The diacylglycerol kinases (DGK) regulate diacylglycerol-based signals by phosphorylating this key lipid intermediate to phosphatidic acid. Here, we have investigated the spatial and temporal regulation of diacylglycerol kinase ζ (DGKζ) in living Jurkat T-cells expressing a muscarinic type I receptor. Using real time confocal videomicroscopy, we show the rapid translocation of a green fluorescent protein-tagged enzyme from the cytosol to the plasma membrane following receptor stimulation. The generation of a panel of truncations, deletions, and point mutations of the enzyme allowed us to examine the requirements of the catalytic domain for full enzymatic activity. Protein kinase C-driven myristoylated alanine-rich C kinase substrate domain phosphorylation and intact zinc fingers are in turn essential for plasma membrane translocation. DGKζ does not translocate to the membrane following stimulation of the endogenous T-cell receptor, and our data demonstrate that the specificity in terms of receptor response is provided by the regulatory motifs present at the C-terminal domain of the protein. This is the first report that shows in vivo DGKζ translocation in response to agonist stimulation and establishes the role of the different domains in enzymatic activity and the selectivity of the response to receptors.

When agonists bind to their cellular receptors, an early signaling event is often the hydrolysis or modification of certain lipids present in cell membranes to produce second messengers. A well known signaling pathway is the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C enzymes to produce diacylglycerol (DAG)1 and soluble phosphatidylinositol 1,4,5-trisphosphate (1). The DAG generated by this mechanism acts as a membrane recruitment signal and activator for a series of C1 domain-containing signaling proteins such as some PKC isoforms (2), UNC-13 (3), RasGRP (4), and chimerins (5). These molecules are key regulators of a broad array of cellular functions including proliferation, differentiation, and/or apoptosis. The correct activation of DAG-regulated enzymes requires signal termination that is provided by the conversion of this lipid to phosphatidic acid (PA) in a reaction catalyzed by diacylglycerol kinase (DGK) (6). As the number of PA-binding proteins continues to grow (7–18), a role for DGK-generated PA must also be considered. Because DGKs can remove a lipid messenger by creating another, the subcellular localization of these enzymes, together with their regulation in response to receptor stimulation, are anticipated to be vital for the correct onset of cellular responses.

To date, nine DGK isoforms have been found in mammalian tissues, and similar genes are present in plants (19), the nematode Caenorhabditis elegans (3, 20), and Drosophila melanogaster (21). Structurally, the DGKs contain at least two N-terminal, zinc finger-like, cysteine-rich domains (CRD) and a conserved catalytic domain. Most mammalian DGKs present other distinct homology domains that have been used to classify them into five subclasses. The presence of different domains and the distinct tissue distribution of DGKs suggest very precise regulation and specific functions for these proteins in different cell types (22).

Most DGKs are cytosolic enzymes, and translocation from the cytosol to other subcellular compartments appears to be a general mechanism for activation of this enzyme family. DGKζ, a type IV DGK, was originally cloned from human endothelial cell cDNA as well as from rat retina and brain (23, 24). Not having the restricted expression pattern described for other isoforms, DGKζ appears much more ubiquitous, and DGKζ RNA is abundant in tissues such as skeletal muscle and lymphoid cells. There are two class IV DGK isoforms (DGKζ and i) that are characterized by a domain homologous to the myristoylated alanine-rich protein kinase C substrate (MARCKS) protein phosphorylation domain, four C-terminal ankyrin repeats, and a PDZ-binding motif. Translocation of DGKζ from the cytosol to the nucleus is proposed to regulate cell growth by modulating nuclear DAG levels (25).

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**The abbreviations used are: DAG, diacylglycerol; PKC, protein kinase C; PA, phosphatidic acid; DGK, diacylglycerol kinase; CRD, cysteine-rich domain; MARCKS, myristoylated alanine-rich C kinase substrate; TCR, T-cell receptor; GFP, green fluorescent protein; EGFP, enhanced GFP; PMSF, phenylmethanesulfonyl fluoride; CT, C-terminal domain; PBS, phosphate-buffered saline.
shown that this enzyme can interact with and regulate either integral or plasma membrane-associated proteins such as the long form leptin receptor (26), syntrophin (27), and RasGRP (28).

Whereas the signaling functions of DGK as a DAG-driven signal attenuator are well characterized (25, 28), the mechanisms by which this enzyme is activated and gains access to its substrate remain undefined. Here, we studied the spatiotemporal regulation of DGK in T-lymphocytes following the stimulation of an ectopically expressed, G-coupled, muscarinic type I receptor known to mimic TCR responses (29, 30). In addition, we examined the requirements of each of the defined structural motifs for both enzymatic activity and translocation. GFP-tagged DGK translocates very rapidly from the cytosol to the plasma membrane in response to the addition of carbachol. Enzymatic activity is not required for movement toward the plasma membrane, whereas phosphorylation of the MARCKS domain is nonessential for enzyme activity but absolutely required for membrane localization. The protein does not translocate following TCR stimulation, indicating that phosphatidic acid-mediated DAG generation and PKC activation are not the only signals required for enzyme translocation. Deletion of the DGK C-terminal domain, which contains four ankyrin repeats and a PDZ-binding sequence, restores membrane translocation following TCR triggering, suggesting that these domains play a restrictive role in the regulation of membrane translocation. All together, these results demonstrate DGK translocation in living cells in response to G protein-coupled signals.
receptors and provide further insight into the role of the protein domains specific to this DGK isoform and those shared by all DGK family members.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP was purchased from Amersham Biosciences, silica gel thin layer chromatography plates (60A LK6D) were from Whatman, and carbachol, orthovanadate, phenylmethylsulfonyl fluoride (PMSF), 1,2-dioctanoyl-sn-glycerol (DiC8), and poly-L-lysine were from Sigma. Leupeptin and aprotinin were from Roche Molecular Biochemicals, and N-Octyl-D-glucopyranoside was from Calbiochem. Anti-GFP monoclonal antibody was purchased from CLONTECH, anti-CD28 antibody (Cat. 555725) was from BD PharMingen, and horseradish peroxidase-conjugated goat anti-mouse IgG was from Dako. Polystyrene 15.0-μm microspheres were from Polysciences Inc. (Warrington, PA; catalog no. 18328).

Plasmids and DNA Constructs—Rat DGK-IV cDNA was a generous gift of K. Goto (Yamagata University School of Medicine, Sendai, Japan). pSREflagDGKIV was excised with EcoRI, and the 3.4-kb fragment encoding the DGK-IV cDNA was subcloned in the pcDNA3Myc vector (pCDNA3MycDGK-IV). For expression experiments, all constructs were subcloned in the pEGFPBos vector in the GFP C terminus. For the MCA construct, pCDNA3MycDGKIV was digested with BstXI/BglII, and the 2.2-kb fragment was subcloned in pEGFPBos. To generate the ankyrin domains deletion (ΔANK), pCMV3MycDGKIV was SacI-digested, blunted, KpnI-digested, and subcloned in pEGFPBos digested with KpnI/SmaI. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). To generate the kinase-dead version of the enzyme, Gly-354 was replaced with Asp as described (25). We designed two mutations in the MARCKS homology domain; in the first we replaced Ser-259, Ser-266, Ser-271, and Ser-272 with Ala (mutant Ser3Ala), and in the second the same residues were replaced with Asp (mutant Ser3Asp). To mutate the CRDs, the first conserved His in each of the two CRDs of the protein, His-98 or His-173, were replaced with Gly either in the wild type protein or in the Ser3Asp mutant. The C-terminal domain (CT) construct, including the four ankyrin repeats and the PDZ-binding motif, was generated by PCR with appropriate primers, which included two restriction sites, SalI and XbaI. The PCR product was subcloned in the pGEM-T easy vector (Promega) and then excised with SalI/XbaI to be subcloned in the expression vector pEFBosCX-HA, which was previously digested with SalI/XbaI.

Cell Lines and Transient Transfections—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM glutamine. For transfection, 60–80% confluent cells were transfected with LipofectAMINE (Invitrogen) ac-

FIG. 2. Phosphorylation of the MARCKS domain Ser residues is required for translocation. A, PKC inhibition prevents carbachol-induced DGKζ translocation. Cells were transfected with GFP-DGKζ; 24 h later they were pretreated for 30 min with 100 nM bisindolylmaleimide or Me2SO before carbachol stimulation for 5 min. The figure shows single confocal frames of cells. B, GFP-DGKζSA and GFP-DGKζSD constructs. In the SA mutant, MARCKS domain residues Ser-259, Ser-266, Ser-271, and Ser-272 were mutated to Ala. In the Ser → Asp mutant, the same Ser residues were mutated to Asp. C, mutations in the MARCKS domain have no effect on enzyme activity. COS-7 cells were transiently transfected with GFP-DGKζSA or GFP-DGKζSD; 24 h later they were plated on poly-L-lysine-coated coverslip chambers in a HEPES-balanced salt solution. Cells were mounted on a 37 °C plate on the confocal microscope and stimulated with 50 μM carbachol. Images correspond to the indicated times after stimulation.
cording to the manufacturer’s instructions. After 24 h cells were harvested, and each sample was divided into two pellets for immunoblot and a DAG activity assay. The J-HM1-2.2 cell line was generated by stable transfection of the human muscarinic subtype 1 receptor in the Jurkat cell line (29). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells (1.2 × 10^7) were transfected by electroporation with 20 μg of DNA using a Gene Pulser (Bio-Rad) at 270 V and a capacitance of 975 microfarads. Cells were immediately transferred to 30 ml of growth medium and assayed 24 h later.

Subcellular Fractionation—Cells were resuspended in an ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.25 mM sucrose) and incubated on ice for 15 min. Cells were lysed by being passed five times through a 30-gauge needle. The lysates were centrifuged at 800 g for 10 min to remove nuclei and cell debris, and the supernatant was collected and centrifuged at 13,000 × g for 60 min at 4 °C. The supernatant (cytosol) and the pellet (membranes) were collected and separated by SDS-PAGE.

Western Blot—Cells were resuspended in lysis buffer (150 mM NaCl, 10 mM NaF, 10 mM NaPO_4 · 12H_2O, 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM PMSF, and 10 μg/ml each aprotinin and leupeptin) and incubated (15 min on ice). After centrifugation (15,000 × g for 15 min at 4 °C), the supernatants were assayed for total protein (DC protein assay, Bio-Rad), and an equivalent amount of protein of each sample was analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose, and the expression of GFP-coupled constructs was determined with an anti-GFP monoclonal antibody and the ECL detection system (Amersham Biosciences).

Diacylglycerol Kinase Activity Assay—The pellet of transfected COS-7 cells was resuspended in lysis buffer (50 mM Tris HCl, pH 7.4, 50 mM NaF, 10 μg/ml each leupeptin and aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate), lysed by sonication, and centrifuged (800 × g, 10 min). The supernatant was removed and assayed for total protein. An equivalent amount of protein from each sample was mixed with DAG as substrate in octyl glucoside, and 32P incorporation into PA was measured. Samples were analyzed by thin layer chromatography using a chloroform, methanol, 4M ammonia (v/v/v) (9:7:2) system. Plates were phosphorimaged in a Molecular Imager Personal FX (Bio-Rad).

Confocal Microscopy Imaging—At 24 h after transfection, J-HM1-2.2 cells were transferred to poly-L-lysine-coated chambered coverslips (LabTek), allowed to attach for at least 15 min at 37 °C, washed, and maintained in HEPES-balanced salt solution. Stimulus was added by replacing the supernatant with a fresh solution containing the appropriate concentration of each stimulant. Cells were imaged with a laser-scanning confocal microscope (TCSS-NT, Leica). A 488-nm krypton-argon laser line was used for excitation, and a 530/30 barrier filter was used for emission. Time series of images were recorded before, during, and after stimulation of the cells.

Stimulation with Antibody-coated Microspheres—Antibodies were adsorbed to microspheres by mixing 0.5 μg of antibody in PBS at 0.5 × 10^6 microspheres in a final volume of 1 ml and incubating (1.5 h at room temperature) it with continuous mixing; 1.5 ml of 1% bovine serum albumin in PBS was then added and mixing continued (30 min). Microspheres were washed three times with PBS and resuspended in PBS for the addition to cells. For stimulation, 10^6 transfected J-HM1-2.2 cells were mixed with antibody-coated beads at a 2:1 cell/bead proportion and plated on chambered coverslips. Time series of images were captured by confocal microscopy.

RESULTS

Translocation of Wild Type and Catalytically Inactive DGKζ in Response to Carbachol—To study the subcellular localization and dynamics of DGKζ in live T-lymphocytes, we linked the cDNA of DGKζ to the EGFP C terminus (Fig. 1A) and expressed this construct (EGFP-DGKζ) in J-HM1-2.2 cells. This is a variant of the Jurkat cell line stably transfected with the human muscarinic type 1 receptor and has been used previously to examine the role of DAG on T-cell responses (29, 30). Transfection of this construct in J-HM1-2.2 and COS-7 cells induced the expression of a protein of the appropriate size with DGK activity in an in vitro assay (Fig. 1B). The transfected EGFP-DGKζ was found in the supernatant after high speed centrifugation of J-HM1-2.2 cells, suggesting a true cytosolic localization of the protein as opposed to localization to vesicular membrane compartments (Fig. 1C). Fluorescent images of live J-HM1-2.2 cells showed that the cytosolic EGFP-DGKζ translocated very rapidly and almost completely to the plasma membrane following carbachol stimulation (Fig. 1E, top). By ~90 s the majority of the protein was located at the plasma membrane, where it remained for at least 45 min after stimulation (see supplemental material, Video 1). We next analyzed whether membrane translocation required enzyme activity. To generate a DGKζ kinase-dead mutant, Gly-354 was mutated to Asp. This residue, which is found within the GGDG motif that is conserved in all DGK family members, has been shown to be essential for enzymatic activity (Fig. 1A). Mutation of this residue in the conserved cluster of the D. melanogaster DGKs induces retinal degeneration (21). Mutation of this same residue in human DGKζ has already been described as essential for enzyme activity (25). The loss of enzymatic activity by mutation of this conserved Gly is therefore a well established observation. As expected, expression of the EGFP-DGKζ (kinase-dead) mutant rendered a protein of the same weight average M_r as the wild type but with impaired activity (Fig. 1D). When examined by confocal videomicroscopy, the catalytically inactive mutant translocated to the membrane with the same kinetics as the wild type protein (Fig. 1E, bottom and supplemental material, Video 2).

Phosphorylation of the DGKζ MARCKS Domain Serine Residues Is Essential for Membrane Translocation—Type IV DGKs bear several domains with a potential role as regulators of enzyme activity and/or localization. One is a highly conserved sequence between the CRDs and the catalytic domain with a similarity to the basic effector domain in MARCKS. This sequence, when examined by confocal videomicroscopy, the catalytically inactive mutant translocated to the membrane with the same kinetics as the wild type protein (Fig. 1E, bottom and supplemental material, Video 2).

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brane. Phosphorylation of the MARCKS domain is not the only necessary signal, however, as the EGFP-DGKζ (Ser \to Asp) mutant requires receptor stimulation for membrane translocation.

**Intact CRD1 and CRD2 Are Required for Enzymatic Activity and Carbachol-induced Translocation of DGKζ**—All DGKs have at least two CRDs of unknown function. Studies (31) of other DGK isoforms suggested that mutants lacking both CRDs are still active and have enzymatic properties similar to those of the intact enzyme. To study the role of DGKζ CRDs, a GFP-fused mutant was generated in which the entire N-terminal domain containing the two CRDs was deleted (DGKζ MCA; Fig. 3A). This mutant showed unique localization when expressed in J-HM1-2.2 cells; unlike intact DGKζ, intense fluorescence was observed mainly at the nucleus with faint cytosolic fluorescence (Fig. 3A). This mutant showed unique localization when expressed in J-HM1-2.2 cells; unlike intact DGKζ, intense fluorescence was observed mainly at the nucleus with faint cytosolic fluorescence (Fig. 3A). When examined, carbachol stimulation of the cells produced no changes in the localization of the truncated enzyme (not shown). To better study the role of the CRDs, we next generated two point mutants in which the first His of each CRD, which is involved in zinc coordination, was replaced with Gly (CRD1 H98G and CRD2 H173G) (Fig. 4A). These mutations were introduced in both the wild type protein and the MARCKS domain Ser \to Asp mutant. All mutant proteins were the same size as the wild type and were inactive in an in vitro assay (Fig. 4B). When examined, the four mutants showed cytosolic localization (Fig. 4C) similar to that found for the wild type protein. None of the mutants translocated to the plasma membrane in response to carbachol addition, however, and they remained cytosolic for the period of the stimulation procedure (Fig. 4C). This indicates that both CRDs in DGKζ are essential for PA production and membrane translocation.

**The Ankyrin Repeats and PDZ-binding Motif Are Not Essential for Enzyme Activity and Membrane Translocation**—Mammalian type IV DGKs are most closely related to the Drosophila DGK2, which is derived from the \textit{rdgA} gene. DGK2 mutants bearing a truncation of the C-terminal ankyrin repeat show retinal degeneration, suggesting that this domain is essential for enzyme function in the retina (21). Using double hybrid technology, DGKζ was found to interact with the leptin receptor through the C-terminal domain containing the ankyrin repeats (26). This C-terminal portion of the enzyme also contains a PDZ-binding motif that interacts with syntrophins (27). Given the apparent relevance of these domains on functions attributed to type IV DGKs, two new EGFP-DGKζ truncation mutants were generated and used to investigate enzyme translocation following receptor stimulation. In one, the four amino acids of the PDZ-binding domain were eliminated. Another
mutant was constructed by deleting the entire C-terminal region of the protein, which contains both the ankyrin repeats and the PDZ-binding motif (Fig. 5A). When analyzed, both proteins were of the appropriate size, and each phosphorylated DAG in an in vitro assay, indicating that these domains are not required for enzyme activity (Fig. 5B). Our study of protein dynamics showed that neither the deletion of the entire C-terminal domain nor the PDZ-binding motif alone had any effect on protein translocation in response to carbachol (Fig. 5C).

The DGKζ C Terminus Contains a Negative Regulatory Domain—We next examined whether, in addition to carbachol, a more physiological stimulus could also induce DGKζ translocation to the plasma membrane. For this, we examined GFP-DGKζ redistribution after stimulation with anti-CD3/anti-CD28 antibody-coated microspheres. GFP-PKCζ was used as a positive control of relocalization to the contact area (32). Whereas PKCζ translocated consistently to the plasma membrane region at which contact with the antibody-coated beads takes place (not shown), wild type GFP-DGKζ did not translocate to the membrane under the same conditions (Fig. 6A). The DGKζ Ser → Asp MARCKS mutant (not shown) also failed to relocate in response to CD3-CD28 stimulation. When the C-terminal deletion mutant GFP-DGKζΔANK was tested, however, we observed rapid translocation to the membrane, which was restricted to the area of contact with the stimulating beads (Fig. 6B). Similar results were obtained when anti-CD3-coated beads were used. This shows that, in DGKζ, the C-terminal region of the protein has a restrictive regulatory role that cannot be circumvented by the signals initiated after TCR triggering.

Expression of the DGKζ C Terminus Prevents Translocation of the Wild Type Protein—The DGKζ C-terminal deletion mutant, lacking the PDZ-binding motif and the four ankyrin repeats, relocalizes to the TCR synapse following stimulation. This indicates the presence of some type of regulatory restriction imposed by the DGKζ C-terminal domain. The presence of ankyrin domains and a PDZ-binding sequence suggests the possibility of a protein-protein interaction through this domain. We next studied the effect of DGKζ CT expression in DGKζ translocation kinetics after carbachol stimulation. As demonstrated, carbachol addition induced rapid translocation of DGKζ, which was found at the membrane as soon as 60 s after receptor stimulation (Fig. 7B, top). When DGKζ was co-expressed with the plasmid encoding the CT domain, however, translocation of the enzyme was fully prevented, and cytosolic localization was still found for the protein 2 min after stimulation (Fig. 7B, bottom). These results further point to a role for the DGKζ CT domain in the regulation of protein translocation.

DISCUSSION
DGKζ mRNA is abundantly expressed in lymphoid tissues, although direct regulation of this enzyme following receptor
stimulation has not been examined previously in T-lymphocytes. Here, we used a Jurkat T-cell line stably transfected with a muscarinic type I receptor; this allows examination of DAG-regulated signals generated by the addition of a soluble ligand. Using this model, we show that DGK\textsubscript{H9256} is located in the cytosol of living T-cells and that it translocates very rapidly to the plasma membrane in response to receptor stimulation. Other DGK isoforms have been shown previously to translocate to the membrane in response to agonists, and the extent and duration of this translocation was dependent both on the isoform examined and the agonist used. With this cell line, we demonstrated previously (33) rapid, transient translocation of DGK\textsubscript{H9251} following triggering of both muscarinic receptors and TCR. In COS-7 cells, this isoform translocates irreversibly to the plasma membrane in response to arachidonate and transiently when cells are ATP-treated (34). DGK\textsubscript{H9253} relocates at the plasma membrane in a sustained manner in response to TPA and transiently in response to arachidonic acid and ATP (34). Translocation to the membrane, where these enzymes gain access to their substrate, thus appears to be a general mechanism of DGK regulation as it is for other lipid-modulating enzymes. As we showed previously for the \(\alpha\)-isoform (33), kinase activity is not necessary for DGK\textsubscript{\(\zeta\)} translocation to the plasma membrane. Enzymatic activity of DGK\(\alpha\), although not required for translocation, regulates enzyme stabilization at the membrane (33). Translocation of the wild type \(\alpha\)-isozyme is a very rapid and transient event, whereas a catalytically inactive mutant remains at the membrane for a longer period of time. This suggests the existence of feedback regulation based on PA generation; we showed that the EF-hand motif, characteristic of type I enzymes, is responsible for this control (33). Subtype IV DGKs are apparently not subject to this type of regulation as might be predicted because of the lack of EF-hand domains. The possibility of PA-driven feedback regulation for DGK\textsubscript{\(\zeta\)} nonetheless cannot be ruled out, because we observe that some agonists that cause transient, partial translocation of the wild type enzyme induce a more sustained, almost complete translocation of the catalytically inactive mutant.\(^2\)

\(^2\)T. Santos, S. Carrasco, D. R. Jones, I. Mérida, and A. Eguinoa, unpublished results.

**FIG. 7.** DGK\textsubscript{\(\zeta\)} C-terminal domain expression prevents carbachol-induced translocation of the wild type enzyme. *A*, the HA-DGK\textsubscript{CT} construct encoded the C-terminal domain of DGK\(\zeta\) containing the ankyrin repeats and the PDZ-binding sequence fused to a hemagglutinin (HA) epitope. *B*, time-lapse confocal imaging of the subcellular localization of GFP-DGK\textsubscript{wt} alone (top) or cotransfected with HA-DGK\textsubscript{CT} (bottom). J-HM1-2.2 cells were transiently transfected with the indicated constructs; 24 h later they were plated on poly-L-lysine-coated chambers and mounted on a 37 \(^\circ\)C plate, and the subcellular localization of GFP-DGK\textsubscript{wt} was determined after carbachol stimulation. *C*, lysates of J-HM1-2.2 cells were analyzed for GFP-DGK\textsubscript{wt} or HA-DGK\textsubscript{CT} expression using anti-GFP (left) or anti-HA (right) antibodies. WT, wild type.
DGKζ localizes both in the cytosol and the cell nuclei in transfected COS-7 cells, and translocation between these two fractions is proposed to be regulated by PKC-dependent phosphorylation of the MARCKS domain (23–25). We did not observe nuclear localization in the J-HM1-2.2 cell subline or in the parental Jurkat T-cells at any stage of the cell cycle. Only deletion of the CRD induced the expression of a truncated mutant with nuclear localization, probably due to greater exposure of the nuclear localization sequence in the MARCKS domain (25). DGKζ thus appears to be a cytosolic enzyme in lymphoid cells, and nuclear localization may be associated with some specific function in the nucleus of certain tissues. A role for DGKζ at the plasma membrane is reinforced by the fact that DGKζ has been found to be associated with transmembrane proteins such as the leptin receptor, with cytosolic proteins that translocate to the plasma membrane such as RasGRP, or with plasma membrane components such as syntrophins (26–28).

DGKζ contains several regulatory domains, and here we analyzed the structural requirements for lipid kinase activity of the enzyme. The results indicate that the domains located at the C-terminal end of the protein are not involved in DAG phosphorylation (Table I). Ankyrin repeats and PDZ-binding sequences are well known protein-protein interaction motifs (35, 36); hence, it is not surprising that their role is unrelated to the regulation of enzyme activity. A model for DGKζ activation by the muscarinic type-1 receptor is shown in Fig. 8. In the absence of ligand, DGKζ is found in the cytosol. Following receptor stimulation, DGKζ binds to a scaffolding protein through the C-terminal domain; this in turn favors phosphorylation of the MARCKS domain and subsequent membrane localization of the protein through the DGKζ membrane-binding domains. PtdIns-4,5-P₂, phosphatidylinositol 4,5-bisphosphate.

**TABLE I**

A summary of the effects of DGKζ domain mutations on receptor-induced translocation and enzyme activity

| Translocation | Activity |
|---------------|----------|
| DGKζ-wt      | +        |
| DGKζ-Kd      | +        |
| DGKζ-SA      | -        |
| DGKζ-SD      | +        |
| DGKζΔAnk     | +        |
| DGKζΔPDZ     | +        |
| DGKζ-MCA     | -        |
| DGKζ-CRD1    | -        |
| DGKζ-CRD2    | -        |

FIG. 8. A model for DGKζ activation by the muscarinic type-1 receptor. In the absence of ligand, DGKζ is found in the cytosol. Following receptor stimulation, DGKζ binds to a scaffolding protein through the C-terminal domain; this in turn favors phosphorylation of the MARCKS domain and subsequent membrane localization of the protein through the DGKζ membrane-binding domains. PtdIns-4,5-P₂, phosphatidylinositol 4,5-bisphosphate.
to the enzymatic activity of DGKζ. PKC-dependent phosphorylation of MARCKS is also involved in the regulation of protein interaction with lipid membranes and/or cytoskeleton components (37, 38). Accordingly, phosphorylation of this motif does not appear to be involved in PA production by DGKζ, as mutation of the critical residues in the MARCKS-like domain produces a fully catalytically active enzyme. The only DGKζ requirements for DAG phosphorylation appear to be an intact CRD and the catalytic domain. The CRDs of most DGKs, including the ζ isofrom, lack the critical residues required for interaction with phorbol esters (39); direct interaction of these conserved domains with DAG thus seems unlikely. The fact that CRD mutations produce an inactive enzyme may reflect a structural change in this domain that prevents correct interaction of the protein with the membranes, although not necessarily with the substrate. Intact CRDs would thus be essential for DGKζ interaction with biological membranes and, in the in vitro assays, with substrate-containing micelles.

This is the first report of DGKζ translocation in response to receptor triggering in living cells. Our results suggest that distinct sequential events are necessary for DGKζ to relocate from the cytosol to the plasma membrane. The generation of mutants at the MARCKS domain indicates that phosphorylation of the Ser residues in the basic cluster domain with homology to Marcks is essential for translocation (Table 1). This domain has been found in proteins that shuttle between the cytosol and the plasma membrane where PKC-dependent phosphorylation promotes cytosolic localization by introducing charges into the basic cluster and releasing cytoskeletal constraints (37, 38). For DGKζ, phosphorylation of the MARCKS domain has the opposite effect, because it is required for membrane association. The MARCKS domain is also found in proteins that interact with the actin cytoskeleton, and phosphorylation was shown to release cytoskeletal constraints (38). We do not know the exact consequence of modulating the charges in the DGKζ MARCKS domain, but we hypothesize that this phosphorylation allows correct protein interaction with the plasma membrane either by releasing some cytoskeletal restriction or inducing a conformational change.

A mutant in which MARCKS domain Ser residues are replaced by amino acids that mimic phosphorylation remains cytosolic and requires receptor stimulation for translocation. This demonstrates that, albeit necessary, the phosphorylation of Ser residues in the DGKζ MARCKS domain is not sufficient to induce membrane localization of the enzyme. Furthermore, the stimulation of T-cells via the endogenous TCR does not promote membrane localization of DGKζ. This may indicate that DGKζ is an enzyme regulated exclusively in response to G protein-coupled receptors and not after the triggering of tyrosine kinase-coupled receptors. The DGKζ C-terminal deletion mutant, which lacks the PDZ-binding motif and the four ankyrin repeats, is nonetheless able to relocalize to the TCR synapse following stimulation. In addition, the expression of the C-terminal domain of DGKζ prevents translocation of the wild-type protein in response to carbachol. This led us to favor a hypothesis in which membrane localization of DGKζ would be regulated by the interaction of these domains with a yet to be characterized protein. In this context, the activation of the type 1 muscarinic receptor promotes the interaction of PLCβ isoforms with multi-PDZ-containing adaptors that induce the scaffolding and membrane localization of other signaling molecules such as NHERF, SHANK, or E3KARP (40). We thus propose a model (Fig. 8) in which cytosolic DGKζ in unstimulated cells would exhibit a folded conformation with the C-terminal end, preventing protein interaction with the membrane. Receptor stimulation would promote DGKζ interaction with a hypothetical scaffolding protein, which would allow PKC-driven phosphorylation of the MARCKS domain and, finally, enzyme accessibility to the membrane. The C-terminal domain of DGKζ is thus not strictly necessary for plasma membrane translocation but is in turn essential for receptor selectivity. Deletion of the C-terminal region would eliminate this receptor-dependent restriction. Accordingly, only the truncated enzyme in which the C-terminal domain is deleted translocates in response to TCR-dependent signals.

The targeting of signaling molecules to specific cellular components is determined not only by signals generated by receptor occupancy, but also by features intrinsic to the proteins. These mechanisms allow a very specific interaction between enzymes and substrates and finally determine the onset of different cellular responses. The detailed analysis presented here of the roles of the DGKζ domains in enzyme activity and membrane translocation in living cells demonstrate that this enzyme is subject to tight regulation following receptor triggering. As we show, whereas membrane localization of DGKζ requires intact CRD and is highly dependent on DAG-regulated signals, the C-terminal domain introduces a means for selectivity of DGKζ activation among receptors.

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Dynamics of Diacylglycerol Kinase ζ Translocation in Living T-cells: STUDY OF THE STRUCTURAL DOMAIN REQUIREMENTS FOR TRANSLOCATION AND ACTIVITY

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