Subtype-specific Translocation of Diacylglycerol Kinase α and γ and Its Correlation with Protein Kinase C*

Received for publication, April 13, 2000, and in revised form, May 2, 2000
Published, JBC Papers in Press, May 24, 2000, DOI 10.1074/jbc.M003151200

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We examined the translocation of diacylglycerol kinase (DGK) α and γ fused with green fluorescent protein in living Chinese hamster ovary K1 cells (CHO-K1) and investigated temporal and spatial correlations between DGK and protein kinase C (PKC) when both kinases are overexpressed. DGKα and γ were present throughout the cytoplasm of CHO-K1 cells. Tetracainophorbon 13-acetate (TPA) induced irreversible translocation of DGKγ, but not DGKα, from the cytoplasm to the plasma membrane. The (TPA)-induced translocation of DGKγ was inhibited by the mutation of C1A but not C1B domain of DGKγ and was not inhibited by staurosporine. Arachidonic acid induced reversible translocation of DGKγ from the cytoplasm to the plasma membrane, whereas DGKα showed irreversible translocation to the plasma membrane and the Golgi network. Purinergic stimulation induced reversible translocation of both DGKα and γ to the plasma membrane. The timing of the ATP-induced translocation of DGKγ roughly coincided with that of PKCγ re-translocation from the membrane to the cytoplasm. Furthermore, re-translocation of PKCγ was evidently hastened by co-expression with DGKα and was blocked by an inhibitor of DGK (R59022). These results indicate that DGK shows subtype-specific translocation depending on extracellular signals and suggest that PKC and DGK are orchestrated temporally and spatially in the signal transduction.

Diacylglycerol (DG) is a second messenger regulating various cellular responses (1, 2). One of the important roles of DG is the activation of protein kinase C (PKC) (1, 3, 4). Thus, DG is very important for regulation of PKC activity and cellular response. DG is produced physiologically as a result of the signal-induced hydrolysis of phosphatidylinositol bisphosphate by phospholipase C and also from phosphatidylincholine by phospholipase D. Generated DG is phosphorylated to phosphatidic acid by diacylglycerol kinase (DGK) or cleavage by DG lipase (2, 5, 6). DGK is an important enzyme for inactivating PKC by attenuation of the DG level, contributing to regulation of the cellular response. In addition, phosphatidic acid itself activates PKCγ and PLCγ1 (7, 8) and modulates Ras GTPase-activating protein (9). DGKs have additional important functions for various cellular responses.

To date at least nine subtypes of mammalian DGKs have been cloned and divided into five groups based on structure (2). Generally, all DGKs have cysteine-rich regions homologous to the C1A and C1B motifs of PKCs in the regulatory domain at the N terminus of the protein and possess a conserved catalytic domain in the C terminus of the protein. Type I DGKs, including DGKγ, -β, and -γ, have EF-hand motifs and two cysteine-rich regions in the regulatory domain (10–12). Type II DGKs such as DGKδ and -η, have a pleckstrin homology domain instead of the EF-hand motif in addition to two cysteine-rich regions (13, 14). Interestingly, the catalytic domains of DGKδ and -η are separated. Type III, consisting of DGKɛ, has only two cysteine-rich regions in the regulatory domain. Type IV, DGKζ and -η, has a unique motif similar to the myristoylated alanine-rich C-kinase substrate (MARCKS) phosphorylation site in the regulatory domain and four ankyrin repeats at its C terminus (15, 16). The final group, type V, includes DGKθ, which has three cysteine-rich regions and a pleckstrin homology domain with an overlapping Ras-associating domain (17). In contrast to the accumulated knowledge of molecular structure, the functions and regulatory mechanism of each DGK subtype are still unknown.

Recently, we developed a system to monitor the translocation of PKCs fused with GFP in living cells (18). Using this system, we demonstrated that each subtype of PKC shows distinct translocation in response to various stimuli, suggesting that each subspecies has a spatially and temporally different targeting mechanism that depends on the extracellular and intracellular signals (19, 20). Thus, we hypothesize that the subtype-specific targeting of PKC contributes to the subspecies-specific functions. Interestingly, in addition to PKC, translocation of DGK by several stimuli such as phorbol ester (21, 22), calcium (23), and carbocapitol (24) has been shown by analysis of immunoblots or of DGK activity, although previous studies concerning the translocation of DGKs have been performed using crude DGKs, and subtype-specific translocation could not be demonstrated. Furthermore, the involvement of PKC in the regulation of activity and localization of DGKα has been described (24–27). These observations suggest that the translocation of DGKs is an effective process to regulate their activity and subtype-specific functions and that DGKs and PKCs are not only functionally but also temporally and spatially correlated in their signal transduction pathways. In this
study, we examined translocations of DGKα and -γ fused with GFP and investigated temporal and spatial correlations between DGK and PKC in addition to their functional correlation.

EXPERIMENTAL PROCEDURES

Materials—Adenosine triphosphate (ATP), uridine triphosphate (UTP), calcium ionophore A 23187, and tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma. Arachidonic acid and 1,2-dioctanoylgllycerol (DiC8) were obtained from Doosan Serdary Research Laboratories (Eaglewood Cliffs, NJ) and Biornol Research Laboratories (Plymouth Meeting, PA), respectively. DGK inhibitor (R59022) was a product of Research Biochemicals International. All the other chemicals used were of analytical grade. A plasmid including cDNA for a variant of blue fluorescence protein (BFP) was donated by Dr. Osumi (Himeji Institute of Technology, Japan). COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium, and CHO-K1 cells were cultured in Ham’s F-12 medium (Life Technologies, Inc.) at 37 °C in a humidified atmosphere containing 5% CO2. Both media contained 25 mM glucose, and both were buffered with 44 mM NaHCO3 and supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). The fetal bovine serum used was not heat-inactivated.

Construct of Plasmids Encoding GFP–DGKγ Fusion Protein—The plasmid bearing cDNA for pig DGKγ (designated as BS 585) (10) was donated by Dr. Kanoh (Sapporo Medical University School of Medicine, Japan). A cDNA fragment of rat DGKγ with a Xhol site in the 5'-terminal and an SmaI site in the 3'-terminal was produced by a PCR with rat DGKγ (12) as the template. The sense and antisense primers were 5'-AAGCTTGCAGTAAGTGGGCTT-3' and 5'-TTCCGGGAGTCCTTTGAACGGCTTTTCCT-3', respectively. The PCR product of DGKγ was first subcloned into a TA cloning vector, pCRTM 2.1 (Invitrogen, San Diego, CA). The plasmid was designated as BS465. The cDNA encoding DGKγ in BS465 was digested with Xhol and SmaI and subcloned into the rat DGKγ expression vector pH292 vector (CLONTECH, Palo Alto, CA) or SfⅡ and SmaI site in pEGFPN1 (CLONTECH) (BS470 and BS561, respectively). Similarly, a cDNA fragment of pig DGKγ with an Xhol site in the 5'-terminal and an SmaI site in the 3'-terminal was produced by PCR with BS585 as the template and subcloned into a TA cloning vector, pCRTM 2.1 (BS597). Finally, the DGKγ was subcloned into the SfⅡ and SmaI site in pEGFP-C1 (BS561), respectively.

Site-directed Mutagenesis of GFP–DGKγ—Site-directed mutagenesis was performed according to the manufacturer’s recommended protocol with ExSite PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA), using BS465 as a template. The sense and antisense primers for producing a mutant DGKγ whose Cys-285 in C1A domain was substituted to Gly (C1A mutant), were 5'-GCCACATGATGCTGATCGGGC-3' and 5'-CAGCGTCACGTGACCAGCAGG-3', respectively. The primers for a mutant DGKγ, whose Cys-345 in C1B region was substituted to Gly (C1B mutant), were 5'-GCCACAAAAAGTCAATAGCTGATGCGT-3' and 5'-CAGCGTCACGTGACCAGCAGG-3'. Mutagenesis was confirmed by verifying sequences. Each C1A or C1B mutant of DGKγ cDNA was subcloned into SfⅡ and SmaI sites in pEGFP-C1, as in the case of BS561 (designated BS691 and BS692, respectively).

Immunoblotting and Kinase Assay of Native DGKs and Their GFP Fusion Proteins—Plasmids (approximately 32 µg) encoding each subtype of DGKs or their GFP fusion proteins were transfected into 6 × 106 COS 7 cells using a Gene Pulser (Bio-Rad, 960 F, 220 V). After being cultured for 2 days, the cells were harvested with PBS(−) and centrifuged. The cell pellet was resuspended in 300 µl of homogenate buffer (250 µl sucrose, 10 µl EDTA, 2 µl Triton X-100, 200 µg/ml leupeptin, 1 µl phenylmethylsulfonyl fluoride, 1 mM Triton X-100, pH 7.4). After the sonication (UD-210 TOMY, Japan; output 3, duty 50%, 10 times, at 4 °C), samples were collected for immunoblotting and kinase assay.

For immunoblotting, the samples were subjected to 7.5% SDS-polyacrylamide gel electrophoresis, followed by acrylamide gel electrophoresis, followed by blotting onto a polyvinyllidine membrane (Millipore, Bedford, MA). Nonspecific binding sites were blocked by incubation with 5% skim milk in 0.01 M PBS-T, the site in the 39-leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, pH 7.3 (Ringer’s solution). Translocation of the GFP fusion protein was triggered by a direct application of various stimuli at 10 times higher concentration into the Ringer’s solution to obtain the appropriate final concentration. The fluorescence of the fusion protein was monitored with a confocal laser-scanning fluorescent microscope (LSM 410 invert, Carl Zeiss, Jena, Germany) at 488-nm argon excitation using a 515–535-nm band pass filter. All experiments were performed at 37 °C.

For simultaneous observation of GFP–DGKγ and PKC-γ-GFP in the same field, a plasmid-bearing variant, GFP, was co-transfected with PKC-γ-GFP into CHO-K1 cells as a marker for detecting PKC-γ-GFP-expressing cells. The GFP–DGKγ or PKC-γ-GFP-expressing cells, which were individually transfected, were spread again and mixed into the same glass-bottomed culture dishes (MatTek Corp., Ashland, MA). Experiments were performed 16–48 h after the transfection.

The culture medium was replaced with HEPES buffer composed of 135 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, 10 mM glucose, pH 7.3 (Ringer’s solution). Translocation of the GFP fusion protein was triggered by a direct application of various stimuli at 10 times higher concentration into the Ringer’s solution to obtain the appropriate final concentration. The fluorescence of the fusion protein was monitored with a confocal laser-scanning fluorescent microscope (LSM 410 invert, Carl Zeiss, Jena, Germany) at 364-nm UV laser excitation using a 397-nm long pass laser filter, whereas that of GFP was monitored at 488-nm argon excitation using a 515–535-nm band pass filter. After confirming, translocation of GFP–DGKγ or PKC-γ-GFP was triggered and observed based on the fluorescence of GFP as described above.

RESULTS

Properties of the Fusion Protein of DGKγ and -γ with GFP—Fig. 1A shows constructs of two fusion proteins of DGKγ with GFP. GFP–DGKγ possesses GFP at the N terminus of the protein, whereas GFP is located at the C terminus of DGKγ–GFP. Immunoblotting using the anti-DGKγ antibody revealed that the molecular size of both fusion proteins is about 120 kDa, which is about 30 kDa larger than intact DGKγ (Fig. 1B).

Furthermore, no significant degraded products were detected, although some specific bands were observed in all lanes. Anti-GFP antibody also recognized the 120-kDa bands of DGKγ–GFP and GFP–DGKγ but not intact DGKγ or other proteins (data not shown). Fig. 1C shows that GFP–DGKγ had kinase activity as significant as that of intact DGKγ, whereas no activity of DGKγ–GFP was detectable. Therefore, we produced only GFP–DGKα–GFP. GFP–DGKα had reasonable molecular mass.
Distinct Translocation of DGK\(_{\gamma}\) and DGK\(_{\alpha}\) — When GFP-DGK\(_{\gamma}\) and -\(\alpha\) were expressed in CHO-K1 cells, the fluorescence of GFP-DGK\(_{\gamma}\) and GFP-DGK\(_{\alpha}\) was observed throughout the cytoplasm and in the nucleus (Figs. 2 and 3). The expression level of GFP-DGK\(_{\gamma}\) and GFP-DGK\(_{\alpha}\) in the nucleus varied in the cells.

As a result of stimulation with 100 \(\mu\)M ATP, DGK\(_{\gamma}\) in the cytoplasm was translocated to the plasma membrane within 0.5–1 min and returned to the cytoplasm within 5–10 min (Fig. 2, top row). As a result of stimulation with 100 \(\mu\)M arachidonic acid, DGK\(_{\gamma}\) in the cytoplasm was translocated to the plasma membrane within 30 s and returned to cytoplasm within 12 min, which was similar to ATP-induced translocation (Fig. 2, middle row). However, these stimuli exerted no effect on DGK\(_{\alpha}\) in the nucleus. Activation by 1 \(\mu\)M TPA induced obvious translocation of DGK\(_{\gamma}\) from the cytoplasm to the membrane (Fig. 2, bottom row). The TPA-induced translocation began within 30 s and remained on the plasma membrane for at least 60 min after the treatment. The TPA-induced translocation was not inhibited by staurosporine, an inhibitor for protein kinases (data not shown).

Target Site of GFP-DGK\(_{\alpha}\) on Stimulation with Arachidonic Acid — To identify the intracellular compartment in which GFP-DGK\(_{\alpha}\) accumulated in response to arachidonic acid, the Golgi network was visualized by Texas red-conjugated wheat germ agglutinin in the CHO-K1 cells expressing GFP-DGK\(_{\alpha}\) after arachidonic acid treatment. Intense GFP fluorescence was present on the plasma membrane and perinuclear area (Fig. 4, left). Fluorescence of Texas red was present around the nucleus, indicating the Golgi network and also the plasma membrane due to glycosylated transmembrane proteins (Fig. 4, center). An overlapping image shows that the fluorescence of Texas-red and of GFP were co-localized in the perinuclear region (Fig. 4, right).

Importance of C1A Domain in the TPA-induced Translocation of DGK\(_{\gamma}\) — Two mutants of DGK\(_{\gamma}\) fused with GFP were produced. In one mutant, Cys-285 in the C1A region was replaced by Gly (C1A mutant), and in the other mutant, Cys-348 in the C1B region was substituted with Gly (C1B mutant). Both
with faint fluorescence in the nucleus (Fig. 5). TPA at 1 µM induced fluorescence was present throughout the cytoplasm with slight green fluorescence and bright green fluorescence in locations were compared. To distinguish DGK from the cytoplasm to the plasma membrane, similar to that before the stimulation within 12 min. Bar, 10 µM. Second row, arachidonic acid (AA) at 100 µM also induced reversible translocation of GFP-DGK from the cytoplasm to the plasma membrane, similar to the ATP-induced one. Bar, 10 µM. Third row, TPA at 1 µM induced irreversible translocation of GFP-DGK from the cytoplasm to the plasma membrane. Bar, 10 µM. Video is available (Video 1).

FIG. 2. Translocation of GFP-DGKγ. Top row, application of 100 µM ATP induced a translocation of GFP-DGKγ from the cytoplasm to the plasma membrane. The translocation was observed within 1 min after the stimulation. Thereafter, the GFP-DGKγ was gradually re-translocated from the membrane to the cytoplasm and was mostly restored to a state similar to that before the stimulation within 12 min. Bar, 10 µM. Second row, application of 100 µM ATP, PKCγ was translocated from the cytoplasm to the plasma membrane, similar to the ATP-induced one. Bar, 10 µM. Second row, significant translocation of DGKγ by arachidonic acid (AA) at 100 µM was detected at 30 s. Subsequently, dotty fluorescence accumulated in the nucleus, and intense fluorescence was detected near the nucleus at 12 min. Bar, 10 µM. Third row, TPA exerted no effects on the localization of GFP-DGKγ in either the cytoplasm or the nucleus. Bar, 10 µM.

FIG. 3. Translocation of GFP-DGKα. Top row, application of 100 µM ATP induced a translocation of GFP-DGKα from the cytoplasm to the plasma membrane. The translocation was observed within 30 s after the stimulation and quickly re-translocated within 1 min. The fluorescence of GFP-DGKα in the nucleoplasm was not changed in response to ATP. Bar, 10 µM. Second row, significant translocation of DGKα by arachidonic acid (AA) at 100 µM was detected at 30 s. Subsequently, dotty fluorescence accumulated in the nucleus, and intense fluorescence was detected near the nucleus at 12 min. Bar, 10 µM. Third row, TPA exerted no effects on the localization of GFP-DGKα in either the cytoplasm or the nucleus. Bar, 10 µM.

Fig. 6 showed the CHO-K1 cells expressing PKCγ and DGKγ, respectively. Intense fluorescence of PKCγ-GFP was observed throughout the cytoplasm with faint fluorescent in the nucleus. As a result of stimulation with 100 µM ATP, PKCγ was translocated from the cytoplasm to the plasma membrane at 10 s after the stimulation, and translocation was most significant at 30 s. Then, PKCγ was re-translocated within 1 min. In contrast to PKCγ, the translocation of DGKγ was initiated around 30 s after the stimulation and was seen most significantly at 2 min. Finally, DGKγ was re-translocated at 5 min. However, there was no significant difference in the time course of ATP-induced translocations of GFP-DGKα and PKCγ-GFP (data not shown).

Comparison of the ATP-induced Translocation of DGKγ and PKCγ—For the purpose of clarifying spatial and temporal correlations between DGKγ and PKCγ, their ATP-induced translocations were compared. To distinguish DGKγ and PKCγ, both cDNA of PKCγ-GFP and variant BFP were simultaneously transfected into the CHO-K1 cells and spread into a glass-bottomed dish together with the CHO-K1 cells that were separately transfected with GFP-DGKγ alone. Accordingly, blue with slight green fluorescence and bright green fluorescence in

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Functional Correlation between DGKγ and PKCγ—Effects of co-expression of DGKγ and of a DGK inhibitor on the translocation of PKCγ-GFP were investigated. Fig. 7 shows the translocation of PKCγ-GFP in different conditions. In normal condi-

mutants showed unique localization when expressed in CHO-K1 cells. Unlike intact DGKγ, in almost of the cells, intense fluorescence was present throughout the cytoplasm with faint fluorescence in the nucleus (Fig. 5). TPA at 1 µM induced irreversible translocation of the CIA mutant similar to that of intact DGKγ. In contrast, the CIA mutant was not translocated in response to TPA.

Comparison of the ATP-induced Translocation of DGKγ and PKCγ—For the purpose of clarifying spatial and temporal correlations between DGKγ and PKCγ, their ATP-induced translocations were compared. To distinguish DGKγ and PKCγ, both cDNA of PKCγ-GFP and variant BFP were simultaneously transfected into the CHO-K1 cells and spread into a glass-bottomed dish together with the CHO-K1 cells that were separately transfected with GFP-DGKγ alone. Accordingly, blue with slight green fluorescence and bright green fluorescence in
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Fig. 5. Effects of TPA on the translocation of DGKγ mutants. CHO-K1 cells expressing C1A or C1B mutant were stimulated with 1 μM TPA. Significant translocation of C1B mutant was observed, whereas C1A mutant showed no translocation. Bars, 10 μm.

Fig. 6. Comparison of ATP-induced translocations of GFP-DGKγ and PKCγ-GFP. Merged image of BFP and GFP fluorescence together with Nomarski image shown at the upper left corner. BFP, which is co-expressed with PKCγ-GFP, was used as a marker for detecting cells expressing PKCγ-GFP. Two cells at the upper left corner show blue fluorescent images, expressing PKC-GFPγ, whereas a rather bigger cell at the lower right corner possesses GFP-DGKγ. PKC-GFPγ was translocated by 100 μM ATP within 10 s and was restored within 1 min. On the other hand, the ATP-induced translocation of GFP-DGKγ occurred at 30 s followed by a maximal translocation at 2 min. Subsequently, GFP-DGKγ was re-translocated from the plasma membrane to the cytoplasm. Bars, 10 μm. Video is available (Video 2).

Although GFP is a useful tool as a marker protein, it should be verified that each GFP fusion protein has the same biological properties as its native protein. Especially, to judge the movement of the fusion protein by GFP fluorescence, it is necessary to confirm that no significant cleavage product of the fusion protein is present. We, therefore, produced two types of fusion proteins, DGKγ-GFP and GFP-DGKγ, and compared their enzymological and immunological properties with native DGKγ. Both DGKγ-GFP and GFP-DGKγ were of appropriate sizes, since the molecular weights of DGKγ and GFP are 88 and 27 kDa, respectively (Fig. 1C). Inhibitors for PKC did not alter the translocation of DGKγ induced by ATP, arachidonic acid, or TPA (data not shown).

DISCUSSION

It has been shown that both PKCγ and DGKγ are abundantly expressed in Purkinje cells (12, 30) and possess not only cysteine-rich regions but also calcium-sensitive domains (2). These findings suggest a functional correlation between this DG-dependent kinase and DG-catalyzing kinase. DGKα has a very similar enzymological character to that of DGKγ but shows glial expression in the brain (11), suggesting that each DGK subtype has a specific function. Thus, we chose DGKα and -γ to study the different functions among many DGK subtypes.
which is a suitable size because DGKα is an 80-kDa protein and has enough kinase activity (data not shown).

To date, we have accumulated knowledge concerning translocations of PKCs in CHO-K1 cells (19, 20, 31). In addition, translocation of DGK by several stimuli such as phorbol ester (21, 22), calcium (23), and also receptor-mediated translocation (24) have so far been reported. Therefore, the effects of TPA (a phorbol ester), calcium ionophore (A23187), and ATP as an agonist of purinergic receptors expressed in CHO-K1 cells (32) on the translocation of GFP-DGKα and GFP-DGKγ were examined in CHO-K1 cells. Moreover, the effect of arachidonic acid was examined based on the finding that fatty acids induce distinct translocation among PKC subtypes (19). Calcium ionophore at 20 μM induced generally similar translocations between GFP-DGKα and GFP-DGKγ. Namely, both DGK fusion proteins in the cytoplasm moved to the plasma membrane at 10–15 s after the ionophore stimulation and were finally restored to the cytoplasm (data not shown). In contrast, ATP, TPA, and arachidonic acid showed distinct effects on the translocations of DGKα and -γ, as described above (Figs. 2 and 3). This is the first report visualizing the subtype-specific translocation of DGKα and -γ in living cells.

Although DGKs have cysteine-rich regions homologous to the C1A and C1B motifs of PKCs, phorbol 12,13-dibutyrate binding in DGKs, unlike PKCs, has never been detected (2, 33). Recently, Hurley et al. (34) compared amino acid sequences of C1 domains of many proteins, including PKCs (α, β, γ, δ, ε, η, θ, ξ, and μ) and DGKs (α, β, γ, δ, ε, and ζ), and divided them into two groups, typical and atypical. C1 domains in the typical group fit the profile for phorbol ester binding, whereas those in the atypical did not. According to Hurley et al. (34), DGK C1 domains have no property to bind DG and TPA except for C1A regions in DGKα and β. Therefore, to elucidate whether the C1A region is responsible for TPA-induced translocation of DGKα, we investigated the translocation of the two mutants. Based on a previous report that mutation on the 17th cysteine in the C1B of PKCδ completely abolished its phorbol ester binding (35), Cys-285 in the C1A region or Cys-348 in the C1B region were replaced by Gly, which corresponds to the 17th cysteine in the C1B region of PKCδ. The C1B mutant showed TPA-induced irreversible translocation, whereas the C1A mutant was not translocated in response to TPA (Fig. 5), indicating that interaction between TPA and the C1A domain of DGKγ resulted in the translocation. Together with the results showing that DGKα does not respond to TPA, these results support Hurley’s classification. Among the C1 domains in Hurley’s typical group, those of PKCs can be further divided into two subgroups based on the data from Irie et al. (36). C1B of all conventional and novel PKCs have a high affinity binding to phorbol ester, whereas all their C1A domains except for the C1A of PKCγ show very low affinity. Since phorbol ester binding of DGKα has not been detected in vitro, the C1A domain of DGKγ is thought to belong to the low affinity group. More interestingly, arachidonic acid induced the translocation of C1A mutant but not the C1B mutant of DGKα (data not shown). These results indicate not only that the loss of TPA-induced translocation observed in the C1A mutant is not due to the loss of translocation ability but also that the domain interacting with arachidonic acid differs from that interacting with TPA. Alternatively, Cys-285 in C1A domain of DGKα may be unnecessary for arachidonic acid binding.

When expressed in the CHO-K1 cells, both GFP-DGKα and GFP-DGKγ were observed in the cytoplasm and the nucleoplasm, although the expression level of the fusion proteins in the nucleoplasm varied from cell to cell. The percentage of cells expressing the fusion protein in the nucleoplasm increased during the culture (data not shown). In contrast, both mutants of DGKγ were detected mainly in the cytoplasm, and a significantly low amount of the mutant was expressed in the nucleus. Since neither mutant showed a significant kinase activity (data not shown), kinase activity may be necessary for expression of DGKα and -α in the nucleoplasm or for their translocation into...
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the nucleoplasm. Alternatively, the different localization of the C1 mutants may be due to loss of interaction with an unknown protein, because it was suggested that the C1 region of DGK is important for protein-protein interaction (2). Furthermore, despite a lack of kinase activity, the mutants showed translocation, indicating that kinase activity is not necessary for the translocation of DGK, as seen in the translocations of PKCs (18, 20).

It is also noteworthy that GFP-DGKγ, but not GFP-DGKα, showed a similar translocation to that of PKCγ-GFP. For example, PKCγ-GFP and GFP-DGKγ were translocated to the plasma membrane reversibly by arachidonic acid (Fig. 2 and 19), whereas GFP-DGKα was targeted to the Golgi network in addition to the plasma membrane (Fig. 4). TPA caused translocation of both GFP-DGKγ and PKCγ-GFP but not GFP-DGKα. Furthermore, DGKγ and PKCγ showed spatially similar but temporally different translocations after purinergic receptor activation (Fig. 6). This phenomenon may indicate a functional correlation of the two kinases, because PKC is probably translocated to the plasma membrane before DGK is targeted there, so PKC is activated by DG for a long time. In other words, the time lag between the translocation of DGK and PKC may regulate the duration of PKC activation. In fact, co-expression of DGKγ hastens the re-translocation of PKCγ from the membrane to the cytoplasm, and the inhibitor of DGK blocked the re-translocation of PKCγ (Fig. 7), indicating clearly the functional correlation of DGK and PKC. Further experiments, however, are necessary to conclude that the temporal and spatial orchestration of PKCγ and DGKγ occurred in vivo, i.e. in Purkinje cells in which both kinases are abundantly expressed.

In the present study, we could not find any evidence that PKC directly regulates the translocation of DGK, although the direct phosphorylation of DGK by PKC was reported previously. For example, Kanoh et al. (25, 26) report in vitro and in vivo phosphorylation of DGK by PKC, and Nobe et al. (24) also show that phosphorylation of DGK by PKC increased kinase affinity to the micell. Furthermore, Topham et al. (27) indicate that PKCα and -γ-regulated nuclear localization of DGKγ. However, in the present study, treatment with staurosporine, a PKC inhibitor, exerted no effect on the initial localization or on any translocation of the two subtypes of DGKs (data not shown). Further experiments are required to elucidate the regulatory mechanism of DGK by PKC.

In conclusion, we demonstrated that DGK as well as PKC shows subtype-specific translocation and that translocation depends on extracellular signals and contributes to the regulation of DGK activity and its subtype-specific function, although the significance of this study may be limited because of using overexpressed enzymes. Our results suggested that functionally correlated proteins such as PKC and DGK are orchestrated temporally and spatially in the signal transduction mediated via both kinases.

Acknowledgment—We thank Dr. Hideo Kanoh for helpful discussions of our work.

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