FEBS 28302 FEBS Letters 564 (2004) 177–182

Thapsigargin stimulated MAP kinase phosphorylation via CRAC channels and PLD activation: inhibitory action of docosahexaenoic acid

Anne Denys, Virginie Aires, Aziz Hichami, Naim Akhtar Khan*

Département de Physiologie, UPRES Lipides et Nutrition, Université de Bourgogne, Faculté des Sciences de la Vie, 6 Boulevard Gabriel, 21000 Dijon, France

Received 13 January 2004; revised 9 March 2004; accepted 11 March 2004

First published online 1 April 2004

Edited by Lukas Huber

Abstract **This study was conducted on human Jurkat T-cells to investigate the role of depletion of intracellular Ca^{2+} stores in the phosphorylation of two mitogen-activated protein kinases (MAPKs), i.e. extracellular signal-regulated kinase (ERK) 1 and ERK2, and their modulation by a polyunsaturated fatty acid, docosahexaenoic acid (DHA). We observed that thapsigargin (TG) stimulated MAPK activation by store-operated calcium (SOC) influx via opening of calcium release-activated calcium (CRAC) channels as tyrphostin-A9, a CRAC channel blocker, and two SOC influx inhibitors, econazole and SKF-96365, diminished the action of the former. TG-stimulated ERK1/ERK2 phosphorylation was also diminished in buffer containing EGTA, a calcium chelator, further suggesting the implication of calcium influx in MAPK activation in these cells. Moreover, TG stimulated the production of diacylglycerol (DAG) by activating phospholipase D (PLD) as propranolol (PROP) (a PLD inhibitor), but not U73122 (a phospholipase C inhibitor), inhibited TG-evoked DAG production in these cells. DAG production and protein kinase C (PKC) activation were involved upstream of MAPK activation as PROP and GF109203X, a PKC inhibitor, abolished the action of TG on ERK1/ERK2 phosphorylation. Furthermore, DHA seems to act by inhibiting PKC activation as this fatty acid diminished TG- and phorbol 12-myristate 13-acetate-induced ERK1/ERK2 phosphorylation in these cells. Together these results suggest that Ca^{2+} influx via CRAC channels is implicated in PLD/PKC/MAPK activation which may be a target of physiological agents such as DHA.**

Key words: Jurkat T-cell; Thapsigargin; Calcium; Docosahexaenoic acid

1. Introduction

It has been established that T-cell activation via T-cell receptors is associated with activation of phospholipase C (PLC) γ1 which cleaves phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate (IP_3) and diacylglycerol (DAG) [1]. Hence, binding of IP_3 to its receptor on the endoplasmic reticulum (ER) releases Ca^{2+} whereas DAG activates protein kinase C (PKC) [1]. According to the capacitative model of calcium homeostasis [2], there is a biphasic rise in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$), hence a calcium release from ER stores is followed by Ca^{2+} influx across the plasma membrane [3]. The rise in $[Ca^{2+}]_i$ constitutes an essential triggering signal for T-cell differentiation and proliferation [4]. Raising $[Ca^{2+}]_i$ has been found to increase the activity of a transcription factor, the nuclear factor of activated T-cells, which, in turn, results in the expression of the *lacZ* gene in transfected murine T-cells [5]. Calcium oscillations with repeated spikes for a period of 100 s are sufficient to activate the transcriptional factors in human T-cells [6]. Following the depletion of intracellular Ca^{2+} stores, the refilling of these stores is brought about by opening of store-operated Ca^{2+} (SOC) channels [2]. SOC channels are modulated by PKC or DAG [7–9]. SOC influx is also brought about by opening of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in human T-cells [10]. It has been reported that activation of these channels is accelerated by a soluble calcium influx factor which is released into the extracellular medium during SOC depletion in Jurkat T-cells [11].

Phospholipase D (PLD) hydrolyzes phospholipids to produce phosphatidic acid (PA) that gives rise to DAG [12]. PLD activity is regulated by many factors [13] and so we investigated whether an increase in free intracellular calcium via SOC activated PLD and mitogen-activated protein kinases (MAPKs) in Jurkat T-cells.

It has been shown that diets enriched with n-6 polyunsaturated fatty acids (PUFAs) stimulate the growth of different cell types and promote metastasis whereas diets containing n-3 PUFAs inhibit cell growth and exert immunosuppressive effects [14,15]. We have recently shown that docosahexaenoic acid (DHA) inhibits MAPK activation in Jurkat T- cells [16,17] and 3T3 fibroblasts [18,19]. We were tempted to ascertain whether SOC influx-stimulated MAPK activation can be further inhibited by this fatty acid.

2. Materials and methods

2.1. Chemicals

Anti-phosphorylated MAPK (extracellular signal-regulated kinase (ERK) 1/ERK2) antibodies and U0126 were obtained from Ozyme (Saint Quentin, France). GF109203X, tyrphostin A9 (TA9) and SKF-96365 were obtained from Calbiochem, France. [γ-^{32}P]ATP (4000 Ci/mmol) and [^{3}H]palmitic acid (57 Ci/mmol) were purchased from Amersham, France. Fura-2/AM was obtained from Molecular Probes

*Corresponding author. Fax: (33)-3-80 39 63 30.
E-mail address: naim.khan@u-bourgogne.fr (N.A. Khan).

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; CRAC, Ca^{2+} release-activated Ca^{2+}; DAG, diacylglycerol; DHA, docosahexaenoic acid; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PROP, propranolol; PUFA, polyunsaturated fatty acid; SOC, store-operated calcium; TA9, tyrphostin A9

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00361-8

(Eugene, OR, USA). All other chemicals including phorbol 12-myristate 13-acetate (PMA), DHA (22:6 n-3), nifedipine, ω-conotoxin and econazole were procured from Sigma (St. Louis, MO, USA).

2.2. Cell culture and preparation of extracts

The Jurkat T-cells were routinely cultured in RPMI 1640 medium supplemented with L-glutamine and 10% fetal calf serum at 37°C in a humidified chamber containing 95% air and 5% CO_2. Cell viability was assessed by trypan blue exclusion test. Cell numbers were determined by hemocytometer.

DHA was dissolved in ethanol at a final concentration of 0.01% (v/v). Prior to determining the MAPK activation, cells were incubated for 6 h in the RPMI 1640 medium without serum. Cells were then suspended in buffer containing NaCl, 110 mM; KCl, 5.4 mM; $NaHCO_3$, 25 mM; $MgCl_2$, 0.8 mM; KH_2PO_4, 0.4 mM; HEPES, 20 mM; $NaHPO_4$, 0.33 mM; $CaCl_2$, 1.2 mM. To make a calcium-free buffer, $CaCl_2$ was replaced in this buffer with EGTA, 2.4 mM. Jurkat T-cells (5×10^6/ml) were incubated or not for 5 min in the presence of n-3 PUFA, DHA (20 μM), and then stimulated with thapsigargin (TG) (500 nM) for 20 min. After incubation at 37°C, cells were lysed with 50 μl of buffer containing the following: HEPES, 20 mM pH 7.3; EDTA, 1 mM; EGTA, 1 mM; NaCl, 0.15 mM; Triton X-100 1%; glycerol 10%; phenylmethylsulfonyl fluoride, 1 mM; sodium orthovanadate, 2 mM; anti-protease cocktail (2 μl in 1 ml of buffer). After centrifugation ($13\,000\times g$; 1 min), cell lysates were used immediately, or stored at −80°C. The protein contents were determined with Folin reagent.

2.3. Western blot detection of phosphorylated ERK1/ERK2

Denatured proteins (20 μg) were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%), transferred to polyvinylidene difluoride membranes and immunodetection was performed by using anti-phosphorylated ERK1/ERK2 antibodies. After incubation of membranes with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies at 1:2000, peroxidase activity was detected using ECL reagents (Amersham, France).

2.4. Measurement of DAG production

Jurkat T-cells were serum-starved during 6 h then preincubated for 15 min in the presence of inhibitors, followed by 20 min incubation with TG (500 nM). Incubation was stopped by the addition of methanol and DAGs were extracted five times with hexane and quantitatively converted to [^{32}P]PA as described elsewhere [20]. Briefly, the dried lipid extract was solubilized in 20 μl of *n*-octyl-β-D-glucopyranoside and cardiolipin solution (7.5% *n*-octyl-β-D-glucopyranoside, 5 mM cardiolipin in 1 mM DETAPAC) and incubated for 5–15 min at room temperature with DAG kinase in the presence of [^{32}P]ATP (specific activity 100 000–500 000 cpm/nmol). The reaction was allowed to proceed at 25°C for 30 min. The resulting PA was then extracted and separated by thin-layer chromatography (TLC) (chloroform/methanol/acetic acid, 65:15:5, v/v). The area corresponding to PA was scraped off and the radioactivity was determined by liquid scintillation counting. In these experiments, tritiated *sn*-1,2-dioleoylglycerol was used as an internal standard to monitor conversion and recovery which was routinely >90% over the entire concentration range (data not shown). TLC analysis of DAG kinase reaction products demonstrated that 99% of the radioactivity was PA.

2.5. Determination of PLD activity

PLD activity was determined by measuring the production of phosphatidylbutanol (Pbut) as described elsewhere [21]. In brief, Jurkat T-cells (10×10^6 cells/ml) were incubated during 16 h in the presence of 5 μCi/ml of [^{3}H]palmitic acid (57 Ci/mmol) diluted with RPMI medium without serum but containing 0.2% bovine serum albumin (w/v) (RPMI-BSA). The cells were washed two times with RPMI-BSA and incubated for 30 min in fresh medium containing butan-1-ol (0.5%, v/v) with or without propranolol (PROP), EGTA or the SOC influx inhibitors, econazole and SKF-96365, before further activation by TG for 20 min. The reaction was stopped by adding 2 ml of methanol and chloroform, and 2 M NaCl plus 0.01 N HCl were added to yield a final methanol/chloroform/aqueous phase, 1:1:0.9 (v/v). The organic phase was separated by TLC in the upper phase of isooctane/ethyl acetate/acetic acid/water (10:65:15:50; v/v), then the radiolabeled areas corresponding to Pbut were scraped off and the radioactivity was measured by liquid scintillation counting.

2.6. Measurement of Ca^{2+} signalling

The cells (2×10^6/ml) were washed with phosphate-buffered saline (PBS), pH 7.4. The composition of PBS was as follows (in mM): KH_2PO_4, 3.5; Na_2HPO_4, 17.02; NaCl, 136. The cells were then incubated with Fura-2/AM (1 μM) for 60 min at 37°C in loading buffer which contained the following (in mM): NaCl, 110; KCl, 5.4; $NaHCO_3$, 25; $MgCl_2$, 0.8; KH_2PO_4, 0.4; HEPES, 20; Na_2HPO_4, 0.33; $CaCl_2$, 1.2 and the pH was adjusted to 7.4.

After loading, the cells were washed three times ($2000\times g$, 10 min) and remained suspended in the identical buffer. $[Ca^{2+}]_i$ was measured according to Grynkiewicz et al. [22]. The fluorescence intensities were measured in the ratio mode in a PTI spectrofluorometer at 340 nm and 380 nm (excitation filters) and 510 nm (emission filter). The cells were continuously stirred throughout the experiment. The test molecules were added into the cuvettes in small volumes with no interruptions in recordings. The $[Ca^{2+}]_i$ was calculated using the following equation: $[Ca^{2+}]_i = K_d\times(R-R_{min})/(F_{max}-F)\times(Sf2/Sb2)$. A value of 224 nM for K_d was added into the calculations. R_{max} and R_{min} values were obtained by addition of ionomycin (5 μM) and $MnCl_2$ (2 mM), respectively. All the experiments were performed at 37°C.

2.7. Statistical analysis

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 one way analysis of variance, followed by a least-significant-difference test.

3. Results and discussion

The present study was conducted to assess the role of SOC influx in the phosphorylation of ERK1/ERK2 in Jurkat T-cells. To induce SOC influx, we employed TG, an inhibitor of Ca^{2+}-ATPase of ER [23].

In the present study, we employed BAPTA, an agent that

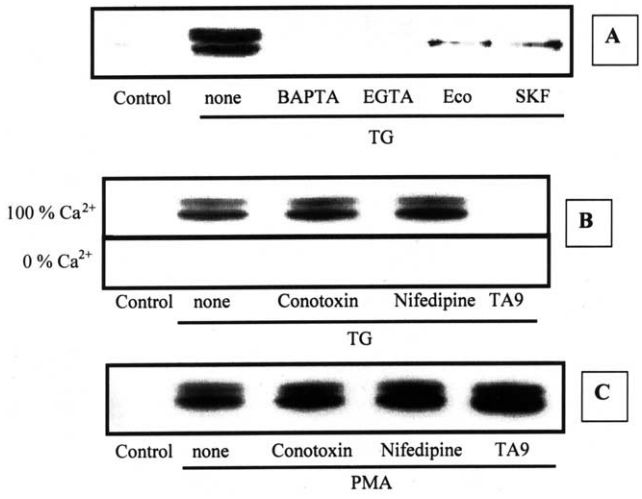

Fig. 1. Effect of calcium fluxes on TG-stimulated ERK1/ERK2 activation. Jurkat T-cells (5×10^6 cells/ml), prior to determining ERK1/ERK2 phosphorylation, were incubated during 6 h in RPMI 1640 medium without serum. After incubation at 37°C, MAPK assays were performed as described in Section 2. A: Intracellular and extracellular Ca^{2+} concentrations were depleted, respectively, by BAPTA, 1 μM and EGTA, 2.4 mM, by incubating cells for 10 min. Cells were also treated with econazole (Eco), 15 μM and SKF-96365 (SKF), 30 μM for 20 min. Later on, the cells were treated or not (control) with TG, 500 nM, for 20 min. B: Cells were pretreated or not for 15 min with calcium channel blockers (nifedipine, 1 μM; ω-conotoxin, 1 μM) and then were further treated or not (control) with TG, 500 nM, for 20 min. In 0% Ca^{2+} medium, $CaCl_2$ was replaced by EGTA, 2.4 mM. Later on, these cells were stimulated or not (control) with TG, 500 nM, for 20 min. C: The same treatments as in B except the cells were treated with PMA.

completely chelates $[Ca^{2+}]_i$. In the presence of BAPTA, TG-recruited intracellular calcium will rapidly bind to this agent and, therefore, SOC influx will not take place. Fig. 1A shows that TG-stimulated ERK1/ERK2 phosphorylation was significantly diminished in the presence of BAPTA. TG again failed to stimulate MAPK activation in the presence of EGTA, an agent that chelates extracellular calcium (Fig. 1A). These observations suggest that this is not a release of calcium from the intracellular pool but rather the calcium influx which is implicated in the phosphorylation of MAPKs in Jurkat T-cells. In order to probe the role of calcium channels, implicated in TG-stimulated MAPK activation, we employed ω-conotoxin and nifedipine, the blockers of L-type and N-type calcium channels respectively. We observed that the incubation of Jurkat T-cells in the presence of ω-conotoxin and nifedipine failed to curtail the TG-stimulated MAPK activation (Fig. 1B). The failure of the inhibition of MAPK activation by ω-conotoxin and nifedipine corroborates our previous observations in which we have demonstrated that these channels are not implicated in calcium influx in Jurkat T-cells [24]. Similarly, these calcium channel blockers could not affect the PMA-induced MAPK phosphorylation (Fig. 1C). Since we employed TA9, and econazole and SKF-96365, SOC inhibitors (see below), the use of ω-conotoxin and nifedipine seemed mandatory as controls.

It has been shown that SOC influx in human T-cells may take place by opening of CRAC channels. The CRAC channels were originally described in mast cells [25]. Human Jurkat T-cells express nearly 10 000 CRAC channels per cell [26,27]. The CRAC channels are not opened directly by depolarization, IP_3 or IP_4 [28]. The depletion-activated CRAC channels underline mitogenic Ca^{2+} influx in T-cells. In order to ascertain the role of these channels, we used TA9, an inhibitor of CRAC channels [10], and econazole and SKF-96365, inhibitors of SOC influx [29,30]. Furthermore, TA9, and econazole and SKF-96365 diminished the action of TG on the activation of MAPK (Fig. 1A,B). In 0% Ca^{2+} medium, the calcium channel blockers could not affect the TG-stimulated ERK1/ERK2 phosphorylation in these cells. These observations suggest that SOC influx via opening of CRAC channels is implicated in the phosphorylation of ERK1/ERK2 in Jurkat T-cells. Our report is in close agreement with the observations of Atherfold et al. [31] who have shown that calcium influx, evoked by calcium ionophores such as A23187, induces ERK1/ERK2 activation in human primary and Jurkat T-cells. Though PMA also induced MAPK activation, its action was not inhibited by TA9 in Jurkat T-cells (Fig. 1C) and it is due to the fact that PMA does not activate MAPKs by inducing calcium influx but rather it acts directly on the Zn-butterfly domain of PKC [32].

It has been established that MAPK activation in Jurkat T-cells is coupled to PKC activation via Raf-1 upstream of MAPK/ERK kinase (MEK) [33]. Hence, we were interested to ascertain whether TG-stimulated MAPK activation is coupled to the PKC/MEK pathway. We therefore employed U0126, an inhibitor of MEK, and GF109203X, a PKC inhibitor. Fig. 2 shows that U0126 and GF109203X completely abolished the TG-stimulated MAPK activation in Jurkat T-cells. On the other hand, TG did not exert additive effects on PMA-induced ERK1/ERK2 activation in these cells (Fig. 2), suggesting that TG and PMA exert their actions via the same pathway. The failure to exert an additive response by TG and

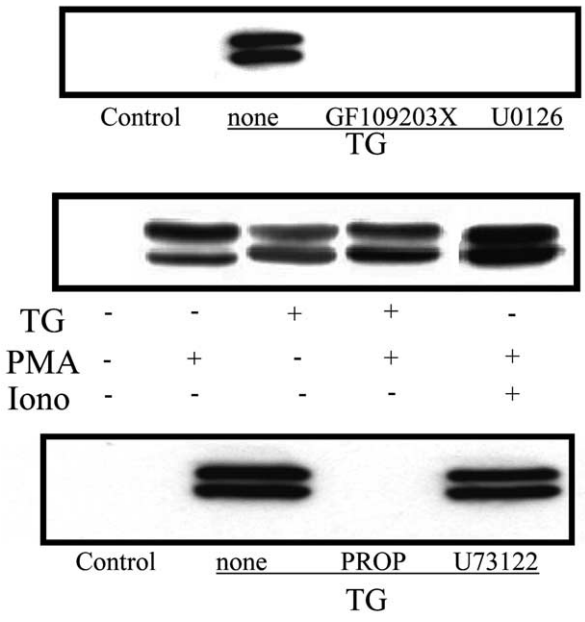

Fig. 2. Effect of inhibitors of PKC, PLC, MEK and PLD on TG-stimulated ERK1/ERK2 phosphorylation. Jurkat T-cells (5×10^6 cells/ml), prior to determining the ERK1/ERK2 phosphorylation, were incubated during 6 h in RPMI 1640 medium without serum. To evaluate the mechanism of action of TG, cells were pretreated or not for 20 min with U73122, 3 μM, GF109203X, 500 nM, PROP, 50 μM or U0126, 10 μM and then stimulated for 20 min with TG, 500 nM, PMA, 200 nM, PMA, 200 nM plus ionomycin (Iono), 500 nM. After incubation at 37°C, cells were lysed and the detection of phosphorylated MAPKs was performed as described in Section 2.

PMA is not due to saturation of MAPK activation by either of the agents as ionomycin in the presence of PMA does exert an additive response in the same experimental conditions (Fig. 2). Hence, the scanner provided the following blot intensities: 200 ± 10% fold increases in PMA plus ionomycin, 100 ± 5% fold increases in PMA, 100 ± 6% fold increases in PMA plus TG as compared to TG alone (100%).

DAG is mainly generated through the actions of PLC and PLD [32]. In order to probe the role of PLC and PLD, we used U73122, a PLC inhibitor, and PROP, a PLD inhibitor. Fig. 2 shows that only PROP, but not U73122, inhibited the activation of ERK1/ERK2, stimulated by TG in Jurkat T-cells. We also measured the production of DAG, evoked by TG. We observed that TG stimulated DAG production after 1 min of stimulation which was sustained for 20 min (Fig. 3, inset). Furthermore, as expected, PROP, but not U73122, inhibited TG-induced DAG production (Fig. 3a). Since PROP is an inhibitor of PA hydrolase, these observations suggest that DAG production by TG via PA is catalyzed by the PLD/PA pathway in these cells. We further detected the PLD activity by measuring the production of Pbut catalyzed by transphosphatidylation in the presence of butan-1-ol. Fig. 3b shows that the production of Pbut was increased by TG. Altogether, these observations suggest that TG-stimulated MAPK activation is dependent on PLD signalling involving DAG production in Jurkat T-cells.

How DAG is produced by TG in Jurkat T-cells is not well understood. However, it is possible that TG-stimulated SOC influx via CRAC channels may contribute to PLD activation. It is noteworthy that EGTA, TA9, econazole and SKF-96365

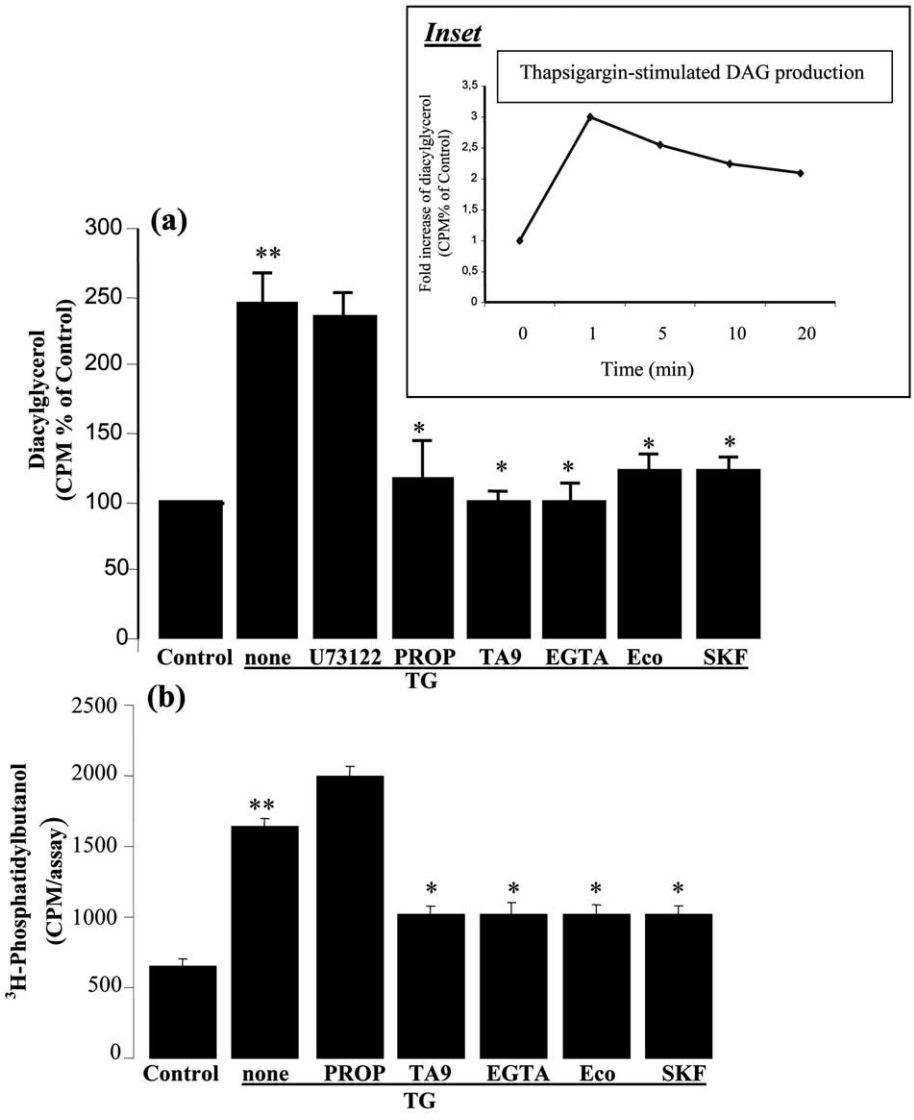

Fig. 3. Effect of inhibitors of PLC and PLD on TG-stimulated [^{14}C]DAG and [^{3}H]Pbut production. Jurkat T-cells (5×10^6 cells/ml) were incubated during 6 h in RPMI 1640 medium without serum. a: The cells were incubated for 20 min with U73122, 3 μM, PROP, 50 μM, TA9, 10 μM, EGTA, 2.4 mM, econazole (Eco), 15 μM, and SKF-96365 (SKF), 30 μM. Cells were treated with TG, 500 nM, in the presence of [^{32}P]ATP. Treatment was arrested with cold methanol. Then, DAG was extracted and quantified as described in Section 2. Results are given as CPM percentage of control in the lipid extract and represent mean ± S.E.M. of three separate experiments, each in duplicate. Data are significantly different as compared to TG-treated (none) ($^*P<0.001$) and control cells ($^{**}P<0.001$). Inset: Cells were treated with TG, 500 nM, for indicated times. b: Jurkat T-cells (10×10^6 cells/ml) were incubated during 16 h in the presence of 5 μCi/ml of [^{3}H]palmitic acid diluted with RPMI 1640 medium without serum containing 0.2% BSA. The cells, containing butan-1-ol (0.5%), were then preincubated or not during 20 min with PROP, 50 μM, TA9, 10 μM, EGTA, 2.4 mM, SKF-96365 (SKF), 30 μM, and econazole (Eco), 15 μM. Cells were then treated for 20 min with TG, 500 nM. The reaction was stopped as described in Section 2. Results are given as mean CPM ± S.E.M. of three separate experiments, each in duplicate. Data are significantly different as compared to TG-treated (none) ($^*P<0.001$) and control cells ($^{**}P<0.001$).

significantly inhibited TG-stimulated production of DAG and Pbut in Jurkat T-cells (Fig. 3a,b). Our observations are supported by the findings of Walter et al. [34] who have reported the role of PLD during TG-induced calcium store depletion in vascular smooth muscle cells. Singh et al. [35] have also demonstrated that agents like ionomycin that increase intracellular calcium do induce PLD basal activity in UMR-106 cells. In this study, EGTA and BAPTA significantly attenuated the PLD activation. The effects of calcium on PLD activation seem specific since A23187 triggered an increase both in calcium concentrations and in PLD activation in PLD1- and PLD2-overexpressing, but not in control, Sf9 cells [36]. Though PLC activation and, consequently, DAG production by TG have also been reported in human peripheral blood lymphocytes [37], TG-stimulated DAG production via PLC and PLD seems to be a cell-specific phenomenon and Jurkat T-cells may differ from freshly isolated human T-cells from the point of view of regulation of several cell functions.

DHA belongs to the n-3 PUFAs which have been shown to exert immunosuppressive actions in vitro and in vivo [14]. DHA has been shown to induce increases in $[Ca^{2+}]_i$ [24] and DAG containing this fatty acid is implicated in the modulation of PKC [38] and MAPK activation [39]. In the present study, we observed that DHA diminished the TG-induced MAPK activation in Jurkat T-cells (Fig. 4A). The action of DHA seems specific as DHA-methyl ester failed to curtail the

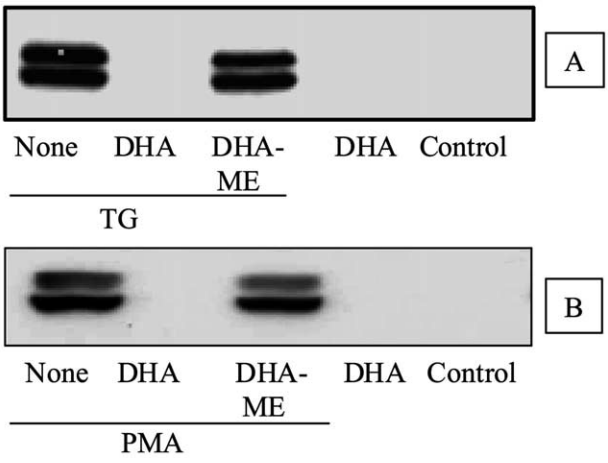

Fig. 4. TG-stimulated ERK1/ERK2 activation is inhibited by DHA. Jurkat T-cells (5×10^6 cells/ml), prior to determining the ERK1/ERK2 phosphorylation, were incubated during 6 h in RPMI 1640 medium without serum. Later on, these cells were pretreated or not with DHA, 10 μM, or DHA-methyl ester (DHA-ME), 10 μM, and then further treated with TG, 500 nM, or PMA, 200 nM, for 20 min. After incubation at 37°C, cells were lysed and the detection of phosphorylated MAPKs was performed as described in Section 2.

TG- and PMA-induced MAPK phosphorylation in these cells (Fig. 4A,B). The inhibitory effects of DHA on TG-stimulated MAPK activation may not be due to the inhibitory action of this fatty acid on TG-induced SOC influx as prior addition of DHA failed to curtail the TG-evoked increases in calcium in these cells (Fig. 5a). Furthermore, addition of DHA after TG induced an additive response on the increases in $[Ca^{2+}]_i$ in these cells (Fig. 5b). The inhibitory effect of DHA on ERK1/ERK2 phosphorylation may be due to its action on TG-induced PKC activation as this fatty acid also inhibited PMA-stimulated MAPK activation in these cells. These observations agree well with our previous findings in which we have shown that DHA inhibits MAPK activation by inhibiting PKC in 3T3 fibroblasts [16] and Jurkat T-cells [17,18].

TA9, econazole and SKF-96365 inhibited TG-evoked SOC influx in these cells (Fig. 5c,d). Another important observation of our study is that TG-evoked increases in $[Ca^{2+}]_i$ are not curtailed by PROP, U0126 or GF109203X (Fig. 5d), indicating that activation of PLD, PKC and MAPK occurs essentially downstream of calcium influx in Jurkat T-cells. In other words, our observations suggest that an increase in $[Ca^{2+}]_i$ stimulates the activation of these enzymes, though some investigators have suggested that TG-stimulated activation of MAPK regulates SOC influx in human platelets [40].

Last, but not least, we would like to underline that Jurkat T-cells represent a good model to study the direct action of fatty acids in T-cell signalling as they do not possess the enzymes of lipoxygenase and cyclooxygenase pathways that account for lipid mediators [41,42]. The novelty of our study lies in the fact that SOC influx, triggered by the depletion of intracellular calcium stores in the presence of TG, can activate PLD, followed by MAPK activation which is a target of

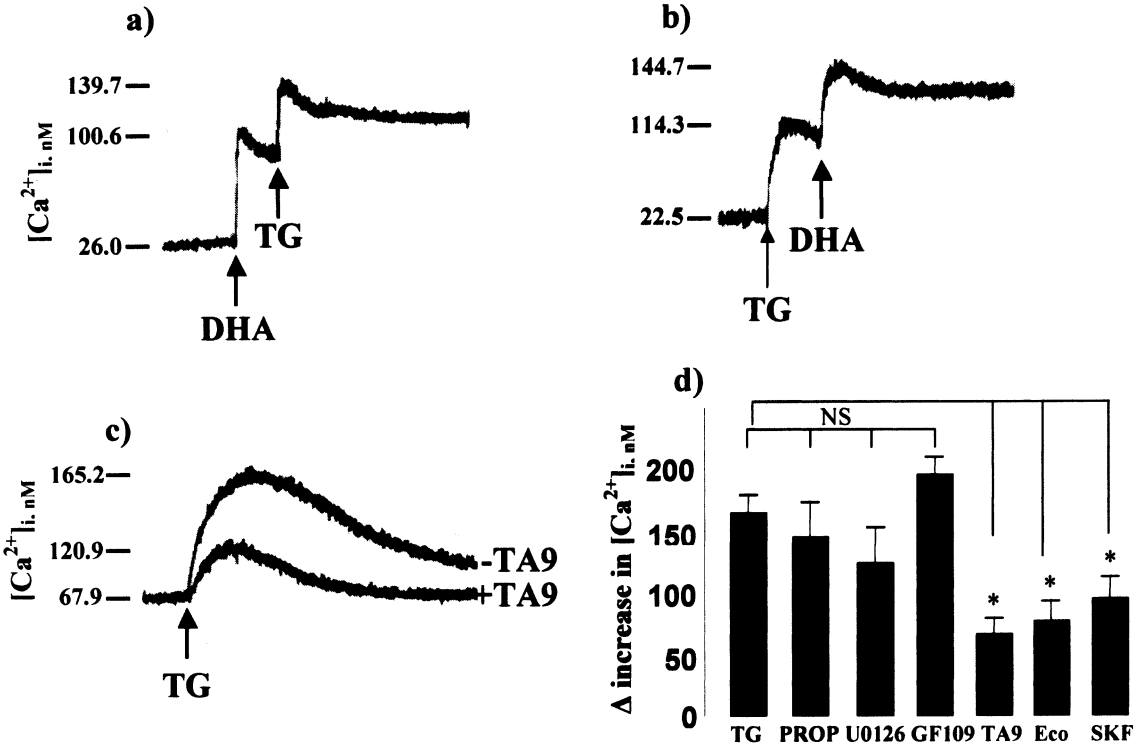

Fig. 5. Effects of TG and DHA on the increases in $[Ca^{2+}]_i$. Cells (4×10^6/assay) were loaded with the fluorescent probe, Fura-2/AM, as described in Section 2. The arrowheads indicate the time when the test molecules were added into the cuvette. The figure shows the single traces of observations which were reproduced independently ($n=4$). a and b represent successive additions of DHA, 10 μM and TG, 500 nM. c: The cells were preincubated in the presence or not of TA9, 10 μM. d: the Δ increase in $[Ca^{2+}]_i$ induced by TG, 500 nM, in the presence or not of various inhibitors at the following concentrations: PROP, 50 μM; U0126, 10 μM; GF109203X (GF109), 500 nM; TA9, 10 μM, econazole, 15 μM; and SKF-96365, 30 μM. Cells were preincubated for 20 min with the various inhibitors before the addition of TG. Values are means ± S.E.M. of three independent experiments, each in triplicate. Data are significantly different as compared to TG-treated cells (*$P<0.001$). NS: insignificant differences as compared with TG-treated cells.

DHA. Hence, inhibition of MAPK activation by DHA may be one of the possible mechanisms of action of immunosuppression, induced by this fatty acid.

Acknowledgements: The authors are grateful to the Région Bourgogne (Dijon) for the sanction of a contingent grant.

References

[1] Cantrell, D. (1996) Annu. Rev. Immunol. 14, 259–274.
[2] Putney Jr., J.W., Broad, L.M., Braun, F.J., Lievremont, J.P. and Bird, G.S. (2001) J. Cell Sci. 114, 2223–2229.
[3] Hichami, A., Joshi, B., Simonin, A.M. and Khan, N.A. (2002) Eur. J. Biochem. 269, 5557–5563.
[4] Dolmetsch, R.E., Xu, K. and Lewis, R.S. (1998) Nature 392, 933–936.
[5] Negulescu, P.A., Shastri, N. and Cahalan, M.D. (1994) Proc. Natl. Acad. Sci. USA 91, 2873–2877.
[6] Li, W., Llopis, J., Whitney, M., Zlokarnik, G. and Tsein, R.Y. (1998) Nature 392, 936–941.
[7] Ma, H.T., Kudlacek, P.E. and Sansom, S.C. (2002) Am. J. Physiol. Cell Physiol. 283, C1390–C1398.
[8] Venkatachalam, K., Ma, H.T., Ford, D.L. and Gill, D.L. (2001) J. Biol. Chem. 276, 33980–33985.
[9] Hofmann, T., Obukhov, A.G., Schaefer, M., Harteneck, C., Gudermann, T. and Schultz, G. (1999) Nature 397, 259–263.
[10] Marhaba, R., Mary, F., Pelassy, C., Stanescu, A.T., Aussel, C. and Breittmayer, J.P. (1996) J. Immunol. 157, 1468–1473.
[11] Randriamampita, C. and Tsein, R.Y. (1993) Nature 364, 809–814.
[12] Exton, J.H. (1994) Biochim. Biophys. Acta 1212, 26–42.
[13] Exton, J.H. (2002) Rev. Physiol. Biochem. Pharmacol. 144, 1–94.
[14] Calder, P.C. (1996) Proc. Nutr. Soc. 55, 127–150.
[15] Collett, E.D., Davidson, L.A., Fan, Y.Y., Lupton, J.R. and Chapkin, R.S. (2001) Am. J. Physiol. Cell Physiol. 280, C1066–C1075.
[16] Denys, A., Hichami, A. and Khan, N.A. (2001) J. Lipid Res. 42, 2015–2020.
[17] Denys, A., Hichami, A. and Khan, N.A. (2002) Mol. Cell. Biochem. 232, 143–148.
[18] Denys, A., Hichami, A., Maume, B. and Khan, N.A. (2001) Lipids 36, 813–818.
[19] Khan, N.A. and Hichami, A. (2002) Recent Adv. Res. Lipids 6, 65–78.
[20] Preiss, J., Loomis, C.R., Bishop, W.R., Stein, R., Niedel, J.E. and Bell, R.M. (1986) J. Biol. Chem. 261, 8597–8600.
[21] Khan, N.A. and Hichami, A. (1999) Biochem. J. 344, 199–204.
[22] Grynkiewicz, G.M., Ponie, M. and Tsein, R. (1985) J. Biol. Chem. 260, 3440–3450.
[23] Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R. and Dawson, A.P. (1990) Proc. Natl. Acad. Sci. USA 87, 2466–2470.
[24] Bonin, A. and Khan, N.A. (2000) J. Lipid Res. 41, 277–284.
[25] Hoth, M. and Penner, R. (1992) Nature 355, 353–356.
[26] Zweifach, A. and Lewis, R.S. (1993) Proc. Natl. Acad. Sci. USA 90, 6295–6299.
[27] Lewis, R.S. and Cahalan, M.D. (1995) Annu. Rev. Immunol. 13, 623–653.
[28] Premack, B.A., McDonald, T.V. and Gardner, P. (1994) J. Immunol. 152, 5226–5240.
[29] Gamberucci, A., Fulceri, R., Benedetti, A. and Bygrave, F.L. (1998) Biochem. Biophys. Res. Commun. 248, 75–77.
[30] Gamberucci, A., Giurisato, E., Pizzo, P., Tassi, M., Giunti, R., MacIntosh, D.P. and Benedetti, A. (2002) Biochem. J. 364, 245–254.
[31] Atherfold, P.A., Norris, M.S., Robinson, P.J., Gelfand, E.W. and Franklin, R.A. (1999) Mol. Immunol. 36, 543–549.
[32] Nishizuka, Y. (1995) FASEB J. 9, 484–496.
[33] Nel, A.E., Hanekom, A., Rheeder, K., William, S., Pollack, S., Katz, R. and Landreth, G.E. (1990) J. Immunol. 144, 2683–2689.
[34] Walter, M., Tepel, M., Nofer, J.R., Neusser, M., Assmann, G. and Zidek, W. (2000) FEBS Lett. 479, 51–56.
[35] Singh, A.T., Bhattacharya, R.S., Radeff, J.M. and Stern, P.H. (2003) J. Bone Miner. Res. 18, 1453–1460.
[36] Siddiqi, A.R., Srajer, G.E. and Leslie, C.C. (2000) Biochim. Biophys. Acta 1497, 103–114.
[37] Nofer, J.R., Tepel, M., Walter, M., Seedorf, U., Assmann, G. and Zidek, W. (1997) J. Biol. Chem. 272, 32861–32868.
[38] Madani, S., Hichami, A., Legrand, A., Belleville, J. and Khan, N.A. (2001) FASEB J. 15, 2595–2601.
[39] Madani, S., Hichami, A., Charkaoui-Malki, M. and Khan, N.A. (2004) J. Biol. Chem. 279, 1176–1183.
[40] Rosado, J.A., Graves, D. and Sage, S.O. (2000) Biochem. J. 351, 429–437.
[41] Goldyne, M.E., Burrish, G.E., Paubelle, P. and Borgeat, P. (1984) J. Biol. Chem. 259, 8815–8819.
[42] Kurland, J.L. and Bockmann, R. (1978) J. Exp. Med. 147, 952–957.