An NMR Investigation of the Changes in Plasma Membrane Triglyceride and Phospholipid Precursors during the Activation of T-Lymphocytes†

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ABSTRACT: Two-dimensional ^{1}H-NMR spectroscopy was used to quantify the level of isotropically tumbling plasma membrane triglyceride and the intracellular concentrations of water-soluble phospholipid precursors during the activation of thymic T-lymphocytes. The concentration of "mobile" triglyceride in the plasma membrane was seen to increase 25-fold during 72 h of activation of murine thymic T-lymphocytes with ionomycin and phorbol myristate acetate. This is the first unequivocal demonstration of such a dramatic increase in mobile plasma membrane triglyceride during T-lymphocyte activation and leads to the suggestion that immune cell activation is associated with increased plasma membrane fluidity. The intracellular concentrations of choline- and ethanolamine-based phospholipid precursors were shown to increase during the early stages of T-lymphocyte activation and then remain at levels above those in resting cells. This may facilitate de novo phospholipid biosynthesis, which is presumably necessary since cell volume, and hence the plasma membrane surface area, was demonstrated to increase significantly during thymocyte activation.

It has been well documented that the one- (1D)[1] and two-dimensional (2D) ^{1}H-NMR spectra of a variety of malignant cells are dominated by resonances from elevated levels of plasma membrane triglycerides [e.g., Mountford et al. (1982, 1984a) and May et al. (1986)]. The nature of these membrane triglycerides remains unclear, but the narrow ^{1}H-NMR linewidths of resonances arising from these molecules can only be explained by a physicochemical model which encompasses at least some degree of isotropic diffusion, otherwise their ^{1}H-NMR spectra would consist of extremely broad and featureless resonances which are characteristic of the restricted motion of membrane phospholipids (Mountford et al., 1984b). Thus, it has been suggested, but not proven, that these triglycerides exist in a proteolipid-like complex in the plasma membrane of malignant cells, within which they are relatively free to tumble (Wright et al., 1986).

Potentially of more general significance is the finding that ^{1}H-NMR spectra remarkably similar to those of malignant cells are obtained from activated immune cells, including mitogen-stimulated T-lymphocytes (Holmes et al., 1988), B-lymphocytes (Holmes et al., 1990), and interferon-γ-stimulated macrophages (King et al., 1991), but not their resting counterparts. Notably, all of these cells, in common with metastatic malignant cells, have the capacity to migrate. Thus, it has been suggested that elevated levels of plasma membrane triglycerides, as measured using ^{1}H-NMR spectroscopy, are associated with an increase in cell motility (Holmes et al., 1990; King et al., 1991) and that their levels may correlate with the ability of malignant cells to metastasize and the capacity of T-lymphocytes to migrate to sites of infection.

The exciting possibility that these ^{1}H-NMR observations indicate a common membrane "activation" pathway for both malignant cells and various immune cells has made it imperative that an attempt be made to quantify the level of plasma membrane triglycerides during immune cell activation. This task is, however, more complicated than at first appears, not least due to the fact that T- and B-lymphocytes increase substantially in volume upon mitogen stimulation [e.g., Speckart et al. (1978)]. Even if the concentration of plasma membrane triglyceride remained unchanged during lymphocyte activation, one would expect to see a significant increase in the intensity of triglyceride resonances in the ^{1}H-NMR spectra of a suspension of a *constant number* of immune cells due to the increasing membrane surface area. In previous NMR reports which demonstrated an increase in the intensity of triglyceride resonances following lymphocyte stimulation [e.g., Holmes et al. (1988)] it was not unequivocally determined whether this represented a significant increase in the concentration of the isotropically tumbling portion of the plasma membrane triglyceride.

Thus, it was the intention of this study to use ^{1}H-NMR spectroscopy to quantify the level of plasma membrane triglycerides and water-soluble phospholipid precursors during the activation of a model immune cell population. A pure population of well-characterized immune cells was considered essential for these studies, and cultured T-lymphocytes from mouse thymus are shown to be ideal. We show that plasma membrane triglyceride levels can be quantified from 2D ^{1}H-NMR spectra of activated T-lymphocytes and that the concentrations of numerous water-soluble phospholipid precursors can be quantified from 2D ^{1}H-NMR spectra of perchloric acid extracts of these cells. It is concluded that the concentration of "mobile" triglyceride in the plasma membrane

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[1] Abbreviations: 1D, one dimensional; 2D, two dimensional; BFCS, bovine fetal calf serum; con A, concanavalin A; COSY, *J*-correlated spectroscopy; $Cr(acac)_3$, chromium acetylacetonate; cpm, counts per minute; TSP-d_4, 3-(trimethylsilyl)[2,2,3,3-^{2}H]propionate; DMEM, Dulbecco's modified Eagle's medium; DQFCOSY, double-quantum-filtered *J*-correlated spectroscopy; FID, free induction decay; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PC, phosphocholine; PHA, phytohemagglutinin; PE, phosphoethanolamine, PMA, phorbol myristate acetate; rf, radio frequency; SCC, standard culture conditions; S/N, signal-to-noise ratio; T_2*, apparent transverse relaxation time; triolein, 1,2,3-tri-*cis*-9-octadecenoylglycerol.

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increases dramatically during T-lymphocyte activation, whereas there is only a marginal increase in the intracellular concentration of water-soluble phospholipid precursors.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (*Mus musculus*) with an average age of 6 weeks were housed in standard laboratory conditions, with free access to food and water, in the animal house, Blackburn Building, University of Sydney, Sydney.

Materials. Chromium acetylacetonate [$Cr(acac)_3$] was a gift from Dr. Jacques Nemorin (Department of Organic Chemistry, University of Sydney). Deuterium oxide (D_2O; 99.97%) was supplied by the Australian Nuclear Science and Technology Organization (Lucas Heights, NSW, Australia), while bovine fetal calf serum (BFCS) was obtained from the Commonwealth Serum Laboratories (Melbourne, Victoria, Australia). Ionomycin and [*methyl*-^{3}H]thymidine were purchased from Calbiochem (La Jolla, CA) and ICN Biomedicals Inc. (Irvine, CA). respectively. Concanavalin A (con A) was from Pharmacia LKB (Uppsala, Sweden). Phorbol myristate acetate (PMA) and 1,2,3-tri-*cis*-9-octadecenoylglycerol (triolein) were obtained from Sigma Chemical Co. (St. Louis, MO), while 3-(trimethylsilyl)[2,2,3,3-^{2}H]propionate (TSP-d_4) was from Fluka A.G. (Buchs, Switzerland). All other reagents were analytical grade.

Activation of Splenic and Thymic T-Lymphocytes. Mice were sacrificed by cervical dislocation, and under sterile conditions (Gelman BH120 biohazard hood; Gelman Sciences, Ann Arbor, MI), the thymus and spleen were removed and placed in sterile Petri dishes. The thymus was excised carefully to avoid removing the parathymic nodes, which would contaminate the thymocyte suspension with peripheral B- and T-lymphocytes.

Excised spleens and thymuses were forced through fine wire mesh, using a glass pestle, into a sterile Petri dish containing a small volume (approximately 10 mL) of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% BFCS, L-glutamine (2 mM), HEPES (20 mM), $NaHCO_3$ (44 mM), penicillin (50 units mL^{-1}), streptomycin (50 μg mL^{-1}), and 10^{-4} M 2-mercaptoethanol. Total live T-lymphocyte numbers were calculated by trypan blue exclusion counts, where dead cells which stained blue because of their permeability to trypan blue were excluded from the count (Hunt, 1987).

The suspension of thymic or splenic T-lymphocytes was diluted to a final concentration of 2.0×10^6 cells mL^{-1} in supplemented DMEM and incubated in a humidified atmosphere of 5% CO_2 in air, at 310 K (CO_2-Auto-Zero incubator; Heraeus, Osterode, Germany), subsequently referred to as "standard culture conditions" (SCC). Thymic T-lymphocytes were activated, unless otherwise indicated, by adding ionomycin (200 ng mL^{-1}) and PMA (0.5 ng mL^{-1}) to the cell culture, while splenic T-lymphocytes were activated by con A at a concentration of 2 μg mL^{-1}.

Tritiated Thymidine Assays. Splenic and thymic T-lymphocytes (hereafter referred to as splenocytes and thymocytes, respectively) were suspended in supplemented medium at a concentration of 2.0×10^6 cells mL^{-1} in bulk culture. Splenocytes were activated with various concentrations of con A ranging from 0 to 10 μg mL^{-1}, while thymocytes were activated with varying final concentrations ranging from 100 to 600 ng mL^{-1} ionomycin and 0.5 to 2.0 ng mL^{-1} PMA. Six replicate aliquots of 200 μL were pipetted into individual wells of sterile 96-well flat-bottom plates and incubated under SCC for the specified experimental time.

Wells containing cultured T-lymphocytes were pulsed with 25 μL (1 μCi/well) of [*methyl*-^{3}H]thymidine in phosphate-buffered saline (PBS) and incubated for 6 h under SCC to allow thymidine incorporation to take place. For splenocytes, well contents were harvested (PHD Cell Harvester, Cambridge Technology) onto filter paper disks, which were placed in plastic scintillation vials and left to dry for 24 h before 3 mL of scintillant was added. Thymidine incorporation was measured using a 1215 Beta II liquid scintillation counter (Pharmacia LKB, Uppsala, Sweden). For thymocytes, well contents were harvested (1295-001 cell harvester, Pharmacia LKB) onto filter mats which were allowed to dry for 4 h before being sealed in a sample plastic bag with 10 mL of scintillant. Thymidine incorporation was measured using a 1205 Betaplate liquid scintillation counter (Pharmacia LKB).

Measurement of Mean Cell Diameter as a Function of Time after Activation of Thymic T-Lymphocytes. Light microscope slides of thymocytes at various stages after activation with PMA/ionomycin were prepared by Giemsa staining (Gurr, 1965). The diameters of 100 cells were measured at each stage of activation using a calibrated ocular grid, and the mean diameter was calculated.

Preparation of T-Lymphocytes for NMR Analysis. Cell cultures were removed from the incubator at the end of the predetermined incubation time and centrifuged in 50-mL plastic centrifuge tubes for 15 min at 1500 rpm and 293 K (Heraeus Omnifuge 2.0RS). Two cell pellets were resuspended in 10 mL of collected supernatant. Resuspended cells were placed on a Ficoll bed (7 mL, 14% Ficoll 400 w/v and 10.5% w/v metrizoic acid) and centrifuged for 20 min at 3000 rpm and 293 K, to separate erythrocytes and dead cells from stimulated live T-lymphocytes. Live T-lymphocytes were collected at the interface between the two layers. The T-lymphocyte suspensions were centrifuged for 15 min at 1500 rpm and 293 K, and the supernatants were discarded, leaving T-lymphocyte pellets.

T-lymphocytes were washed three times in 3 mL of PBS prepared in D_2O (PBS/D_2O) to substantially reduce the percentage of protonated water (^1H_2O), and to remove any traces of culture medium. Lymphocytes were counted at this stage by a trypan blue exclusion assay, and viability of >95% was always observed. Lymphocytes were transferred to a PP-507 5-mm o.d. NMR tube (Wilmad, Buena, NJ) using a 100-μL positive displacement pipet. Cells were collected at the bottom of the NMR tube using a hand-operated centrifuge (Newcastle Scientific, Newcastle, NSW, Australia), and PBS/D_2O was added where required to make the final cell volume at least 450 μL. The sample was either used directly in NMR experiments or a coaxial capillary (Wilmad WGS-5BL) containing a chemical shift and/or intensity reference (ethanol) was added.

Preparation of Perchloric Acid Extracts of T-Lymphocytes for NMR Analysis. Thymocytes were removed from the NMR tube on completion of the live cell 2D NMR experiments and transferred to a microcentrifuge tube(s). To ensure all thymocytes were recovered, the NMR tube was thoroughly rinsed with PBS/D_2O. Thymocyte viability, as tested by trypan blue exclusion, was >85% at the completion of all NMR experiments. The cell suspensions were centrifuged for 1 min at 12 000 rpm and ambient temperature. Two volumes of 8% (v/v) perchloric acid were added to the cell pellet(s), and the solution was vigorously mixed and then centrifuged at 12 000 rpm for 10 min. The supernatants (extracts) were collected and the extraction was *repeated*. The extracts were pooled and neutralized with 4 M KOH,

and the pH was adjusted to 7.0–7.5 with 0.1 M NaOH and 0.1 M HCl. Precipitated $KClO_4$ was pelleted by centrifugation for 15 min at 12 000 rpm and 277 K, and the supernatant was collected and lyophilized.

The lyophilized residue was dissolved in 480 μL of D_2O and centrifuged for 10 min at 12 000 rpm and ambient temperature to remove any residual $KClO_4$ precipitate. The supernatant was collected, the pD adjusted to 7.40 ± 0.05 using DCl and NaOD, and the solution was centrifuged for 15 min at 12 000 rpm and 277 K to remove any $KClO_4$ precipitate formed during the pD adjustment. The extract was transferred to a PP-528 5-mm o.d. NMR tube (Wilmad), and the internal chemical shift reference TSP-d_4 was added to an approximate final concentration of 1 mM. Finally, a coaxial capillary (Wilmad WGS-5BL) containing an intensity reference (ethanol) was added to the sample.

NMR Spectroscopy. ^{1}H-NMR spectra were acquired at 600 MHz (14.1 T) or 400 MHz (9.4T) using a Bruker AMX 600 narrow-bore spectrometer or AMX 400 wide-bore spectrometer, respectively. Spectra were collected in the pulsed Fourier transform mode and processed on Aspect X32 computers using UXNMR software (Bruker A.G., Karlsruhe, Germany). The temperature of the samples was controlled at 310 K. Chemical shifts (δ) were referenced to the methyl protons of ethanol, which resonate at 1.24 ppm relative to internal TSP-d_4 at 0.00 ppm.

1D spectra were acquired using a 90° radio-frequency (rf) pulse, a spectral width of 6667 Hz, and a relaxation delay of 1 s prior to the rf pulse, during which time the water resonance was coherently irradiated. Free induction decays (FIDs) were the result of 32 transients summed into 8192 (8K) data points. These were zero-filled to 32K data points and multiplied by a decaying exponential with a line-broadening of 1 Hz prior to Fourier transformation to improve the signal-to-noise ratio (S/N).

The type of 2D *J*-correlated spectra collected of *live T-lymphocytes* represented a compromise between the required S/N and the length of time for which the cells will survive in the NMR tube (typically ~85% viability as assessed by trypan blue exclusion after 3 h in the NMR probe). Non-phase-sensitive COSY spectra (Nagayama et al., 1980) of satisfactory S/N were acquired in ~2.75 h using a spectral width of 6667 Hz in both *F*2 and *F*1, with 256 incremented values of the evolution time (t_1). Acquisition incorporated a relaxation delay of 1 s prior to the first radio-frequency pulse, during which time the water resonance was coherently irradiated. FIDs were collected after 8 unwritten dummy transients, and each FID consisted of 32 transients digitized into 2K data points (real and imaginary). Chemical shifts were referenced to the cross-peak at (1.24, 3.72) ppm arising from spin–spin coupling between the methyl and methylene protons of the external ethanol standard. Double-quantum-filtered (DQF) COSY spectra (Rance et al., 1983) of sufficiently good S/N could not be acquired in less than 3 h due to the theoretically predicted 2-fold reduction in sensitivity compared with the non-phase-sensitive COSY experiment (Rance et al., 1983).

The raw data for all COSY spectra were zero-filled to 1K and 2K real data points in *F*1 and *F*2, respectively. Prior to Fourier transformation, the dataset was multiplied by unshifted sine bell functions in both *F*1 and *F*2 dimensions to remove the "star" line shapes characteristic of magnitude mode spectra (Delikatny et al., 1991). Base lines were corrected using a third-order polynomial function.

2D DQFCOSY spectra (Marion & Wüthrich, 1983) of *thymocyte extracts* were collected using a spectral width of 6667 Hz (600 MHz) or 4424 Hz (400 MHz) in both *F*2 and *F*1. All spectra were acquired using time-proportional phase incrementation to give pure absorption mode phase-sensitive spectra. The water resonance was coherently irradiated for 1.3 s prior to the first rf pulse, and 512 FIDs were collected with incremented values of t_1. FIDs were collected after 8 unwritten dummy scans, and each consisted of 64 summed transients digitized into 2K data points (real and imaginary). Chemical shifts were referenced to the methyl protons of the internal standard TSP-d_4 at 0.00 ppm. The raw data for all spectra were double zero-filled to 1K real data points in *F*1 and single zero-filled to 2K real data points in *F*2. Prior to Fourier transformation, the data were multiplied by a pure cosine bell function in *F*1 and a Lorentz–Gauss function (LB = –20 Hz, GB = 0.2) in *F*2 to improve spectral resolution. Base-line correction, using a third-order polynomial, was applied in both dimensions.

Calculation of Triglyceride Concentrations. Triolein, a triglyceride which resembles those identified in the plasma membrane of malignant cells and activated immune cells (May et al., 1986), was prepared at a known concentration and added to an NMR tube along with a coaxial capillary containing ethanol as an intensity and chemical shift reference. This solution was then titrated with a paramegnetic line-broadening reagent, $Cr(acac)_3$, until the triolein linewidths resembled those of the triglyceride resonances in the thymocyte suspensions (see Results). A 2D COSY spectrum was then collected using the same acquisition and processing parameters as for thymocyte suspensions. The triolein and ethanol cross-peak volumes were integrated, and these were used in combination with cross-peak intensities from COSY spectra of the thymocyte suspensions to calculate the plasma membrane triglyceride concentrations. For the purposes of quantification, the volumes of related (or "brother") cross-peaks on either side of the diagonal were averaged when the two values were within 30%, otherwise the higher of the two values was taken. Furthermore, cross-peaks obscured by t_1 and t_2 noise artifacts (Mehlkopf et al., 1984) were excluded from the calculations.

The concentration of triglyceride in the lymphocyte suspensions ($[T]_{cell}$) was calculated using the equation:

$$[T]_{cell} = \frac{CE_s(10^6x)}{EC_sN} \pm \frac{CE_s(10^6x)}{EC_sN} \times \sqrt{(\delta E/E)^2 + (\delta C/C)^2 + (\delta E_s/E_s)^2 + (\delta C_s/C_s)^2 + (\delta N/N)^2 + (\delta x/x)^2} \quad (1)$$

where $[T]_{cell}$ is the concentration of the triglyceride in nmol/10^6 cells; C and C_s are the integrals of the triglyceride cross-peaks in the lymphocyte suspension and appropriately line-broadened triolein standard solution, respectively; E and E_s are the integrals of the ethanol cross-peak in the lymphocyte suspension and the line-broadened triolein standard solution, respectively; N is the cell count; and x is nmol of triolein in the triolein standard. $\delta E/E$, $\delta E_s/E_s$, $\delta C/C$, $\delta C_s/C_s$, $\delta N/N$, and $\delta x/x$ are the respective percentage errors of the measured parameters, where $\delta E/E$, $\delta E_s/E_s$, $\delta C/C$, and $\delta C_s/C_s$ were 5%, and $\delta N/N$ and $\delta x/x$ were 1%, of the measured parameter values.

Calculation of the Concentrations of Water-Soluble Phospholipid Precursors. Phospholipid precursors, identified in perchloric acid extracts of thymocytes, were prepared at a known concentration, and this "standard solution" was then added to an NMR tube along with a coaxial capillary

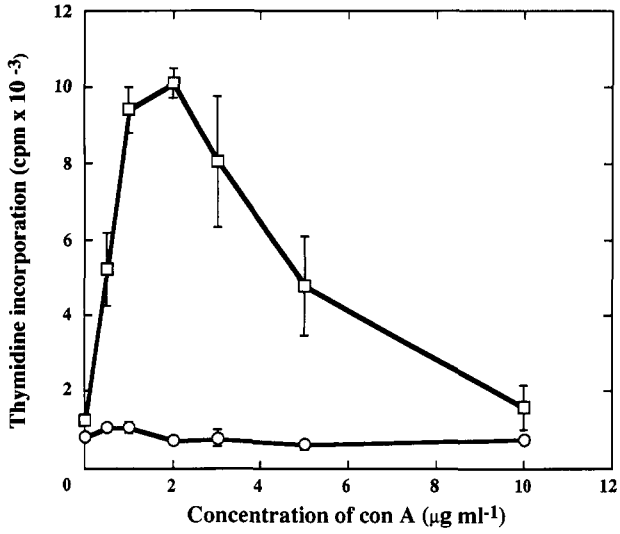

FIGURE 1: Effect of varying concentrations of con A on the incorporation of [*methyl*-^{3}H]thymidine by cultured thymic and splenic T-lymphocytes. The counts per minute values are the means of six separate experiments, and the vertical error bars denote ±1 standard error. Thymocytes (○) were unresponsive to con A, while splenocytes (□) were optimally stimulated at a con A concentration of 2 μg mL^{-1}.

containing ethanol as an intensity and chemical shift reference. A DQFCOSY spectrum was collected using the same acquisition and processing parameters used to obtain DQF-COSY spectra of extracts. The cross-peak volumes from ethanol and the phospholipid precursors were integrated and used in combination with the cross-peak intensities from the thymocyte extracts to calculate the concentrations of phospholipid precursors ([P]$_e$) in the extracts using the equation:

$$[P]_e = \frac{C_e E_s (10^6 x V_c)}{E_e C_s N V_e} \pm \frac{C_e E_s (10^6 x V_c)}{E_e C_s N V_e} \times \sqrt{(\delta E_e/E_e)^2 + (\delta C_e/C_e)^2 + (\delta E_s/E_s)^2 + (\delta C_s/C_s)^2 + (\delta N/N)^2 + (\delta x/x)^2} \quad (2)$$

where [P]$_e$ is in nmol/10^6 cells; V_c is the volume of thymocytes used in the live-cell COSY analysis and V_e is the volume of thymocytes used to prepare the extract; C_e and C_s are the integrals of the metabolite cross-peaks in the extract and the standard solution consisting of the phospholipid precursors, respectively; E_e and E_s are the integrals of the ethanol cross-peak in the extracts and the standard solution, respectively; and x is nmol of metabolite in the standard solution. All other parameters and errors were the same as above. It was assumed that there was negligible error in the measurement of V_c and V_e.

RESULTS

Determination of Optimal Growth Conditions for Proliferation of Thymocytes and Splenocytes. NMR analysis of live tissue, such as lymphocytes, requires large numbers of cells (~10^8; Sze & Jardetzky, 1990) due to the relative insensitivity of the technique. Therefore, it was imperative to determine the optimal concentrations of stimuli to ensure that maximum proliferation, and thus the highest yield of cultured T-lymphocytes, was attained.

Splenocytes were strongly activated by con A, with the highest proliferative response occurring at a con A concentration of 2 μg mL^{-1} (see Figure 1). Thymocytes were not activated by con A at any of the concentrations tested (see Figure 1) as the thymus cell suspension has very few co-

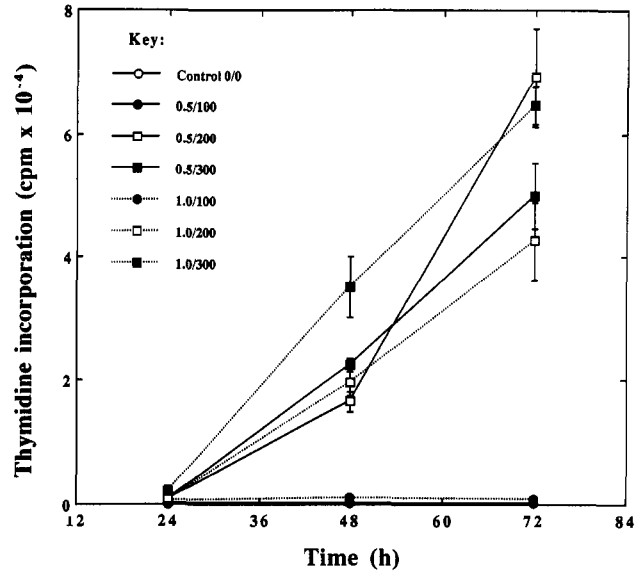

FIGURE 2: Effect of various combinations of the concentrations of PMA and ionomycin on [*methyl*-^{3}H]thymidine incorporation into cultured thymic T-lymphocytes. The key gives PMA/ionomycin concentrations in ng mL^{-1}. The counts per minute values are the means of six separate experiments, and the vertical error bars denote ±1 standard error. Thymocytes were optimally stimulated by a combination of PMA and ionomycin at concentrations of 0.5 and 200 ng mL^{-1}, respectively.

stimulatory cells required to elicit activation (<1%; Smith et al., 1980). The small amount of thymidine incorporation that was observed for the thymocytes was probably due to the high level of growth hormones in the BFCS stimulating a small fraction of the cell population.

Since thymocytes were not activated by con A, an alternative stimulus of ionomycin and PMA was employed. Thymocytes were activated with various combinations of these stimuli, with the optimal proliferative response occurring after 72 h of activation with 0.5 ng mL^{-1} PMA and 200 ng mL^{-1} ionomycin (see Figure 2). Note the higher ordinate scale in Figure 2 compared with Figure 1, indicating that higher T-lymphocyte numbers were obtained by culturing PMA/ionomycin-activated thymocytes than con A-activated splenocytes. This observation, combined with the knowledge that the thymus provides an inherently purer source of T-lymphocytes than the spleen, made thymocytes an ideal "model" immune cell population for subsequent NMR experiments.

1D ^{1}H-NMR Spectra of Activated Splenic and Thymic T-Lymphocytes. The time course of thymocyte activation was examined using 1D ^{1}H-NMR spectroscopy, with emphasis placed on the early part of the time course as a consequence of previous observations on splenocytes (Holmes et al., 1988). 1D ^{1}H-NMR spectra of resting thymocytes (0 h) showed no distinct resonance at 1.30 ppm corresponding to the methylene groups in the acyl chain of triglyceride(s) (see Figure 3A). The resonance at ~3.25 ppm is from the N(CH$_3$)$_3^+$ groups of choline, phosphocholine (PC), glycerophosphocholine (GPC), and phosphatidylcholine (Williams et al., 1985). After 6 h of activation a small resonance at 1.30 ppm (Figure 3B) indicated the presence of triglyceride, and by 12 h this resonance was quite distinct (spectrum not shown). Triglyceride HC=CH and CH$_3$ resonances at 5.30 and 0.85 ppm, respectively, were also identified in the 6- and 12-h spectra. The 1D spectra of 48- and 72-h thymocyte cultures were dominated by the 1.3 ppm triglyceride resonance (Figure 3C). The 1D ^{1}H-NMR spectra of activated splenocytes and thymocytes exhibited distinct similarities with regard to the

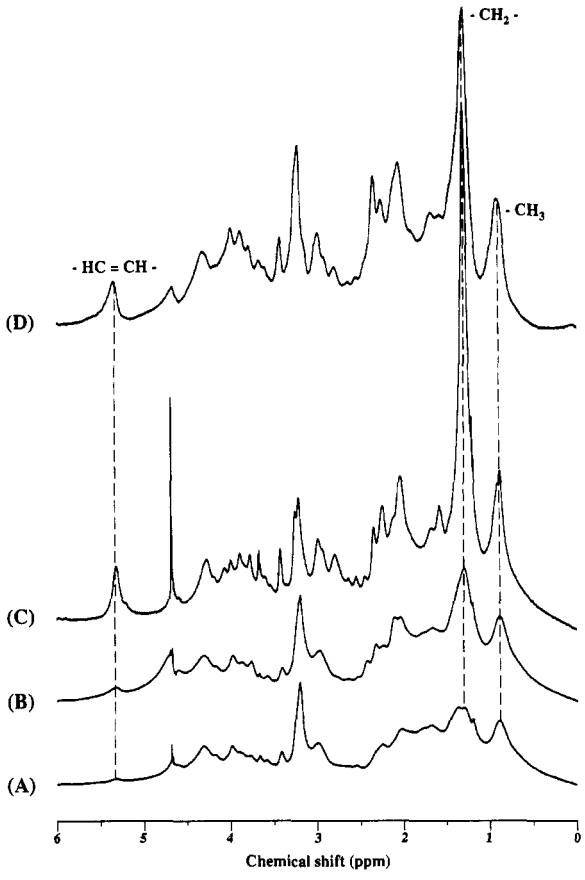

FIGURE 3: 1D ^{1}H-NMR spectra of cultured splenic and thymic T-lymphocytes at various stages after the onset of activation. Splenocytes and thymocytes were activated with con A and PMA/ionomycin, respectively. The characteristic resonances from plasma membrane triglyceride at 5.30 (CH=CH), 1.30 (CH_2), and 0.85 ppm (CH_3) were barely present in the spectrum of nonactivated thymocytes (A), but began to increase in intensity after 6 h of activation (B) and dominated the 1D ^{1}H-NMR spectrum after 72 h (C). After 72 h of activation, the 1D ^{1}H-NMR spectra of thymocytes (C) and splenocytes (D) were similiar, despite the use of different stimuli for these cell types.

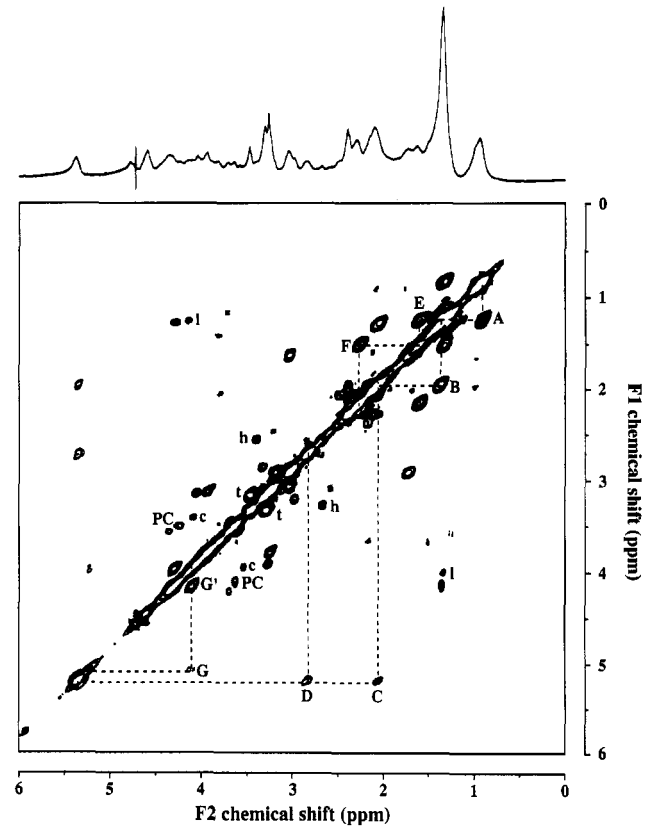

FIGURE 4: 2D COSY spectrum of thymic T-lymphocytes after 72 h of stimulation with PMA/ionomycin. Cross-peaks arising from plasma membrane triglyceride are labeled A–G as previously documented (May et al., 1986), and the respective scalar couplings are indicated by dotted lines. The spectrum was symmetrized, and the upper trace represents the *F*2 projection. Nonsymmetrized spectra were used in the calculation of plasma triglyceride concentrations. Other labeled cross-peaks arise from taurine (t), hypotaurine (h), lactate (l), choline (c), and phosphocholine (PC).

presence of resonances arising from plasma membrane triglyceride (Figure 3D).

Quantification of Triglyceride Concentrations in Activated Thymocytes using 2D ^{1}H-NMR COSY Spectra. Figure 3 demonstrates that 1D ^{1}H-NMR spectra provide useful qualitative indications of changes in plasma membrane triglyceride levels during the activation of T-lymphocytes. However, they cannot be used for calculating triglyceride concentrations since magnetic field gradients (Kuchel & Bulliman, 1988) resulting from the inhomogeneous thymocyte suspensions accentuate the large linewidths of these membrane-bound molecules, causing severe peak overlap and making it impossible to accurately integrate the areas of the triglyceride resonances.

Figure 4 shows a 2D COSY spectrum of a suspension of thymocytes 72 h after activation with PMA/ionomycin. The cross-peaks are a manifestation of scalar coupling between protons on adjacent chemical groups no more than three chemical bonds apart, and those arising from plasma membrane triglyceride are labeled A–G as previously described (May et al., 1986). A–F denote the acyl chains of the triglyceride, while G and G′ arise from scalar couplings between the protons on the glycerol backbone (May et al., 1986). Other metabolites identified in the 2D COSY spectrum include taurine, hypotaurine, choline, PC, and lactate, as indicated in the legend to Figure 4.

The increased spectral resolution available in the 2D COSY experiment meant that the triglyceride cross-peaks could be accurately integrated. However, these cross-peak volumes could not be converted to concentrations by simple comparison with the cross-peak volume of an internal or external reference of known concentration because (i) the time constraints imposed by the limited life span of the cells in the NMR tube necessitated use of an interscan delay of insufficient duration to enable full relaxation of nuclear magnetization between transients and (ii) the unshifted sine bell apodization function used to reduce twisted line-shape artifacts in the non-phase-sensitive COSY spectra effectively acted as a T_2 filter, thus differentially affecting the intensity of resonances on the basis of their transverse relaxation times (Delikatny et al., 1991).

Thus, we chose an alternative approach which involved comparing the ratios of triglyceride cross-peak volumes to an external ethanol standard, with the corresponding ratios obtained from a triglyceride standard of known concentration containing the same external standard. COSY spectra of the triglyceride standard were collected using the same acquisition and processing parameters as for the thymocyte suspensions. However, since the triglyceride (triolein) in the standard was free in solution rather than membrane-bound, the transverse relaxation times were considerably longer and the cross-peak volumes were correspondingly larger. Figure 5 illustrates that decreasing the apparent transverse relaxation time (T_2*) of the triglyceride protons by addition of the paramagnetic

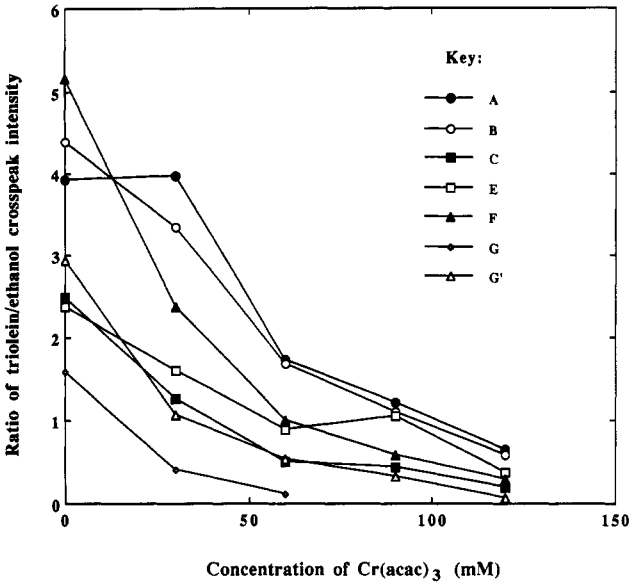

FIGURE 5: Effect of transverse relaxation rate on the intensity of triolein cross-peaks. A standard solution of triolein was titrated with the line-broadening agent $Cr(acac)_3$, and the ratio of the intensity of triolein cross-peaks to that of an external standard, ethanol, is shown as a function of the $Cr(acac)_3$ concentration. The key identifies data corresponding to the cross-peaks labeled in Figure 4. Clearly, decreasing the apparent transverse relaxation time (T_2^*) of the triglyceride by addition of the paramagnetic reagent $Cr(acac)_3$ caused a dramatic reduction in the triglyceride/ethanol cross-peak volume ratios.

reagent $Cr(acac)_3$ to the standard triglyceride solution caused a dramatic reduction in the triglyceride/ethanol cross-peak volume ratios. This can be explained as resulting from less magnetization being sampled by the observe pulse in the COSY experiment and from a larger proportion of the magnetization being filtered by the sine bell apodization function as the transverse relaxation time decreases.

Thus, for the proposed quantification technique to be successful, the transverse relaxation times of the triglyceride protons in the standard solution and the thymocyte suspension should be equal. We consequently added $Cr(acac)_3$ to the standard solution until the linewidths of the triglyceride resonances were as close as possible to those in the lymphocyte suspension and then acquired a COSY spectrum for the purposes of quantification using eq 1; the linewidths were approximately matched when the $Cr(acac)_3$ concentration in the standard solution reached 120 mM. Figure 6 shows that the concentration of the isotropically tumbling portion of plasma membrane triglyceride increased monotonically from a very low level in resting thymocytes to $\sim$0.68 nmol/10^6 cells in those which had been activated for 72 h with PMA/ionomycin. Using chemical analysis, Mackinnon et al. (1989) showed that the total triglyceride content of malignant Chinese hamster ovary cells (EOT cell line) was 2.99 nmol/10^6 cells. These malignant cells were similar in size [$\sim$14-μm diameter measured from the electron micrograph in Figure 4 of Mackinnon et al. (1989)] to the activated thymocytes used in this study (see Table I), and they also yielded a 1D ^{1}H-NMR spectra dominated by triglyceride resonances. Our triglyceride values for activated thymocytes are predictably lower than those measured chemically by Mackinnon et al. (1989) for EOT cells and by Holmes et al. (1987) for con A-activated murine splenocytes (3.88 nmol/10^6 cells) because we used NMR to selectively measure only the "mobile" plasma membrane triglyceride, whereas the chemical method non-selectively measures total cell triglyceride.

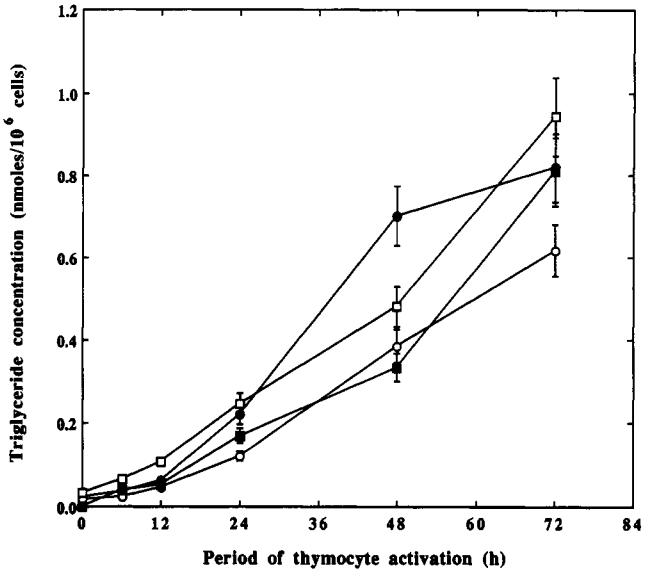

FIGURE 6: Changes in the concentration of the isotropically tumbling portion of plasma membrane triglyceride during the stimulation of thymocytes with PMA/ionomycin. Concentrations were calculated independently from the intensities of triglyceride cross-peaks A (○), B (●), C (□), and E (■) using eq 1 to give some indication of the precision of the data. Error bars denote ±1 standard deviation.

Quantification of the Concentrations of Phospholipid Precursors Using 2D ^{1}H-NMR DQFCOSY Spectra of Perchloric Acid Extracts of Activated Thymocytes. It is not possible to use ^{1}H-NMR spectrocopy to quantify the level of membrane-soluble phospholipids during lymphocyte activation since the diffusion of these molecules within the membrane is restricted and anisotropic, which leads to extremely broad and featureless ^{1}H-NMR resonances. However, numerous water-soluble lipid precursors such as PC and phosphoethanolamine (PE) can be readily identified in 2D DQFCOSY spectra of perchloric acid extracts of these cells (Sze & Jardetzky, 1990), although no attempt has been previously made to use these spectra for the purposes of metabolite quantification. 1D ^{1}H-NMR spectra of perchloric acid extracts of human peripheral lymphocytes have been used in an attempt to quantify the levels of various metabolites (Sze & Jardetzky, 1990), but the method is unreliable due to the high degree of resonance overlap.

Figure 7 shows that cross-peaks arising from choline, PC, GPC, ethanolamine, PE, and glycerophosphoethanolamine (GPE) were well separated from other cross-peaks and the diagonal in DQFCOSY spectra of activated thymocytes and thus could be accurately integrated, unlike the corresponding resonances in the 1D ^{1}H-NMR spectra. It was our aim to use these cross-peaks to quantify the levels of phospholipid precursors during thymocyte activation. However, in 2D DQFCOSY spectra, significant cancellation of the antiphase components of a cross-peak occurs if the active coupling constants are of similar magnitude to the resonance linewidths (Neuhaus et al., 1985). This precluded quantification of metabolites by comparing their cross-peak intensities with that of an internal or external reference of known concentration, since the extent of antiphase cancellation varies from one metabolite to another. Moreover, as discussed above, use of this technique is further precluded by the use of an inter-transient delay of insufficient duration to allow complete nuclear relaxation and apodization functions which effectively act as a T_2 filter.

Thus, phospholipid precursors were quantified by comparing the ratios of their cross-peak intensities to an external standard

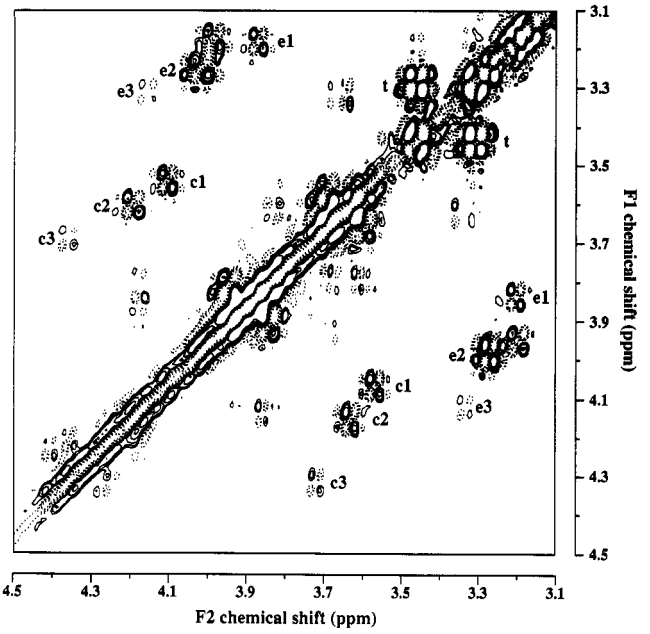

FIGURE 7: 2D DQFCOSY spectrum of a perchloric acid extract of thymic lymphocytes which had been activated for 48 h with PMA/ionomycin. Cross-peaks are antiphase square arrays, with positive and negative contours represented by solid and dotted lines, respectively. Peaks labeled c1, c2, c3, e1, e2, and e3 arise from the lipid precursors choline, PC, GPC, ethanolamine, PE, and GPE, respectively. Taurine (t) was also detected in high concentration.

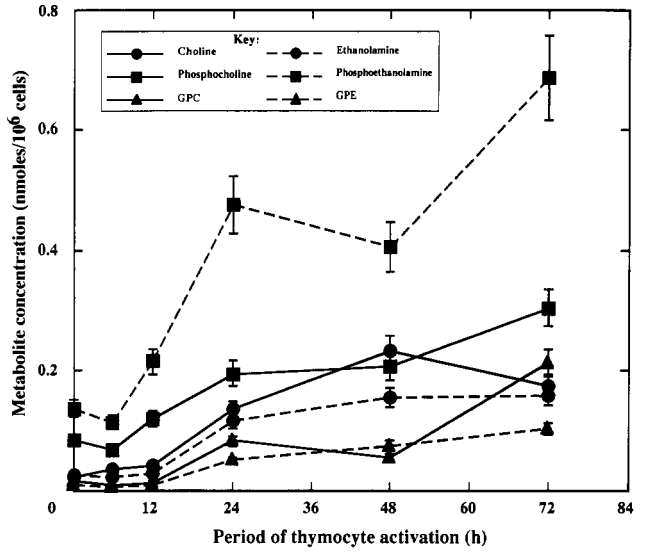

FIGURE 8: Changes in the levels of water-soluble phospholipid precursors during the stimulation of thymic T-lymphocytes. The key identifies data corresponding to choline, PC, GPC, ethanolamine, PE, and GPE.

(ethanol) with the corresponding ratios obtained from a standard solution containing each of these metabolites at a known concentration; Berkowitz and Balaban (1989) used a similar method, except with TSP-d_4 as an internal standard, to measure the inositol concentration in perchloric acid extracts of rabbit kidney. The linewidths (and thus T_2*) were similar in the standard mixture and the perchloric acid extracts of thymocytes, thus obviating the need for addition of a line-broadening reagent to the standard mixture. Figure 8 shows that the concentrations of the choline- and ethanolamine-based phospholipid precursors generally increased monotonically during the activation of thymic lymphocytes with PMA/ionomycin.

Table I: Changes in Mean Cell Diameter, Cell Volume, and Membrane Surface Area during the Activation of Cultured Thymic T-Lymphocytes with PMA/Ionomycin[a]

time after activation (h)	mean cell diameter (μm)	cell volume (fL)	membrane surface area (μm^2)
0	10.8	660	366
24	10.9	678	373
48	11.8	860	437
72	14.4	1563	651

[a] Cell volume and membrane surface area were calculated from the mean cell diameter assuming that the cells are spherical.

DISCUSSION

We have demonstrated that the amount of "mobile" plasma membrane triglyceride can be quantified from 2D COSY spectra of thymic T-lymphocytes and that the concentrations of various water-soluble phospholipid precursors can be quantified from 2D DQFCOSY spectra of perchloric acid extracts of these cells. The former result highlights a unique advantage of NMR spectroscopy, since no other technique is capable of selectively measuring just the *mobile* triglyceride fraction as opposed to the *total* triglyceride content of the plasma membrane. The results, as presented in Figures 6 and 8 using the conventional units of nmol of metabolite/10^6 cells, show that the amounts of these compounds increase essentially monotonically during the activation of cultured thymic T-lymphocytes with PMA/ionomycin. This is perhaps not surprising, since lymphocytes are known to increase in volume upon activation by various chemical stimuli (Speckart et al., 1978). We measured the mean cell diameter during a 72-h period of activation of thymocytes with PMA/ionomycin in an attempt to interpret these metabolite concentrations in a more rigorous way. These results are summarized in Table I, along with the mean cell volume and surface area calculated assuming that thymocytes are spherical.

The cell volumes were subsequently used with the data presented in Figure 8 to calculate the changes in the intracellular molar concentrations of choline- and ethanolamine-based phospholipid precursors in activated thymocytes. These data, shown in Figure 9, indicate that the intracellular concentrations of phospholipid precursors, particularly PE, increased sharply during the first 24 h of activation with PMA/ionomycin, presumably to facilitate de novo phospholipid biosynthesis which is required to stock the expanding plasma membrane bilayer. After 24 h the concentrations of these compounds varied significantly, but always remained at levels above those of resting murine T-lymphocytes.

Interestingly, the intracellular concentrations of choline (0.04 mM), phosphocholine (0.13 mM), and glycerophosphocholine (0.03 mM) in resting murine thymic T-lymphocytes were significantly less than the reported concentrations of these compounds (2.71, 6.77, and 4.19 mM, respectively) determined from 1D ^{1}H-NMR spectra of a heterogeneous mixture of resting human peripheral lymphocytes, but similar to the intracellular concentrations of these compounds (0.07, 0.03, and 0.14 mM, respectively) estimated from 1D ^{1}H-NMR spectra of human erythrocytes (Sze & Jardetzky, 1990). Furthermore, in contrast to the observations made in this study, the intracellular choline concentration was observed to decrease significantly during the activation of human peripheral lymphocytes with either phytohemagglutinin (PHA) or con A (Sze & Jardetzky, 1990). The reasons for these discrepancies are not clear, but the T-lymphocytes were obtained from different sources and were activated by different

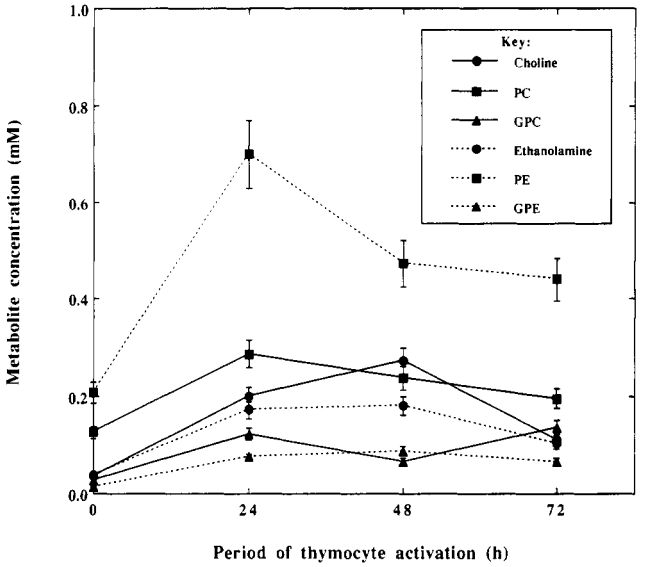

FIGURE 9: Changes in the intracellular molar concentrations of water-soluble phospholipid precursors during the stimulation of thymic T-lymphocytes. The key identifies data corresponding to choline, PC, GPC, ethanolamine, PE, and GPE.

chemical stimuli in each of the studies. Some of the metabolite concentrations reported in the study of Sze and Jardetzky (1990) may be ovestimates because of the problems of integrating severely overlapped resonances in 1D ^{1}H-NMR spectra of lymphocytes.

The mean membrane surface area increased from 366 μm^2 in resting murine thymocytes to 651 μm^2 in thymocytes which had been activated for 72 h with PMA/ionomycin (see Table I). The latter value is very similar to the membrane surface area of 564 μm^2 calculated from measurements of the cell volume of human peripheral lymphocytes which had been activated with PHA or con A (Speckart et al., 1978). However, both of these values might be slight underestimates, since microvillus formation has been reported to accompany lymphocyte activation (Criswell et al., 1975).

Whereas the membrane surface area increased approximately 1.8-fold over 72 h of thymocyte activation, the *amount* of isotropically tumbling triglyceride in the plasma membrane increased approximately 45-fold (see Figure 6). Thus, the *density* of mobile triglyceride (i.e., amount of triglyceride/unit membrane surface area) increased by 45/1.8 = 25-fold during the 72 h of activation. This is the first unequivocal demonstration of a dramatic increase in the density of the isotropically tumbling portion of the plasma membrane triglyceride during immune cell activation. Thus, the use of NMR spectroscopy to monitor plasma membrane triglyceride levels may serve as a noninvasive means for identifying cells of the immune system undergoing activation. The observed accumulation of plasma membrane triglyceride during this process supports the suggestion that it may be associated with increases in membrane fluidity (Mountford et al., 1982) which may assist in the movement of T-lymphocytes to a site of inflammation where they can proliferate and combat the pathogen more directly. The increased fluidity of the membrane may also be important in permitting movement of cell surface proteins to reveal otherwise functionally cryptic domains which are used as receptors for lymphokines or for communication between cells during an immune response.

Whether the observed accumulation of "mobile" triglyceride in the plasma membrane of activated thymocytes results from de novo triglyceride synthesis or from mobilization of triglyceride from a preexisting, NMR-invisible store remains to be determined. The possibility that triglyceride is assimilated from the extracellular fluid was eliminated by using bovine serum albumin in the culture medium instead of BFCS. Under these circumstances, cells survived for 24 h after activation with PMA/ionomycin, and 1D ^{1}H-NMR spectra of these cells showed a similar increase in triglyceride levels to that observed in spectra of activated cells which were cultured for 24 h in BFCS medium (data not shown).

The possibility that triglyceride is assimilated into the plasma membrane of activated immune cells from a preexisting intracellular (NMR-invisible) store is attractive because there are numerous precedents for the incorporation of intracellular membranous vesicles into the plasma membrane of cells in response to a chemical stimulus. For example, the surface area of a mast cell plasma membrane increases dramatically in response to various chemical stimuli as a result of regulated exocytosis of histamine-containing vesicles (Alberts et al., 1989). There are also numerous reports of receptors and membrane transport proteins being exocytotically recruited into the plasma membrane following binding of a growth factor or hormone; an example is the insulin-stimulated recruitment of glucose transporters into the plasma membrane of insulin-sensitive cells (Lienhard et al., 1992). However, no definitive conclusions can be made at this stage about the source and mechanism of accumulation of isotropically tumbling triglyceride in the plasma membrane of malignant cells and activated immune cells. We are currently continuing our NMR investigations into the metabolic changes which occur during thymocyte activation in an attempt to address this question.

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Registry No. PC, 107-73-3; GPC, 563-24-6; PE, 1071-23-4; GPE, 1190-00-7; choline, 62-49-7; ethanolamine, 141-43-5.