 3B). Similar to that caused by the activation of the AT1AR, this ETaR-promoted redistribution of GFP-PKCβII was transient and readily reversible. In contrast, when cells were transfected to coexpress GFP-PKCβII with either β2AR or D2R, the activation of neither receptor by its cognate ligand affected the distribution of GFP-PKCβII fluorescence (Fig. 3, C and D). These results indicate that the mobilization of GFP-PKCβII in live cells are selectively mediated by receptors activating the phospholipase C signaling pathway including GPCRs coupled to Gαo but not Gαo and Gαo.

Desensitization of Gαi-coupled Receptor Signaling Revealed by GFP-PKCβII Trafficking—The observation that there was only one brief peak of PKC membrane translocation even in the continuous presence of angiotensin II or endothelin suggests that the transitory nature of PKC trafficking in response to these receptor agonists might be associated with the rapid desensitization of AT1AR or ETaR signaling. To further investigate this phenomenon, HEK 293 cells transfected with GFP-PKCβII and AT1AR were sequentially exposed to two pulses of stimulation by angiotensin II separated by 10 min (Fig. 4A). Whereas the first exposure resulted in a transient GFP-PKCβII trafficking between the cytoplasm and plasma membrane and GFP-PKCβII returned to the cytoplasm within 1 min following its membrane translocation, there was no apparent PKC mobilization in response to the second angiotensin II stimulation, indicating the AT1AR signaling was turned off as a result of initial receptor activation at a step prior to PKC translocation or at the level of PKC. The possibility that PKC itself might be desensitized and loses its ability to respond to further receptor activation was examined by exposing AT1AR...
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FIG. 2. Characterization of GFP-PKCβII fusion protein expressed in HEK 293 cells by immunoblotting analysis, protein kinase C assay, and confocal microscopy. A, shown are immunoblots of homogenates from HEK 293 cells transfected with 1 μg of pEGFP-N1 (lane 1), pBK-CMV-GFP-PKCβII (lane 2), and pBK-CMV-PKCβII (lane 3) probed with polyclonal antibody against PKCβII (left) and monoclonal antibody against GFP (right). 20 μg of homogenate was loaded in each lane. As expected, the homogenate from cells transfected with pBK-CMV-GFP-PKCβII showed strong immunoreactivity to both the PKCβII antibody and GFP antibody. The band seen at approximate 50 kDa in each lane with anti-GFP represents nonspecific immunoreactivity. B, the above described HEK 293 cell homogenates (100 μg) were immunoprecipitated with PKCβII antibody, and kinase activity of the immunocomplexes was assayed. Protein kinase C assay was performed under standard conditions in the presence (+) and absence (−) of PKC activator sn-dioctanoyl-glycerol (DhC8), and cofactors Ca2+ and phosphatidyserine. Shown is a representative autoradiograph indicating GFP-PKCβII and wild-type PKCβII comparably phosphorylated substrate histone IIIS. C, shown are the confocal micrographs of HEK 293 cells transfected with 1 μg of pBK-CMV-GFP-PKCβII (top) or pEGFP-N1 (bottom) before (control) and after 5 min of PMA treatment (PMA). The transfected cells were stimulated with 1 μM PMA at 30 °C to activate PKC. The redistribution of GFP-PKCβII started within 1 min, and almost 90% of fluorescence was located at the plasma membrane after 5 min of stimulation. In contrast, there was no change of the distribution of GFP itself in response to 1 μM PMA stimulation. Shown in the middle are the confocal micrographs of HEK 293 cells transfected with 1 μg of pBK-CMV-PKCβII before (control) and after 5 min of treatment with 100 nM PMA (PMA) at 37 °C. The cells were fixed and stained for wild-type PKCβII as described under “Experimental Procedures.” The experiments were performed independently on three different occasions, and each time 3–5 cells from independent stimulation by each agonist were recorded. All of the micrographs are representative of more than 70% of the cells observed. Bar, 10 μm.

FIG. 3. Confocal microscopy of the time-dependent GFP-PKCβII redistribution in response to G protein-coupled receptor activation. HEK 293 cells were transfected with 1 μg of pBK-CMV-GFP-PKCβII as well as 10 μg of one of the following G protein-coupled receptor constructs: AT1R, ETAR, β2AR, or D2R. All cells were visualized at 30 °C with a confocal microscope. Before receptor activation (control), GFP-PKCβII was evenly distributed in the cytoplasm. When cells transfected with AT1Rs or ETARs were stimulated with corresponding receptor agonists, 0.5 μM angiotensin II (A) or 0.1 μM endothelin (B), GFP-PKCβII underwent a transient redistribution between the cytoplasm and plasma membrane. In contrast, no GFP-PKCβII mobilization was observed in cells transfected with either β2AR (C) stimulated with 10 μM isoproterenol or D2R (D) stimulated with 10 μM dopamine. Also indicated in the micrographs are the time points following agonist stimulation for each receptor. The experiments were performed independently on three different occasions, and each time 3–5 cells from independent stimulation by each agonist were recorded. All of the micrographs are representative of more than 70% of the cells observed. Bar, 10 μm.
same Gaα-mediated signaling pathway in which Gaα activates phospholipase Cβ and results in the hydrolysis of phosphoinositide lipid to generate IP3 and DAG, the latter serving as a second messenger for activation of PKC. To further study the rapid receptor desensitization as well as the relationship between signaling pathways of different receptors, AT1AR and ET2AR were cotransfected with GFP-PKCβII into HEK 293 cells, and the effect of their potential cross-talk on the trafficking of GFP-PKCβII was examined and visualized by confocal microscopy. In initial experiments, the cells were pretreated with angiotensin II to activate the AT1AR and induce PKC response. As described above, the resulting GFP-PKCβII trafficking was transient and GFP-PKCβII returned to the cytoplasm within 1 min following its membrane translocation. No second peak of PKC translocation was observed in the continuous presence of agonist for as long as 30 min. However, when endothelin was subsequently added to the angiotensin II-pretreated cells to activate the ET2AR, a second peak of GFP-PKCβII translocation was apparent within 1 min, the time profile and extent of which were indistinguishable from the first peak (Fig. 5A). As both AT1AR and ET2AR induce PKC response by activating Gaα, which increases the activity of phospholipase C, the ability of endothelin to mobilize PKC in cells prestimulated with AT1AR agonist further indicates that the activation of AT1AR did not desensitize Gaα-mediated signaling pathways, but instead the desensitization occurred at the level of the ET2AR receptor itself. Moreover, these results also demonstrate that the activation of AT1ARs does not contribute to the desensitization of the ET2AR.

To further investigate the effect of ET2AR activation on AT1AR signaling, we reversed the order of introducing agonists and sequentially added endothelin and then angiotensin II to the cells cotransfected with both receptors. Surprisingly, when the cells were preexposed to endothelin for 5 min, no additional peak of GFP-PKCβII cellular movement was detected by confocal microscopy in response to a subsequent stimulation with angiotensin II (Fig. 5B). This result indicates that in contrast to the inability of AT1AR to cause ET2AR desensitization in HEK 293 cells, the activation of ET2AR indeed led to the heterologous desensitization of the AT1AR in the same cells, possibly mediated by the activation of PKC and subsequent phosphorylation of the receptor by PKC. This is consistent with the observation that inhibition of activation of PKC by 0.5 μM staurosporine, a potent protein kinase inhibitor, resulted in a 42% loss of total agonist-stimulated phosphorylation of the AT1AR but had no effect on that of the ET2AR (16, 36). The specificity of the heterologous desensitization was further assessed by examining the effect of activation of another second messenger-dependent kinase, cyclic AMP-dependent protein kinase (PKA) on AT1AR signaling in cells cotransfected with 10 μg of pcDNA I/amp-ET AR and stimulated with 1 μg of pcDNA I/ET AR and stimulated with 1 μg of pcDNA I/ET AR and stimulated with 0.1 μM endothelin (C and D) to induce GFP-PKCβII transient trafficking between the cytoplasm and plasma membrane. 10 min after the first stimulation, the cells were restimulated with additional 1.5 μM angiotensin II (A), 0.5 μM endothelin (C), or 1 μM PMA (B and D).

Indicated in the micrographs are the time points following the first agonist stimulation. All cells were visualized at 30 °C by confocal microscope. The experiments were performed independently on three different occasions, and each time 3–5 cells from independent stimulation by each drug were recorded. The confocal micrographs are representative of more than 70% of the cells observed. Bar, 10 μm.

**FIG. 4.** Effects of angiotensin II (AgII), endothelin (ET), and PMA on the redistribution of GFP-PKCβII in cells preexposed to angiotensin II or endothelin. HEK 293 cells were cotransfected with 1 μg of pBK-CMV-GFP-PKCβII and 10 μg of pcDNA I/amp-AT1R and stimulated with either 0.5 μM angiotensin II (A and B) or cotransfected with 1 μg of pBK-CMV-GFP-PKCβII and 10 μg of pcDNA I/ET AR and stimulated with 0.1 μM endothelin (C and D) to induce GFP-PKCβII transient trafficking between the cytoplasm and plasma membrane. 10 min after the first stimulation, the cells were restimulated with additional 1.5 μM angiotensin II (A), 0.5 μM endothelin (C), or 1 μM PMA (B and D). Indicated in the micrographs are the time points following the first agonist stimulation. All cells were visualized at 30 °C by confocal microscope. The experiments were performed independently on three different occasions, and each time 3–5 cells from independent stimulation by each drug were recorded. The confocal micrographs are representative of more than 70% of the cells observed. Bar, 10 μm.

In the present work, the development of a fully functional green fluorescent protein conjugated PKCβII has allowed visualization in live cells of the real time interaction of PKC with the plasma membrane in response to extracellular signals. Whereas stimulation by phorbol esters (e.g. PMA) causes a persistent localization of PKC to the plasma membrane, our results reveal a dynamic nature of PKCβII trafficking between the cytoplasm and plasma membrane in response to physiological signals such as those activating G protein-coupled receptors. PKCβII responds selectively only to specific signals that
activate receptors coupled to Gq,α but not Gs,α and Gα,α proteins. The membrane translocation of PKCβII triggered by the activation of Gα,α-coupled receptors is rapid and transient and is followed immediately by the returning of PKC to the cytoplasm within minutes, indicating a desensitization of the signaling pathway. Moreover, when GFP-PKCβII trafficking in response to the sequential activation of two distinct Gα,α-coupled receptors (i.e., AT1AR and ETAR) was studied, the results indicated that the signaling desensitization occurs at the level of receptors and that there is cross-talk between the two receptors. Thus, GFP conjugated PKC fusion proteins serve as a novel useful tool not only for studying the dynamic localization of PKCs in signal transduction in live cells but also for detecting the activation and desensitization of receptors coupled to phospholipase C such as Gα,α-coupled receptors.

PKC cellular localization has been extensively studied in culture cells using antibody staining and immunofluorescent microscopy (19, 20). However, many signal transduction events involving PKC are rapid, transient, and difficult to follow in fixed cells. Green fluorescent protein, because of its inherent bioluminescence and stoichiometric labeling, represents a sensitive optical reporter to follow the real time localization of many proteins in live cells and is thus well suited for the study of transitory and dynamic distribution of molecules in the process of signal transduction (26). Recently, the GFP technique has been used in the study of cellular distribution of PKCy (28). In our study, when EGF was fused to the N terminus of PKCy, GFP-PKCy conjugate displayed Ca2+ and phospholipid-dependent kinase activity comparable with that of wild-type PKCy and was localized mainly in the cytoplasm and excluded from the nuclei. Moreover, in cells stimulated with PMA, like wild-type PKCy, GFP-PKCy redistributed to the plasma membrane. The stable association of GFP-PKCy with the plasma membrane is a true reflection of the persistence of PMA in the plasma membrane as well as the stable interaction of PKCy to PMA. Therefore, although GFP is about one-third the size of PKCy (Fig. 1), as reported for many other proteins, our data demonstrate that GFP-PKCy conjugate retains the function of native PKCy in terms of biochemical behavior and cellular localization, although the possibility cannot be ruled out that the coupling of GFP to PKCy may alter the affinity of the enzyme to its interacting molecules.

The activation of PKC is triggered by a large number of extracellular signals including hormones, neurotransmitters, and growth factors that act through cell surface receptors. The activation of these receptors regulates the intracellular level of various PKC activators including DAG, Ca2+, and many other lipid mediators. G protein-coupled receptors were used in this work as an example to address PKC cellular trafficking and distribution in response to various physiological stimuli. Four receptors were examined for their ability to stimulate GFP-PKCβII redistribution, including Gα,α-coupled β2AR, Gα,α-coupled D2R, and Gα,α-coupled AT1AR and ETAR. The physiological relevance of PKC activation mediated by these receptors is apparent since PKC responded selectively only to signals activating Gα,α-coupled receptors (i.e., AT1AR and ETAR) but not Gα,α- and Gα,α-coupled receptors, corresponding to the fact that among the three major G proteins only Gα,α mediates the production of DAG at the plasma membrane and a rise of intracellular Ca2+. More importantly, unlike PMA-stimulated PKC translocation, physiological signals activating AT1ARs and ETARs induce a redistribution of GFP-PKCy to the plasma membrane, which appears to be transient, consistent with the

![Figure 5](image-url)
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biochemical studies indicating that DAG is one of the plasma membrane stimuli for PKC and that the interaction between DAG and PKC is rapid and reversible (1, 34).

However, it is somewhat unexpected that only one rapid cycle of PKC translocation from the cytoplasm to plasma membrane and back was observed although receptor agonists were present continuously. This lack of further PKC responsiveness is probably the result of a rapid desensitization of the agonist-mediated signaling pathway upstream of PKC, since PKC itself still retains the ability to respond normally when subsequently exposed to PMA. In addition, sequential stimulation of the AT_{1}A receptor and then the ET_{A}R with their corresponding agonists induced consecutive transient cycles of PKC translocation, one after each stimulation. As the AT_{1}A and ET_{A}R presumably share the same signaling components downstream of the receptors, this suggests that the desensitization of AT_{1}A signaling occurs at the level of the receptor itself. More interestingly, although activation of the AT_{1}A does not affect the activity of the ET_{A}R, activation of the ET_{A}R not only shuts off its own signaling but also causes the desensitization of the AT_{1}A, indicating differential regulation of the two receptors.

The rapid desensitization of G protein-coupled receptors is achieved mainly through phosphorylation of the receptors by two classes of serine/threonine protein kinases: the second messenger activated protein kinases, PKA and PKC; and the G protein-coupled receptor kinases that specifically phosphorylate agonist-activated receptors (15). Although the role of PKC in regulating AT_{1}A desensitization in different tissues is still variable depending on experimental conditions, recently it was reported that the AT_{1}A was phosphorylated by both G protein-coupled receptor kinases and PKC in response to short term angiotensin II stimulation in HEK 293, a cell line widely used in the study of receptor desensitization (16). In contrast, in the same cell line, the ET_{A}R was mainly phosphorylated by G protein-coupled receptor kinases but not by activated PKC in response to agonist (36). These studies suggest that while both receptors serve as substrates for G protein-coupled receptor kinases, only the AT_{1}A (but not ET_{A}R) has the unique biochemical property to undergo agonist-dependent phosphorylation by PKC. The homologous desensitization of both receptors probably involves G protein-coupled receptor kinase or possibly PKC in the case of the AT_{1}A. However, our results suggest that cross-talk (i.e. heterologous desensitization) between the two receptors is mainly mediated by PKC, and the distinct ability to be phosphorylated by PKC might underlie the observed differential desensitization properties of the two receptors. For instance, the inability of the ET_{A}R to be phosphorylated by PKC might account for its lack of heterologous desensitization by activation of the AT_{1}A. In addition, despite the finding that the AT_{1}A was phosphorylated by PKA in intact aortic vascular smooth muscle cells (RASM) (37), we demonstrate that activation of PKA by stimulating the β_{2}AR does not lead to desensitization of the AT_{1}A, consistent with the lack of PKA phosphorylation of the AT_{1}A found in HEK 293 cells (16).

A large variety of signaling pathways are known to culminate in the activation of PKC, including those mediated by G_{α} protein-coupled receptors (1, 34). The numbers of such receptors are expected to expand rapidly with the progress of genomic sequencing and challenge the conventional biochemical measurements that assess individual receptor-specific properties for defining their corresponding ligands, detecting signaling activation and measuring change of second messenger levels. In this study, by combining the inherent fluorescence of GFP with the translocation property of PKC, we have developed a potential live cell biosensor that may provide simple, sensitive, and rapid assessment of the involvement of PKC activation in the signaling pathways of these receptors. In addition, since GFP-PKC redistribution can serve as a sensitive indicator for receptor activation, it may provide a simple and universal tool for screening new ligands for receptors coupled to PKC as well as for associating newly discovered receptors with their cognate ligands and physiological functions. Moreover, by monitoring inhibition of PKC translocation, it may be potentially also applicable to the identification of the inhibitors of PKC itself as well as inhibitors that block the signaling components leading to activation of PKC. In the case of GPCRs, GFP-PKC translocation has been shown in this study to be a specific measure of G_{α}-coupled receptor activation. The transitory nature of the translocation makes GFP-PKC a useful tool for studying G_{α}-coupled receptor desensitization. Compared with detecting Ca^{2+} signals, measuring PKC trafficking represents a more direct and accurate assessment of the properties of the plasma membrane receptors without concerns from the permeability, solubility, and compartmentalization of Ca^{2+} indicators and interference from plasma membrane and intracellular Ca^{2+} channels (e.g. the IP_{3} receptor) (7, 18, 38). Furthermore, with the discovery of numerous novel G protein-coupled receptors by genomic sequencing, GFP-PKC should also be extremely useful in quickly identifying those receptors coupled to G_{α} and their ligands and functions.

The visualization of GFP-PKC/GFP dynamic translocation in this study provides a direct real time assessment of the distribution of a PKC in live cells in response to changes of intracellular PKC activators triggered by physiological stimuli. To date, 12 members are identified as belonging to the PKC superfamily, associated with a wide variety of cellular signaling events, such as mitogenesis and tumorigenesis (2, 8). The use of GFP conjugates as optical reporters should provide valuable information concerning not only the specific cellular distribution of different PKC isozenzymes but also their dynamic trafficking in response to various physiological stimuli. Therefore, GFP-PKC conjugates may represent ideal optical tools in the study of specific functions and kinetics of each PKC isozenzyme in different signal transduction systems. Furthermore, when employed as a biosensor, GFP-PKC fusion proteins may also provide a unique and sensitive means for studying the kinetics and components of signal transduction pathways in which PKCs are involved.

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