Recent studies have highlighted the existence of discrete microdomains at the cell surface that are distinct from caveolae. The function of these microdomains remains unknown. However, recent evidence suggests that they may participate in a subset of transmembrane signaling events. In hematopoietic cells, these low density Triton-insoluble (LDTI) microdomains (also called caveolae-related domains) are dramatically enriched in signaling molecules, such as cell surface receptors (CD4 and CD55), Src family tyrosine kinases (Lyn, Lck, Hck, and Fyn), heterotrimeric G proteins, and gangliosides (GM₁ and GM₃). Human T lymphocytes have become a well established model system for studying the processing of phorbol ester-induced down-regulation of CD4. Here, we present evidence that phorbol 12-myristate 13-acetate (PMA)-induced down-regulation of the cell surface pool of CD4 occurs within the LDTI microdomains of T cells. Localization of CD4 in LDTI microdomains was confirmed by immunoelectron microscopy. PMA-induced disruption of the CD4-Lck complex was rapid (within 5 min), and this disruption occurred within LDTI microdomains. Because PMA is an activator of protein kinase C (PKC), we next evaluated the possible roles of different PKC isoforms in this process. Our results indicate that PMA induced the rapid translocation of cytosolic PKCs to LDTI microdomains. We identified PKCα as the major isoform involved in this translocation event. Taken together, our results support the hypothesis that LDTI microdomains represent a functionally important plasma membrane compartment in T cells.

Recent studies have highlighted that the plasma membrane is not homogenous but instead consists of a variety of discrete microdomains (1–4). CD4 is an 55–59-kDa membrane glycoprotein expressed on the surface of T helper cells and to a lesser extent on monocytes/macrophages. It is the human receptor for HIV, whose binding allows the entrance of HIV to target cells (5).

CD4 is considered to be the TCR co-receptor in T-cell activation and thymic selection (6). In this regard, it binds to major histocompatibility complex class II epitopes, thereby strengthening cell-to-cell contact and TCR-major histocompatibility complex formation (7). In addition, CD4 itself mediates intracellular signals that influence TCR-CD3 complex formation and augments the cellular response (8). It is well established that this effect is due to CD4 interaction with Lck (9), a member of the Src family of tyrosine kinases. Lck is anchored to the cytoplasmic side of the membrane via lipid modifications and is involved in CD3 γ, ε chain phosphorylation (10).

Lck also plays a role in regulating the endocytic properties of CD4, thereby controlling the cellular distribution of this co-receptor (11). Treatment of T cells with phorbol esters such as PMA is one of the methods used to mimic modulation of CD4 that occurs during the antigen encounter (12). Upon activation, cytoplasmatic serine residues of CD4 are phosphorylated most likely via an isoform or isoforms of PKC (13, 14).

PKCs are a family of at least 12 isoenzymes, whose 8 isotypes (α, β₁, β₂, δ, ε, η, ζ, and θ) are expressed in T cells (15, 16) and are responsible for CD4 down-modulation by endocytosis through clathrin-coated pits (17). In this regard, CD4 lacking intracellular serine residues (a possible target for PKC phosphorylation) is not down-regulated by phorbol esters (18, 19). CD4 expression returns to normal only upon prolonged PMA stimulation, which exhausts cytoplasmic stores of PKC (20).

PKCs differ in substrate specificity, cofactor requirements, tissue and cellular distributions, subcellular localizations (21), and regulatory mechanisms (22) that lead to their differential translocation in the cell following stimulation (23). In T lymphocytes, PMA activation induces PKCα, β₁, and β₂ redistribution from a diffusely cytoplasmatic localization to a discrete focal distribution around the plasma membrane and nucleus (24).

Little is known about the specific role played by each of the

1 The abbreviations used are: HIV, human immunodeficiency virus; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; LDTI, low density Triton-insoluble; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; FITC, fluorescein isothiocyanate.
multiple isoforms present in a given cell type, although the involvement of PKC\(\theta\) in T cell activation following stimulation by antigen presenting cells has been recently described. In this regard PKC\(\theta\) was spatially restricted to the site of contact, where receptors on the T cells encounter their counterparts on antigen presenting cells (25).

The subcellular localization of a given PKC isoform may represent an important clue in determining the specific function of a given PKC isoform. Electron microscopy and plasma membrane fractionation in the absence of detergent have demonstrated that the PKC\(\alpha\) isoform is enriched with caveolae (26, 27). The co-existence in the same cell of caveolae and membrane “rafts” enriched in glycolipids has been described (28–30) as well as membrane rafts in cells devoid of morphologically recognizable caveolae such as neuronal and hematopoietic cells (2, 31, 32). These rafts have also been termed LDTI or “caveola-related domains.”

We recently purified and characterized these low density Triton-insoluble (LDTI) microdomains from hematopoietic cells. These LDTI microdomains morphologically and biochemically resemble raft domains and were highly enriched in signal transducing molecules such as a subset of cell surface receptors, Src family tyrosine kinases, heterotrimeric G proteins, and gangliosides (GM3) (2, 4).

New insights into the dynamic clustering of raft domains have highlighted their potential role as a starting point for many membrane-linked processes, including certain transmembrane signaling events (30). However, in the hematopoietic system, the exact function of these domains has not yet been established. To address this issue, we have analyzed the well described process of CD4 internalization in human T cells that is induced by activation with PMA.

Here, we show that this process takes place within LDTI microdomains. More specifically, we demonstrate that upon PMA treatment (i) the CD4-Lck complex is disrupted within LDTI domains; (ii) CD4 shifts from LDTI domains to a Triton-insoluble particulate fraction in a time-dependent manner, whereas Lck remains within the LDTI domain; and (iii) many PKC isoforms are activated and translocated from the cytosol to LDTI domains but at different rates. In addition, PKC\(\alpha\) appears to be the most abundant isoform within LDTI domains, suggesting that it plays an important role in this process.

EXPERIMENTAL PROCEDURES

Materials—Anti-CD4 monoclonal antibody used for Western blotting was purchased from Novocastra (Newcastle-upon-Tyne, UK). Anti-CD4 monoclonal antibody used for immunoprecipitation was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-Lck, anti-PKC\(b_1, b_2, d\), and \(\eta\) polyclonal antibodies were purchased from Santa Cruz Biotechnologies; anti-Fyn, anti-PKC\(\alpha\), and anti-PKC\(\delta\) monoclonal antibodies were purchased from Transduction Laboratories, Inc. (Lexington, KY); anti-PKC\(\theta\) was purchased from Calbiochem (La Jolla, CA). Anti-monoclonal or polyclonal secondary antibodies horseradish peroxidase-conjugated were purchased from Bio-Rad. Biotin-NHS was purchased from Calbiochem, and streptavidin-horseradish peroxidase conjugated was from Pierce. Go 6976 and Go 6850 were obtained from Calbiochem. PKC\(\alpha\) purified enzyme was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). PMA and Histone H1, Type IIIS, were obtained from Sigma. Anti-\(\gamma\) antibody was a generous gift of Dr. Tommaso Costa.

Isolation of Cell Membranes (M) and Detection of Surface Proteins—An established protocol was followed to prepare total cell membranes (or particulate), enriched in plasma membrane, from human peripheral lymphocytes, with some modifications (65). Briefly, \(1 \times 10^9\) lymphocytes were surface labeled by incubation with biotin-NHS (0.5 mg/ml) for 30 min at 4 °C. After washing with ice-cold serum-free Dulbecco’s modified Eagle’s medium and then with PBS, cells were incubated with PMA (100 ng/ml) at 37 °C for the indicated times in warm RPMI medium. After three washes with ice-cold PBS, cells were harvested, washed, and resuspended in 2 ml of lysis buffer (20 mM Tris, pH 8.0, 2 mM EGTA containing 0.1 mg/ml phenylmethanesulfonyl fluoride, 2 \(\mu\)g/ml aprotinin, 2 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin A). The homogenate was then centrifuged at 1,000 \(\times\) \(g\) for 5 min at 4 °C, and then the supernatant was centrifuged at 2,000 \(\times\) \(g\) for 5 min to remove nuclear debris. The resulting supernatant was centrifuged at 60,000 rpm for 30 min to produce a nucleous-free membrane fraction (pellet) and a cytosol fraction (supernatant). Proteins were quantified by Peterson method (33) and then resolved by SDS-PAGE, transferred to a 0.22-\(\mu\)m nitrocellulose filter (Amersham Life Science, Buckinghamshire, UK), and blocked with 4% nonfat milk and 1% BSA in TBS (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20). The membrane was then incubated with streptavidin-horseradish peroxidase conjugated at 1:30,000 dilution in TBS for 1 h at room temperature. The membrane was washed six times with TBS and incubated with SuperSignal chemiluminescence ULTRA (Pierce) according to the manufacturer’s instructions. Reactive proteins were detected by autoradiography on Kodak T-Mat G/R film (Eastman Kodak, Rochester, NY).

Western Blotting Analysis and Immunoprecipitation—LDTI, total cell lysates or total membrane proteins were resolved by 8% SDS-PAGE under reducing conditions and transferred to nitrocellulose filter. The blots were blocked using 5% nonfat milk in TBS for 1 h at room temperature, followed by incubation with anti-Lck polyclonal (dilution 1:100), anti-Fyn monoclonal (dilution 1:400), anti-Gi polyclonal (dilution 1:2000), anti CD4 monoclonal (dilution 1:200) or anti-PKC-specific antibodies in TBS for 1 h at room temperature. PKCa was 1:4000 diluted, PKC\(\theta\) was 1:250 diluted, and the other PKC isoforms were 1:100 diluted. After washing with TBS, each filter was incubated with the appropriate secondary antibody-horseradish peroxidase conjugated at 1:3000 dilution for 1 h at room temperature. Reactive proteins were detected as described above. For immunoprecipitation experiments LDTI domains or membrane fraction from biotin-labeled lymphocytes were prepared as described above. 1–5 \(\mu\)g of protein were precleared with 30 \(\mu\)l of a 50% slurry protein A/G-agarose (Pierce) and 1 \(\mu\)g of nonimmune serum in 0.5 ml of lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.1 mg/ml aprotinin, 2 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin A) for 1 h at 4 °C. CD4 antibody (0.5 \(\mu\)g/ml) or PKC\(\alpha\) (1 \(\mu\)g/ml) or PKC\(\theta\) (1 \(\mu\)g/ml) or PKC\(b\) (1 \(\mu\)g/ml) were then added to the sample and kept overnight at 4 °C, followed by incubation with prewashed beads (40 \(\mu\)l), 1 h at 4 °C. The beads were spun down and washed four times with lysis buffer, resuspended in 30 \(\mu\)l of SDS-PAGE sample buffer under reducing conditions, boiled, and spun down. The supernatant was loaded on a 8% SDS-PAGE. Cell surface CD4 was detected by blotting the filter with streptavidin-horseradish peroxidase conjugated under the conditions described for detection of surface proteins. Using this procedure only one band corresponding to 55-kDa CD4 was detectable.

Kinase Assay—1 \(\mu\)g of LDTI complexes isolated from PMA-treated cells were resuspended in 20 \(\mu\)l of kinase reaction buffer (20 mM Hepes, pH 7.4, 5 mM MgCl\(_2\), and 1 mM MnCl\(_2\)) supplemented with Go 6976 (10 \(\mu\)M) and 5 \(\mu\)Ci of \([\gamma-32P]\)ATP for 10 min at room temperature. The diaphosphatidylerosine inactivated PKC\(\alpha\) was then phosphorylated under reducing conditions. The mixture was boiled, separated on 10% SDS-PAGE that was then dried, and exposed to Kodak XAR film. In other experiments, to test the activity of PKC\(\delta\), 5 \(\mu\)g of Histone type IIIS and 25 \(\mu\)g of PKC\(\delta\) purified enzyme were incubated with 10 \(\mu\)g of phosphatidylinerine in Adb buffer (1 mM sodium orotate, 25 mM \(\beta\)-glycerophosphate, 20 mM MOPS, pH 7.2, 1 mM diithiothreitol, 1 mM Ca\(_2+\), 5 mM MgCl\(_2\)), prior to the addition of 5 \(\mu\)Ci of \([\gamma-32P]\)ATP.
Immunofluorescence Staining—CD4⁺ lymphocytes (1 × 10⁶ in 1 ml of PBS) were incubated with PMA as reported above. PMA-treated and untreated cells were then fixed with acetone/methanol 1:1 (v/v) for 10 min at 4 °C. Cells were then labeled with anti-PKCα, δ, or ε polyclonal antibodies (Santa Cruz Biotechnology) for 1 h at 4 °C. After three washes in PBS, cells were then incubated with FITC-conjugated goat anti-rabbit IgG (Sigma) for 30 min at 4 °C. After washing the three times in PBS, pH 7.4, cells were then incubated for 1 h at 4 °C with anti-Lck monoclonal antibody, followed by 3 washes in PBS and the addition (30 min at 4 °C) of goat anti-mouse IgG (γ-chain-specific) conjugated with Texas Red (Calbiochem Biochem). Cells were finally washed three times in PBS and then mounted upside down onto a glass slide in 5 ml of glycerol/Tris-HCl, pH 9.2. The coverslips were sealed with nail varnish to prevent evaporation and stored at 4 °C before imaging. The images were acquired through a confocal laser scanning microscope (Sarastro 2000, Molecular Dynamics) equipped with a NIKON OPTIPHOT microscope (objective 60/1.4 oil) and an Argon Ion Laser (25 mW output). Simultaneously, the green (FITC) and the red (Texas Red, which reduces greatly overlapping) fluorophores were excited at 488 and 518 nm. Acquisition of single
FITC-stained samples in dual fluorescence scanning configuration did not show contribution of green signal in red. Images were collected at 512 × 512 pixels (0.08 μm/pixel lateral dimension, 0.48 μm/pixel axial dimension). Serial optical sections were assembled in Depth-Coding (Molecular Dynamics) mode. Acquisition and processing were carried out using Image Space software (Molecular Dynamics).

Flow Cytometry Analysis—CD4 expression on untreated and PMA-treated lymphocytes was investigated using monoclonal antibody CD4 (OKT4) fluorescein conjugated (1:20 in PBS, 1% BSA for 30 min at 4 °C). After washing with PBS/BSA cells were fixed with 1% formaldehyde in PBS. Green fluorescence intensity was analyzed with FACS scan cytometer (Becton Dickinson). For every histogram 5000 cells were counted to evaluate the percentage of CD4⁺ cells. The percentage of surface expression at different times of incubation with PMA was calculated using the mean fluorescence divided by the mean fluorescence at time 0 minus the background fluorescence.

Immunoelectron Microscopy—To assess CD4 localization at the cell plasma membrane of Triton-treated cells we followed a previously reported protocol with some modifications (35). Briefly, CD4⁺ cells were isolated from peripheral lymphocytes using the IsoCell™ human CD4 Isolation Kit (Pierce) according to the manufacturer’s instruction. CD4⁺ cells were then incubated with monoclonal antibody (OKT4) at 1:5 dilution in 0.5 ml of PBS/BSA for 1 h at 4 °C, followed by two washes with ice-cold PBS. Cells were resuspended with 1 ml of paraformaldehyde 3% in PBS, pH 7.2 for 30 min at 4 °C. After washing, cells were left untreated or 1% Triton X-100-treated for 30 min at 4 °C, followed by a second incubation with monoclonal antibody (OKT4) for 1 h at 4 °C. After incubation with rabbit anti-mouse IgG (Sigma) (1:10 in PBS for 1 h at 4 °C), cells were fixed with glutaraldehyde (1% in PBS for 1 h at 4 °C), extensively washed, and then labeled with colloidal gold (18 nm, prepared by the citrate method) conjugated with protein A (Amersham Pharmacia Biotech) for 3 h at 4 °C. Control experiments were performed omitting the incubation with OKT4 monoclonal antibody in both untreated and 1% Triton X-100-treated lymphocytes. All samples were postfixed in 1% osmium tetroxide in Veronal acetate buffer, pH 7.4, for 2 h at 4 °C, stained with uranyl acetate (5 mg/ml), dehydrated in acetone, and embedded in Epon 812.

Morphometry—Morphometric analysis of the length of plasma membrane in Triton X-100-treated cells and in untreated controls as well as quantification of colloidal gold granules were performed on 30 micrographs printed at the same magnification. The results were expressed as the means ± S.D.

RESULTS

Characterization of CD4 in LDTI Domains of Resting Lymphocytes—The detergent insolubility of caveolae and caveolae-related domains is based on their high content of cholesterol and sphingolipids; many distinct classes of lipid-modified signaling molecules are retained within these detergent-resistant membrane domains. However, certain caveola- and caveolin-1-associated proteins are dissociated from these domains as a consequence of detergent treatment; these include receptor tyrosine kinases (such as epidermal growth factor receptor) and a variety of prenylated proteins (such as Ha-Ras) (36). In addition, a small amount of caveolin-1 is found in the Triton-soluble fraction, and this fraction of caveolin-1 is associated with the Golgi complex (37). More interestingly, recent evidence indicates that proteins can move in and out of caveolae-related domains, depending on their activations state and that this dynamic movement can be monitored by changes in Triton solubility and partitioning into low density Triton-insoluble domains (LDTI, a biochemically descriptive term for caveolae-related domains) (38).

In a previous report, we demonstrated that CD4 is dramatically enriched within these LDTI domains in human lymphocytes (4). Here, we analyze the changes in CD4 localization after cell activation by phorbol esters (PMA). To study changes in CD4 localization following phorbol ester cell activation, we first analyzed the Triton sensitivity of CD4 in resting lymphocytes. Thus, we experimentally defined the optimal Triton X-100
CD4-Lck Complexes and Caveolae-related Domains

Concentration that is required for the isolation of Triton-insoluble CD4 from the bulk of soluble plasma membrane proteins (Fig. 1a). To this end, \(\sim 1 \times 10^6\) lymphocytes were surface labeled with sulfo-NHS-biotin. Two mg of the cell membrane fraction (M) were prepared from these labeled lymphocytes and homogenized with increasing amounts of Triton X-100, followed by equilibrium density gradient centrifugation. Each tube was then divided into twelve 375-\(\mu\)l fractions, and 10 \(\mu\)l/fraction was subjected to SDS-PAGE and blotting with streptavidin-horseradish peroxidase. Only biotin-labeled cell surface proteins were detected by this procedure (Fig. 1a, left).

Treatment of the membrane fraction with increasing concentrations of Triton X-100 resulted in differential solubilization of the plasma membrane. As expected, in the absence of detergent, all of the biotin-labeled cell surface proteins partitioned exclusively at the bottom of the gradient (fractions 7–12); none of the labeled proteins attained buoyancy. In contrast, increasing concentrations of Triton X-100 (0.02 or 0.1%) led to differential protein solubilization resulting in bands migrating at many different gradient densities. Note that a concentration of 1% Triton X-100 allowed optimal isolation of the LDTI microdomains (fractions 4–6), which migrated to the upper 20% sucrose region of the gradient (Fig. 1a, left).

In parallel experiments, each fraction was diluted, and the sedimentable material was collected by centrifugation and used to assay the distribution of CD4 by immunoblotting (Fig. 1a, right). Analysis of the distribution of CD4 demonstrated selective partitioning of CD4 into the LDTI fraction at a concentration of 1% Triton. We identified a 55-kDa biotin-labeled cell surface protein as CD4 by immunoprecipitation with CD4-specific antibodies (Fig. 1a, inset).

In addition, the protein profile of the sucrose gradient fractions indicated that \(\sim 99\%\) of total cellular protein was recovered in fractions 8–12, whereas only \(\sim 1\%\) of the total protein was recovered as LDTI domains (Fig. 1b). Densitometric analysis of the distribution of CD4 indicated that it quantitatively co-distributed with the LDTI domains (Fig. 1b).

The enrichment of CD4 in LDTI domains was estimated by comparing the amount of CD4 at different steps during the fractionation procedure (total lysate, total membrane fraction, and the LDTI fraction) (Fig. 1, c and d). Our results indicate that CD4 is \(\sim 66\)-fold enriched in total membranes (M) and \(\sim 1000\)-fold enriched in LDTI domains, relative to the total cell lysate. A representative blot is shown in Fig. 1d.

Detergent-resistant Plasma Membrane Microdomains Are Present in the Native Plasma Membrane of Lymphocytes—To assess the native distribution of molecules found associated with detergent-insoluble membrane domains, other laboratories have used an electron microscopy approach. In one such study, cells were first detergent extracted, fixed, and analyzed by standard transmission electron microscopy. These authors elegantly demonstrated that caveolae and caveolae-related domains exist in intact cells (35).

Here, we have applied this type of approach to localize CD4 in lymphocytes. Our ultrastructural observations reveal for the first time that in Triton-treated lymphocytes, although most of the plasma and intracellular membranes were almost completely dissolved, small portions of lipid bilayer were still present (Fig. 2a). These microdomains of the plasmalemma provide morphological evidence for the existence of intrinsically detergent-insoluble membrane domains characterized by the typical trilaminar unit membrane appearance (Fig. 2a, arrowheads).

To verify the presence of CD4 molecules localized on these Triton-insoluble membrane microdomains, we applied an immunocytochemical approach to Triton-treated lymphocytes. Briefly, after detergent treatment, cells were incubated with the OKT4 monoclonal antibody followed by anti-IgG antibodies and protein A-covalid gold conjugates. Our results show that most of the gold particles corresponding to immunolabeled CD4 molecules are steadily associated within these Triton-insoluble microdomains (Fig. 2b). Gold particles were observed both on the nonvillous portion of the retained membrane (Fig. 2b) and on microvilli (not shown). Similarly, in cell surface biotinylated lymphocytes the immunolabeling corresponding to Triton-insoluble biotinylated membrane proteins showed that gold particles were restricted to domains retaining the bilayer unit membrane appearance (Fig. 2, c and d). In further support of these observations, in lymphocytes that were not treated with detergent, the distribution of CD4 was clearly uneven over the plasma membrane (Fig. 2e). This uneven distribution is in accordance with previous reports (39).

Quantitative morphometric analysis revealed that the portions of unextracted membrane retaining the trilaminar unit membrane appearance measured approximately \(3.5 \pm 0.3 \mu m\)}
Kinetics of PMA-induced CD4 down-regulation at the plasma membrane and within LDTI microdomains. Lymphocytes (1 × 10^6) were cell surface labeled with biotin and incubated with 100 ng/ml PMA for 0, 5, 30, and 60 min at 37 °C. Total particulate plasma membrane fractions (M) were then prepared and either analyzed directly or used to purify LDTI microdomains. a, CD4 was monitored by immunoprecipitation with antibodies directed against CD4 and streptavidin blotting. LDTI (0.5 μg) and M (10 μg) were separated on 8% SDS-PAGE and transferred to nitrocellulose. b, cell surface CD4 was detected by immunoprecipitation with antibodies directed against CD4 and streptavidin blotting. LDTI (3 μg) and M (2 μg) were immunoprecipitated with 1 μg/ml anti-CD4 IgG. A representative blot is shown for each condition. For Lck detection, LDTI (2 μg) were analyzed by 10% SDS-PAGE and immunoblotted with anti-Lck. LDTI (5 μg) were immunoprecipitated with anti-CD4 and assayed for Lck by immunoblotting with anti-Lck antibody. FACS analysis was also performed as an independent measure of CD4 cell surface expression. For the graphs in panels a and b, the results shown are the means ± S.E. of three separate experiments. c, LDTI (1 μg) from untreated or 5 min PMA-treated cells were separated on a 10% SDS-PAGE, transferred to nitrocellulose and blotted with anti-Fyn or anti-Gi antibodies. d, total particulate membrane fraction from 1 × 10^6 untreated or 5 min PMA-treated lymphocytes were homogenized in 1% Triton X-100 and subjected to sucrose density gradient centrifugation. 0.5 μg/fraction was then analyzed by CD4 immunoblotting.

**FIG. 3.** Kinetics of PMA-induced CD4 down-regulation at the plasma membrane and within LDTI microdomains. Lymphocytes (1 × 10^6) were cell surface labeled with biotin and incubated with 100 ng/ml PMA for 0, 5, 30, and 60 min at 37 °C. Total particulate plasma membrane fractions (M) were then prepared and either analyzed directly or used to purify LDTI microdomains. a, CD4 was monitored by immunoprecipitation with antibodies directed against CD4 and streptavidin blotting. LDTI (0.5 μg) and M (10 μg) were separated on 8% SDS-PAGE and transferred to nitrocellulose. b, cell surface CD4 was detected by immunoprecipitation with antibodies directed against CD4 and streptavidin blotting. LDTI (3 μg) and M (2 μg) were immunoprecipitated with 1 μg/ml anti-CD4 IgG. A representative blot is shown for each condition. For Lck detection, LDTI (2 μg) were analyzed by 10% SDS-PAGE and immunoblotted with anti-Lck. LDTI (5 μg) were immunoprecipitated with anti-CD4 and assayed for Lck by immunoblotting with anti-Lck antibody. FACS analysis was also performed as an independent measure of CD4 cell surface expression. For the graphs in panels a and b, the results shown are the means ± S.E. of three separate experiments. c, LDTI (1 μg) from untreated or 5 min PMA-treated cells were separated on a 10% SDS-PAGE, transferred to nitrocellulose and blotted with anti-Fyn or anti-Gi antibodies. d, total particulate membrane fraction from 1 × 10^6 untreated or 5 min PMA-treated lymphocytes were homogenized in 1% Triton X-100 and subjected to sucrose density gradient centrifugation. 0.5 μg/fraction was then analyzed by CD4 immunoblotting.
Moreover, the interaction of Lck with CD4 is mediated via a reciprocal interaction between the cytoplasmic domain of CD4 and the N-terminal domain of Lck. A cysteine within the N-terminal domain of Lck is critical for this interaction, and this cysteine residue is distinct from the cysteine that undergoes palmitoylation (47). Thus, we would predict that disruption of the CD4-Lck complex would not affect the localization of Lck, because dual acylation of other Src family kinases is sufficient to mediate caveolar localization in other cell systems. In this sense, Lck would serve as an internal control for these studies and a stable marker for the LDTI fraction.

Thus, we next analyzed the amount of Lck in the LDTI fraction before and after PMA treatment. The same protein amounts were separated by SDS-PAGE and subjected to immunoblotting for Lck. As shown in Fig. 3, no differences in the distribution of Lck were observed before or after PMA treatment.

Disruption of the CD4-Lck complex was also monitored by performing a series of co-immunoprecipitation experiments. Antibodies directed against CD4 were used to recover the CD4-Lck complex, and the presence of Lck in this complex was then visualized by Western blotting. Note that Lck is only present in the CD4-Lck complex and not in the corresponding CD4+ fraction.

**Fig. 4.** PMA induces recruitment of PKC isoforms to LDTI microdomains. a, lymphocytes were treated with PMA for 1 h (++) or left untreated (−) and then used to prepare LDTI microdomains by sucrose density gradient centrifugation. 5 µg of each fraction were analyzed by 8% SDS-PAGE and immunoblotted with antibodies directed against PKC isoforms. Note that PMA induces a significant recruitment of PKCs to fractions 4–6 that represent LDTI microdomains. Results shown are representative of three independent experiments. b, kinetics of the recruitment of PKC isoforms to LDTI microdomains. Total lymphocytes (1 × 10⁶) were incubated with 100 ng/ml PMA for 0, 5, 30, or 60 min at 37 °C and used to prepare LDTI microdomains by sucrose density gradient centrifugation. LDTI microdomains (2 µg of protein for each condition) were analyzed by 8% SDS-PAGE and immunoblotted with antibodies directed against PKC isoforms (α, δ, or ζ). Alternatively, LDTI microdomains (5 µg for each condition) were immunoprecipitated with 1 µg of anti-CD4, -PKCα, or -PKCζ, and blotted with the same antibodies. WB, Western blot; IP, immunoprecipitation; N.D., not done.

**Fig. 5.** Relative LDTI/particulate ratio of the PKC isoforms. Total particulate membrane fractions or LDTI microdomains (purified using the total particulate membrane fraction as the starting material) were prepared from lymphocytes that were left untreated (a) or treated with PMA for 30 min (b). Immunoblots containing 1 µg of protein of either particulate membrane fractions or LDTI microdomains were quantitated by densitometry and values expressed as a ratio (LDTI/particulate) for each isoform. Values are expressed as the means ± S.E. of three independent experiments.
FIG. 6. Immunolocalization of PKC isoforms by scanning confocal microscopy. a, scanning confocal microscopic analysis of PKC redistribution after PMA treatment of CD4+ human peripheral blood lymphocytes. Cells were fixed with acetone/methanol and then labeled with anti-PKCa, δ, or ε polyclonal antibodies followed by incubation with FITC-conjugated goat anti-rabbit IgG. After washing three times in PBS, cells were then incubated with an anti-Lck monoclonal antibody followed by the addition of goat anti-mouse IgG conjugated with Texas Red. Panel 1, untreated cell stained with anti-PKCa revealed a diffuse cytoplasmic immunolabeling. Panel 2, untreated cell stained with anti-PKCa revealed a diffuse cytoplasmic immunolabeling. Panel 5, untreated cell stained with anti-Lck revealed uneven immunolabeling on the plasma membrane. Panel 4, PMA-treated cells stained with anti-PKCa showed a clustered distribution of immunolabeling on the plasma membrane. Panel 5, PMA-treated cells stained with anti-PKCa showed a clustered distribution of immunolabeling on the plasma membrane. Panel 6, PMA-treated cells stained with anti-PKCa revealed an uneven immunolabeling on the plasma membrane. Panel 6, PMA-treated cells stained with anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 9, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 4, PMA-treated cells stained with anti-PKCa showed a clustered distribution of immunolabeling on the plasma membrane. Panel 5, PMA-treated cells stained with anti-Lck revealed an uneven immunolabeling on the plasma membrane. Panel 6, PMA-treated cells stained with anti-PKCa revealed an uneven immunolabeling on the plasma membrane. Panel 7, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 8, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 9, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 4, PMA-treated cells stained with anti-PKCa showed a clustered distribution of immunolabeling on the plasma membrane. Panel 5, PMA-treated cells stained with anti-Lck revealed uneven immunolabeling on the plasma membrane. Panel 6, PMA-treated cells stained with anti-Lck revealed uneven immunolabeling on the plasma membrane. Panel 7, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 8, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 9, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 4, PMA-treated cells stained with anti-PKCa showed a clustered distribution of immunolabeling on the plasma membrane. Panel 5, PMA-treated cells stained with anti-Lck revealed uneven immunolabeling on the plasma membrane. Panel 6, PMA-treated cells stained with anti-Lck revealed uneven immunolabeling on the plasma membrane. Panel 7, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 8, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 9, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 4, PMA-treated cells stained with anti-PKCa showed a clustered distribution of immunolabeling on the plasma membrane. Panel 5, PMA-treated cells stained with anti-Lck revealed uneven immunolabeling on the plasma membrane. Panel 6, PMA-treated cells stained with anti-Lck revealed uneven immunolabeling on the plasma membrane. Panel 7, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 8, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane. Panel 9, dual immunolabeling of anti-PKCa and anti-Lck revealed nearly complete co-localization yellow-stained portions of the plasma membrane.

During PMA time course experiments no appreciable differences in LDTI total protein (data not shown) or the Lck content of the LDTI fraction were observed. In addition, two other acylated LDTI marker molecules, Fyn and Gα, were analyzed and found unchanged after short PMA incubations (5 min) (Fig. 3c). These critical control experiments clearly indicate that the down-modulation of CD4 with LDTI microdomains is a highly selective event.

The acquired detergent solubility of plasma membrane CD4 at 5 min is confirmed by the persistence of CD4 at this time point in total plasma membrane (Fig. 3, a and b, see fraction). The delayed disappearance of CD4 from the plasma mem-

the CD4 immunoprecipitates in untreated cells, as expected (Fig. 3b) (17).

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brane indicates a shift of CD4 from its initial location within LDTI microdomains to the detergent-sensitive areas of the plasma membrane. This PMA-induced shift was also observed in an independent experiment in which the amount of CD4 in LDTI (fraction 4–6) and soluble plasma membrane (fractions 8–12) was monitored by sucrose density gradient centrifugation (Fig. 3d).

PMA-induced Recruitment of PKC Isoforms to LDTI Microdomains—in lymphocytes, PMA activates PKCs and induces their translocation from the soluble to particulate fraction consisting of the plasma membrane proper and other subdomains of the plasma membrane (for review see Refs. 23 and 50).

To establish whether a particular PKC isoform was involved in the CD4 internalization event, we analyzed the distribution of PKC isoforms by immunoblotting before and after PMA treatment. We found that all seven PKC isoenzymes (α, β1, β2, γ, δ, ε, η, and θ) shifted, although in variable amounts, from Triton-soluble fractions (8–12) to the LDTI microdomains (fractions 4–6) (Fig. 4a).

In line with previous studies showing an immediate PKC redistribution to plasma membrane following activation (50), our data with PKCs (α and δ isoforms) show a complete recruitment of PKC α and δ isoforms to LDTI microdomains within 5 min of PMA addition (Fig. 4b).

Surprisingly PKC ζ, which lacks the phorbol ester binding region, was also recruited to LDTI microdomains, although at later times, reaching maximum recruitment between 5–30 min (Fig. 4b). In Jurkat and peripheral blood T cells translocation of PKCζ from cytosol to membrane fraction after 15 min of PMA stimulation has been described (51, 52). As has been suggested by other laboratories using Jurkat cells, the delayed recruitment of PKCζ is likely due to an indirect effect of PMA activation (51).

In an attempt to better quantitate this translocation event, we compared the amount of PKC immunoreactivity present in total membranes (M, particulate) and within LDTI microdomains, before and after stimulation with PMA (Fig. 5). Thus, 1 µg of protein of both preparations was resolved by SDS-PAGE and subjected to immunoblotting with PKC isoform-specific antibodies. Results are expressed in arbitrary units as a ratio between the amount within LDTI domains and the amount within total membranes (LDTI/particulate ratio). The results from densitometric analysis of three independent experiments are shown.

When we evaluated the translocation event as a ratio of LDTI to total membrane levels PKCα showed a remarkably high translocation ratio to LDTI microdomains. Its relative enrichment was 6–32 times greater compared with the other PKC isoforms analyzed (Fig. 5b).

In the total membrane fraction, PKC isoforms β1, β2, δ, ε, and η were poorly represented at steady state (α and θ were completely absent) (data not shown), in agreement with results obtained by others (51, 53, 54). However, most of these PKC isoforms underwent translocation after PMA-induced activation, resulting in a noticeable increase of 0.5–4-fold within total membranes (data not shown).

Since PKC isoforms may have an intrinsically different Triton solubility, we separately analyzed the co-localization of PKCα and two lesser involved isoforms PKCδ and ε with Lck in CD4⁺ intact cells, by scanning confocal fluorescence microscopy (Fig. 6a). PKCs appeared in an uneven and punctate distribution at the plasma membrane after PMA addition (Fig. 6a, panels 4 and 5), whereas in untreated cells PKC isoforms were mostly diffuse in the cytoplasm (Fig. 6a, panels 1 and 2). The clustered distribution of the PKCs indicates that these enzymes translocate mostly to specific microdomains of the plasma membrane. Membrane distribution of Lck in untreated cells (Fig. 6a, panel 3) was confirmed to be unaffected by PMA treatment (Fig. 6a, panel 6).

Double-labeling studies with Lck and PKCα revealed nearly complete co-localization of the two proteins at the plasma membrane, appearing as yellow-stained membrane microdomains (Fig. 6a, panel 7). This finding indicates that after treatment with PMA a large fraction of translocated PKCs effectively co-localized and was associated with Lck. In striking contrast, only a few areas of co-localization were evident with Lck and PKCδ or Lck and PKCε in PMA-treated lymphocytes (Fig. 6a, panels 8 and 9).

Parallel experiments were performed to verify whether, after treatment with PMA, PKCα became associated with monosialoganglioside GM3, which represents the main ganglioside constituent of human peripheral blood lymphocytes (55) and is selectively recovered in LDTI microdomains of cell plasma membrane (4).

In agreement with our previous observations (4), the GM3 signal appeared uneven and punctate over the plasma membrane, indicating a clustered distribution of GM3 molecules (Fig. 6b, panel 2). Anti-PKCα and anti-GM3 double labeling revealed yellow areas, corresponding to nearly complete co-localization, indicating that PKCα molecules were localized in membrane microdomains enriched in GM3.

These observations independently demonstrate that PMA treatment induces a preferential translocation of PKCα to discrete microdomains of the plasma membrane where glycosphingolipids and Lck molecules are highly enriched.

PMA Treatment Up-regulates the Kinase Activity Associated with LDTI Domains—To further elucidate the activation state of PKCα, we examined the in vitro kinase activity associated with LDTI microdomains before and after stimulation of intact
cells with PMA for 1 h. Our results indicate that the amount of kinase activity associated with LDTI domains was increased, as assessed by the increased phosphorylation of endogenous substrates (Fig. 7a) or an exogenously added substrate (Histone III) (Fig. 7b).

To investigate whether this enhanced kinase activity might be due to the presence of activated PKCs, we tested the effect of a new PKC inhibitor (Gö 6976) that selectively affects only
Ca\(^{2+}\)-dependent isomers of PKC (\(\alpha, \beta_1, \beta_2, \gamma\)) (56). Treatment of LDTI domains with Gö 6976 (10\(^{-6}\) M for 1 h at 37 °C) blocked the enhanced proteins phosphorylation (Fig. 7a). These results support the hypothesis that enhanced kinase activity associated with LDTI domains is due to PKC translocation.

Finally, to evaluate the proposed role of PKC\(\alpha\) in down-modulation of CD4, we incubated CD4\(^1\) cells with PKC inhibitors (Gö 6750 or Gö 6976), followed by PMA stimulation. We then examined CD4 expression in intact cells by FACS analysis (Fig. 8a) and within LDTI microdomains by immunoprecipitation of surface-labeled cells (Fig. 8b).

Treatment with either PKC inhibitor greatly reduced (to 30–35%; with Gö 6976) or completely abolished (with Gö 6850) CD4 internalization. Virtually identical results were obtained by FACS analysis and immunoprecipitation of LDTI domains.

Taken together, these data suggest a preferential involvement of PKC\(\alpha\), and in to lesser extent of PKC\(\beta\), in this process of CD4 internalization.

**DISCUSSION**

The existence of plasma membrane microdomains distinct from caveolae is now generally accepted. A biochemical definition of these membrane compartments has been derived from their unusually high glycolipid and cholesterol content, whose interaction creates “the detergent-resistant platform” (1, 57, 58). Receptors (31), Src family tyrosine kinases (2, 31, 44), heterotrimeric G proteins (2, 31), and Ha-Ras (31) have all been found associated with these microdomains. However, the precise function of these membrane rafts or caveolae-related domains still remains to be defined.

We recently described that the CD4-Lck complex is localized within LDTI microdomains isolated from lymphoid cells (2). Lck, among the PTKs, represents the best characterized lymphocyte-specific tyrosine kinase, whose unique N-terminal binding region is responsible for CD4 anchoring and surface expression on the plasma membrane (59). At steady state, it has been estimated that only ~5–7% of cell surface CD4 is endocytosed by coated pits in T cells (11).

Conversely, in phorbol ester-activated T cells, disruption of CD4-Lck complex occurs following serine phosphorylation of CD4 (12, 18) and Lck (60, 61). This sequence of events initiates the rapid cell surface down-modulation of CD4 that occurs through an increased uptake via coated pits (17).

We report here data on the molecular events leading to PMA-induced down-regulation of CD4 in lymphocytes. We find that (i) plasma membrane CD4 is steadily confined to and enriched within LDTI microdomains (15-fold versus plasma membranes); (ii) in agreement with previous studies, PMA-induced disruption of the CD4-Lck complex occurs rapidly within 5 min; and (iii) analysis of total membrane fractions revealed that CD4 was down-modulated by 20% at 5 min and by 90% at 60 min (17, 41). Interestingly, CD4 levels within LDTI microdomains decreased by 60% within 5 min (Fig. 3). This time lag indicates that the disappearance of CD4 from LDTI microdomains occurs more rapidly than from plasma membrane.

One possible explanation for this difference in the CD4 content of LDTI microdomains and total plasma membrane is that down-modulation of CD4 requires it to move from LDTI microdomains to another region of the plasma membrane that is Triton-soluble. This movement may be initiated by a conformation change in CD4 that is due to PMA-induced serine phosphorylation of CD4. Release of CD4 from the confines of LDTI microdomains and Lck may then expose a putative endocytosis signal that mediates its uptake via clathrin-coated pits and targets it for lysosomal degradation (41).

Additionally, we demonstrate that Lck, which at steady state in T cells is 60–70% complexed with CD4 (62), and other LDTI marker proteins (Fyn and G\(\alpha\)) (2, 48, 49), remain within the LDTI microdomains during PMA treatment. Thus, Lck is firmly restricted to these LDTI microdomains, and its localization within LDTI microdomains is independent of CD4. This may be due to the observation that Lck, Fyn, and G\(\alpha\) all undergo N-terminal dual acylation (62). It has been proposed that dual acylation targets Src family kinase to detergent-resistant membrane domains (63).

A dissection of the novel CD4-Lck molecular events observed within the LDTI microdomains of activated T cells has been hindered by a lack of methods to separate distinct microdomains of the plasma membrane. Moreover, the analysis of these LDTI microdomains provide a tool to evaluate the specific role of PKC in this process by the elucidation of kinase selected substrates. Here, we have used LDTI microdomains to assess the translocation and compartmentalization of the PKC isoforms (\(\alpha, \beta_1, \beta_2, \delta, \epsilon, \zeta, \eta, \theta\) known to be expressed in T cells (64).

Involvement of PKC in the PMA-induced phosphorylation of CD4 has been hypothesized based on (i) *in vitro* experiments suggesting that CD4 serves as substrate for PKC (18) and (ii) the observation that prolonged PMA treatment exhausts cytoplasmic reserves of PKC and inhibits down-modulation of CD4 (20). However, evidence for the involvement of a specific PKC isoform in this process has been lacking.

Here, we provide evidence that PKCs is the most abundant isoform recovered within LDTI microdomains upon PMA treatment (5 min to 1 h). This specificity was independently confirmed by (i) *in vivo* inhibition of CD4 down-modulation by a PKC\(\alpha\) selective inhibitor (Gö 6976); (ii) a series of *in vitro* phosphorylation experiments using purified LDTI microdomains; and (iii) immunofluorescence data showing that PKCs, GM3, and Lck share significant co-localization at the level of the plasma membrane after PMA treatment.

As seen by FACS analysis, other PKCs partially contribute to the phenomenon (Fig. 8a). Future studies with PKC selective inhibitors will elucidate the specific involvement of a given PKC isoform.

Similarly, in the present study we observed that CD4 is enriched within low density membranes that were purified in the absence of detergent. These membranes had the same buoyant density as LDTI microdomains. Furthermore, we show by immunogold labeling and transmission electron microscopy that CD4 is localized within detergent-resistant areas of the plasma membrane of intact cells. Taken together, these data provide strong evidence that purified LDTI domains correspond to native plasma membrane compartments where signals involving CD4-Lck complex may be transduced. In addition, while this paper was under review, another paper appeared demonstrating that the entire T-cell receptor complex is recruited after its engagement to plasma membrane domains resembling LDTI domains (38).

These observations highlight the importance of these caveolae-related microdomains in lymphocyte signal transduction. Moreover, this system provides a new tool to assess the translocation of PKC and other signal molecules in response to a variety of stimuli presented to the hematopoietic cell system.

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