We elucidated the effects of different diacylglycerols (DAGs), i.e. 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), 1-stearoyl-2-docosahexaenoyl-sn-glycerol (SDG), and 1-stearoyl-2-eicosapentaenoyl-sn-glycerol (SEG), on [3H]PDBu binding to RasGRP. The competition studies with these DAGs on [3H]PDBu binding to RasGRP revealed different K_i values for these DAG molecular species. Furthermore, we transfected human Jurkat T cells by a plasmid containing RasGRP and assessed the implication of endogenous DAGs on activation of MAP kinases ERK1/ERK2, induced by phorbol-12-myristate-13-acetate (PMA). In control cells, GF109203X, a protein kinase C inhibitor, inhibited ERK1/ERK2 activation. However, this agent curtailed but failed to completely diminish ERK1/ERK2 phosphorylation in RasGRP-overexpressing cells, though calphostin C, a DAG binding inhibitor, suppressed the phosphorylation of MAP kinases in these cells. In cells incubated with arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), PMA induced the production of endogenous DAGs containing these fatty acids, respectively: DAG-AA, DAG-DHA, and DAG-EPA. The inhibition of production of DAG-AA and DAG-DHA significantly inhibited MAP kinase activation in RasGRP overexpressing, but not in control, cells. Our study demonstrates that three DAG molecular species bind to RasGRP, but only DAG-AA and DAG-DHA participate in the modulation of RasGRP-mediated activation of MAP kinases in Jurkat T cells.

The Ras family of small GTPases is comprised of the classical Ras GTPases (H-Ras, N-Ras, and K-Ras) as well as a more divergent group of Ras-related GTPase (TC21, R-Ras, R-Ras3, RasG, and Raps) (1). These proteins cycle between an inactive form bound to GDP and an active GTP-bound state. To reach the active GTP-bound state, Ras proteins must first release bound GDP. This rate-limiting step in GTP binding is catalyzed by guanine-nucleotide exchange factors (GEFs). Several mammalian GEFs have been identified so far, including SOS (2), RasGRF (3), and RapGDS (4). A new class of GEFs, expressed mainly in brain and T cells (5, 6), is composed of at least four members: 1) RasGRP, the first member characterized as a GEF for Ras (7); 2) CalDAG-1 or RasGRP2, which possesses GEF activity for N-Ras, K-Ras, and Rap1 (8); 3) CalDAGIII or RasGRP3, which can activate both Ras and Rap 1 (9); 4) RasGRP4, recently discovered, has been shown to activate H-Ras in a cation-dependent manner (10, 11). All these GEF members have a pair of atypical EF-hands (a calcium-binding motif), and the C1 domain, which represents a signature motif that is involved in the recognition of phorbol ester and diacylglycerol (DAG) (12–14). Although calmodulin-like EF-hands are present in RasGRP, [3H]phorbol 12,13-dibutyrate (PDBu) binding is independent of calcium (12). This is in marked contrast to the calcium-dependence observed for conventional protein kinase C (PKC) (15). For many years, DAG was believed to act solely through the PKC family of isoenzymes. Now, RasGRP provides a direct link between DAG generation and Ras activation (12, 14). Ebina et al. (7) monitored the subcellular fractionation of RasGRP in Rat2 cells, and they observed that PMA binding increased the recruitment of RasGRP to plasma membrane where it interacted efficiently with ras. Activated RasGRP and PKC, in turn, stimulate the mitogen activated protein (MAP) kinase-signaling pathway (12, 16). Propagation of these signaling cascades promotes the transcription of numerous genes including those encoding cytokines critical for T cell development, activation, and proliferation. In fact, deletion of C1 domain of the RasGRP not only abolishes phorbol ester binding but also MAP kinase activation (7, 17).

Numerous reports have described a beneficial effect of omega 3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acids (EPA) on cell-mediated immunity. Fish oil supplementation of human and animal diets has been shown to decrease interleukin-2 production, T cell mitogen-induced proliferation, and delayed type hypersensitivity, indicating an overall decrease in T cell-mediated immune function (17). Hence, the omega 3 fatty acids incorporated into cell phospholipids could affect cell signaling via DAG production after phospholipid hydrolysis. In T cells, DAG is produced in a biphasic way (18). The transient production of DAG is assured by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), catalyzed by phospholipase C (PLC).

The second sustained phase of DAG generation is associated with phosphatidatic acid; PC, phosphatidycholine; PLD, phospholipase D; PC-PLD, PC-specific PLD; PDBu, phorbol 12,13-dibutyrate; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PI-PLC, PI-specific-PLC; PS, phosphatidylserine; PKC, protein kinase C; PMMA, phorbol 12-myristate 13-acetate; PUFAs, polyunsaturated fatty acids; HPLC, high pressure liquid chromatography; BSA, bovine serum albumin; ANOVA, analysis of variance.
with an increase in the activation of phospholipase D (PLD)-catalyzed phosphatidylcholine (PC) hydrolysis, producing phosphatic acid (PA), which can be converted to DAG by the action of phosphatidate phosphohydrolase (19, 20). In cardiomyocytes, addition of DHA has been shown to modulate cell contractility via the production of DAG, containing this fatty acid (21). On the other hand, EPA incorporated into DAG has been found to reduce vascular complications associated with diabetes (22). In the same way, we have recently demonstrated that different isoforms of conventional PKCs (α, β1, and γ) and novel PKCs (ε and δ) differ in their response to DAGs containing omega 6 or omega 3 PUFAs in a cell-free system (23). As far as the modulation of RasGRP by DAG is concerned, to our knowledge, a few studies are available. Lorenzo et al. (15) have shown that 1-oleoyl-2-acetyl-sn-glycerol was 20-fold more potent for binding to rat C1 RasGRP than with PKCζ. These authors also examined the translocation pattern of RasGRPs in response to 1,2-diacyl-sn-glycerol in HEK-293 cells (12). They found that 1,2-diacyl-sn-glycerol induced RasGRPs redistribution to the perinuclear region, and to a lesser extent, to the plasma membrane. Nonetheless, no comparative study is available on the affinity of RasGRPs to bind DAGs containing either omega 6 or omega 3 PUFAs. Therefore, it was thought worthwhile to elucidate 1) the comparative effects of different DAGs on RasGRPs and 2) the implication of endogenously produced DAGs containing these fatty acids in the regulation of MAPK activation in human Jurkat T cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—[^H]AA (217 Ci/mmol) and [3H]DHA (50 Ci/mmol) were purchased from PerkinElmer Life Sciences, and [3H]EPA (100 Ci/mmol) was from ICN Biomedicals (Orsay, France). Phosphatidyl-t-serine (PS) from bovine brain, 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-lysophosphatidylcholine, phosphatidylcholine, lysolecithin, PMA, AA, DHA, and EPA were obtained from Sigma. [3H]PDBu (629 GBq/mmol) and [γ-32P]ATP (3000 Ci/mmol) were purchased from PerkinElmer Life Sciences and Amersham Biosciences, respectively. Anti-phosphorylated MAP kinase (ERK1/ERK2) antibodies were obtained from New England BioLabs (Beverly, MA). Anti-RasGRP antibodies (R20) were obtained from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). GF109203, calphostin C, and U73122 were obtained from Calbiochem (La Jolla, CA). The MAP kinase assay kit (BIOTRAK) was purchased from Amersham Biosciences.

**Expression and Purification of Rat RasGRP Proteins in Echerichia coli**—The cDNA coding for RasGRP was cloned from rat brain mRNA (7). Recombinant RasGRP produced in E. coli was used for the assays. Briefly, BL21 bacteria, transformed with the RasGRP-pMAL-c2 construct, were induced using 0.5 mM isoprropyl-β-D-thiogalactopyranoside for 2 h at 37°C. The bacterial culture was then centrifuged for 20 min at 4,000 x g at 4°C, and the pellet was resuspended in column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and protease inhibitors). Cell lysis was completed by six 15-s pulses of sonication over 2 min. The lysate was clarified by centrifugation at 14,000 x g at 4°C for 20 min, and the supernatant was used as the crude extract for the subsequent purification. The maltose basic protein-RasGRP fusion protein was purified using an amylose resin according to the manufacturer's instructions (New England BioLabs). Depending on the batch, 10 μg of partially purified protein per tube were used for the binding assay.

**Synthesis of SAG, SDG, and SEG**—SAG, SDG, and SEG were obtained by the action of phospholipase C from Bacillus cereus on 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (1-stearoyl-2-arachidonoyl-PC), 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (stearyl-2-docosahexaenoyl-PC), and 1-stearoyl-2-eicosapentaenoyl-PC, respectively. The synthesis was performed as previously described (23). Briefly, DAGs after their synthesis were purified using straight-phase HPLC on a 30 cm x 8 mm column (Millipore, Saint-Quentin, France) and eluted isocratically with hexane/isopropyl alcohol, 100:1 (v/v). The purified DAGs were identified by comparing their retention times with those of standards [3H]DAGs.

Standards of radiolabeled DAGs were obtained as follows: [3H]AA, [1-CDHA], and [3H]EPA were esterified by lsoPC using rat liver microsomes. The radiolabeled phospholipids obtained were hydrolyzed by phospholipase C, and DAGs were purified using straight-phase HPLC as described in this paragraph. HPLC-purified DAGs were quantified after transesterification at 80°C for 20 min by BF3/methanol, using dinonadecanoin as internal standard. Fatty acid methyl esters were extracted with 2 ml hexane, separated by gas-liquid chromatography in a Packard Model 417 gas-liquid chromatograph, and equipped with a flame ionization detector and a 30-m capillary gas column coated with carbowax 20 m. The analysis conditions were as follows: oven at 194°C, injector and ionizing detector at 240°C. Helium was used as carrier gas, with a flow rate of 0.4 ml/min. Quantification of fatty acid methyl esters was achieved with the internal standard by using DELSI ENICA 31 (Delsi Nermag, Rungis, France).

**Preparation of Vesicles**—The required amounts of PS, DAG, labeled [3H]PDBu and unlabeled-PDBu in chloroform or ethanol were dried under a stream of nitrogen in a glass tube and solubilized in Tris-HCl 20 mM by vortexing and sonication at 30°C for 5 min.

**Binding of [3H]PDBu**—Binding of [3H]PDBu was measured using polyethylene glycol precipitation assay as described by Sharkey and Blumberg (24). The assay mixture contained 50 mM Tris HCl (pH 7.4), 1 mg/ml IgG, 0.1 mM CaCl2, RasGRP protein and the corresponding lipid mixture or sonicated PS dispersion. Incubations were carried out for 5 min at 18°C. Nonspecific binding was measured using an excess of unlabelled PDBu (50 μM), and specific binding was performed in triplicate at each ligand concentration (0.125–30 mM).

**For competition experiments on [3H]PDBu binding to RasGRP by different DAG molecule species, the reactions were performed under similar conditions, but using a fixed concentrations of [3H]PDBu (6 nM) and increasing concentrations of the nonradioactive ligands. In atypical competition assay, ten different concentrations (in triplicate) of the competing ligand were used from the IC50 by using the relationship \( K_c = IC_{50}/(1 + L/K_c) \), where L is the concentration of free [3H]PDBu used and \( K_c \) is the dissociation constant for [3H]PDBu for RasGRP. The Hill coefficient (nH) was calculated according to the following equation: \( \%B_{\text{max}} = a \times \log [c] \), which will provide a straight line, y = ax + b; where a is the slope of the straight line and is equal to Hill number; c is the concentration of the ligand. Saturation and competition studies were analyzed using the software RADLIG 4.0, by non-linear curve-fitting LIGAND ( Biosoft, Cambridge, UK).

**Cell culture and transfection**—To assess the effects of DAGs on the expression of RasGRPs and MAP kinase signaling, Jurkat T cells, which possess a little RasGRP activity (25), were engineered to overexpress the rat RasGRP cDNA using PQE-TriSystem vector. The Jurkat T cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified chamber containing 95% air and 5% CO2. Cells were counted and assessed for viability by trypan blue exclusion test. Cells (7.5 x 10⁶) were then plated in 24-well plates and transient transfection of Jurkat cells with RasGRP-PQE-TriSystem construct was performed using X-tremeGENE Q2 Transfection Reagent (Roche Applied Science). Non-transfected cells (control) received all transfection reagents except RasGRP DNA. 48 h after, control and RasGRP-transfected cells were used for MAPK assay. Lysozyme activity in supernatants of transfected cells were transfected with pHCI 110 vector (Amersham Biosciences) containing lacZ gene, and β-galactosidase expression was monitored using a photometric assay as described by Flenger et al. (25).

**Detection of RasGRP by Immunoblotting**—The protein lyasease was prepared from non-transfected and rasGRP-transfected cells. Protein lyase was prepared by SDS-PAGE (10%) and transferred to polyvinylidene difluoride membranes, and immunodetection of RasGRP was performed by using goat polyclonal anti-RasGRP antibodies raised against a peptide mapping at the carboxyl terminus of RasGRP of rat origin. This antibody reacts with both rat and human RasGRP. After treating the membrane with peroxidase-conjugated rabbit anti-goat secondary anti-PC, the peroxidase activity was detected with ECL reagents (Amersham Biosciences).

**Preparation of Fatty Acid-Albumin Complexes**—Free AA, DHA, and EPA were stored at −20°C in ethanolic solution under nitrogen. Aliquots of the ethanolic solution were evaporated to dryness under reduced
pressure. 0.2% fatty acid-free bovine serum albumin (BSA) in RPMI 1640 medium was added to give a final fatty acid concentration of 10 μM. The mixtures were incubated under nitrogen for 4 h at 37 °C.

Measurement of DAG Production—To determine the contribution of phospholipases and PKC in DAG production, we used specific inhibitors of phospholipases and PKC. U73122 was used for inhibition of phosphoinositide-specific-PLC (PI-PLC), propranolol for phosphatidylcholine-specific PLD (PC-PLD) and GF109203X for PKC.

Both control and RasGRP-transfected Jurkat T cells were incubated for 3 h in RPMI without serum. Cells were further incubated for 3 h at 37 °C in RPMI 1640 (0.2% BSA) in the presence or not (none) of [3H]AA (10 μM, 17 mCi/mmol), [1-14C]DHA (10 μM, 20 mCi/mmol) or [3H]EPA (10 μM, 20 mCi/mmol). After the incubation period, cells were washed three times in RPMI 1640 medium (0.2% BSA). Cells (4 × 10^6/ml) were then incubated for 30 min with either U73122, propranolol, U73122 + propranolol, or GF109203X before incubation for 10 min at 37 °C in the absence (Unstim.) or presence of PMA. Incubation was stopped by the addition of methanol and DAGs were extracted three times with hexane and quantitatively converted to [32P]ATP. The resulting PA was then extracted and separated by TLC (chloroform/methanol/acetic acid, 65:15:5 (v/v)). The area corresponding to PA was scraped and the radioactivity spectrum was counted by liquid scintillation.

MAP Kinase Assay—Prior to experiments, both control and RasGRP-transfected Jurkat T cells were incubated for 3 h in RPMI without serum. Cells were further incubated for 3 h at 37 °C in RPMI 1640 (0.2% BSA) in the presence or not (none) of non-radioabeled PUFA: (each at 10 μM) AA, DHA, or EPA. Later on, cells were washed three times in RPMI 1640 medium (0.2% BSA). Cells (4 × 10^6/ml) were then incubated for 30 min with either U73122 (10 μM), propranolol (100 μM), U73122 + propranolol, GF109203X (500 nM), or calphostin C (1 μM), and again incubated for 10 min at 37 °C in the absence (Unstim.) or presence of PMA (200 nM). After incubation, the cells were lysed and centrifuged (2,500 × g for 1 min). The protein contents of cell lysate were determined with a Sigma Bradford reagent kit. Denatured protein (20 μg) was separated by SDS-PAGE (10%) and transferred to polyvinylidene difluoride membranes, and immunodetection was performed by using anti-phosphorylated anti-rat ERK1/ERK2 antibodies. After treating the membrane with peroxidase-conjugated goat anti-rabbit secondary antibodies, peroxidase activity was detected with ECL reagents (Amersham Biosciences).

MAP kinase enzyme activity was determined according to the instructions, furnished with the kit. The enzyme activity was assessed by monitoring the incorporation of [32P]P into a peptide fraction of EGFr receptor containing the PL5T8 sequence, which is a more specific substrate for MAP kinases than the myelin basic protein. Assays were terminated by spotting cell lysates onto P-81 filter paper followed by extensive washing with 75 mM orthophosphoric acid. Radioactivity was counted by liquid scintillation.

Statistical Analysis—Results are shown as means ± S.E. Statistical analysis of data was carried out using STATISTICA (version 4.1, Statsoft, Paris, France). The significance of the differences between mean values was determined by employing one way ANOVA.

RESULTS

Binding of [3H]PDBu to RasGRP and Its Displacement by SAG, SDG, and SEG—As shown in Fig. 1, [3H]PDBu bound to RasGRP (∆max 152 ± 1.66 pmol/mg protein) with high affinity, the dissociation constant (KD) being 1.5 ± 0.35 nM (n = 6). To determine the structure-activity relationship for ligand recognition by RasGRP, we performed competition binding studies using different DAGs in which sn-2 position contained either omega 6 or omega 3 fatty acids. The ligands included SAG, an omega 6 PUFAs, and SDG and SEG, two omega 3 PUFAs (Fig. 2 and Table I). The displacement of [3H]PDBu by all non-radioabeled DAG molecular species revealed nH values of 0.79, 1.03, and 0.87 for SAG, SDG, and SEG, respectively (Table I). The competition curve of the displacement of [3H]PDBu by SAG begins at 0.5 μM with an IC50 of 4.52 μM (Fig. 2, Table I). In the case of SDG, the IC50 is 8.41 μM and this DAG begins to displace the binding of [3H]PDBu beyond 2.5 μM (Fig. 2). The displacement of [3H]PDBu by SEG begins at 1 μM and provides an IC50 of 5 μM (Fig. 2). The KI values for SAG, SDG, and SEG were, respectively, 4.49 ± 0.01 μM, 8.37 ± 1.02 μM, and 4.97 ± 1.04 μM. These results indicate a high affinity of RasGRP for SAG and SEG and low affinity for SDG.

Exogenous AA, DHA, and EPA Contribute to the Production of DAG—Cells were preincubated with 10 μM of radiolabeled AA, DHA, or EPA in a medium containing 0.2% of fatty acid-free BSA for 3 h to obtain a measurable enrichment of membrane phospholipids. The presence of three fatty acids in cell lipids was confirmed with a higher incorporation of DHA (10% of the amount present in the culture medium), compared with AA (8%) and EPA (2%) (results not shown). Fatty acid incorporation into total phospholipids was 52, 48, and 28% for AA, DHA, and EPA, respectively, of total lipids including phospholipids cholesterol esters and triglycerides (results not shown). On the other hand, the three fatty acids, i.e. DHA, AA and EPA, being incorporated at a similar rate in PC, phosphatidylethanolamine (PE) and PI/PS, varied from 80, 11, and 8% of total.

![Fig. 1](http://www.jbc.org/Downloaded from http://www.jbc.org) Specific binding of [3H]phorbol 12,13-dibutyrate to RasGRP. Recombinant RasGRP, produced in E. coli, was used for the assays. The myelin basic protein-RasGRP fusion protein was purified using an amylose resin as described under “Experimental Procedures.” Binding assays were performed on the purified protein (10 μg per assay) using sonicated vesicles of PS (100 μg/ml). Inset shows Scatchard plot. Data are mean ± S.E. (n = 6).

![Fig. 2](http://www.jbc.org/Downloaded from http://www.jbc.org) Displacement of [3H]PDBu binding to RasGRP by different DAG molecular species. Lipid vesicles were prepared with different DAGs (SAG, SDG, or SEG) and the displacement of [3H]PDBu binding was determined as a function of increasing concentrations of DAG as described under “Experimental Procedures.” Data are the mean ± S.E. (n = 6).
phospholipids, respectively (results not shown). In the neutral lipids, AA, DHA, and EPA incorporation was 45, 50, and 70%, respectively, of total lipids including phospholipids cholesterol esters and triglycerides.

To determine the optimal production of DAG by PMA, the cells were incubated for different periods (0, 5, 10, 20, and 30 min) in the absence of PUFAs. We observed that PMA incubation of cells for 10 min exerted optimal stimulatory effects on DAG production as shown in Fig. 3.

Basal DAG production was not different between control and transfected cells whether or not cells were incubated with PUFAs (Fig. 4A). We noticed that after incubation in the presence of AA, DHA, or EPA, unstimulated (Unstim.) cells contained DAGs acylated with these fatty acids (Fig. 4B). DAG containing radiolabeled AA, DHA, and EPA represent 18, 25, and 20% of total endogenous DAG, respectively (Fig. 4). The basal level of 1-acyl-2-(DHA)glycerol (DAG-DHA) was higher (30 ± 2 pmol/million cells), compared with 1-acyl-2-(AA)glycerol (DAG-AA) or 1-acyl-2-(EPA)glycerol (DAG-EPA) (6 ± 0.50 and 2 ± 0.10 pmol/million cells, respectively) (Fig. 4B). We found that DHA, but not AA or EPA, significantly increased by 35% basal total DAG production (Fig. 4A), which is principally due to the rise in DAG-DHA formation (Fig. 4B). PMA activation significantly increased total DAG mass in cells (Fig. 4A). In DHA-treated cells, PMA induced a lower DAG production compared with AA-treated cells, and PMA increased DAG mass production by 2- and 6-fold for AA and DHA, respectively. PMA stimulation also enhanced the relative amounts of DAGs containing [3H]AA and [3H]EPA in both control and RasGRP-transfected cells without modifying those containing [3H]AA and [3H]EPA incorporation into cell phospholipids.

**PMA Induced DAG Production by Activating PLC and PLD**—To determine the contribution of PKC-activated-phospholipases C and D in DAG production, we used U73122, an inhibitor of PI-PLC, and propranolol, an inhibitor of PC-PLD. Fig. 4 shows that GF109203X, a PKC inhibitor, abolished PMA-induced DAG production both in control and transfected cells, suggesting that PMA increased DAG production via PKC activation. In the absence of PMA, propranolol and U73122 had no effect on basal DAG production (data not shown). On the other hand, these two agents significantly diminished PMA-stimulated DAG production. Furthermore, U73122 and propranolol together exerted additive effects on the inhibition of PMA-stimulated DAG in both control and RasGRP overexpressing Jurkat T cells and inhibitory effect was up to the level of GF109203X-treated cells (Fig. 4). U73343, an inert analogue of PI-PLC inhibitor, completely failed to curtail PMA-induced DAG production in either of cells (results not shown). These results suggest that both PC-PLD and PI-PLC are activated by PMA in Jurkat T cells, and PKC may be the principal activator of phospholipases and a key mediator of DAG production.

**RasGRP Overexpression Is Coupled to Increased ERK1/ERK2 Phosphorylation**—Evaluation of RasGRP expression by immunoblotting using its specific antibody shows a detectable expression of Ras GRP in Jurkat T cells. On the other hand, Ras GRP protein is highly expressed in RasGRP-transfected cells (Fig. 5A).

As shown in Fig. 5, b and c, PMA induced ERK1/ERK2 phosphorylation. Functional RasGRP overexpression in Jurkat T cells is confirmed by using GF109203X, a specific PKC inhibitor, and calphostin C, a specific inhibitor of binding of DAG and phorbol ester to C1 domain present on PKC and RasGRP (15). We observed that GF109203X diminished the PMA-induced ERK1/ERK2 phosphorylation in control cells, whereas it curtailed, but did not completely diminish, the phosphorylation of MAPK in RasGRP-transfected cells, suggesting that PMA induced MAP kinase activation via PKC in control cells, whereas in transfected cells, the activation of MAP kinase is mediated via both a PKC-dependent and -independent pathways.

Endogenously Produced DAG-AA and DAG-DHA, but Not DAG-EPA, Are Implicated in ERK1/ERK2 Activation—As we have observed in Fig. 4 that incubation of Jurkat T cells in the presence of PUFAs (AA, DHA, or EPA) can give rise to DAG-containing these fatty acids, we further examined the effects of inhibitors of phospholipases on MAPK enzyme activation. Fig. 6 shows that PMA-induced ERK1/ERK2 enzyme activity was higher in transfected cells compared with control cells whether or not they were incubated in the presence of DHA and AA. It is noteworthy that PMA-treated RasGRP-transfected Jurkat T cells after incubation with AA and DHA exhibited higher MAPK enzyme activation than those cells having no fatty acid treatment.

The inhibitory effects of U73122, an inhibitor of PI-PLC, and propranolol, an inhibitor of PC-PLD, on MAPK activation were higher in transfected cells than in control cells, which were
incubated with DHA and AA (Fig. 6). The association of these two phospholipase inhibitors potentiated their inhibitory effect. This inhibition is more pronounced in RasGRP-transfected cells, indicating that DAGs are involved in RasGRP/MAP kinase activation pathway. U73343, an inert analogue of PI-PLC inhibitor, failed to diminish MAPK activation (results not shown). Furthermore, no difference in the effects of U73122 and propranolol was observed in EPA-treated cells in either of the group of cells (Fig. 6).

**DISCUSSION**

RasGEF called RasGRP has been shown to possess a DAG and phorbol ester-binding motif, which possesses strong homology to DAG-binding site, or C1 domain, of PKC (7, 15, 25). In the present work, we analyzed the properties of RasGRP as a DAG/phorbol ester receptor. We observed that $[^{3}H]$PDBu bound to RasGRP with high affinity with a dissociation constant ($K_d$) of 1.5 ± 0.35 nM. These results agree well with the findings of Lorenzo et al. (12) who have reported, using human RasGRP3, a $K_d$ value equal to 1.53 ± 0.33 nM. To our knowledge, no study is available on the affinity of binding of different DAG molecular species to RasGRP. In the present work, our findings show for the first time the differences in the affinity of RasGRP to bind DAGs-containing AA, EPA, and DHA. The displacement of $[^{3}H]$PDBu by different DAG molecular species reveal a high affinity binding site of RasGRP for SAG, SEG, and SDG. Hence, SDG ($K_i$, 8.37 ± 1.02 μM) seemed to be less potent than SAG ($K_i$, 4.49 ± 0.01 μM) and SEG ($K_i$, 4.97 ± 1.04 μM). The structural similarities (acyl chain length) between AA and EPA could explain the differences in their binding affinity to RasGRP compared with DHA. Taken together, these findings lead to the hypothesis that AA, DHA, and EPA could affect cell function through their substitution into DAG and through a specific effect of 1-acyl-2-(AA)glycerol (DAG-AA), 1-acyl-2-(DHA)glycerol (DAG-DHA), and 1-acyl-2-(EPA)glycerol (DAG-EPA) on RasGRP and, subsequently, on MAP kinase signaling. We, therefore, addressed the question whether these DAGs could be produced by cells, and if so, what would be their effects on MAP kinase activation. To test this hypothesis, human Jurkat T cells that present a detectable RasGRP activity (26) were engineered to overexpress the rat RasGRP cDNA. We
observed that RasGRP-transfected cells expressed a high level of RasGRP proteins compared with control Jurkat T cells. The overexpression of functional RasGRP in Jurkat T cells was confirmed by using GF109203X and calphostin C. Hence, calphostin C, specific inhibitor of DAG, and phorbol ester-binding domain inhibited PMA-induced ERK1/ERK2 phosphorylation in both types of cells, whereas GF109203X, a specific PKC inhibitor, curtailed but did not completely diminish, the action of PMA on ERK1/ERK2 phosphorylation in RasGRP overexpressing Jurkat T cells compared with control cells. These results indicate that PMA directly stimulates RasGRP and, thereby, promotes PKC-independent but Ras-dependent signaling in transfected Jurkat T cells.

In the present study, both control and RasGRP overexpressing Jurkat T cells incorporated exogenous fatty acids (AA, DHA, and EPA) into PC (80% of total phospholipids, data not shown). Such predominant incorporation of fatty acids into PC has been described in a variety of cell types (28, 29). Due to the fact that PMA-treatment of Jurkat T cells can give rise to the production of DAGs via activation of PI-PLC and PC-PLD (19, 20, 29), this phorbol ester was used to activate these phospholipases in our study. We observed that DHA-incubated cells exhibited high production of DAG-DHA whether or not treated with PMA. This observation is in accordance with the report of Beshoua et al. (29) who have also noticed high production of DAG-DHA in peripheral blood mononuclear cells. These authors have shown that DHA, compared with AA or EPA, stimulates both phospholipase D and phosphatidate phosphohydrolase activities, which results into an increased DAG production at the expense of phosphatidate. Alternatively, it is also possible that a high basal level of DAG-DHA could result form the failure of this DAG to be converted to phosphatidic acid, via DAG kinase, and further assimilated into phospholipids; however, to our knowledge there is no report on the selective enzymatic activities of DAG kinase on DAG-DHA. We also observed that the basal level of DAG-DHA was higher as compared with DAG-AA. The level of DAG-EPA was not significantly elevated in both control and transfected Jurkat T cells. Furthermore, PMA treatment significantly increased the relative amounts of DAG-AA and to a lesser extent DAG-DHA in both control and RasGRP-transfected cells without modifying those containing DAG-EPA, probably because of the low EPA incorporation into cell phospholipids. In RasGRP-transfected cells, we observed that the percentage of increase in DAG mass was 2- and 0.6-fold for AA and DHA, respectively, after PMA treatment. These results indicate that PMA does not stimulate proportionally as is the case of AA-treated cells. Hence, PMA will activate PKC, which in turn will activate PLD and, consequently, DAG production. This could explain the attenuated production of DAG-DHA in PMA-stimulated cells. The source of radiolabeled DAG molecular species is likely to be PC and PIP2 as U73122 and propranolol, the respective inhibitors of PI-PLC and PC-PLD, significantly curtailed the PMA-induced DAG-AA and DAG-DHA formation in control and RasGRP overexpressing Jurkat T cells.

Finally, we determined whether a cell enrichment in DAG-containing AA and DHA could modulate ERK1/ERK2 enzyme activation via RasGRP. Hence, we measured the ERK1/ERK2 enzyme activity in place of detecting phosphorylation state, and this due to the fact that the phosphorylation status of MAPK determines its enzymatic activity that goes on under complex regulation by different factors like kinases and phosphatases (30). Secondly, the small variations in MAPK activation in Western blot can not be detected so precisely as we can measure them in enzyme assays. Moreover, we would like to recall that previously we have shown that incubation of Jurkat T cells with DHA (and also EPA) significantly diminished the PMA-stimulated ERK1/ERK2 activation (31), whereas in the present study DHA incubation was found to potentiate the action of PMA (see “Discussion”). In fact, the two experimental conditions are different: in our previous study, the incubation of cells with PUFAs was for 30 min without BSA, whereas in the present study the cells are incubated for 3 h with BSA plus PUFAs in order to enrich their phospholipids and then PMA is employed to activate the MAPK.

Though the production of DAG-DHA was higher than that of DAG-AA, the potentiation of PMA-induced MAPK activation did not follow the same order of magnitude in cells incubated with DHA and AA. On the contrary, stimulation of PMA-induced MAPK activation in cells treated with DHA and AA is identical. It seems that DAG-AA, being produced in small amounts, can exert high stimulatory effect. Indeed, DAG-AA seems to be more potent than DAG-DHA to bind to RasGRP as is revealed by K values (SDG, 8.37 ± 0.01; SAG, 4.49 ± 0.01). This suggests that the DHA-DAG is less potent activator of effectors (RasGRP and PKC) involved in MAPK activation. We have also previously reported that SAG and SDG are more potent activator of PKC and SDS in a cell free system (23).

As far as DAG-EPA is concerned, it does not seem to be implicated in MAPK activation as EPA was poorly incorporated into phospholipids and Jurkat T cells did not produce DAG-EPA in sufficient amounts (see “Discussion”). As expected, PMA induced MAPK activation in cells treated with EPA was similar to the cells which received no fatty acid in both the group of cells (control and transfected). These observations corroborate the results of Marignani et al. (32) who have also shown that SAG and SDG are more potent than SEG for whole PKC activation.

Furthermore, the inhibition of the production of DAG-AA and DAG-DHA by U73122 and propranolol significantly curtailed, but did not completely diminish, the PMA-induced ERK1/ERK2 activation in RasGRP-transfected, but not in control, Jurkat T cells. This could be explained by the strong dependence of RasGRP on DAG for its activation, compared with PKC. In the same way, Lorenzo et al. (15) have shown that DAG was 20-fold more potent for binding to rat C1 RasGRP
than with PKCs. Inhibition of both PLC and PLD completely curtailed PMA-induced DAG production either in transfected and control cells and markedly inhibited MAP kinase activity in transfected cells. This inhibition does not reach MAP kinase activity to the level of control cells, and this is due to the direct PMA activation of RasGRP and PKC. Because MAPK activation was still apparent in the presence of phospholipase inhibitors, these results suggest that two mechanisms, i.e. PKC-independent, but DAG-dependent, and PKC-dependent, are implicated in MAPK activation in these cells. PC-PLD inhibitor, propranolol, known to inhibit DAG production, also increases PA levels (33) and one can envisage that an increase in PA contents may influence MAPK activation. Hence, the implication of PA in inhibition of MAPK activation can be ruled out as Bradshaw et al. (34) have shown that in Jurkat T cells, PA failed to influence ERK1/ERK2 phosphorylation.

In the present study, it is also possible that DAG-DHA may bypass its action on PKC and regulate preferentially the RasGRP-coupled signaling. In fact, under physiological conditions, DHA is preferentially incorporated into PC (29). Leach et al. (35) have reported that DAG derived from PC-PLD does not participate in the activation of PKC in IIC9 fibroblasts. Similarly, Pettitt et al. (36) have concluded that DAG generated from PI(2) hydrolysis by PI-PLC activates PKC, whereas that produced from PC hydrolysis by PLD is not able to activate this enzyme. However, we cannot rule out the possibility of action of endogenous DAG on PKC. Nonetheless, the following observations strongly suggest that endogenous DAGs do participate in Ras-mediated MAPK activation: 1) addition of AA and DHA significantly potentiated PMA-induced MAPK activation as compared with respective transfected and PMA-treated (having no fatty acid treatment) cells and 2) inhibitors of phospholipases diminished this stimulatory effect up to the extent that was seen in RasGRP-transfected (and PMA-treated) cells that were not treated with AA and DHA. Though the degree of MAPK activation was still higher in these cells (RasGRP-transfected, treated with AA/DHA, PMA, and inhibitors of phospholipases) as compared with control cells (treated with PMA), this is due to action of this phorbol ester on PKC and RasGRP in transfected Jurkat T cells.

We conclude that RasGRP, in a cell-free system, bound phorbol ester and DAG with a high affinity. Moreover, the affinity of the DAG binding seems to be dependent on the nature of PUFAs (AA, DHA, and EPA) present at sn-2 position. Incubation of RasGRP overexpressing Jurkat T cells with these PUFAs, through phospholipase C- and D-mediated pathways, gave rise to the production of DAG containing these PUFAs. These DAGs, particularly those containing DHA and AA, seem to exert similar effects on PMA-induced MAP kinase activation (via RasGRP). Our study is certainly of physiological relevance as PUFAs ingested in food may affect health and disease by interfering with cell signaling.

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REFERENCES
1. Bokoch, G. M., and Der, C. J. (1993) FASEB J. 7, 759–769
2. Chandim, P., Canouis, J. H., Gale, N. W., van Arkel, L., Schlessinger, J., Wigler, M. H., and Bar-Sagi, D. (1993) Science 260, 1338–1343
3. Shou, C., Farnsworth, C. L., Neel, B. G., and Feig, L. A. (1992) J. Biol. Chem. 267, 10351–10354
4. Albright, C. F., Giddings, B. W., Liu, J., Vito, M., and Weinberg, R. A. (1993) EMBO J. 12, 339–347
5. Pierrot, P., Dunn, R. J., Djordjevic, B., Stone, J. C., and Richardson, P. M. (2000) J. Neurocytol. 29, 485–497
6. Pierrot, P., Vallee, A., Mechawar, N., Dower, N. A., Stone, J. C., Richardson, P. M., and Dunn, R. J. (2001) Neuroscience 108, 381–390
7. Ebina, Y., Botterell, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, J. C. (1998) Science 280, 1082–1086
8. Rebhun, J. F., Castro, A. F., and Quilliam, L. A. (2000) J. Biol. Chem. 275,
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9. Rebhun, J. F., Chen, H., and Quilliam, L. A. (2000) J. Biol. Chem. 275, 13406–13410
10. Yang, Y., Li, L., Wang, G. W., Kritio, S. A., Madhusudhan, M. S., Sali, A., and Stevens, R. I. (2002) J. Biol. Chem. 277, 25756–25774
11. Reuther, G. W., Lambert, Q. T., Rebhun, J. F., Caligiuri, M. A., Quilliam, L. A., and Der, C. J. (2002) J. Biol. Chem. 277, 30508–30514
12. Lorenzo, P. S., Rung, J. W., Bottruff, D. A., Garfield, S. H., Stone, J. C., and Blumberg, P. M. (2001) Cancer Res. 61, 943–949
13. Reuther, G. W., Lambert, Q. T., Rebhun, J. F., Caligiuri, M. A., Quilliam, L. A., and Der, C. J. (2002) J. Biol. Chem. 277, 30508–30514
14. Lorenzo, P. S., Kung, J. W., Bottorff, D. A., Garfield, S. H., Stone, J. C., and Blumberg, P. M. (2000) Mol. Pharmacol. 57, 840–846
15. Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M. F., and Marshall, C. J. (1998) Science 280, 109–112
16. Harbige, L. S. (1998) Proc. Nutr. Soc. 57, 555–562
17. Khan, N. A., and Hichami, A. (2002) in Recent Advances in Research in Lipids (Pandali, G., ed) Vol. 6, pp. 65-78, Transworld Publications, Trivendrum
18. Sebaldt, R. J., Adams, D. O., and Uhing, R. J. (1999) Biochem. J. 344, 199–204
19. Sebaldt, R. J., Adams, D. O., and Uhing, R. J. (1992) Biochem. J. 284, 367–375
20. Bordoni, A., Biagi, P. L., Parchetti, E., Rossi, C. A., and Hrelia S. (1992) Cardiologia 37, 631–634
21. Kuroki, T., Inoguchi, T., Umeda, F., and Nawata, H. (1998) Biochem. Biophys. Res. Commun. 24, 473–477
22. Madani, S., Hichami, A., LeGrand, A., Belleville, J., and Khan, N. A. (2001) FASEB J. 15, 2595–2601
23. Sharkey, N. A., and Blumberg, P. M. (1986) Cancer Res. 45, 19–24
24. Flenger, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M., and Felgner, P. L. (1994) J. Biol. Chem. 269, 2550–2561
25. Ebinu, J. O., Stang, S. L., Teixeira, C., Bottruff, D. A., Hooton, J., Blumberg, P. M., Barry, M., Bleakley, R. C., Ostergaard, H. L., and Stone, J. C. (2000) Blood 95, 3199–3203
26. Preiss, J., Louns, C. R., Bishop, W. R., Stein, R., Niedle, J. E., and Bell, R. M. (1986) J. Biol. Chem. 261, 8597–8600
27. Marignani, P. A., and Sebaldt, R. J. (1996) J. Nutr. 126, 2738–2745
28. Marignani, P. A., and Sebaldt, R. J. (1996) J. Biol. Chem. 261, 8597–8600
29. Marignani, P. A., and Sebaldt, R. J. (1996) J. Lipid Res. 37, 873–883
30. Amaral, M. C, Casillas, A. M., and Nel, A. E (1993) Immunology 79, 24–31
31. Denys, A., Hichami, A., and Khan, N. A. (2001) J. Lipid Res. 42, 2015–2020
32. Marignani, P. A., Epand, R. M., and Sebaldt, R. J. (1996) Biochem. Biophys. Res. Commun. 225, 469–473
33. Ha, K. S., and Exton, J. H. (1993) J. Biol. Chem. 268, 10534–10539
34. Bradshaw, C. D., Ellis, K. M., Heather, Qi, C., Salisbury, H. M., Wisehart-Johnson, A. E., and Meier, K. E. (1996) Immunol. Lett. 53, 69–76
35. Leach, K. L., Ruff, V. A., Wright, T. M., Pessin, M. S., and Raben, D. M. (1991) J. Biol. Chem. 266, 3215–3221
36. Pettitt, T. R., Martin, A., Horton, T., Liossis, C., Lord, J. M., and Wakelam, M. J. (1997) J. Biol. Chem. 272, 17354–17359
Diacylglycerols Containing Omega 3 and Omega 6 Fatty Acids Bind to RasGRP and Modulate MAP Kinase Activation
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Identification of cathepsin B as a mediator of neuronal death induced by Aβ-activated microglial cells using a functional genomics approach.

Li Gan, Shiming Ye, Alan Chu, Kristin Anton, Sailing Yi, Valerie A. Vincent, David von Schack, Daniel Chin, Joseph Murray, Scott Lohr, Laszlo Pathy, Mirella Gonzalez-Zulueta, Karoly Nikolich, and Roman Urfer

Page 5571, Fig. 4C: The first value shown at bottom of Fig. 4C should be 0.5 μM. A corrected figure is shown at right.

Also, in the Fig. 4 legend, second line from the last, within the parentheses “[em],” should be deleted. It should read “(p < 0.05) . . . .”
Diacylglycerol (DAG)-lactones, a new class of protein kinase C (PKC) agonists, induce apoptosis in LNCaP prostate cancer cells by selective activation of PKCα.

Maria Laura Garcia-Bermejo, Federico Coluccio Leskow, Teruhiko Fujii, Qiming Wang, Peter M. Blumberg, Motoi Ohba, Toshio Kuroki, Kee-Chung Han, Jeewoo Lee, Victor E. Marquez, and Marcelo G. Kazanietz

The structures of compounds HK654 and HK602 studied in this paper were incorrectly reported as N-hydroxylamides in Lee et al. (Lee, J., Han, K.-C., Kang, J.-H., Pearce, L. L., Lewin, N. E., Yan, S., Benzaria, S., Nicklaus, M. C., Blumberg, P. M., and Marquez, V. E. (2001) J. Med. Chem. 44, 4309–4312; Correction (2003) J. Med. Chem. 46, 2794). The compounds correspond instead to esters HK434 and HK204. The reader should be aware that the biological properties described for HK654 correspond instead to HK434. When taking into consideration the small difference in molecular weight (HK654, $M_r$ = 397.55, and HK434, $M_r$ = 382.54), the values reported remain virtually unchanged. The slightly greater potency for the alleged HK654 in Fig. 2 is due to this difference, which resulted in testing a slightly more concentrated solution of HK434. Since discovering the problem, authentic samples of HK654 and HK602 have been synthesized and tested. They showed a nearly 1000-fold reduction in binding affinity towards PKCα. The correct structures appeared in Choi et al. (Choi, Y., Kang, J. H., Lewin, N. E., Blumberg, P. M., Lee, J., and Marquez, V. E. (2003) J. Med. Chem. 46, 2790–2793). HK434 has the attributes of isozyme specificity and apoptotic-inducing activity originally associated with the N-hydroxylamide, and therefore the main conclusions of our paper remain unchanged.

Diacylglycerols containing omega 3 and omega 6 fatty acids bind to RasGRP and modulate MAP kinase activation.

Sihem Madani, Aziz Hichami, Mustapha Cherkaoui-Malki, and Naim A. Khan

Page 1176: Dr. Cherkaoui-Malki’s name was misspelled in this article. The correct spelling is shown above.