Cell membranes contain sphingolipids and cholesterol, which cluster together in distinct domains called rafts. The outer-membrane leaflet of these peculiar membrane domains contains glycosylphosphatidylinositol-anchored proteins, while the inner leaflet contains proteins implicated in signalling, such as the acylated protein kinase p56\(^{ck}\) and the palmitoylated adaptor LAT (linker for activation of T-cells). We present here an approach to study the lipid composition of rafts and its change upon T-cell activation. Our method is based on metabolic labelling of Jurkat T-cells with different precursors of glycerophospholipid synthesis, including glycerol and fatty acids with different lengths and degrees of saturation as well as phospholipid polar head groups. The results obtained indicate that lipid rafts isolated by the use of sucrose density-gradient centrifugation after Triton X-100 extraction in the cold, besides sphingolipids and cholesterol, contain unambiguously all classes of glycerophospholipids: phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine. Fatty acid labelling shows that lipid rafts are labelled preferentially with saturated fatty acids while the rest of the plasma membrane incorporates mostly long-chained polyunsaturated fatty acids. To see whether the raft composition as measured by metabolic labelling of phospholipids is involved in T-cell activation, we investigated the production of sn-1,2-diacylglycerol (DAG) in CD3-activated cells. DAG production occurs within rafts, confirming previous demonstration of protein kinase C translocation into membrane microdomains. Our data demonstrate that raft disorganization by methyl-\(\beta\)-cyclodextrin impairs both CD3-induced DAG production and changes in cytosolic Ca\(^{2+}\) concentration. These lines of evidence support the conclusion that the major events in T-cell activation occur within or due to lipid rafts.

**Key words:** Ca\(^{2+}\), cholesterol, diacylglycerol, fatty acid, sphingomyelin.

**INTRODUCTION**

Engagement of the T-cell receptor (TCR) by specific antigen–MHC complexes or antibodies which bind the CD3 subunits of the TCR results in activation of multiple biochemical pathways, leading to cytokine production and cell proliferation. Jurkat cells are widely used as a model of T-cell activation because in these cells triggering the TCR results in interleukin-2 production and expression of CD25 and CD69 at the cell surface. Transduction of CD3–TCR-induced signals in these cells involves the phosphorylation and activation of numerous protein tyrosine kinases, the best characterized being the p56\(^{ck}\) and the ZAP-70 (zeta-associated protein of 70 kDa) tyrosine kinases. Their substrates, such as p36–38 LAT (linker for activation of T-cells of 36–38 kDa) and the ZAP-70 (zeta-associated protein of 70 kDa) tyrosine kinases. Their substrates, such as p36–38 LAT (linker for activation of T-cells of 36–38 kDa) and phospholipase Cy1 (PLCy1), are phosphorylated rapidly and participate in a large protein complex necessary to obtain full cell activation [1,2]. Activation of PLC\(_{y1}\) is known to result in the generation of two intracellular second messengers, d-myoi-Ins(1,4,5)\(_{P_3}\) (IP\(_{3}\)) and sn-1,2-diacylglycerol (DAG), arising from the hydrolysis of membrane phosphatidylinositol[3]. The discovery of membrane domains that remain intact after detergent extraction of cell membranes has contributed to a re-examination of T-cell signalling in this new and exciting context. These membrane domains, called rafts or detergent-insoluble membranes, are characterized by their cholesteryl content and the presence of glycolipids and, as a consequence, were also called glycolipid-enriched membranes (GEMs) or detergent-insoluble glycolipid-enriched domains [4–7]. To date, studies of the membrane raft composition indicate the presence of high amounts of glycosylphosphatidylinositol-anchored proteins (GPI-APs), cholesterol, PtdIns(4,5)\(_{P_2}\) (PIP\(_{2}\)), sphingolipids (sphingomyelins) and glycosphingolipids (GSLs; gangliosides) [8–10].

In raft models, sphingolipids associate laterally with one another, probably through weak interactions between the carbohydrate heads of GSLs. The sphingolipid head group occupies a larger area than the lipid hydrocarbon chain and the volume between the chains is filled with cholesterol, which functions as a spacer molecule. The close-packed cholesterol–sphingolipid clusters interact with phospholipids on the cytoplasmic side of the membrane; however, the microdomain composition of the cytoplasmic side remains unknown, although it has been suggested that this leaflet might contain saturated fatty acids to optimize raft packing [8,11]. This lateral organization allows comparison with moving platforms, which is the reason why GEMs are also called rafts. From a biophysical point of view, the plasma-membrane bilayer is made of a liquid-crystalline or liquid-disordered phase, characterized by the presence of fluid and disordered acyl chains [12]. Rafts exist in membranes in the liquid-ordered phase; in this phase, the physico-chemical properties of lipids are intermediate between the liquid-disordered phase and a theoretical gel phase that does not exist in biological
membranes [13]. Thus, lipids present in rafts are extended and ordered, as in the gel phase, but have high lateral mobility in the bilayer, as in the liquid-disordered phase [14]. Model membrane studies reveal a good correlation between the liquid-ordered phase and detergent insolubility [15,16]. These biophysical characteristics may explain why lipid rafts are resistant to solubilization at low temperatures by non-ionic detergents, such as Triton X-100 or Brij 58, and due to their low buoyant density, they can be readily isolated by flotation in sucrose gradients [12,17].

The presence of glycerophospholipids in rafts has been shown in Madin-Darby canine kidney cells [12], but has never been studied in T-lymphocytes. This lack of data is probably due to the low level of glycerophospholipids in this membrane fraction. Indeed, classical phospholipid measurements, such as phosphorus determination or specific coloration on TLC plates, are unusable due to the low concentration of phospholipids in the samples. According to Brown and London [6], a comparison between the very low level of protein in rafts and the protein content of detergent-soluble fractions would lead to misinterpretation of the results, especially when the lipid concentration is expressed as a function of the protein content, “because the rafts have a higher lipid:protein ratio, even if the insoluble and the soluble fractions have a similar composition, every lipid would appear enriched in a light membrane sampled for an equal amount of proteins” [6]. Even the enrichment in cholesterol in rafts has been questioned by some authors and is discussed with respect to the lipid/protein ratio by Brown and London in their review [7]. Given the above remarks, we decided to study raft composition by using cell metabolic labelling. The results described in this paper indicate an enrichment in cholesterol, sphingomyelin and phosphatidylinerine (PtdSer) in Jurkat rafts. Moreover, the presence of the other glycerophospholipids, such as phosphatidycholine (PtdCho), phosphatidylserine (PtdIns) and phosphatidylethanolamine (PtdEtN) is reflected with saturated fatty acids is evident. In addition, the formation of DAG in the Triton-insoluble fraction in CD3-activated cells and its preferential localization in rafts indicate that signalling via the TCR might involve lipid rafts, as indicated by previous studies on protein phosphorylation [18].

**EXPERIMENTAL**

**Cells**

The human leukaemic T-cell line Jurkat clone JE 6.1, obtained from the A.T.C.C. (Manassas, VA, U.S.A.), was cultured in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine and 1 mM pyruvate (Life Technologies, Rockville, MD, U.S.A.) in a humidified incubator (Heraeus, Hanau, Germany) under 5% CO₂.

**Antibodies and reagents**

Rabbit polyclonal anti-ZAP-70 tyrosine kinase (sc-574) and rabbit polyclonal anti-PLCγ1 (sc-81) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-p56lk (06-583) and anti-LAT (06-807) IgGs were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-paxillin monoclonal antibody (mAb; P13520, IgG1) was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-CD59 (P258, IgG2a) and anti-CD3 (X3; IgG2a) mAbs were produced in our laboratory. Horseradish peroxidase-labelled anti-rabbit IgG was from Rockland (Gilbertsville, PA, U.S.A.). Goat anti-mouse IgG coupled to horseradish peroxidase and rabbit anti-mouse IgG conjugated with FITC were from Dako (Glostrup, Denmark). Methyl-β-cyclodextrin (m-β-CD), ionomycin, pepstatin, leupeptin and chymostatin were purchased from Sigma (St Louis, MO, U.S.A.). α2-Macroglobulin was purchased from Roche (Indianapolis, IN, U.S.A.) and Indo-1 was from Molecular Probes (Leiden, The Netherlands).

**Radioactive products**

[1,2,3-(n)-H]Cholesterol (1.3–1.85 TBq/mmol), [U-14C]glycerol, l-[3-3H]serine (37 MBq/mmol), 1-d-myO-[2-3H]inositol (370–740 GBq/mmol), [l-3H]ethanol-1-ol-2-amine hydrochloride (37 MBq/mmol), [methyl-3H]choline chloride (2.22–3.14 TBq/mmol), [1-14C]oleic acid (185 MBq/mmol), [1-14C]linoleic acid (1.85–2.20 GBq/mmol), [9,10-(n)-3H]palmitic acid (37 MBq/mmol), [1-14C]stearic acid (1.85–2.20 GBq/mmol) and [5,6,8,9,11,12,14,15-(n)]arachidonic acid (5.55–8.51 TBq/mmol) were purchased from Amersham Bioscience (Arlington Heights, IL, U.S.A.; see Table 1).

**Disruption of membrane rafts with m-β-CD**

Jurkat JE 6.1 cells were suspended at 2 × 10⁶ cells/ml in a buffer (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 1 mM Na₂HPO₄·12H₂O, 2.5 mM glucose, 20 mM Heps, 1 mM MgCl₂, 1 mM CaCl₂ and 0.1% BSA. Cholesterol extraction was achieved by treating cells with 10 mM m-β-CD for 7 min at 37 °C, followed by washing. Under these experimental conditions, cell viability was above 95%, as measured by the Trypan Blue-exclusion method.

**Cell labelling**

Cells were washed and then incubated for 4 h in buffer (pH 7.4; as detailed in the previous section) at 37 °C in the presence of 4 μCi of the different radioactive lipid precursors. Aqueous or alcoholic solutions of the different ¹H or ¹⁴C radioactive markers were used directly; by contrast, toluene solutions of some labelled fatty acids were first evaporated under N₂ and dissolved in ethanol just prior to utilization.

**Lipid raft isolation**

Raft isolation was accomplished using a combination of published protocols [18]. After labelling and/or treatment, JE 6.1 cells [(80–100) × 10⁶] were sonicated with a VibraCell sonicator (five bursts of 5 s, 5 W; Biorad Scientific) in ice-cold buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 mM sodium pyrophosphate and 10 mM NaF) supplemented with a mixture of protease inhibitors (1 mg/ml leupeptin, 1 mg/ml pepstatin, 2 mg/ml chymostatin and 5 mg/ml a2-macroglobulin) and centrifuged at 800 g at 2 °C for 10 min to remove nuclei and large debris. The resulting supernatant, called post-nuclear
supernatant (PNS), was incubated with 0.5% Triton X-100 for 30 min at 2°C. The lysate was then adjusted to 1.33 M sucrose by the addition of 2 ml of 2 M sucrose and placed at the bottom of an ultracentrifuge tube (Ultra-Clear; Beckman Instruments, Palo Alto, CA, U.S.A.). A stepped sucrose gradient (0.2–0.9 M with 0.1 M steps, 1 ml each) was placed on top. The tubes were centrifuged at 270000 g for 16 h (L8-70M Ultracentrifuge; Beckman Instruments) with a SW41Ti rotor (Beckman Instruments) at 2°C. Fractions of 1 ml were harvested from the top. GEMS were recovered from low-density fractions 2 and 3 while the soluble material was recovered from high-density fractions 8 and 9 at the bottom of the ultracentrifuge tube [18].

**Immunoblot analysis**

Aliquots (50 µl) of each sucrose-density-gradient fraction were solubilized in 50 µl of 2 × Hoessli buffer (150 mM Tris/HCl, pH 8.5, 20% glycerol, 5 mM EDTA, 5% SDS and 10% β-mercaptoethanol) and resolved by SDS/PAGE (10% gel) under reducing conditions. Proteins were then transferred on to PVDF membranes (Immobilon-P; Millipore, Saint Quentin en Yvelines, France). Membranes were blocked for 2 h at room temperature in blocking buffer pH containing 5% (w/v) non-fat dried milk in Tris-buffered saline (10 mM Tris/HCl/140 mM NaCl, pH 7.4) and then incubated for 1 h with the appropriate antibody diluted 1000-fold in the same buffer. The membranes were washed extensively in Tris-buffered saline containing 0.4% (v/v) Tween-20. Detection was performed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse mAbs and enhanced chemiluminescence reagents (ECL®; Amersham Bioscience, Little Chalfont, Bucks., U.K.) according to the manufacturer’s instructions.

**Lipid analysis**

To determine the distribution of different radioactive labels, an aliquot (50 µl) of each fraction obtained after ultracentrifugation on the sucrose density gradient was mixed with Picofluor and counted by liquid scintillation in a Beckman Tricarb scintillation spectrometer. Lipids from the nine fractions obtained were extracted with chloroform/methanol according to Bligh and Dyer [19] and then separated by mono-dimensional TLC on LK6D gel 60 A plates (Whatman, Clifton, NJ, U.S.A.) in a solvent system composed of chloroform/methanol/acetic acid/water (75:45:12:3, by vol.). Authentic phospholipid standards (Sigma) were run in parallel and detected with iodide vapours. Radioactivity in lipid spots was determined by using an automatic linear radiochromatography analyser, Tracemaster 20 (Berthold), equipped with an 8 mm window and the integration software supplied by the manufacturer.

**DAG measurements**

Cells pre-labelled with either [3H]palmitic acid or [3H]arachidonic acid were first treated for 5 min with 2 µg/ml CD3 mAb, and then the mAb was cross-linked with 1 µg/ml rabbit anti-mouse IgG (Dako). After 5–60 min of incubation cells were solubilized first to the second fluorescence reading allows the evaluation of intracellular [Ca2+] independently of cell size and the intracellular Indo-1 concentration [20]. The flow rate was set to approx. 1000 cells/s and the mean ratio of 3000 cells was noted every 30 s. Damaged cells and debris were gated out according to the dual-scatter dot plot from the blue laser.

**RESULTS**

**Characterization of membrane microdomains/rafts**

The plasma membrane of many cell types, including T-cells, contains glycosphingolipid microdomains that are commonly referred to as lipid rafts. These are biochemically and biophysically distinct from the rest of the plasma membrane: they are enriched in GPI-APs, sphingolipids, GSLs and cholesterol and are resistant to solubilization at low temperature by non-ionic detergents. Due to their low buoyant density, rafts can be isolated by density-gradient centrifugation [16,21,22].

In order to characterize lipid rafts in the human leukemic Jurkat T-cell line, we first prepared Triton X-100 cell extracts at 2°C and then analysed the distribution of CD59, also termed membrane inhibitor of reactive lysis (MIRL), and paxillin in the nine different fractions obtained after sucrose density-gradient ultracentrifugation. CD59 is a GPI-AP which is found to be localized exclusively in the low-density buoyant membrane fractions and is considered, for that reason, to be an excellent marker of rafts [23]. As shown in Figure 1(B), CD59 was found exclusively in the buoyant membrane fraction. Paxillin is a cytoskeletal component which binds to vinculin, α-actinin and talin, and it localizes to the focal adhesions at the ends of actin stress fibres. Contrary to GPI-APs, paxillin is never found in the low-density fractions. It is always localized with soluble material [24]. As shown in Figure 1(B), paxillin was recovered exclusively from high-density fraction 9 at the bottom of the ultracentrifuge tube. CD59 was used as a positive control for the GEM fractions, whereas the absence of paxillin in GEMs was a negative control. The dual-acylated protein tyrosine kinase p56lck (which is both a

**Indirect immunofluorescence**

Jurkat JE 6.1 cells (1 × 10⁶) were washed in cold PBS supplemented with 0.1% BSA, pH 7.3, and incubated (30 min at 4°C) in 100 µl of the same buffer with anti-CD3 X3 mAb (10 µg/ml). Cells were then washed and incubated (30 min at 4°C) in 100 µl of a 1/25 dilution of rabbit anti-mouse IgG conjugated to FITC. Cells were washed again and fixed with 0.37% paraformaldehyde. The mean fluorescence intensity of 3000 cells was determined by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA, U.S.A.).

**Measurement of changes in cytosolic Ca2+ concentration**

Cytosolic Ca2+ concentration was measured using Indo-1 and flow cytometry. Cells (5 × 10⁶/ml) were loaded with 4 µM Indo-1-AM at 37°C for 1 h and resuspended at 1 × 10⁶ cells/ml in Heps-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 20 mM Hepes and 0.1% BSA, pH 7.4). Analysis were performed at room temperature using a fluorescence-activated cell sorter (FacStar+; Becton Dickinson) fitted with two argon lasers, one tuned to 488 nm and the other to the UV wavelength. The fluorescence excited by UV light was measured at 400 nm (corresponding to the Indo-1–Ca2+ complex) and at 480 nm (corresponding to free Indo-1). The ratio of the first to the second fluorescence reading allows the evaluation of intracellular [Ca2+] independently of cell size and the intracellular Indo-1 concentration [20]. The flow rate was set to approx. 1000 cells/s and the mean ratio of 3000 cells was noted every 30 s. Damaged cells and debris were gated out according to the dual-scatter dot plot from the blue laser.
A PNS preparation of Jurkat cells \((80–100) \times 10^6\) labelled with \(^{3}H\)cholesterol was treated with Triton X-100 at 2 °C and fractionated on a sucrose density gradient as described in the Experimental section. After ultracentrifugation, 1 ml fractions were harvested from the top. The nine fractions obtained were either assayed for radioactivity by liquid scintillation (A) or submitted to PAGE and transferred on to PVDF membranes (B). (A) The distribution of \(^{3}H\)cholesterol in the gradient fractions is shown for control (□) and m-β-CD-treated (▲) cells; this graph is representative of three independent experiments. The values represent means ± S.E.M. (where not visible, error bars smaller than the symbols). (B) The distribution of PLC\(_{c1}\), ZAP-70, paxillin, p56\(\text{Lck}\), LAT and CD59. The buoyant fractions (rafts) at the top of the gradient correspond to fractions 2 and 3 and the soluble material at the bottom of the centrifuge tube to fractions 8 and 9.

Figure 1 Characterization of membrane microdomains (rafts)

Previous work [5–7] has shown that the buoyant vesicles are enriched in cholesterol, so Jurkat cells were incubated with radioactive cholesterol for 4 h, washed and lipid rafts isolated. The distribution of \(^{3}H\)cholesterol in the nine different fractions of the sucrose density gradient is shown in Figure 1(A). As expected, cholesterol preferentially labels buoyant membranes.

\[^{3}H\]Glycerol-labelled glycerophospholipids are detected in lipid rafts

In order to detect the eventual presence of glycerophospholipids in membrane microdomains, cells were first labelled for 4 h with \[^{3}H\]glycerol, and the membrane fractions were separated on a sucrose density gradient as described above. A simple analysis of the distribution of the tritiated marker by scintillation counting (results not shown) indicates the presence of \[^{3}H\]glycerol-labelled material in the buoyant membrane fraction. Further analysis by TLC of the \[^{3}H\]glycerol-labelled material (Figure 2) confirmed
Rafts exhibit phospholipids implicated in CD3 T-cell receptor signalling

Figure 3 Distribution of the major phospholipids in rafts and soluble fractions

A PNS preparation of Jurkat cells (80–100 × 10⁶) metabolically labelled with [3H]choline, [3H]inositol, [3H]ethanolamine or [3H]serine was treated with Triton X-100 at 2 °C and fractionated on a sucrose density gradient as described in the Experimental section. Phospholipids from each fraction were extracted with chloroform/methanol and analysed by TLC on silica gel plates. The amount of radioactivity found in phospholipids labelled with their respective polar head groups is shown from the top to the bottom of the density gradient. These graphs are representative of three independent experiments and the values represent means ± S.E.M. (where not visible, error bars are within the symbols).

the presence of different radioactive peaks, corresponding to PtdCho, PtdIns/PtdSer (these two glycerophospholipids were not separated in the solvent system used for chromatography; they appeared as a single spot) and PtdEtn as well as other undetermined glycerol-containing lipids in the buoyant fraction (results not shown).

Phospholipids with ³H-labelled phospholipid polar head group were detected in rafts

To confirm the presence of glycerophospholipids in rafts, Jurkat cells were labelled for 4 h with tritiated phospholipid polar head groups. [³H]Choline, [³H]ethanolamine, [³H]serine- or [³H]-dmyo-inositol-labelled cells were submitted to Triton X-100 extraction at 2 °C. Then, Triton extracts were submitted to sucrose density-gradient ultracentrifugation and analysed by TLC after lipid extraction for the presence of PtdCho, PtdEtn, PtdSer and PtdIns. Figure 3 indicates clearly that all the phospholipids were present in both the soluble and buoyant insoluble fractions. The distribution of the phospholipids with ³H-labelled polar head groups in the lipid rafts was 19, 9, 28 and 13 % for PtdCho, PtdIns, PtdSer and PtdEtn respectively.

[³H]Palmitic acid and [³H]arachidonic acid distribution

To further study the fatty acid composition of the different lipid classes found in membrane microdomains, Jurkat cells were labelled with either [³H]palmitic acid (C₁₆), a saturated fatty acid, or [³H]arachidonic acid (C₂₀), a polyunsaturated fatty acid. The distribution of radioactivity, determined by liquid scintillation after incubation of 2–18 h (results not shown), indicates that the ratio (c.p.m. measured in rafts/total c.p.m.) remained stable at each time point. On that account, a convenient 4 h incubation period was chosen for further experiments. Analysis of [³H]fatty acid-labelled fractions presented a completely different profile. [³H]Palmitic acid-labelled lipids were found preferentially in the buoyant fraction whereas [³H]arachidonic acid-labelled lipids were mostly found in the soluble fraction at the bottom of the ultracentrifuge tube (Figure 4). The percentage of tritiated label in the buoyant fraction was 9 and 34 % for arachidonic acid and palmitic acid respectively.

Analysis by TLC of the extracted lipids indicated unambiguously the presence of PtdCho, PtdEtn and the couple PtdIns/PtdSer, as well as other unidentified lipids, in rafts (Figure 5). Interestingly, [³H]palmitic acid, which is known to label sphingomyelin, allowed us to detect this lipid almost exclusively in the buoyant fraction (Table 2 and Figure 5). This fact confirms the preferential distribution of sphingolipids in rafts, as determined by other methods [30,31].

Rafts are labelled preferentially with saturated fatty acids

Given the results obtained with cells labelled with either palmitic or arachidonic acid, and to confirm the preferential labelling of lipid rafts with saturated fatty acids, we pursued our analysis by studying Jurkat cells labelled with three C₁₆ fatty acids with different degrees of saturation: [³¹⁴]stearic, [³¹⁴]oleic and [³¹⁴]linoleic acids (see Table 1). We found that the saturated fatty acid, i.e. stearic acid, was the best marker for rafts among this series of C₁₆ fatty acids (Figure 4). The distribution of labels in
Figure 4  Comparison of the distribution profile of five different ³H- or ¹⁴C-labelled fatty acids

A PNS preparation of Jurkat cells [(80–100) × 10⁶] metabolically labelled with radioactive arachidonic, linoleic, oleic, palmitic or stearic acid was treated with Triton X-100 at 2 °C and fractionated on a sucrose density gradient as described in the Experimental section. The distribution of radioactivity in each fraction determined by liquid scintillation is shown from the top to the bottom of the density gradient. A further analysis of each radioactive fatty acids-labelled phospholipid was performed and is presented in Table 2. These graphs are representative of three different and independent experiments. The values represent means ± S.E.M. (where no visible, errors bars are within the symbols).

The buoyant fraction was 7, 10 and 17% for linoleic, oleic and stearic acids, respectively. Analysis of the different phospholipid classes by TLC confirmed these results (Table 2). Of the three C₁₈ fatty acids studied, labelling of rafts decreased with the degree of unsaturation, indicating that these buoyant membrane preparations were enriched in saturated fatty acids.

Effect of m-β-CD on cholesterol level and CD3 expression

An approach to perturb raft functions is based on cholesterol extraction from cell membranes [9,32]. m-β-CD is generally used for this purpose [10,33]. As shown in Figure 1, m-β-CD treatment of Jurkat cells resulted in a strong diminution of [³H]cholesterol in rafts. Actually, 78% of [³H]cholesterol was released after 7 min of incubation with 10 mM m-β-CD. FACS analysis of control and m-β-CD-treated cells indicated a small but reproducible increase of CD3 in m-β-CD-treated cells (Figure 6A): this difference was probably due to better recognition of CD3 by the anti-CD3 X3 mAb. Accordingly, CD3-induced second-messenger transduction mechanisms can be studied in m-β-CD-treated cells.

m-β-CD treatment reduces Ca²⁺ mobilization and DAG production

Engagement of the TCR complex with the anti-CD3 X3 mAb generated IP₃-induced Ca²⁺ mobilization from intracellular stores followed by a Ca²⁺ influx through calcium-release-activated channels. In m-β-CD-treated cells, the CD3-induced change in cytosolic Ca²⁺ concentration was strongly reduced (Figure 6B), indicating that rafts are necessary for T-cell activation. By contrast, ionomycin (10⁻⁶ M), a calcium ionophore able to mobilize calcium via a CD3–TCR-independent pathway, still caused calcium mobilization in cholesterol-depleted cells (Figure...
Rafts exhibit phospholipids implicated in CD3 T-cell receptor signalling

Figure 5 Distribution profile of $^{3}$H]palmitic acid-labelled lipids

Top panel: chromatograms of fractions 2 (raft) and 9 (soluble material). A PNS preparation of Jurkat cells ($80–100 \times 10^6$) metabolically labelled with $^{3}$H]palmitic acid was treated with Triton X-100 at 2 °C and fractionated on a sucrose density gradient as described in the Experimental section. Lipids from each fraction were extracted with chloroform/methanol and analysed by TLC on silica gel plates. The distribution of $^3$H-labelled compounds detected by the TLC scanner is shown. The different peaks are characterized by superposition with standard phospholipid spots detected with iodide vapours. SF, solvent front; SM, sphingomyelin.

Bottom panel: distribution of $^{3}$H]palmitic acid-labelled glycerophospholipids and sphingomyelin in rafts and soluble fractions. The amount of radioactivity found in phospholipids or sphingomyelin labelled with $^{3}$H]palmitic acid is shown from the top to the bottom of the density gradient. These graphs are representative of three independent experiments and the values represent means ± S.E.M. (where not visible, errors bars are within the symbols).
Table 2 Distribution of the different radioactive fatty acid-labelled glycerophospholipid species in rafts

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Palmitic acid</th>
<th>Stearic acid</th>
<th>Arachidonic acid</th>
<th>Linoleic acid</th>
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<td>29</td>
<td>ND</td>
<td>11</td>
<td>ND</td>
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<td>6</td>
<td>7</td>
<td>9</td>
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<tr>
<td>PtdIns/PtdSer</td>
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<td>7</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>PtdIns</td>
<td>32</td>
<td>17</td>
<td>ND</td>
<td>3</td>
<td>9</td>
</tr>
</tbody>
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6C). Ca\(^{2+}\)-mobilization is attributed to the phosphorylation and activation of PLC\(_{\gamma}1\), a phospholipase which cuts PIP\(_2\) into two second messengers, IP\(_3\) and DAG. IP\(_3\) is a water-soluble cytoplasmic messenger involved in Ca\(^{2+}\) movements whereas DAG is a lipophilic molecule that is generated in the plasma membrane. In animal tissues, plasma membranes often contain high proportions of arachidonic acid, which is the reason why we studied DAG production in cells pre-labelled with either \(^{3}H\)arachidonic acid or \(^{3}H\)palmitic acid. Studies on Triton X-100-soluble and -insoluble fractions indicated clearly that both CD3-induced arachidonoyl-DAG and palmitoyl-DAG were recovered in the Triton X-100-insoluble fraction of the plasma membrane (Figure 7). DAG production reached its maximum after 10 min of stimulation. The fact that m-\(\beta\)-CD treatment impaired CD3-induced generation of both arachidonoyl- and palmitoyl-DAG (Figure 8A) supports the idea that Triton X-100-insoluble fractions look like lipid rafts. This is illustrated by the sucrose-gradient ultracentrifugation method, which showed that CD3-induced DAG production was localized mainly in rafts (Figure 8B).

**DISCUSSION**

Recent studies have demonstrated that T-cell activation induced by anti-CD3 X3 mAb stimulation leads to the redistribution of some surface proteins into two membrane compartments, one soluble and the other insoluble in Triton X-100 at 2 °C. This detergent-insoluble membrane fraction is called a GEM or raft. The raft outer leaflet is composed of peculiar proteins, such as GPI-anchored surface receptors, sphingolipids and GSLs. The inner membrane fraction of rafts contains some proteins involved in signal transduction, such as the tyrosine kinase p56\(^k\) and LAT; these proteins are bound to the rafts due to their acylated amino acids [34]. The lipid composition of rafts is not well known but previous work has established that this membrane fraction is enriched in cholesterol and sphingomyelin. However, when the lipid composition of high- and low-density membrane fractions were examined, some discrepancies were noted (for a review, see Brown and London [7]). Because rafts might represent a small part of the plasma membrane, classical assays for phospholipids, such as phosphorus determination or specific coloration after TLC, are not sensitive enough to accurately determine the lipid composition of rafts. In order to visualize more precisely the lipid composition of rafts, we have undertaken metabolic labeling studies by using many \(^3H\)- or \(^{14}C\)-labelled precursors of phospholipid biosynthesis. In agreement with Brown and London [6], and in order to avoid artifacts introduced by calculating the lipid/protein ratio, our results are presented in terms of c.p.m. for each phospholipid in the different fractions obtained after sucrose-density-gradient ultracentrifugation and TLC.
Our results demonstrate clearly that \[^{3}H\]cholesterol is found in higher quantities in rafts than in the soluble fraction of the plasma membrane (Figure 1), confirming pioneering work in this domain. Metabolic labelling with \[^{3}H\]glycerol, which results in the radioactive labelling of all classical phospholipids, indicates that the insoluble membrane fraction (rafts) contains the four major classes of phospholipid, i.e. PtdCho, PtdEtn, PtdIns and PtdSer. These data were confirmed by using cell metabolic labelling with tritiated phospholipid polar head groups, \[^{3}H\] ethanolamine, \[^{3}H\]d-myoinositol and \[^{3}H\]serine, which specifically label their respective phospholipids, PtdCho, PtdEtn, PtdIns and PtdSer. A marked preference was noted for the presence of PtdCho and PtdSer in the buoyant vesicles.

Cell labelling with different \(^{3}H\)- or \(^{14}C\)-labelled fatty acids, either saturated or unsaturated, allowed us to determine (within the presence of PtdCho and PtdSer in the buoyant vesicles. A marked preference was noted for

All together, our results give a clear indication that besides cholesterol and sphingomyelin, which are essentially found in the light membrane fraction, all the major glycerophospholipids are present in this buoyant membrane fraction (rafts). This is worth remembering that our results corroborate previous data obtained in Caco-2 cells [35]. These glycerophospholipids were labelled preferentially with saturated fatty acid, supporting the observation that the partitioning of a saturated fluorescent phospholipid analogue into the raft phase, after cross-linking by an antibody, is increased significantly, whereas cross-linking of a doubly unsaturated phospholipid analogue does not change its redistribution [36]. In addition, cell labelling with the fluorescent lipid analogues Dil-C18 and BODIPY-sphingomyelin have shown that rafts are labelled preferentially with the fluorescent probes provided that they contain saturated acyl chains [37]. All together, these data confirm the idea that rafts are composed of packed lipids with long saturated acyl chains interspersed with cholesterol and acylated proteins. Our study on the saturation degree of fatty acids strengthens the idea of the existence of the liquid-ordered phase in the plasma membrane. Indeed, lipids from ‘classical’ (i.e. detergent-soluble) plasma membranes are mainly glycerophospholipids. Because of these membranes’ content of fatty acids, both saturated and unsaturated, they generally have very low melting points, whereas rafts, due to sphingolipids (especially GSLs), have much higher melting points because of the predominance of saturated fatty acids. This disparity suggests that phase separation between glycerolipid and glycosphingolipid domains might occur in biological membranes. Phase separation may explain the observation that cellular-membrane lipids are not completely solubilized by non-ionic detergents such as Triton X-100; today this biophysical particularity is commonly used to purify GEMs.

The role of lipid rafts in T-cell activation was studied by using m-\(\beta\)-CD, which depletes plasma-membrane cholesterol [9,32]. We found that m-\(\beta\)-CD strongly diminished the cholesterol content of lipid rafts (Figure 1) without changing CD3 expression on the T-cell surface (Figure 6). As already reported [38,39], CD3-induced Ca\(^{2+}\) movements are dramatically decreased in m-\(\beta\)-CD-treated cells (Figure 6). In many cell types, PI3P has been shown to be enriched in lipid rafts. Disruption of rafts with m-\(\beta\)-CD results in an inhibition of PI3P turn 40,41]. Inhibition of the production of the second messenger DAG, probably explains the decreased CD3-induced Ca\(^{2+}\) response. By contrast, production of the counterpart of IP\(_3\), the second messenger DAG, has never been studied. Our work proves that m-\(\beta\)-CD treatment of Jurkat cells results in markedly decreased CD3-induced DAG production. In addition, it demonstrates for the first time that in CD3-treated cells both arachidonoyl-DAG and palmitoyl-DAG are localized preferentially in the buoyant membrane fraction. This biochemical evidence helps to explain the translocation and redistribution of protein kinase C isoenzymes during T-cell activation. Protein kinase Cs are a family of at least 12 isoenzymes, whose eight isotypes (\(\alpha\), \(\beta1\), \(\beta2\), \(\delta\), \(\epsilon\), \(\xi\), \(\eta\) and \(\theta\)) are expressed in T-cells [42]. It has been shown that seven protein kinase C isoenzymes shifted from Triton-soluble fractions to membrane microdomains after PMA treatment of Jurkat cells [43]. The preferential distribution of CD3-induced DAG into rafts is likely to explain changes in protein kinase C activity and localization in rafts during T-cell activation. Furthermore, another variety of lipids, GSLs, has been shown to play an important role in T-cell activation. Indeed, data indicate that engagement of surface gangliosides on T-cells by either bacterial toxins or anti-ganglioside antibodies can trigger T-cell effector functions such as proliferation, cytokine release or apoptosis [44]. For example, it has been shown that the trisialoganglio-
Figure 8  m-β-CD inhibits CD3-induced DAG production

(A) Cells pre-labelled with either [3H]palmitic acid or [3H]arachidonic acid were left untreated (controls) or treated with cross-linked CD3 mAb (CD3 XL). The DAG production was measured after lipid extraction and TLC in cells previously submitted or not to m-β-CD treatment. These graphs are representative of three independent experiments and values represent means ± S.E.M. (B) A PNS preparation of Jurkat cells (80–100 × 10⁶) pre-labelled with [3H]palmitic acid and stimulated for 10 min with cross-linked CD3 mAb was treated with Triton X-100 at 2 °C and fractionated on a sucrose density gradient as described in the Experimental section. DAG production was measured after lipid extraction and TLC in cells previously treated (■) or not (□) with anti-CD3 mAb. This panel shows the distribution of [3H]palmitic acid-labelled DAG in the nine fractions obtained after sucrose-gradient centrifugation. The majority of [3H]palmitic acid-labelled DAG was found in the buoyant fraction corresponding to lipid rafts. This graph is representative of three independent experiments and the values represent means ± S.E.M. (where not visible, errors bars are within the symbols).

side GT1b can mediate TCR-dependent activation of T-cells by binding to the tetanus toxin. Given the observations that the GT1b-dependent tetanus toxin response was independent of the fine specificity of the TCR, this ganglioside seems to be involved in innate immunity [45]. In addition, exposure of TCR-negative Jurkat T-cells to the B subunit of cholera toxin, which binds specifically to the monosialoganglioside GM1, results in tyrosine phosphorylation of PLCγ1 and p56lk, inducing calcium flux. These results indicate that GM1-mediated signalling can occur in the absence of surface TCR expression [46]. In addition, cross-linking of GPI-APs also causes TCR-independent activation of T-lymphocytes [47,48]. Accordingly, it becomes evident that GSLs and GPI-APs play an important role in immune response through glycosphingolipidic microdomains.

The whole data set allows us to conclude that the wide variety of lipids found in membranes and particularly in membrane rafts actively participates in signal-transduction pathways. The rafts should therefore not only be considered as a platform that maintains close relationships with the different receptors and protein tyrosine kinases necessary for the transmission of signals, but also as a more complex structure, from the point of view of lipids, from which glycerolipid messengers can emanate.

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REFERENCES


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