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Arachidonoyl-Phospholipid Remodeling in Proliferating Murine T Cells

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Abstract

Backgound: Previous studies have shown that the functional capacity of T cells may be modulated by the composition of fatty acids within, and the release of fatty acids from membrane phospholipids, particulary containing arachidonic acid (AA). The remodeling of AA within membrane phospholipids of resting and proliferating $CD4^+$ and $CD8^+$ T cells is examined in this study.

Results: Splenic T cells were cultured in the presence or absence of anti-CD3 mAb for 48 h then labeled with [^{3}H]AA for 20 min. In unstimulated cells, labeled AA was preferentially incorporated into the phosphoglycerides, phosphatidylcholine (PC) followed by phosphatidylinositol (PI) and phosphatidylethanolamine (PE). During a subsequent chase in unlabeled medium unstimulated $CD4^+$ and $CD8^+$ T cells demonstrated a significant and highly selective transfer of free, labeled AA into the PC pool. In contrast, proliferating $CD4^+$ and $CD8^+$ T cells distributed labeled [^{3}H]AA predominantly into PI followed by PC and PE. Following a chase in AA-free medium, a decline in the content of [^{3}H]AA-PC was observed in association with a comparable increase in [^{3}H]AA-PE. Subsequent studies revealed that the cold AA content of all PE species was increased in proliferating T cells compared with that in non-cycling cells, but that enrichment in AA was observed only in the ether lipid fractions. Finally, proliferating T cells preincubated with [^{3}H]AA exhibited a significant loss of labeled arachidonate in the PC fraction and an equivalent gain in labeled AA in 1-alk-1'-enyl-2-arachidonoyl-PE during a chase in unlabeled medium.

Conclusion: This apparent unidirectional transfer of AA from PC to ether-containing PE suggests the existence of a CoA-independent transacylase system in T cells and supports the hypothesis that arachidonoyl phospholipid remodeling may play a role in the regulation of cellular proliferation.

Background

T lymhocytes occupy a pivotal role in immune regulation by virtue of their ability to promote B cell maturation, drive antibody production and influence the growth and proliferation of macrophages, neutrophils, mast cells, eosinophils and other T cells. Previous studies have shown that the functional capacity of T cells may be modulated by the composition of fatty acids within, and the release of fatty acids from membrane phospholipids. Alterations in the content of membrane phospholipids, particularly those containing arachidonic acid (AA) have been shown to modify the cytotoxic capacity of T cells [1],

membrane transport processes [2], enzymatic activity [3] and the expression of programmed cell death [4]. AA promotes the activation of protein kinase C [5], stimulates T cell mitogenesis [6] and serves as a precursor of prostaglandins and leukotrienes [3].

In T lymphocytes, which lack significant 5-lipoxygenase and cyclooxygenase activities, the level of free arachidonic acid (AA) is tightly regulated. AA comprises approximately 25% of fatty acids within the plasma membranes of resting T cells [7]. It is found primarily esterified at the *sn*-2 position of the phophoglycerides, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE). In unstimulated T cells, PC and PE each contain approximately 38%, PI 20% and PS 4% of the total AA content of plasma membrane phospholipids. Less than 3% of AA exists free in T cell membranes.

AA is introduced into phospholipid molecular species largely after *de novo* synthesis, through remodeling pathways. The remodeling of arachidonoyl phospholipids is orchestrated by acyltransferases and transacylases [8]. Acyl-CoA synthetase catalyzes the ATP-dependent production of arachidonoyl-CoA. Acyl-CoA:lysophosphatidyl acyltransferase may then transfer AA to the *sn*-2 position of 1-acyl-glycerophospholipids. In resting lymph node T cells, 1-acyl-PC is the preferred acceptor species [7]. Whether 1-acyl-, 1-alkyl- and 1-alk-1'enyl-lysophospholipid acceptors require different acyltransferases in T cells is currently unknown. Acyltransferase activities are thought to be coupled to phospholipase A_2, which generates lysophospholipids thereby completing a deacylation-reacylation cycle. A number of reports have suggested that several members of the phospholipase A_2 family are capable of exhibiting acyltransferase and transacylase activities in membranes. In this connection, cytosolic phospholipase A_2, which is specific for AA-containing phospholipids, has been shown to express both phospholipase A_2 and lysophospholipase/transacylase activities [9,10]. Thus, phospholipase A_2s may be important in the remodeling of AA-containing phospholipids in certain cells with inflammatory properties.

CoA-dependent transacylases provide another mechanism by which AA esterified in phospholipids may be transferred to lysophospholipids, in this case without the generation of free fatty acids [11]. CoA-dependent transacylation occurs in T cells in the absence of ATP and Mg^{2+} and may be mediated by the backward reaction of acyl-CoA:lysophospholipid acyltransferase [7,12-14].

CoA-independent transacylase activity has been found in cells that contain significant concentrations of ether-linked phospholipids, such as macrophages and neutrophils [15,16]. This activity catalyzes the transfer of C20 and C22 polyunsaturated fatty acids from the *sn*-2 position of diacylphospholipids to lysophospholipids in the absence of CoA or other cofactors [4]. A preferred substrate of CoA-independent transacylase is diacyl-PC [17]. Potential acceptor lysophospholipids include 1-alkenyl, 1-acyl and 1-alkyl species of both PC and PE [17,18].

The incorporation and distribution of AA in a variety of cells with proinflammatory potential such as neutrophils, macrophages and mast cells follows a predictable pattern. As stated above, AA is initially incorporated into specific phospholipids and subsequently remodeled under the influence of acyl-CoA synthetase, acyl-CoA transferase and acyltransferases. In such cells, the AA content of the ether phospholipids, 1-alkyl-2-acyl-PC and 1-alk-1'-enyl-2-acyl-PE is particularly high [17,19,20] and the arachidonoyl plasmalogen pool increases further following activation [19,21-24]. It has been hypothesized [25] that these ether phospholipids may serve as reservoirs for key lipid mediators (e.g. platelet activating factor) and/or AA derivatives (i.e. prostaglandins and leukotrienes). On the bases of fatty acid and donor specificities, it has been suggested further that the transfer of AA from 1-acyl-2-arachidonoyl-PC to 1-alkyl-2-lyso-PC and 1-alk-1'-enyl-2-lyso-PE is mediated by a CoA-independent transacylase activity [8,25].

T cells possess certain biological and chemical similarities with neutrophils, macrophages and mast cells in that they have proinflammatory potential and contain relatively high levels of ether phospholipids [19]. However, unlike macrophages, neutrophils or mast cells resting T cells fail to accumulate significant levels of ether-containing phospholipids following short-term incubation with labeled AA. This leaves open the question of whether a CoA-independent transacylase system is a common feature of cells with inflammatory properties, including those that possess a limited capacity to synthesize eicosanoids and platelet activating factor, such as T cells. The issue is of immunological interest since significant expression of CoA-independent transacylase activity is in part a commitment to the generation of plasmalogens, which may play a role in T cell function. To test the hypothesis that T cells possess CoA-independent transacylase activity *in vivo*, we took advantage of the fact that the arachidonoyl-phospholipid remodeling rate may be greatly accelerated in proliferating cells. Our results suggest that proliferating T cells possess a CoA-independent transacylase system as evidenced by an apparent transfer of AA from diacyl-PC to 1-alk-1'-enyl-lyso-PE and support the hypothesis [25] that the remodeling of AA-phospholipids and the generation of plasmalogens play a possible role in cell growth.

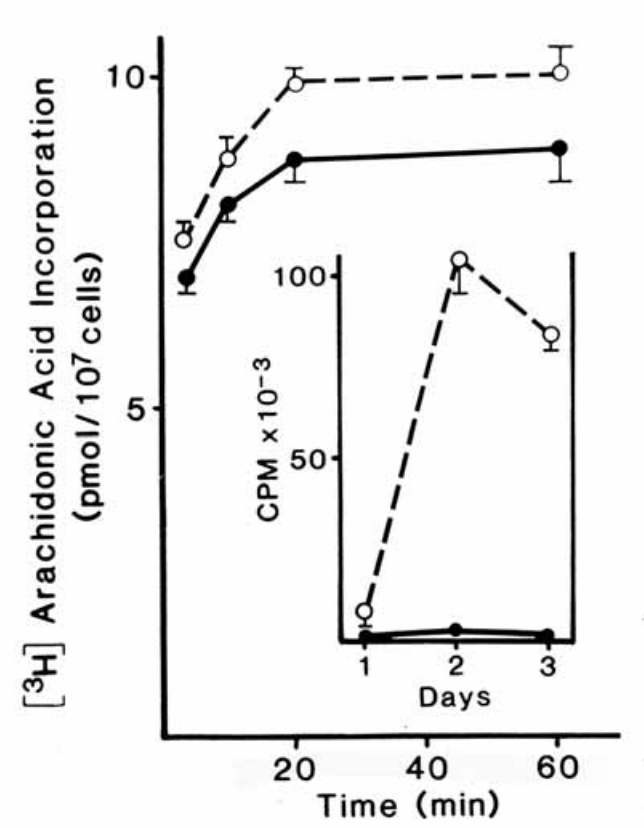

Figure 1
[3H]AA incorporation in T cells. Splenic T cells were cultured for 48 hr with (open circles) or without (closed circles) anti-CD3 mAb, then incubated with [3H] AA (1 μCi/ml) for up to 60 min. At varying times, cells were harvested and labeled AA incorporation was quantified. *Inset*, Tritiated thymidine incorporation, assessed daily for 3 days, in T cells cultured with (open circles) or without (closed circles) anti-CD3 mAb. The results are the mean and standard error of 3 separate experiments.

Results and Discussion

[3H]AA incorporation, distribution and turnover in T cells stimulated with anti-CD3 mAb

Initial studies were directed toward comparing the distribution of radioactive AA of high specific activity in proliferating and resting T cells. The monoclonal anti-CD3 antibody was chosen as the stimulant. Anti-CD3 transduces a signal that mimics antigen binding to the T cell receptor. Preliminary experiments revealed that splenic T cells activated with anti-CD3 mAb exhibited maximal tritiated thymidine incorporation at 48 hrs [Fig 1, inset]. To examine differences in the distribution of [3H]AA within phosphoglycerides of cycling versus quiescent T lymphocytes, cells were cultured in the presence or absence of anti-CD3 mAb for 48 hrs, then harvested, washed and incubated with labeled arachidonate for up to 60 min. At varying times, lipid extracts were prepared, phospholipids were resolved by TLC and [3H]arachidonoyl-phospholipid content within T cell membranes was quantified by liquid scintillation counting. Optimal incorporation of labeled material was found to occur in cultured cells at 20 min [Fig. 1]. At that time, 65.4 ± 3.2% of added label was cell associated in unstimulated cells and 76.0 ± 4.5% in proliferating T cells.

After 20 min. of culture, unstimulated cells preferentially incorporated [3H]AA into PC followed by PI and PE [Fig. 2, left panel, 0 minute]. Approximately 20% of labeled AA remained free in the membrane. In contrast, at 20 min proliferating T cells distributed [3H]AA predominantly into PI followed by PC and PE and showed an approximate 67% reduction in the level of free AA relative to unstimulated T cells [Fig. 2, right panel, 0 minute]. Less than 3% of [3H]AA was present in the PS fraction of both proliferating and control T cells (not shown).

The cells were then washed and recultured in BME containing 1% BSA for an additional 60 min. The molar quantities of cold arachidonate and the radioactivity within the total phosholipid pool did not significantly change during the chase period (data not shown). Over time, unstimulated T cells displayed a small but significant decline in the level of free [3H]AA and an increase in the level of [3H]AA-PC [Fig 2, left panel]. In proliferating T cells, a marked decline in the content of [3H]AA-PC (1.15 pmol/10^7 cells) was observed in association with an increase in the level of [3H]AA-PE (1.22 pmol/10^7 cells) when cells were chased for 60 min in AA-free medium [Fig 2, right panel].

[3H]AA incorporation into CD4+ and CD8+ T cell subsets

To determine whether the differences in [3H]AA distribution in proliferating versus unstimulated T cells was limited to a particular T cell subset, splenic CD4+ and CD8+ T cells, negatively selected following a 48 hr culture in the presence and absence of anti-CD3 mAb, were incubated with [3H]AA for up to 20 min. The cells were then washed and further cultured for 60 min, as above in BME with 1% BSA. Membrane phospholipids were then extracted, separated by TLC and radioactivity was quantified by liquid scintillation counting. Proliferating CD4+ and CD8+ T cells exhibited quantitatively similar reductions in the levels of [3H]AA-PC and corresponding increases in those of [3H]AA-PE over time [Fig. 3]. Because differences in arachidonoyl-phospholipid remodeling were not apparent between CD4+ and CD8+ T cells, subsequent experiments were carried out using unfractionated T cells.

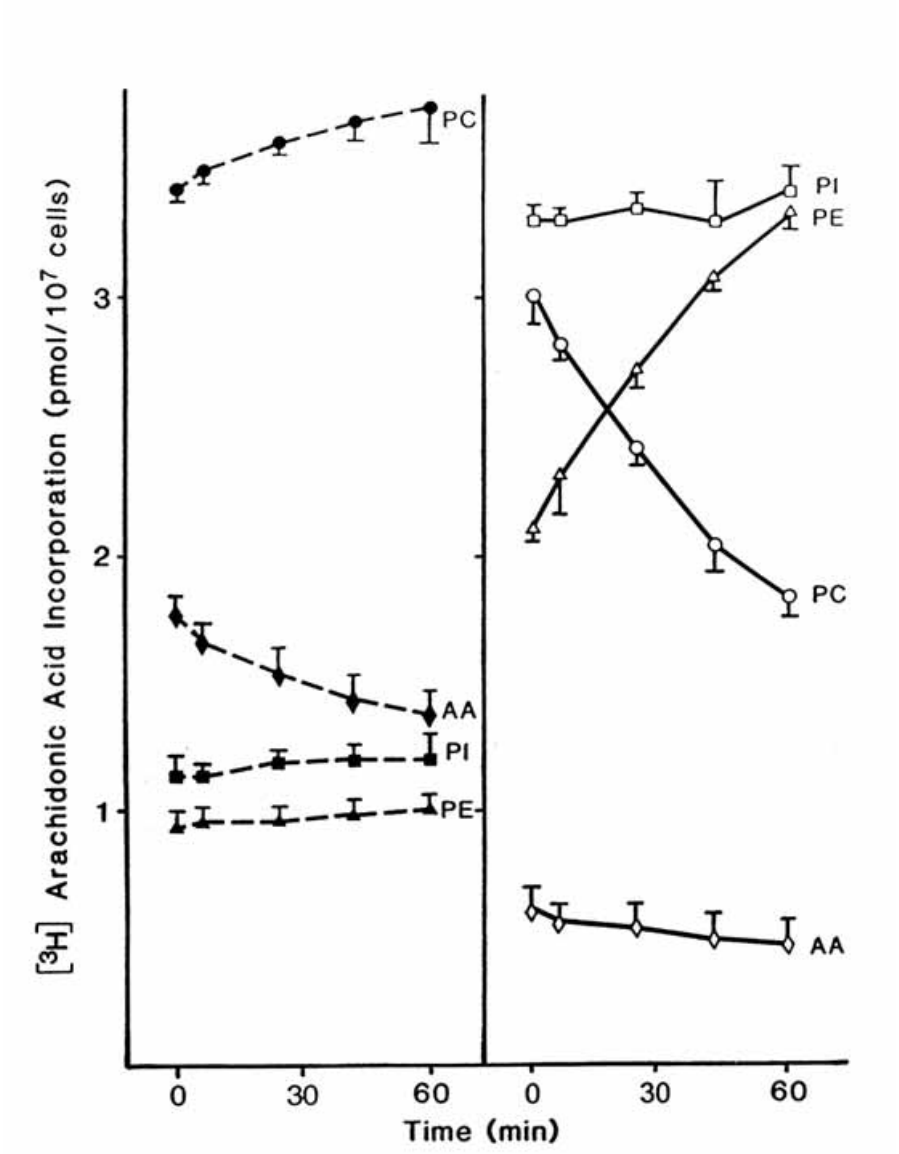

Figure 2
The distribution and turnover of [^{3}H]AA in proliferating and unstimulated T cells. Splenic T cells, previously cultured as above for 48 hours with (right panel, open circles) or without (left panel, closed circles) anti-CD3 mAb, were labeled with [^{3}H]AA (1 μCi/ml) for 20 min at 37°C. The cells were then washed and cultured at 37°C for up to 60 min in BME with 1% BSA. At the initiation of culture (zero time) and at various times thereafter, cells were harvested, membrane lipids were extracted and free AA and phosphoglycerides were resolved by TLC. The concentration of free [^{3}H]AA and of [^{3}H]AA within different phospholipid molecular species was measured by liquid scintillation counting. Data are the mean and standard error of 4 separate experiments.

Arachidonic acid content in proliferating and resting T cells

We next compared the AA content in membrane phosphatides of proliferating and control T cells. Splenic T cells were cultured with and without anti-CD3 mAb for 48 hr. Membrane lipids were extracted, separated by TLC, glycerolipids were isolated, fatty acids were derivatized to methyl esters and AA content was measured by gas chromatography. Relative to controls, proliferating cells demonstrated a marked increase in AA-PE (9.5 nmol/10^7 cells)

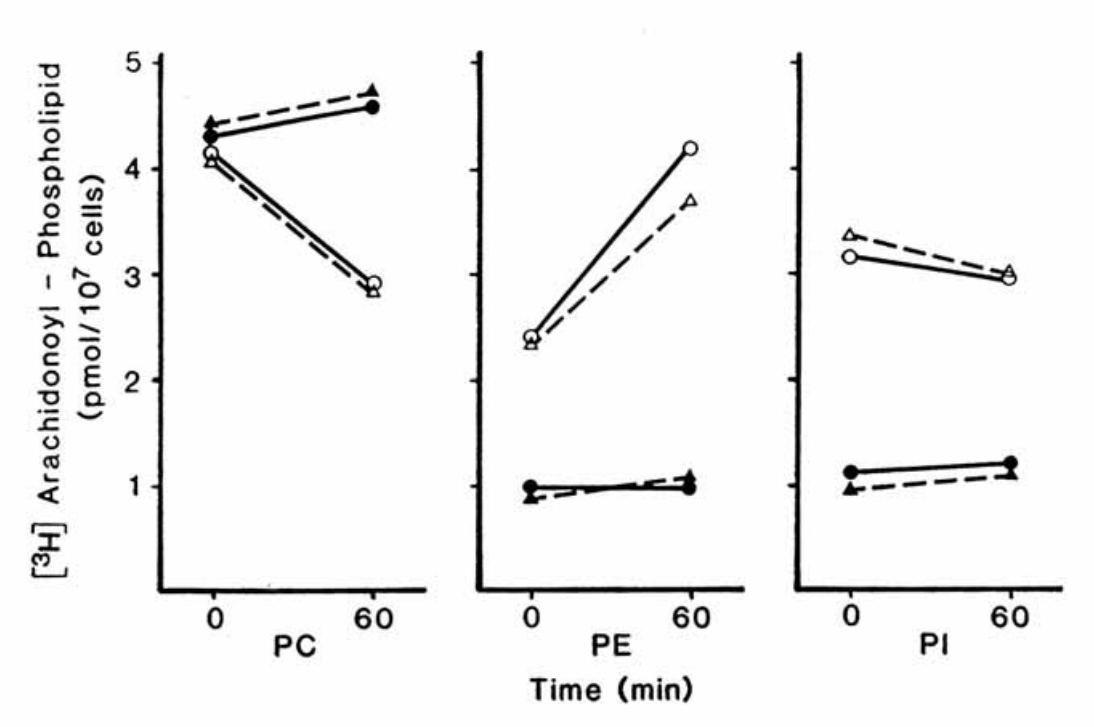

Figure 3
Incorporation and turnover of [^{3}H] AA within membrane phospholipids of proliferating and resting CD4$^+$ and CD8$^+$ T cells. Splenic T cells were culture as above for 48 hr with (open symbols) or without (closed symbols) anti-CD3 mAb, then enriched for CD4$^+$ T cells (dashed triangles) or CD8$^+$ T cells (circles) by negative selection. Following labeling with [^{3}H]AA (1 μCi/ml) for 20 min, the cells were then washed and cultured at 37°C for 60 min in BME with 1% BSA. At the initiation (zero time) and conclusion of culture (60 min), cells were harvested, membrane lipids were extracted and free AA and phosphoglycerides were resolved by TLC. The concentration of free [^{3}H]AA and of [^{3}H]AA within different phospholipid molecular species was measured by liquid scintillation counting. Data are the mean of 2 experiments.

and a smaller increase in AA-PI (2.0 nmol/10^{7}cells) [Fig 4]. No significant differences were found in the levels of arachidonoyl-PC and -PS.

Arachidonic acid content in diacyl and plasmalogen species of PC and PE

The AA content within individual species of PC and PE was next determined in proliferating and control T cells. Membrane glycerolipids of T cells previously cultured for 48 hr with anti-CD3 mAb or medium were resolved by TLC, separated into diacyl-, 1-alkyl-2-acyl- and 1-alk-1'-enyl-2-acyl-phospholipids and arachidonoyl-phospholipid levels were quantified by gas chromatography. Equivalent levels of arachidonate were found in individual species of PC in proliferating and control T cells [Fig 5]. In contrast, the AA content of all PE species was increased in proliferating T cells compared with that in non-cycling cells.

The changes in the individual species of PC and PE, which accompany T cell proliferation were examined. After 48 hr of culture with anti-CD3 mAb, a significant increase was found in the level of diacyl PE [Fig 6]. The levels of other species of PE and of all species of PC were comparable in resting and stimulated T cells. Based on earlier results (see Fig 5) we conclude that although diacyl PE expanded in proliferating T cells, the percentage of arachidonate in the latter fraction (i.e. ~30%) was equivalent in resting and cycling lymphocytes. In proliferating T cells, enrichment in AA was observed only in the 1-alkyl-2-acyl and in the 1-alk-1'enyl-2-acyl fractions of PE, where increases of 1.4 nM/10^7 cells and 4 nM/10^7 cells, respectively, were found [Fig 6].

[^{3}H]AA incorporation into diacyl and plasmalogen species of PC and PE

To further define the changes in arachidonoyl phospholipid content that accompany T cell proliferation, the movement of [^{3}H]AA within individual species of PC and

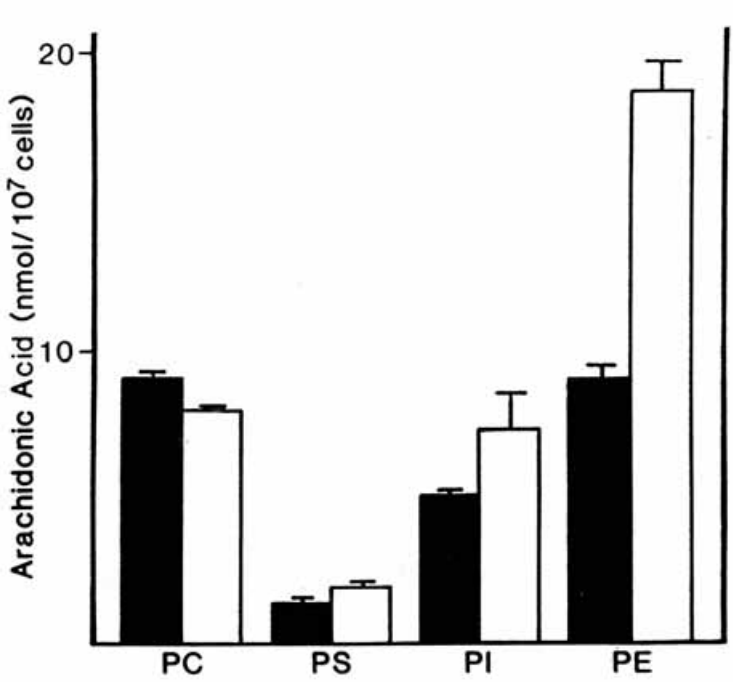

Figure 4
AA content within phospholipids of proliferating and unstimulated splenic T cells. Splenic T cells, previously cultured as above for 48 hours with (open bars) or without (closed bars) anti-CD3 mAb were harvested, phospholipids were isolated by TLC, fatty acids were derivatized to methyl esters and arachidonoyl-methyl ester content was determined by gas chromatography. Data are the mean and standard error of 4 experiments.

PE was investigated. Splenic T cells cultured with and without anti-CD3 mAb were incubated with [^{3}H]AA for 20 min. The cells were then harvested, washed and recultured for an additional 60 min in BME and 1% BSA. Membrane phospholipids were subsequently extracted, separated by TLC and individual species of PC and PE were quantified. In unstimulated cells, over time, significant difference in the distribution of [^{3}H]AA was found only in the diacyl fraction of PC [Fig 7]. This may reflect a transfer of free AA to lyso PC (see Figure 2). In proliferating T cells, a loss of [^{3}H]AA in diacyl-PC was associated with a quantitatively comparable and highly selective increase of [^{3}H]AA into 1-alk-1'-enyl-2-acyl-PE.

Discussion

The current study compares the incorporation, distribution and remodeling of AA within membrane phospholipids of resting and activated CD4$^+$ and CD8$^+$ T cells. The importance of understanding the movement of AA in T cells inheres in the fact that the AA content within, and the release of AA from the plasma membranes of lymphocytes can significantly modulate T cell activation and the signals provided by T cells for the activation and growth of other immune cells, including macrophages, neutrophils, B lymphocytes and other T cells. It should be noted, however, that the data presented herein reflect the net changes that occur in the sum of all cell membranes, not just plasma membranes. As previously described [7], in intact resting T cells following a short incubation, newly incorporated, radiolabeled AA is primarily distributed into PC or found free in the membrane. Over 60 min, a decrease in the level of free [^{3}H]AA is accompanied by a comparable increase in the level of [^{3}H]AA-PC. In lymph node T cells and thymocytes, the main route of incorporation of AA into diacyl-PC during short incubations is catalyzed by the sequential reactions of acyl-CoA synthetase and acyl-CoA:1-acyl-2-lyso-glycerophosphocholine acyl transferase [7]. In contrast to resting cells, following a brief exposure to radiolabeled AA, CD4$^+$ and CD8$^+$ T cells activated by 48 hr of stimulation with anti-CD3 mAb, preferentially incorporate [^{3}H]AA into PI followed by PC and then PE. Less than 3% of labeled AA is found free in membranes. As was true of resting T cells, the initial incorporation of radiolabeled AA into the PC and PI pools of activated T cells presumably involved the sequential activities of AA-CoA synthetase followed by 1-acyl-2-lyso-glycerophospholipid acyl transferases. During the subsequent 60 min chase in AA-free medium, a decrease in the [^{3}H]AA

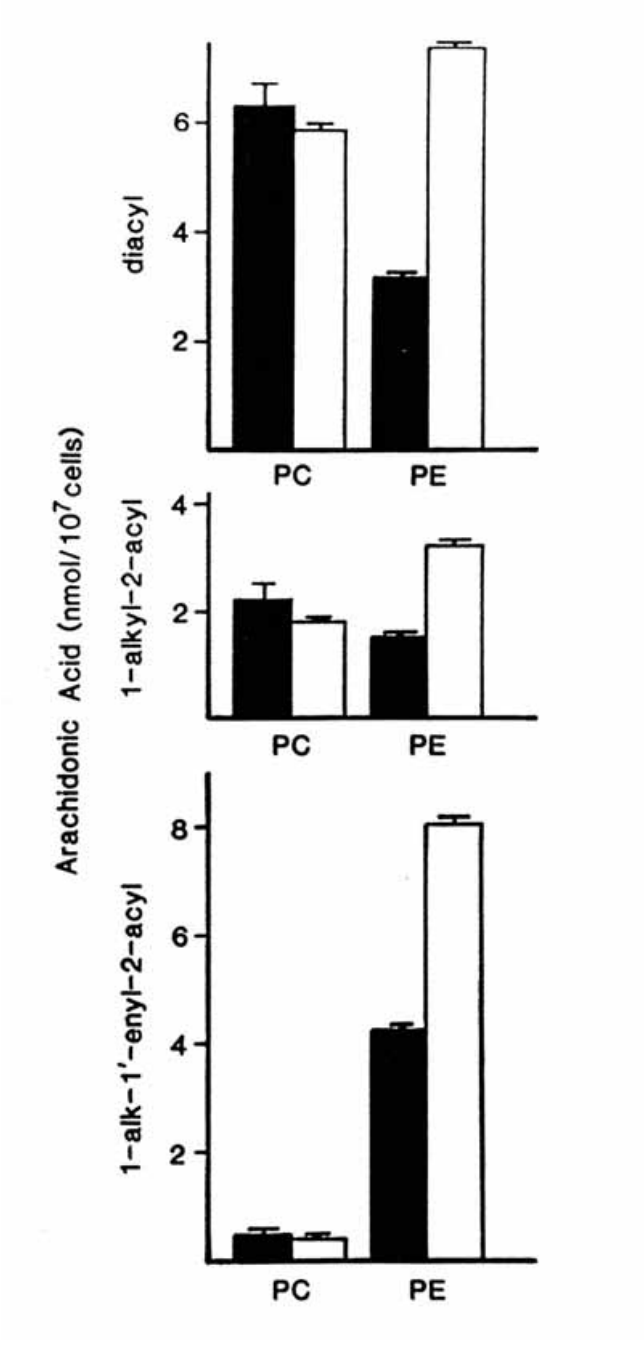

Figure 5
AA content within PC and PE molecular species of proliferating and quiescent T cells. Splenic T cells, previously cultured as above for 48 hours with (open bars) or without (closed bars) anti-CD3 mAb, were harvested and PC and PE species were resolved by TLC. Phospholipids were further separated into diacyl-, 1-alkyl-2-acyl- and 1-alk-1'enyl-2-acyl-diaglycerides. Fatty acids were derivatized to methyl esters as above and arachidonoyl-methyl ester content was determined by gas chromatography. Data are the mean and standard error of 3 experiments.

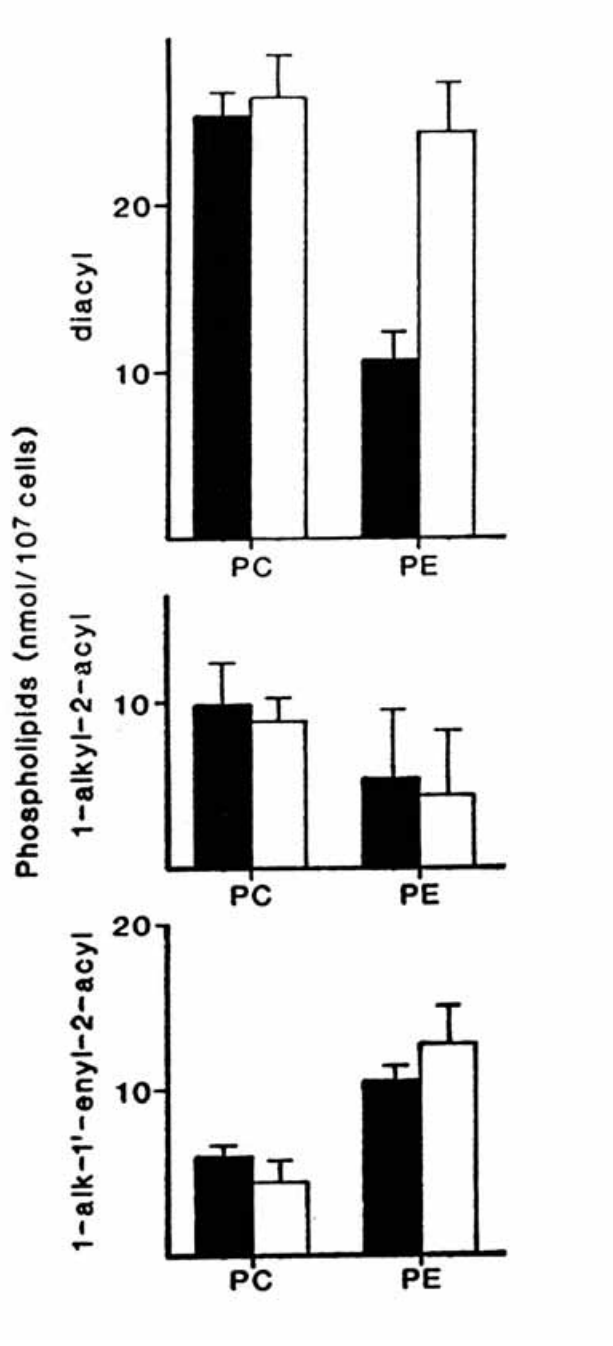

Figure 6
A comparison of the content of diacyl- and ether-lipids within PC and PE species of proliferating and unstimulated T cells. Splenic T cells pooled from 4 mice, previously cultured as above for 48 hours with (open bars) or without (closed bars) anti-CD3 mAb, were harvested and PC and PE species were resolved by TLC. Phospholipids were further separated into diacyl-, 1-alkyl-2-acyl- and 1-alk-1'enyl-2-acyl-diaglycerides and the content of diacyl- and ether-lipids was quantified by elemental phosphorous measurement. Data are the mean and standard error of three experiments.

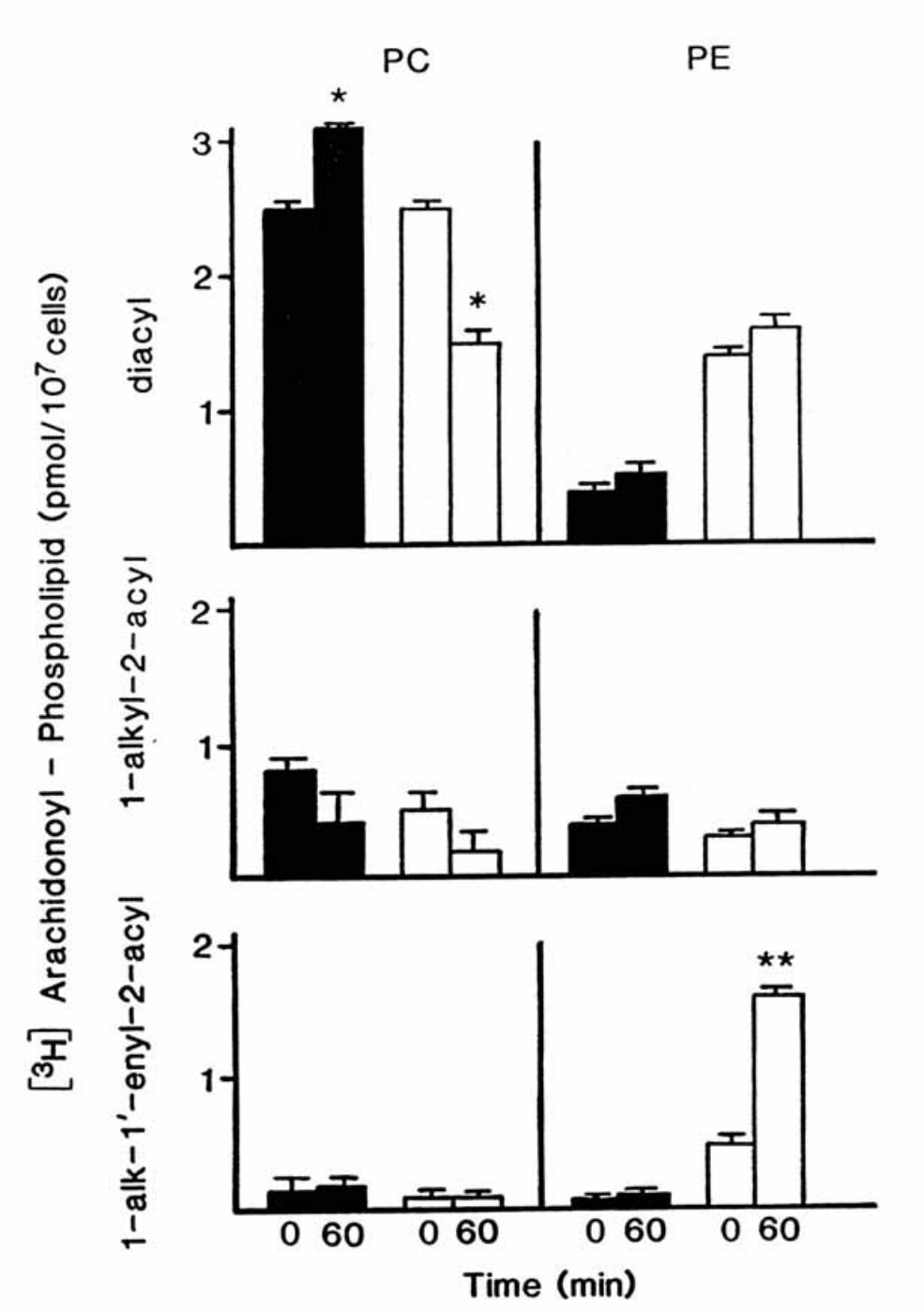

Figure 7
Movement of [^{3}H]AA within PC and PE species of proliferating and unstimulated T cells. Splenic T cells pooled from 6 mice, cultured as above for 48 hr with (open bars) or without (closed bars) anti-CD3 mAb, were labeled with [^{3}H]AA (1 μCi/ml) for 20 min. The cells were then washed and cultured at 37°C for 60 min in BME with 1% BSA. At the initiation (zero time) and conclusion of culture, cells were harvested and PC and PE species were resolved by TLC. Phospholipids were further separated into diacyl-, 1-alkyl-2-acyl- and 1-alk-1'enyl-2-acyl-diaglycerides and the content of diacyl- and ether-lipids was quantified by liquid scintillation counting. Data are the mean and standard error of 3 experiments. *$p < .05$ compared to arachidonoyl phospholipid content at the initiation of culture. **$p < .01$ compared to arachidonoyl phospholipid content at the initiation of culture.

content of PC was observed. The reduction in labeled AA from PC was accompanied by an equivalent increase in the [^{3}H]AA content of PE. Further analysis demonstrated that within the PE pool, all species (i.e. diacyl, alkyl-acyl and alk-1-enyl-acyl) exhibited an increase in the cold AA content following T cell activation. However, the percentage of AA relative to all fatty acids was increased only in the alkyl-acyl and 1-alk-1'-enyl-acyl species of PE. Finally, the loss of [^{3}H]AA within the diacyl-PC fraction of proliferating T cells was accompanied by an equivalent gain in the level of [^{3}H]AA in 1-alk-1'-enyl-acyl-PE. The data suggest an apparent transfer of AA from the diacyl-PC pool to the PE plasmalogen pool in proliferating CD4$^+$ and CD8$^+$ T cells. Regardless of AA remodeling, a highly selective net increase of diacyl-PE content accompanied T cell proliferation suggesting that de novo diacyl-PE synthesis, or PL synthesis coupled to head group exchange to yield diacyl-PE may be an important mechanism turned on by anti-CD3 mAb.

The mechanism by which AA was putatively transferred from PC to PE remains speculative. Short term incubation of alveolar and peritoneal macrophages [26,27] and neutrophils [23] with labeled AA is accompanied by a transfer of esterified AA from diacyl-PC to alkyl-PC and 1-alk-1'-enyl-acyl-PE. The transfer of AA was shown to exhibit fatty acid specificity and donor/acceptor specificity resembling that observed *in vitro* in the CoA-independent transacylation system [25]. A similar pattern of labeled AA movement into 1-ether lipids has been reported in mast cells [28], platelets [18] and in the brain [29].

As stated earlier, inflammatory cells such as macrophages and neutrophils contain a high content of ether-phospholipids [15,16]. In such cells, Co-A independent transacylase activity has, under certain conditions been coupled to the production of platelet activating factor (PAF) and eicosanoids [30]. PAF, in turn, derives principally from 1-alkyl-2-arachidonoyl-PC [23]. In contrast, the phospholipid molecular species which serves as a source of AA to be released or to be utilized for eicosanoid synthesis appears to depend on cell type. For example, the specific activity of free AA released from mast cells correlates best with that of 1-alk-1'-enyl-2-acyl-GPE [31,32], suggesting that AA may derive from that phospholipid pool, whereas, the specific activity of released AA in platelets best correlates with the specific activity of 1-alkyl-2-arachidonoyl-GPC. Interestingly, AA utilized in leukotriene synthesis in mast cells appears to emanate not from PE, but rather from PI or from PC subclasses [31,32]. In neutrophils, AA has a specific activity in leukotriene B4 that matches AA in 1-alkyl-2-arachidonoyl-GPC suggesting that it may derive from that phospholipid [33].

It has been argued that the channeling of AA into ether phospholipids through the remodeling activity of CoA-independent transacylase may be necessary for both eicosanoid production and the synthesis of lipid mediators such as PAF. In support of this thesis, pretreatment with a CoA-independent inhibitor produced a dose-related decrease in clinical markers of cellular infiltration (e.g. edema) in an experimental model of inflammation [34]. It is somewhat more difficult to rationalize a role for a CoA-independent transacylase system in T cells, which have a limited capacity to produce PAF and eicosanoids. A potential explanation is suggested by the finding that a number of inhibitors of CoA-independent transacylase induce apoptosis, or programmed cell death in certain cell lines [35]. One such inhibitor, 1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine (ET-18-O-CH3), is an alkyllysophospholipid with known antiproliferative effects on a variety of transformed cell lines including those derived from promyelocytes, carcinomas, leukemias and sarcomas [35-38]. Two additional, structurally distinct classes of CoA-independent transacylase inhibitors also induce apoptosis in HL60 cells, which suggests that disarming the enzyme may lead to programmed cell death [35]. Inhibition of CoA-independent transacylase activity in HL60 cells is associated with accumulation of AA in 1-acyl-2-arachidonoyl-GPC and a loss of arachidonate in 1-acyl and 1-alk-1'-enyl-2-arachidonoyl-GPE[35]. Based on these data, it has been postulated that inhibition of CoA-independent transacylase leads to a failure to appropriately redistribute AA, which in turn predisposes to apoptosis.

It has been argued that the antiproliferative effect of alkyl-lysophospholipids is selective in that, while cancer cell lines are susceptible to ET-18-0-CH3, bone marrow cells, neutrophils and skin fibroblasts are unaffected by this CoA-transacylase inhibitor [39]. An alternative explanation is that CoA-independent transacylase inhibitors preferentially cause cell programmed cell death in rapidly cycling cells. Viewed in this manner, transformed cells may undergo apoptosis on exposure to CoA-synthetase inhibitors simply because they represent one extreme case of proliferating cells. Conversely, bone marrow cells, neutrophils and fibroblasts may resist programmed cell death because they are either not cycling or are cycling at a relatively slow rate. The therapeutic implications of CoA-inhibitors, therefore, may transcend their potential use as oncological agents. Indeed, such inhibitors may have a therapeutic role in cell-mediated diseases that are orchestrated by rapidly proliferating lymphocytes cells, such as rheumatoid arthritis and systemic lupus erythematosus.

Conclusion

In summary, the current study suggests that T cells possess CoA-independent transacylase activity *in vivo*, which may

be responsible for the high plasmalogen content of GPE. The physiological relevance of this enzyme activity and the role of ether lipids in immune function remain to be fully clarified.

Methods

Mice

Six to 8 week old, male C3H/HeN (H-2^k) and Balb/C (H-2^d) mice were obtained from Harlan (Indianapolis, IN).

Materials

Fetal Bovine Serum (FCS) was purchased from Hyclone Laboratories Inc. (Logan, UT). Eagle's Basal Media (BME), arachidonic acid, Type XIII phospholipase C, Coomasie Blue R, triethylamine, Tris (hydroxymethyl) aminomethane (Tris), EGTA, mannitol phenyl methlysulfonyl fluoride (PMSF) and heptadecanoic acid (17:0) were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI-1640, L-glutamine, Hepes, and penicillin/streptomycin were purchased from Gibco (Grand Island, NY). Monoclonal antibodies (mAbs) against CD8 (clone 53-6.7), CD4 (clone H129.19), and CD3-epsilon (clone 145-2C11) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Fluorescein-conjugated MARK-1, a murine antibody to rat *kappa* chains, was obtained from AMAC, Inc.(Westbrook, ME). Rabbit complement was purchased from Cederlane (Westbury, NY). Goat anti-mouse Ig was obtained from Tago, Inc.(Burlingame, AL). Thymidine, [methyl-^{3}H] ([^{3}H]dTd)(6.7 Ci/mmol), [5,6,8,9,11,12,14,15,-^{3}H] arachidonic acid ([^{3}H]AA) (76 Ci/mmol), and [choline-methyl-^{14}C] sphingomyelin ([^{14}C] Sph) were purchased from New England Nuclear (Boston, MA). Lyophilized PC, PS, PI, PE and phosphatidic acid (PA) were obtained from Avanti Polar Lipid Co. (Birmingham, AL). Silica gel G TLC plates were purchased from Analtech Inc. (Newark, DE). The alk-1'-enyl-2-acyl-glycerol and 1-O-alkyl-2-acyl standards were obtained by phospholipase C hydrolysis of 1-O-alkyl-2 acyl-sn-glycerol-3-phosphoethanolamine and 1-O-alkyl-2-acyl-sn-glycerophosphocholine respectively. Phospholipids were obtained from Serdary Research Laboratories (London, Ontario, Canada).

Preparation of the cells

Single cell suspensions from the spleens of 4 to 6 mice were freed of erythrocytes by osmotic lysis. T cell-enriched preparations were obtained by suspending cells in BME plus 0.02% NaN_3 and 5% FCS and incubating the mixture on 100 × 15 mm plastic dishes (Falcon Labware, Oxnard, CA) precoated with 5 μg of anti-mouse Ig at 4°C for 70 min to eliminate B cells. The nonadherent cells were recovered, washed and the procedure was repeated. The preparations contained 92–96% Thy-1.2$^+$ cells by immunofluorescence and cytofluorographic analysis, as previously described [40]. CD4$^+$ T cells and CD8$^+$ T cells [41,42] were prepared by cytotoxic depletion of T cell-enriched preparations using anti-CD8 or anti-CD4 mAb, respectively, as reported earlier [42,43].

Cell proliferation assay

Two × 10^5 cells (0.2 ml) were optimally stimulated with 5 μg/ml of anti-CD3 mAb in 96 well flat bottom plates (Costar, Cambridge, MA) for varying periods of time in medium consisting of RPMI-1640 supplemented with FCS (5%), L-glutamine (2 mM), penicillin (100 U)/streptomycin (50 mg) and 2-mercaptoethanol (10^{-5} M). Proliferation was assessed daily for 3 days using tritiated thymidine incorporation [42].

Incorporation and remodeling of [^{3}H]AA within membrane glycerolipids

T cells (10^7/ml) were incubated in BME at 37°C in a humidified atmosphere containing 5% CO_2 with [^{3}H] AA (1 μCi/ml) for up to 60 min. After varying periods of time, duplicate samples containing labeled cells were harvested, washed and recultured in acid-washed, glass tubes at 10^7 cells/ml in BME containing 1% BSA, as previously described [44]. At the conclusion of culture, suspensions were centrifuged at 400 × *g*. The supernatant was removed, cell pellets were washed once in BME then resuspended in PBS. The suspensions were then spiked with [^{14}C] sphingomyelin (100,000 dpm; 0.9 nmol) to assess recoveries and membrane lipids were extracted by the method of Bligh and Dyer [45]. Organic and aqueous phase radioactivities were quantified by liquid scintillation counting. The organic phase was dried under a stream of nitrogen, membrane lipids were reconstituted in chloroform/methanol (3:1) and spotted onto Silica gel G plates. The individual phospholipid molecular species were resolved by TLC using a solvent system (number I) consisting of chloroform/methanol/2-propanol/0.25% KCl/ triethylamine (90:27:75:18:54) [44]. The radioactive glycerolipids were located on TLC plates using a Bioscan System 200 Imaging Scanner (Washington DC). Areas corresponding to authentic Sph (R_f 0.19), PC (R_f 0.22), PS (R_f 0.34), PI (R_f 0.46), PA (R_f 0.50), PE (R_f 0.54), AA (R_f 0.77) and neutral lipids (R_f 0.86) were scraped from the plate and radioactivity was quantified by liquid scintillation counting.

Quantification of [^{3}H]AA phospholipid molecular species

[^{3}H]AA glycerophospholipids were separated as described above then scraped and eluted from TLC plates with methanol/chloroform(2:1). Silica gel was removed by passage through a empty filtration column with 20 μm PDVF frits (BAKERBONDspe, J.T. Baker Co.). The eluate was dried under a stream of nitrogen, reconstituted in diethylether (2 ml) plus KH_2PO_4(0.5 M) and incubated for 60 min at room temperature in the presence of 2 units (for PC) or 4 units (for PE) of phospholipase C (type XIII) to generate diglycerides. The ether layer was recovered and

the aqueous phase was washed twice with diethylether. After drying under nitrogen, the combined ether layers were reconstituted in chloroform/methanol (3:1) and spotted onto Silica gel G plates. Diacyl-, 1-alkyl-2-acyl- and 1-alk-1'-enyl-2-acyl-diglycerides were resolved by TLC in a solvent system (number II) consisting of diethylether/hexane/acetic acid (60:50:0.75). The radioactive diglycerides were localized as described above. Areas corresponding to authentic diacyl-, 1-alkyl-2-acyl- and 1-alk-1'enyl-2-acyl-diglycerides were scraped from the plate and radioactivity was quantified by liquid scintillation counting.

Quantification of membrane phospholipids

The membrane phospholipid content of T cells was quantified as described earlier [46]. T cells (2.5×10^6/ml) were suspended in a buffer (pH 7.4) consisting of mannitol (300 mM), Tris (15 mM), EGTA (5 mM) and PMSF and the cells were cavitated at 4°C using a cell disruption bomb (Parr Instrument Co., Moline, IL). The homogenate was centrifuged at 48,000 × *g* for 30 min. The supernatant was discarded and the membrane pellet was resuspended in cold PBS. Membrane homogenates were spiked with [^{14}C] sphingomyelin (100,000 dpm) to assess recoveries and lipids were extracted. Membranes were spotted onto Silica Gel G plates and lipids were resolved in solvent system number I. Areas corresponding to authentic sphingomyelin, PC, and PE were scraped and eluted from TLC plates. To determine overall recoveries, total PC and PE levels were quantified by elemental phosphorous measurement [47] in an aliquot of eluate. Diglycerides were generated from the remaining portion of the phospholipid eluates and the diacyl and plasmalogen fractions were resolved in solvent system number II using Silica Gel G plates as above. Areas corresponding to authentic standards were scraped and eluted from the plates, and the diacyl-, 1-alkyl-2-acyl-, and 1-alk-1'enyl-2-acyl contents of PC and PE were quantified by elemental phosphorous measurement.

Assessment of the molar concentration of AA within phospholipids

The molar concentration of AA within membrane phospholipids was measured as previously described [44]. Lipid extracts were prepared from T cells which had been cultured for 48 hrs with and without anti-CD3 mAb and membrane phospholipids were isolated. In some experiments, phospholipids were further separated into diacyl-, 1-alkyl-2-acyl- and 1-alk-1'-enyl-2-acyl-diglycerides. Glycerolipids were scraped from TLC plates, eluted from silica into chloroform/methanol (2:1) and the silica was removed by passage through a filtration column. The remaining silica was washed with chloroform/methanol (2:1). Lipids were spiked with heptadecanoic acid (17:0) (10 μg) as an internal standard and dried under a stream of nitrogen. Fatty acid methyl esters (FAMEs) were made by heating in 1 ml hydrochloric acid/methanol (10:90) at 100°C for 12 min. Mixtures were cooled then neutralized using 5 N NaOH (0.5 ml). FAMEs were extracted twice from the aqueous phase into hexane (1 ml) and the FAME-hexane was dried under nitrogen. FAMEs were then resuspended in hexane (50 μl) and resolved from 140 to 220°C using a Hewlet-Packard (Palo Alto, CA) 5890A gas chromatography system equipped with a DB355 column (0.25 mm × 30 m) (J&W Scientific, Folsom, CA). Individual FAMEs were detected by a flame ionization detector and identified by comparison of R_f of FAME standards. The quantity of arachidonoyl-methyl ester was determined relative to the heptadecanoyl-methyl ester peak area.

Expression of data

Data are expressed as the mean ± SEM. Significance was defined as a *p* value of less than .05 using Student's t test.

List of abbreviations

AA, arachidonic acid, PC, phosphatidyl choline, PS phosphatidyl serine, PI phosphatidyl inositol, PE phosphatidyl ethanolamine, PA, phosphatidic acid, Sph, sphingomyelin, GPC, glycerophosphocholine, GPE, glycerophosphoethanolamine, FAMEs, fatty acid methyl esters.

Author's contributions

MT participated in all of the techniques used in this manuscript including preparation of T cell subsets. SA participated in fatty acid analysis by gas chromatography. RB gave the technical supports and fatty acid standards necessary for this study. TS conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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