Interleukin-2 Causes an Increase in Saturated/Monounsaturated Phosphatidic Acid Derived from 1,2-Diacylglycerol and 1-O-Alkyl-2-acylglycerol*

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Phosphatidic acid generation through activation of diacylglycerol kinase α has been implicated in interleukin-2-dependent T-lymphocyte proliferation. To investigate this lipid signaling in more detail, we characterized the molecular structures of the diradylglycerols and phosphatidic acids in the murine CTLL-2 T-cell line under both basal and stimulated conditions. In resting cells, 1,2-diacylglycerol and 1-O-alkyl-2-acylglycerol subtypes represented 44 and 55% of total diradylglycerol, respectively, and both showed a highly saturated profile containing primarily 16:0 and 18:1 fatty acids. 1-O-Alk-1-ethyl-2-acylglycerol represented 1–2% of total diradylglycerol. Interleukin-2 stimulation did not alter the molecular species profiles, however, it did selectively reduce total 1-O-alkyl-2-acylglycerol by over 50% at 15 min while only causing a 10% drop in 1,2-diacylglycerol. When radiolabeled CTLL-2 cells were challenged with interleukin-2, no change in the cellular content of phosphatidylcholine nor phosphatidylethanolamine was observed thereby ruling out phospholipase C activity as the source of diradylglycerol. In addition, interleukin-2 failed to stimulate de novo synthesis of diradylglycerol. Structural analysis revealed approximately equal amounts of 1,2-diacyl phosphatidic acid and 1-O-alkyl-2-acyl phosphatidic acid under resting conditions, both containing only saturated and monounsaturated fatty acids. After acute (2 and 15 min) interleukin-2 stimulation the total phosphatidic acid mass increased, almost entirely through the formation of 1-O-alkyl-2-acyl species. In vitro assays revealed that both 1,2-diacylglycerol and 1-O-alkyl-2-acylglycerol were substrates for 1,2-diacylglycerol kinase α, the major isoform in CTLL-2 cells, and that the lipid kinase activity was almost totally inhibited by R59949. In conclusion, this investigation shows that, in CTLL-2 cells, 1,2-diacylglycerol kinase α specifically phosphorylates a pre-existing pool of 1-O-alkyl-2-acylglycerol to form the intracellular messenger 1-O-alkyl-2-acyl phosphatidic acid.

Signal transduction by a wide variety of extracellular stimuli involves phospholipid hydrolysis and/or the activation of lipid kinases and phosphatases as a means to generate biologically active lipid second messengers (LSMs). The second messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate are now well established as modulators of protein kinase C (PKC) activity and intracellular calcium ion concentration, respectively (1). In recent years, investigations have revealed that phospholipids distinct from phosphatidylinositol 4,5-bisphosphate (PIP2) can also act as substrates for activated phospholipases. One of these is phosphatidylcholine (PC), the principal substrate for phospholipase D (PLD) which catalyzes its hydrolysis to form phosphatidic acid (PA) and free choline (2). The other route for the formation of PA is through the phosphorylation of DAG by DAG kinase (DGK) (3). Structural analysis of precursor phospholipids and LSMs have demonstrated that phospholipid hydrolysis by activated phospholipases is not a random process, but rather is highly organized and selective for particular lipid classes leading to the formation of specific LSMs (4–7). DAG and PA exist in biologically active and inactive forms depending on their fatty acid compositions. Biologically active DAGs are those which possess polyunsaturated fatty acids (i.e. those containing three or more double bonds) (6, 7). Phosphorylation by DGK attenuates their activity, generating polyunsaturated PAs which are thought to be biologically inactive. In contrast, biologically active PAs are those containing saturated, monounsaturated, and to a lesser extent diunsaturated fatty acids (6, 8). Dephosphorylation by PA phosphatase (PAP) switches off this PA signal by converting it to inactive, primarily monounsaturated, DAG (7, 9). The conversion of biologically active DAGs and PAs into their inactive counterparts is important for the cessation of the incoming signal and for the resynthesis of their precursor phospholipids to maintain membrane integrity. As the interconversion between biologically active and inactive DAGs and PAs is through DGK and PAP this implies their generation through two distinct routes: via PLD for the generation of biologically active PA and via phospholipase C (PLC) for the generation of biolog-

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The abbreviations used are: LSM, lipid second messenger; DAG, 1,2-diacylglycerol; AAG, 1-O-alkyl-2-acylglycerol; AEAG, 1-O-alk-1-ethyl-2-acylglycerol; PA, phosphatidic acid; IL-2, interleukin-2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP2, phosphatidylinositol 4,5-bisphosphate; DAG-PA, 1,2-diacyl-PA; AA-PA, 1-O-alkyl-2-acyl-PA; AAG-PC, 1-O-alk-1-ethyl-2-acyl-PC; AAG-PE, 1-O-alkyl-2-acyl-PE; FAME, fatty acid methyl ester; PLC, phospholipase C; PLD, phospholipase D; DGK, diacylglycerol kinase; PAP, PA phosphatase; DNB, dinitrobenzoyl derivative; PKC, protein kinase C; DB2, diradylglycerol; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; BSA, bovine serum albumin; HA, hemagglutinin.

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ically active DAG. This model is supported by both the kinetics of accumulation of species-specific DAGs and PAs and additionally through the fatty acid analysis of their precursor phospholipids which show a close match to DAG and PA (4). From the information in the literature it has now become apparent that the generation of LSMs is a highly ordered sequence of events that displays a high degree of specificity (9).

Interleukin-2 (IL-2) is the cytokine responsible for T-cell proliferation. After binding to its high affinity receptor on the cell surface, the activated receptor initiates a variety of signal transduction pathways of which those involving protein and lipid kinases have been the best characterized. IL-2 neither causes the hydrolysis of PIP2 nor provokes the generation of cyclic nucleotides as a means to generate second messenger molecules (10–12). In contrast, a rapid elevation of PA levels is seen in T-lymphocytes (13) due to the activation of DGK (14). Further investigations revealed that this acute activation of DGK, specifically that of the DGKα isozyme, was essential for subsequent cell cycle progression and in addition, DGKα was rapidly translocated to the perinuclear region of T-cells (15). The increase in PA is thought to be entirely due to the activation of DGKs as no PLD activity at equivalent times is detected in IL-2-stimulated cells (16). These observations underline the importance of DGKα activation for the generation of biologically active PA species during T-cell proliferation and distinguish it from other signal transduction mechanisms.

Our goal in this work was to identify the fatty acid composition of both basal and IL-2-elevated PA in CTLL-2 cells to demonstrate that despite the lack of IL-2-stimulatable PLD activity, IL-2 could generate biologically active PA species via DGKs instead. In addition, the molecular species analysis of the diradylglycerols (DRGs) (consisting of DAG, 1-O-alkyl-2-acetylglycerol (AAG) and 1-O-alk-1'-enyl-2-acetylglycerol (AEAG)) was determined in order to define the substrates for the IL-2-stimulated DGKs in CTLL-2 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant IL-2 was a generous gift from Hoffman-LaRoche Inc. [3H]oleic acid (specific activity 2–10 Ci/mmol), and [9,10-3H]oleic acid (specific activity 9-10 Ci/mmol) were purchased from Amersham (Amersham, United Kingdom). Fetal calf serum and all cell culture media were bought from Life Technologies, Inc. (Paisley, U.K.). Silica gel (60 Å) TLC plates were from Whatman (Ham, U.K.). 3,5-Dinitrobenzoylchloride and all HPLC grade/analytical reagents were from Fisher Scientific U.K. Ltd. (Loughborough, U.K.). Anti-HA antibody was purchased from Berkeley Antibody Co. (Richmond, CA). R59949 was obtained from Calbiochem (Nottingham, U.K.). 3,5-Dinitrobenzoylchloride and all HPLC grade/analytical reagents were from Fisher Scientific U.K. Ltd. (Loughborough, U.K.).

**Cell Culture and Stimulations**—CTLL-2 cells were cultured as published previously (16). Upon reaching a density of 1 × 10⁵/ml they were washed twice in incomplete medium (RPMI buffered with 10 mM HEPES pH 7.2, supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin) before reincubating for a starving period of 6 h in the presence of 0.5% BSA. At this point experiments were initiated after washing the cells once with incomplete medium. Cells were incubated at 500 units/ml IL-2 for various periods, pelleted, and immediately frozen on dry ice.

**PA Isolation**—Cellular phospholipids, together with 1 μg of 17:0/17:0-PA internal standard, were extracted by the method of Bligh and Dyer (17). After drying under a gentle stream of N₂, the total lipid extract was resuspended in 20 μl of chloroform/methanol/NH₄OH (50/482, v/v/v) before injection onto a Kromasil 100–55SIL HPLC column (5 μm, 2.1 × 250 mm; Hichrom Ltd., Reading, U.K.). PA was separated from all other neutral and phospholipids using a linear gradient changing from 70% solvent A (chloroform/methanol/NH₄OH, 70/28/2, v/v/v) plus 30% solvent B (chloroform/methanol/NH₄OH, 50/48/2, v/v/v) to 100% solvent B over 50 min at 0.5 ml/min. Detection was with a Varex MKIII evaporative light scattering detector (Alltech, Carnforth, U.K.). At all times before the detection was started the PA extract was to be diverted into a glass vial for structural analysis. The fatty acid content of PA was quantified by GC-MS relative to 17:0/17:0-PA internal standard (insignificant amounts of naturally occurring 17:0 fatty acid were found in the samples).

**DGK Isolation and HPLC Molecular Species Analysis**—Total cellular lipids were extracted using 12/0/23 of chloroform/methanol/2% aqueous buffer for 4 × 24 h, the lipid extract was evaporated to dryness before TLC analysis. After concentration, separation of the isolated DRG classes was performed by reverse-phase HPLC (Spherisorb S5ODS2 EXCEL column, 5 μm, 2.1 × 250 mm, Hichrom Ltd.) employing a linear gradient of 100% cyclohexane, 2,4,trimethylpentane, diethyl ether/propan-2-ol (49/49/20, v/v/v) by volume) changing to 100% cyclohexane-diethyl ether/propan-2-ol (9/1, v/v) over 45 min at 0.4 ml/min with absorbance detection at 254 nm. Fractions corresponding to the AAG-DNB + AEAG-DNB and DAG-DNB classes were collected. AAG-DNB and AEAG-DNB were separated from each other by TLC using hexane/diethyl ether (9/1, v/v) after ligating spraying the TLC plate with 0.1% 1,6-diphenyl-1,3,5-hexatriene in hexane visualized under UV light, the bands corresponding to AAG-DNB and AEAG-DNB were scraped off and eluted from the silica with diethyl ether. After concentration, separation of the isolated DRG-DNB classes into their component molecular species was performed by reverse-phase HPLC (Spherisorb S5ODS2 EXCEL column, 5 μm, 2.1 × 250 mm, Hichrom Ltd.) employing a linear gradient of 100% acetotrinite/propan-2-ol (9/1, v/v) changing to 100% acetotrinite/propan-2-ol (1/1, v/v) over 50 min at 0.5 ml/min with absorbance detection at 254 nm. To verify the identification of individual DRG species, HPLC peaks were collected into glass vials, derivatized, and subjected to GC-MS.

**Phospholipid Digests**—An exhaustive PLC digest of PC and PE isolated from CTLL-2 cells was performed overnight at 25 °C in 300 μl of reaction buffer (100 mM sodium borate, pH 7.4, 1 mM CaCl₂, 1 mM ZnCl₂). The lipid was dried under a stream of N₂ then transmethylated with 3 M HCl in dry methanol in a sealed vial for 2 h at 70 °C. After cooling to room temperature, the contents of the vial were dried and re-dissolved in 100 μl of hexane. Fatty acid methyl esters (FAMEs) were identified by gas chromatography-mass spectrometry (GC-MS; 5890GC/5972MSD, Hewlett-Packard) using authentic FAME standards as described previously (6). Transmethylation was performed without the use of internal standard FAMES (from hydrolysis of the fatty vinyl alcohol at the sn-1-position), the latter being identified by their retention times relative to known standards and by the appearance of a characteristic fragmentation ion at m/z 75. For the 1-O-alkyl-2-acyl lipids a two-step procedure was used; transmethylolation of the fatty acid at the sn-2-position of the glycerol backbone followed by trimethylsilylation of the free hydroxyl groups at the sn-2- and sn-3-positions of the resultant 1-O-alkylglycerol using 100 μl of bis(trimethylsilyl)trifluoroacetamide for 30 min at 60 °C. The FAMES and 1-O-alkyl-2,3-bis(trimethylsilyl) (TMS) glycerols generated were identified by electron impact GC-MS using a DB-23 column (0.25 mm × 30 m; J & W Scientific) with a temperature program holding at 55 °C for 2 min, ramping to 140 °C in 9 min then to 240 °C at 2 °C/min, an injector temperature of 280 °C, a detector temperature of 270 °C and a helium head pressure of 12 p.s.i. A characteristic fragmentation ion at m/z 252, corresponding to [TMS-O-CH₂-CH₂-O-TMS]⁺ formed by cleavage of the C1-C2 bond within the glycerol backbone of 1-O-alkyl-2,3-bis-TMS glycerol, was used for identification (18).

In Vitro DGK Assays—250 μg of 1-O-hexadecyl-2-oleoyl-PC and 1-palmitoyl-2-oleoyl-PC were separately hydrolyzed with PLC in order to generate a mixture of AAG, AEAG, and DAG for use as standards. The DRG was ether-extracted from the aqueous buffer four times, dried under N₂, then DNB-derivatized, as described earlier.
Novel Species of Phosphatidic Acid in T-lymphocytes

In vivo metabolic labeling—During the last 24 h of exponential growth, CTLL-2 cells were labeled with 1 μCi/ml of 1-O-[3H]octadecyl lysophosphatidylcholine. Thereafter, the cells were washed twice with incomplete medium before reincubating for a starving period of 6 h in the presence of 0.5% BSA. At this point experiments were initiated after washing cells once with incomplete medium. Cells (2 x 10⁶) were incubated ± 500 units/ml IL-2 for various times, pelleted and immediately frozen on dry ice. Total lipids were extracted, (17), followed by the separation of [3H]-1-alkyl-2-acyl-PC (AAG-PC) and [3H]-1-acyl-2-acyl-PE (AAG-PE) by TLC using a solvent system consisting of propane-1-ol/propanolic acid/chloroform/water (60/40/40/20, by volume). Radioactivity within the AAG-PC and AAG-PE fractions of CTLL-2 cells (comigrating with authentic phospholipid standards) was determined after scraping the silica into vials followed by liquid scintillation counting.

Incorporation of [1-14C]Glucose and [9,10-3H]Oleic Acid into Glycerolipids—CTLL-2 cells starved of both serum and IL-2 for 6 h were washed twice with Krebs-HEPES buffer (118.46 mM NaCl, 4.74 mM KCl, 2.54 mM NaHCO₃, 1.18 mM KH₂PO₄, 1.18 mM MgSO₄, 24.88 mM NaCl, 10 mM HEPES buffered to pH 7.35) containing 0.1% (v/v) BSA before incubation in the latter for 1 h. Cells (5 x 10⁶) were incubated ± 500 units/ml IL-2 for 10 min followed by a 4-min incubation with 5 μCi of [1-14C]glucose. Cell incubations were terminated by rapidly pelleting the cells and their immediate freezing on dry ice. Total lipids were extracted, (17), followed by their separation using two-dimensional TLC (19). Radioactivity within the lipid fractions of interest (DRG, PA, triacylglycerol, PC, and PE, identified using authentic lipid standards) was determined as described above. The incorporation of oleic acid into the DRG fraction of CTLL-2 cells involved incubating IL-2- and serum-starved CTLL-2 cells (1 x 10⁶) with 1 μCi of [9,10-3H]oleic acid for various periods of time (from 30 s to 2 h) in the presence or absence of 500 units/ml IL-2. Cell incubations were terminated by the rapid addition of 9 volumes of ice-cold phosphate-buffered saline, containing 1% BSA followed by cell centrifugation and freezing of the pellets on dry ice. After total lipid extraction (17), the DRG was separated from all other neutral lipids by TLC using the solvent system composed of petroleum ether/diethyl ether/glacial acetic acid (70/30/2, v/v/v). Radioactivity within the DRG fraction of the CTLL-2 cells (comigrating with authentic 1,2-diacylglycerol standard) was determined as described above.

RESULTS

In T-lymphocytes, IL-2 has been reported to both stimulate DGK activity (14, 15) and raise PA levels (13) at early time points (less than 30 min). To investigate the molecular species composition of this PA we initially analyzed its fatty acid content in resting CTLL-2 cells. Fatty acids identified as 14:0, 15:0, 16:0, 16:1n-7, 16:1n-5, 18:0, 18:1n-9, and 18:1n-7 were represented 44% of the total DRG, AAG represented 55%, while the fatty acid composition of saturated and monounsaturated species was very similar to that seen in lysophosphatidic acid-stimulated porcine aortic endothelial cells where the PA was generated by a PLD (6), we determined the DRG (DAG, AAG, and AEAG) composition of CTLL-2 cells. DAG represented 44% of the total DRG, AAG represented 55%, while AEAG accounted for the remaining 1–2%, under resting conditions. Molecular species analysis by reverse-phase HPLC revealed 16 major DAG peaks corresponding to distinct molecular species (Fig. 24 and Table I). 16:0/18:1n-9 (peak 11) was the most abundant species. The most abundant polyunsaturated species, 18:0/20:4n-6 (peak 8), was present as only 1% of total DAG. Reverse-phase HPLC of AAG demonstrated 10 major peaks (Fig. 2a), the most abundant being identified as 1-O-hexadecyl-2-oleoyl glycerol (1-O-16:0/18:1n-9; peak 8). Other AAG molecular species detected are listed in Table II. No polyunsaturated AAGs were detected. Although AEAG mass was too low for a full species analysis, transmethylation of total cellular DRG followed by GC-MS detected only 16 and 18 carbon-saturated dimethylecetals derived from the corresponding alk-1’-enyl groups.

IL-2 stimulation caused no obvious changes in the DRG molecular species profiles, however, at 15 min, it did cause a small drop in total DAG mass (from 2498 ± 46 to 2245 ± 248 pmol/10⁶ cells) and a halving of total AAG mass (from 3059 ± 26 to 1416 ± 70 pmol/10⁶ cells). Preincubation with the DGK inhibitor, R59949, blocked this IL-2-induced DRG response by approximately 90% (data not shown).

The results of the molecular species analyses of DAG and AAG prompted us to reinvestigate the short-term IL-2-stimulated changes in PA. Since AAG represented approximately half of the total DRG in CTLL-2 cells, we envisaged that an

\[ \text{FIG. 1. IL-2 stimulates acute accumulation of saturated and monounsaturated PA. Arrested CTLL-2 cells were incubated ± IL-2 (500 units/ml) for the times indicated. PA was isolated by HPLC, transmethylated, and the resultant FAMEs identified and quantified by GC-MS. The data are represented as mean ± S.D. (n = 4).} \]

\[ \text{204% of control, respectively, indicative of a rise in total PA mass. The monounsaturated fatty acids were found to increase above their respective control levels to a proportionately greater extent as compared with their saturated counterparts, although in total mass terms the saturated remained predominant (Fig. 1). The increase at 2 min was to 134% of control for total saturated fatty acids and 187% of control for total monounsaturated fatty acids, while at 15 min it was 177 and 364%, respectively. No new fatty acids appeared in the PA fraction after any IL-2 stimulation conditions.} \]

Since the IL-2-stimulated increase in PA at short times is thought to be entirely through the action of DGK (15, 16), yet the fatty acid composition of saturated and monounsaturated species was very similar to that seen in lysophosphatidic acid-stimulated porcine aortic endothelial cells where the PA was generated by a PLD (6), we determined the DRG (DAG, AAG, and AEAG) composition of CTLL-2 cells. DAG represented 44% of the total DRG, AAG represented 55%, while AEAG accounted for the remaining 1–2%, under resting conditions. Molecular species analysis by reverse-phase HPLC revealed 16 major DAG peaks corresponding to distinct molecular species (Fig. 24 and Table I). 16:0/18:1n-9 (peak 11) was the most abundant species. The most abundant polyunsaturated species, 18:0/20:4n-6 (peak 8), was present as only 1% of total DAG. Reverse-phase HPLC of AAG demonstrated 10 major peaks (Fig. 2a), the most abundant being identified as 1-O-hexadecyl-2-oleoyl glycerol (1-O-16:0/18:1n-9; peak 8). Other AAG molecular species detected are listed in Table II. No polyunsaturated AAGs were detected. Although AEAG mass was too low for a full species analysis, transmethylation of total cellular DRG followed by GC-MS detected only 16 and 18 carbon-saturated dimethylecetals derived from the corresponding alk-1’-enyl groups.

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alkyl, acyl structure could also be present in the PA fraction. By initially transmethylating PA and then trimethylsilylating the products we were able to identify, using GC-MS, both the component fatty acids and any alkyl ethers present. Fig. 3A shows the electron impact mass spectrum for 1-alkyl-2,3-bis-TMS glycerol. The C1-C2 bond within the 1-alkylglycerol following trimethylsilylation to 1,2-DAG-DNB molecular species; various fragmentation and re-arrangement products tentatively identified to O-TMS glycerol. The C1-C2 bond within the 1-alkylglycerol following trimethylsilylation to 1,2-DAG-DNB molecular species; various fragmentation and re-arrangement products tentatively identified to O-TMS glycerol is susceptible to cleavage and generates a major ion fragment at m/z 205 corresponding to [TMS-O-CH₂(CH₃)₂Si⁻]⁺. The ions at m/z 73, 103, 117, and 147 correspond to various fragmentation and re-arrangement products tentatively identified as [TMS]⁻ (or [(CH₃)₂Si]⁻), [TMS-O-CH₂(CH₃)₂]⁻, [TMS-O-CH₂(CH₃)₂ - CH₃]⁻, and [CH₃₂Si - O-Si(CH₃)_₃]⁺, respectively. No molecular ion could be detected. As the ion at m/z 205 could only arise from the decomposition of the 1-O-alkyl-2,3-bis-TMS glycerol we used this to quantify the alkyl, acyl content of PA.

In resting cells, 1-O-alkyl-2-acyl-PA (AAG-PA) represented over 50% of total PA and consisted of a mixture of 1-O-16:0, 1-O-16:0, 1-O-18:0, and 1-O-18:1 alkyl structures based on retention times relative to an authentic standard (data not shown). 1-O-16:0 was the predominant form, representing approximately one-third of the total AAG-PA. IL-2 stimulation for 2 and 15 min increased the level of AAG-PA by 2- and 4-fold, respectively (Fig. 3B). At 15 min almost all the PA is of the AAG-PA form since there is approximately a 1:1 ratio of total fatty acid to alkyl groups at this time point (Figs. 1 and 3B). The increases in AAG-PA are similar to those for the monounsaturated fatty acids (Fig. 1) suggesting that DGKα may preferentially phosphorylate AAG containing unsaturated acyl chains when available.

When the CTLL-2 PC molecular profile was analyzed following PLC hydrolysis and DNB derivatization, 16:0/18:1-9 PC and 1-O-16:0/18:1-9 PC were found to be the most abundant species (data not shown). The whole profile was similar to that for DRG, suggesting PC as a possible source of DRG. We decided to investigate this possibility by radiolabeling CTLL-2 cells with 1-O-[^3H]octadecyl lysophosphatidylcholine which is rapidly and specifically incorporated into the AAG-PC and AAG-PE phospholipid fractions (87 and 12% of total radioactivity incorporated, respectively). In addition, we found a minor proportion (approximately 1%) of the radiolabel associated with AAG-PE, AAG-PE, nor AAG (Fig. 4 and data not shown), suggesting that hydrolysis of AAG-PC and AAG-PE was not taking place in order to generate AAG. Another route for the generation of DRG is that through de novo synthesis. To assess if IL-2 increased the de novo synthesis of DRG, we measured the incorporation of radioactivity from [9,10-^3H]oleic acid into the DRG fraction in CTLL-2 cells. IL-2 treatment of CTLL-2 cells did not cause an increase in the incorporation of oleic acid (over a period of 30 s to 2 h) compared with control cell treatment (data not shown).
response to IL-2. The data are represented as mean ± S.D. (n = 3).

FIG. 3. IL-2-stimulated formation of AAG-PA. Panel A, electron impact-mass spectrum of 1-O-hexadecyl-2,3-bis-TMS glycerol. Inset shows the cleavage producing the characteristic m/z 205 ion. Panel B, arrested CTLL-2 cells were incubated ± IL-2 (500 units/ml) for the times indicated. PA was isolated by HPLC, transmethylated, the 1-O-alkylglycerols formed were then trimethylsilylated to form the corresponding 1-O-alkyl-2,3-bis-TMS glycerols before analysis by GC-MS using selected ion monitoring at m/z 73, 103, 117, 147, and 205. The 205 ion was used to quantify changes in 1-O-alkyl-2-acyl-PA mass in response to IL-2. The data are represented as mean ± S.D. (n = 3).

FIG. 4. Lack of effect of IL-2 on the hydrolysis of AAG-PC and AAG-PE in CTLL-2 cells. Arrested radiolabeled CTLL-2 cells were incubated ± IL-2 (500 units/ml) for the times indicated. AAG-PC and AAG-PE were isolated by two-dimensional TLC. Radioactivity associated with the lipid fractions was determined by scraping the silica into vials followed by liquid scintillation counting. The data are represented as mean ± S.D. (n = 2). The figure is representative of a further independent experiment with identical results.

not shown). We also measured the incorporation of d-[U-14C]glucose into DRG, triacylglycerol, PA, PC, and PE in control- and IL-2-stimulated CTLL-2 cells. When radioactivity from a glucose tracer was determined in the glycerol backbone of DRG (which incorporated by far the greatest proportion of radioactivity at the end of a 4-min period of linear d-[U-14C]glucose uptake) from cells acutely treated with IL-2, there was no increase with respect to control-treated cells confirming that IL-2 did not affect de novo synthesis (Table III). In addition, we found that IL-2 did not increase de novo synthesis of triacylglycerol nor of PE. Very small changes (less than 10%) in the de novo synthesis of PC and PA were observed after treatment of the cells with IL-2 compared with control medium. However, these were not sufficient to alter the value of the estimated flux (the sum of DRG, triacylglycerol, PC, and PE (19)) through PAP, an enzyme involved in glycerolipid synthesis, shown in Table III.

We have previously shown IL-2-dependent short-term activation of DGKα leading to PA generation (14, 15). In order to complement the results of the in vitro AAG-PA generation, we tested the ability of DGKα to phosphorylate DAG and AAG in vitro. COS cells were either transfected with an empty plasmid or a plasmid containing HA-tagged murine DGKα. The DGKα was immunoprecipitated through its tag. Only those cells transfected with the HA-tagged DGKα expressed the protein of approximately 80 kDa as determined by Western blotting (Fig. 5A). DGKα immunoprecipitates were preincubated with or without R59949 before incubation with the substrates, 1-O-18:1n-9 (AAG) or 16:0/18:1n-9 (DAG), in the presence of [γ-32P]ATP. Fig. 5B shows that the HA-tagged murine DGKα was able to phosphorylate both the AAG and DAG substrates to the same extent. This enzymatic activity was inhibited by over 90% by preincubation of the immunoprecipitates with the DGK inhibitor II, R59949.

DISCUSSION

It is becoming increasingly important when examining mitogen-stimulated cells not only to measure the mass generation of LSMs, such as DRG and PA, but to define the molecular species of each LSM generated. By reference to the molecular structure of precursor phospholipids (such as PC and PIP2), the DRG and PA species formed may be mapped to their appropriate sources thereby fully characterizing a signaling pathway. At present, there exists a limited amount of information on this subject, but together it forms a model describing the formation and clearance of the LSMs DRG and PA through the tight regulation of at least the four enzymes: PLC, PLD, DGK, and PAP. These appear to function in many, but not all, signal transduction events delivered by mitogens (9). One of the exceptions to this general rule is IL-2, the T-lymphocyte growth factor, which deploys nonconventional signal transduction pathways (10–16). It therefore offers a unique opportunity to study novel LSM generation following cytokine receptor stimulation in T-lymphocytes which up to now has received little attention. To this end we investigated the effect of IL-2 on PA and DRG metabolism in mouse CTLL-2 cells and show that in vivo stimulated DGKα preferentially utilizes a putative PKC inhibitor, AAG, to form AAG-PA with putative second messenger functions.

The fatty acid composition of PA in resting CTLL-2 cells

### Table III

| Lipids          | Radioactivity incorporated (cpm) |
|-----------------|----------------------------------|
|                 | Control IL-2                     |
| PA              | 760 ± 32 826 ± 31                |
| PC              | 1,261 ± 51 1,459 ± 8              |
| PE              | 1,092 ± 247 1,216 ± 72            |
| DRG             | 15,953 ± 191 16,396 ± 292         |
| Triacylglycerol | 441 ± 41 456 ± 25                 |
| Flux through PAP| 18,632 ± 190 19,523 ± 340         |

Acute incorporation of radioactivity from d-[U-14C]glucose into DRG, triacylglycerol, PA, PC, and PE in CTLL-2 cells

CTLL-2 cells starved of both serum and IL-2 for 6 h were preincubated in glucose-free Krebs-HEPES buffer for 1 h before stimulation ± IL-2 (500 units/ml) for 10 min followed by the addition of d-[U-14C]glucose for a further 4 min. After lipid extraction, DRG, triacylglycerol, PA, PC, and PE were isolated by two-dimensional TLC. Radioactivity associated with the lipid fractions was determined by scraping the silica into vials followed by liquid scintillation counting. The data are represented as mean ± S.D. (n = 3). The table is representative of a further independent experiment with identical results.
revealed only saturated and monounsaturated fatty acids (Fig. 1), similar to that we have previously reported for porcine aortic endothelial and Swiss 3T3 cells (6). The fatty acids found in PA did not change following IL-2 stimulation although their relative abundance did alter, with an increase in monounsaturated species as compared with that seen in resting cells. Since IL-2 stimulates acute PA generation through the activation of DGKα (14, 15), rather than through breakdown of PC by PLD (16), then the PA must be derived from DRG already present in the CTLL-2 cells.

Using DNB derivatization followed by HPLC, we were able to separate DAG, AAG, and AEAG families of DRGs. AAG represented approximately 55% of the total DRG in resting CTLL-2 cells which is rather more than that seen in some other cell types (4, 6). Molecular species profiling of DAG (Fig. 2) showed similarities to that of 3T3 cells (4), but was distinct from that of porcine aortic endothelial cells (6) where polyunsaturated forms (containing 20:3n-9, 20:4n-6, or 20:5n-3 at the sn2-position) represented over 25% of the total DAG. This was over 20 times higher than that seen in CTLL-2 cells where polyunsaturated species are very minor components which do not change significantly following IL-2 treatment. Previous work has failed to detect any PIP2-PLC activation by IL-2 in CTLL-2 cells which is rather more than that seen in some other cell types (4, 7). Unfortunately these low levels made a comprehensive analysis impractical, although very small amounts of saturated 16 and 18 carbon alk-1'-enyl structures were detected. Stimulated increases in AAG and AEAG have been observed in other cell types. In response to interleukin-1 (28, 29) and cholecytokinin (30) in mesangial and pancreatic cells, respectively, a high proportion of AAG and/or AEAG arising from PE and PC hydrolysis, is generated as part of the total DRG production. In contrast to DAGs, the function of both AAGs and AEAGs is poorly understood and early reports concerning their properties compared with those known about DAGs were rather confusing (31–33), however, the current consensus is that if they have any PKC modulating function then it is as inhibitors rather than activators (29, 34).

As IL-2 is known to signal through the activation of DGKα and the finding that AAG represented a high proportion of the total DRG, it was necessary to assess if within the PA fraction there were AAG-PA species. Using the electron impact-mediated decomposition properties of the 1-O-alkyl-2,3-bis-TMS derivatives of AAG-PA, we were able to identify structures containing 1-O-14:0, 1-O-16:0, 1-O-18:0, and 1-O-18:1 alkyl groups. Quantification revealed that while approximately half of the PA was of the AAG-PA form in resting cells, this increased to almost 100% following acute stimulation. This fits with the observations that acute IL-2 stimulation caused a large drop in AAG levels but had little effect on DAG and that these changes could be blocked by R59949. Thus IL-2-activated DGKα (14, 15) preferentially uses AAG as a substrate in CTLL-2 cells.

In order to show that the IL-2-induced formation of AAG-PA could be attributable to DGKα, we transfected murine DGKα into COS cells which do not normally express this isoform (3). This provided us with an essentially pure enzyme preparation, after immunoprecipitation, for studies on its substrate specificity. In vitro DGKα assays revealed that it phosphorylated both AAG and DAG to the same extent (Fig. 4). Furthermore, the DGKα activity was blocked by R59949, a recognized inhibitor of this enzyme (3, 35), confirming our previously published observations (15, 16). The proportionately greater utilization of AAG for stimulated PA synthesis by DGKα in vivo as compared with that seen in vitro suggests that other factors effect selectivity within the cell such as restrictions on substrate accessibility.

FIG. 5. Expression and in vitro activity of murine DGKα. COS cells were transfected with either an empty plasmid or a plasmid containing HA-tagged murine DGKα. Panel A shows that only cells transfected with a plasmid for HA-tagged murine DGKα (molecular mass approximately 80 kDa) expressed this protein as revealed by Western blotting. HA-tagged DGKα was immunoprecipitated from transfected cells then kinase activity was assessed using AAG and DAG as substrates with or without preincubation with the specific DGK inhibitor II R59949. The resultant [32P]PA was separated by TLC and as substrates with or without preincubation with the specific DGK inhibitor II R59949. The resultant [32P]PA was separated by TLC and

3 D. R. Jones and I. Mérida, unpublished observations.
Thus in CTLL-2 cells IL-2 delivers a mitogenic signal which provokes saturated/monounsaturated PA generation, an event previously described as being absolutely essential for cell cycle entry and ultimately cell proliferation (15). PA is known to exhibit numerous biological properties (36), however, its intracellular targets remain to be identified. Now with the finding that in addition to DAG-PA there exists AAG-PA in T-lymphocytes and the fact that these species are positively regulated by IL-2, there exists the possibility that the biological properties of PAs are perhaps broader than first thought and it is likely that the PA-binding proteins show selectivity for certain classes and/or molecular species, similar to that seen with the DRGs. Future work revealing the biological properties of the IL-2-stimulated PA species, by examining the activation and inhibition of various enzymes, will further extend our knowledge concerning downstream elements of IL-2 signaling. This work has shown for the first time that a member of the DGK family is able to phosphorylate AAG to the same extent as that of DAG in vitro. In vivo, the physiological relevance of IL-2-stimulated DGKs activation could be that of the clearance of AAG to form AAG-PA thereby reducing the intracellular level of the former which is considered to have senescent and growth inhibitory properties (34). For that reason the commonly used term “dioxyglycerol kinase” should be used with caution in order to both remove previous concepts of DAG being its unique substrate and at the same time not to ignore AAGs as valid substrates. The term “diradylglycerol kinase” would be a more appropriate substitute for previous DGK nomenclature.

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