Regulation of calcium signalling by docosahexaenoic acid in human T-cells: implication of CRAC channels

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Abstract **We elucidated the role of docosahexaenoic acid (DHA) on the increases in free intracellular calcium concentrations, $[Ca^{2+}]i$, in human (Jurkat) T-cell lines. DHA evoked an increase in $[Ca^{2+}]i$ in a dose-dependent manner in these cells. Anti-CD3 antibody, known to stimulate increases in Ca^{2+} from endoplasmic reticulum (ER) via the production of inositol trisphosphate, also evoked increases in $[Ca^{2+}]i$ in Jurkat T-cells. We also used thapsigargin which inhibits Ca^{2+}-ATPase of the ER and, therefore, increases Ca^{2+} in the cytosol. Interestingly, addition of DHA during the thapsigargin-induced peak response exerted an additive effect on the increases in $[Ca^{2+}]i$ in human T-cells, indicating that the mechanisms of action of these two agents are different. However, the DHA-induced calcium response was not observed when this agent was added during the anti-CD3-induced calcium peak, though its addition resulted in a prolonged and sustained calcium response as a function of time, suggesting that DHA recruits calcium, in part, from the ER pool and the prolonged response may be due to Ca^{2+} influx. In the medium containing 0% Ca^{2+}, the DHA-evoked response on the increases in $[Ca^{2+}]i$ was significantly curtailed as compared to that in 100% Ca^{2+} medium, supporting the notion that the response of the DHA is also due, in part, to the opening of calcium channels. Furthermore, preincubation of cells with tyrphostin A9, an inhibitor of Ca^{2+} release–activated Ca^{2+} (CRAC) channels also significantly curtailed the DHA-induced sustained response on the increases in $[Ca^{2+}]i$ in these cells. These results suggest that DHA induces an increase in $[Ca^{2+}]i$ via the ER pool and the opening of CRAC channels in human T-cells.**—Bonin, A., and N. A. Kahn. **Regulation of calcium signalling by docosahexaenoic acid in human T-cells: implication of CRAC channels.** ***J. Lipid Res.*** **2000.** 41: **277–284.**

Supplementary key words polyunsaturated fatty acid • CD3 • thapsigargin • Jurkat T-cells

The T-cell activation mechanisms can be divided into two events, early events and late events (1). The early events are rapid and involve mobilization of intracellular Ca^{2+}, protein kinase C (PKC) activation, expression of immediate early genes (c-fos and c-jun), and activation of mitogen-activated protein (MAP) kinases (2), whereas the late events, which assure the transition of cells from S phase to G2/M phase of the cell cycle, are sustained responses implicating the prolonged PKC activation, expression of receptors for interleukin-2 (IL-2 R), and signalling via CD28 (3). In T-lymphocytes, a biphasic rise in concentrations of free calcium, $[Ca^{2+}]i$, is elicited by the binding of antigen or polyclonal mitogens to the T-cell receptor, TCR (4, 5). According to the capacitative model of calcium entry, first calcium is released via receptor activation from the intracellular pool, mainly endoplasmic reticulum and then it is extruded into the extracellular medium. In turn, the cells refill their intracellular emptied pool by opening calcium channels (6, 7). These calcium channels have been termed as Ca^{2+} release–activated Ca^{2+} (CRAC) channels in Jurkat T-cells (8). Randriamampita and Tsein (9) have demonstrated that the refilling mechanism via the opening of CRAC channels is regulated by the calcium influx factor (CIF) which is released into the extracellular medium during calcium release in Jurkat T-cells.

Hence, the rise in $[Ca^{2+}]i$ constitutes an essential triggering signal for T-cell differentiation and proliferation (5). Raising $[Ca^{2+}]i$ from resting 70 nm to 200 nm has been found to increase the activity of a transcription factor, the nuclear factor of activated T-cells (NF-AT), which in turn results in the expression of lacZ gene in transfected murine T-cells (10). Calcium oscillations with spikes of nearly 300 nm for a period of 100 sec are sufficient to activate the transcriptional factors in human T-cells (11). In another study conducted on caged IP_3 molecules, the trains of short ultraviolet pulses (from 0.3 to 1.5 sec) which induced calcium oscillations promoted the activity of NF-AT in RBL-2H3 lymphocytes (12).

During the recent past, there has been an upsurge of information on the role of polyunsaturated fatty acids

Abbreviations: AA, arachidonic acid (20:4 n–6); DHA, docosahexaenoic acid (22:6 n–3); bis-oxonol, bis-5(1,3-diethylthiobarbiturate) tirmethinoxonol; CRAC, Ca^{2+} release–activated Ca^{2+}; Con A, concavalin A; EPA, eicosapentaenoic acid (20:5 n–3); $[Ca^{2+}]i$, free intracellular calcium concentrations; PMA, 12-phorbol-13-myristate acetate; PBS, phosphate-buffered saline, pH 7.4 ; PUFA, polyunsaturated fatty acid; TCR, T-cell receptor.

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(PUFA) in the regulation of immune cell functions (13). The PUFAs of n−3 series, having the last double bond on the three carbons from the methyl or omega end of the acyl chain, have been considered immunosuppressors (13). The n−3 PUFA exert curative effects in autoimmune diseases like rheumatoid arthritis (14), psoriasis (15), atopic dermatitis (16), and multiple sclerosis (17). The precursor of n−3 PUFA is linolenic acid (18:3 n−3) which is metabolized to the most unsaturated fatty acid, docosahexaenoic acid (DHA, 22:6 n−3) in microsomes by an altering series of position specific desaturase and malonyl-CoA-dependent chain elongation steps (18). In fact, DHA is the most interesting PUFA as it is found in large amounts in different parts of human body (19). Diets containing fish oil are rich in DHA and eicosapentaenoic acid (20:5 n−3), an intermediate of DHA synthesis. DHA has also been shown to alter the expression of CD8 and Thy-1 on murine splenocytes (20).

Most of the studies on calcium signalling have dealt with the PUFAs of n−6 category, particularly, arachidonic acid (AA) whose exogenous addition has been shown to induce increases in $[Ca^{2+}]i$ in a number of cells, like cardiac myocytes (21, 22) and cultured rat oligodendrocytes (23). In fact, AA is the natural PUFA which is present on the *sn*-2 position of the membrane lipids and can be released in non-esterified form by the action of phospholipase A_2. Some studies also elucidated the role of DHA on the increases in $[Ca^{2+}]i$ in Jurkat T-cells (24, 25). These investigators were mainly interested in assessing the implication of the intracellular pool. However, the exact mechanism of action of DHA in the regulation of T-cell calcium signalling from extra- and intra-cellular pools, particularly in the light of a capacitative model of calcium homeostasis, is not well understood.

As DHA exerts immunosuppressive effects and the PUFAs influence the calcium signalling, we undertook the present study to elucidate role of DHA in the regulation of T-cell signalling via CRAC channels and intracellular stores.

MATERIALS AND METHODS

Materials

The culture medium RPMI 1640 and l-glutamine were purchased from Biowhitaker, Belgium. Fura-2/AM and bis-oxonol, bis-5(1,3-diethylthiobarbiturate)tirmethinoxonol, were procured from Molecular Probes, Eugene, OR. [^{3}H]thymidine (sp act, 20 Ci/mmol) was purchased from Amersham Radiochemicals, France. All other chemicals including arachidonic acid, AA (20:4 n−6), docosahexaenoic acid, DHA (22:6 n−3), eicosapentaenoic acid, EPA (20:5 n−3), EPA were obtained from Sigma Chemicals, St. Louis, MO.

Cell culture

The human (Jurkat) T-cells (TCR^+ wild-type and TCR^- mutants) were kindly provided by Dr. Bent Rubin, Head, UMR-CNRS Research Unit at CHR of Toulouse (France). The cells were 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.

Measurement of Ca^{2+} signalling

The cells (2×10^6/ml) were washed with phosphate-buffered saline, pH 7.4, and 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; KCI, 5.4; $NaHCO_3$, 25; $MgCl_2$, 0.8; KH_2PO_4, 0.4; HEPES-Na, 20; $NaHPO_4$, 0.33; $CaCl_2$, 1.2, and the pH was adjusted to 7.4.

After loading, the cells were washed three times (2000 $g \times 10$ min) and remained suspended in the identical buffer. $[Ca^{2+}]i$ was measured according to Grynkiewicz, Ponie, and Tsein (26). The fluorescence intensities were measured in the ratio mode in PTI spectrofluorometer at 340 nm and 380 nm (excitation filters) and 510 nm (emission filters). 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 intracellular concentration of free Ca^{2+}, $[Ca^{2+}]i$, were calculated by using the following equation: $[Ca^{2+}]i = K_d \times (R - R_{min}) / (F_{max} - F)(Sf2/Sb2)$. A value of 224 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 35°C instead of 37°C in order to minimize the leakage of Fura-2. The PUFAs were dissolved in ethanol (w/v, 0.1%) and used immediately or kept at −20°C in ampules, tightly sealed under the stream of nitrogen.

Measurement of membrane potential, V_m

The probe bis-oxonol has been used to measure membrane potential in lymphocytes as this probe is chemically unrelated to the cyanines and is not toxic to these cells (27). For these experiments, the cells were prepared as described for $[Ca^{2+}]i$ determinations. After washing, the cells (2×10^6/ml) were transferred to the fluorimeter cuvette and 150 nm of bis-oxonol was added. The stock solution of bis-oxonol was prepared in dimethyl sulfoxide. The cells were allowed to equilibrate with the dye and, after 10 min, different test molecules were added. The fluorescence intensities were determined at 540 nm (excitation filter) and 580 nm (emission filter). Downward and upward deflections represent, respectively, hyper- and de-polarizations.

T-cell proliferation assay

The human (Jurkat) T-cells (10×10^4) were cultured in 96-well flat-bottom tissue culture plates (Nunc) in the presence of mitogens, like Con-A (5 μg/ml) or/and PMA (10 nm) plus ionomycine (500 nm). Cells were distributed in quadruplicate as follows: 140 μl, cell suspension; 20 μl, mitogen or medium and 20 μl, docosahexaenoic acid, DHA (as indicated in legends). Plates were incubated at 37 ± 2°C (90% humidity). After 36 h, 20 μl of [^{3}H]thymidine (20 Ci/mmol, 0.8 μCi/well) was added and, 12 h later, the cells were harvested using a cell harvester (Dynatech, Vienna, VA), trapping their DNA onto glass filtermats. Dried filter circles were placed in plastic minivials (Packard), 3.5 ml Optifluor-O (Packard) was added, and the radioactivity was recorded in a scintillation counter (Packard). Index of T-cell stimulation (IS) was calculated as: cpm in the presence of mitogens/cpm of unstimulated cultures. In these studies, we used DHA at 20 μm as this was the optimal concentration deduced from the calcium studies.

RESULTS

DHA evokes increases in $[Ca^{2+}]i$ in Jurkat T-cells

Figure 1a shows that the DHA-induced response was marked by a calcium spike, followed by the diminution

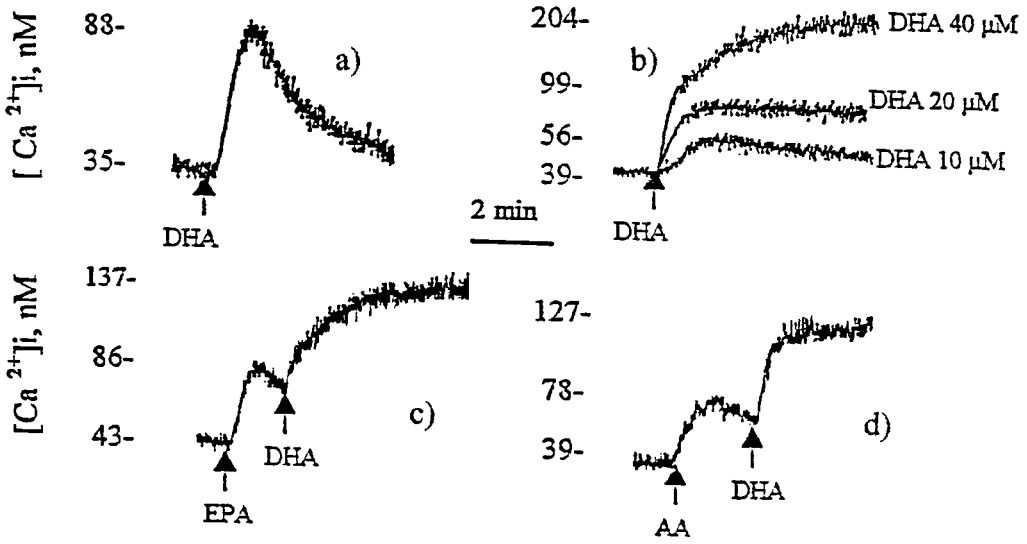

Fig. 1. Effects of DHA on the increases in $[Ca^{2+}]i$ in Jurkat T-cells. Cells (4×10^6/assay) were loaded with the fluorescent probe, Fura-2/AM, as described in Materials and Methods. The arrow heads indicate the time when the test molecules, PUFAs, were added into the cuvette without interruptions in the recordings. All PUFA were added at 20 μm (a, c, d) except the Fig. 1b where DHA was added at increasing concentrations as indicated in the figure. Upward deflections indicate increases in $[Ca^{2+}]i$ in these cells. The figure shows the single traces of observations which were reproduced several times independently.

as a function of time (**Table 1**). While comparing the plateau responses, we observed that DHA induced increases in $[Ca^{2+}]i$ in a dose-dependent manner (Fig. 1b). Addition of DHA after the addition of EPA or AA further evoked additive increases in $[Ca^{2+}]i$ in Jurkat T-cells (Fig. 1c, d).

DHA increases $[Ca^{2+}]i$ from anti-CD3-, but not thapsigargin-, stimulated intracellular pool

The T-cell receptor (TCR) is coupled to CD3 molecules, responsible for the signal transduction in T-cells (1). Upon antigenic stimulation, the TCR/CD3 complex activates the phospholipase Cγ1 which hydrolyzes phosphatidylinositol (28), thus producing diacylglycerol (DAG) and inositol trisphosphate (IP_3). This action can be mimicked in vitro by using anti-CD3 antibody whose interaction with the TCR/CD3 complex will result in the production of DAG and IP_3.

Figure 2a demonstrates that addition of anti-CD3 to Jurkat T-cells stimulates increases in $[Ca^{2+}]i$ (Table 1). Addition of DHA, when the response of anti-CD3 was in decline, further evoked a small rise in $[Ca^{2+}]i$ (Fig. 2b, Table 1). Furthermore, we failed to notice a DHA-induced calcium spike when this PUFA was added during the peak response, evoked by anti-CD3, though a sustained calcium response was apparent (Fig. 2c). It is also noteworthy that the DHA-induced response in the mutant TCR^- cells, lacking the expression of TCR, was not diminished, rather it was higher in these cells as compared to that in wild-type TCR^+ Jurkat T-cells used in the present study (TCR^+, 55.2 ± 8.1 nm, n = 10 vs. TCR^-, 170.8 ± 31.4 nm, n = 4).

The increases in $[Ca^{2+}]i$ can also be achieved by using thapsigargin (29). In fact, thapsigargin is a specific inhibitor of Ca^{2+}-ATPase, present on the ER, and thus increases intracellular cytosolic calcium by inhibiting the refilling of the ER pool. **Figure 3a** shows that thapsigargin induces an increase in $[Ca^{2+}]i$ (Table 1, Fig. 3a). Addition of DHA after thapsigargin or vice versa evoked additive effects on the increases in $[Ca^{2+}]i$ in these cells (Figs. 3b, c, Table 1).

TABLE 1.

PUFA + Other Compounds	n	Increase Δ in $[Ca^{2+}]i$
+ DHA	10	55.2 ± 8.1[a]
+ Anti-CD3	10	38.5 ± 3.1
+ DHA (during the decline of anti-CD3 response, see Fig. 2b)	8	11.2 ± 1.6[b]
+ Thapsigargin (see, Fig. 3a)	9	40.2 ± 3.4
+ Thapsigargin (after addition of DHA, see Fig. 3b)	11	43.1 ± 5.0
+ DHA (after the addition of thapsigargin, see Fig. 3c)	9	60.2 ± 5.1
+ DHA (pre-addition of TA9)	8	15.3 ± 2.2[c]
+ DHA (pre-addition of diltiazem)	9	48.2 ± 5.3
+ DHA (pre-addition of ω-conotoxin)	8	47.3 ± 4.4

For legends and concentrations of the test compounds, see Figs. 2 and 3. Diltiazem and ω-conotoxin were used at 1 μm.

Values were evaluated using Student's *t*-test of significance: [a] versus [b], $P < 0.001$; [a] versus [c], $P < 0.001$.

Inhibition of IP_3 production does not diminish the DHA-induced rise in $[Ca^{2+}]i$

In order to evaluate whether DHA evokes increases in $[Ca^{2+}]i$ via production of IP_3, we used U-73122, an agent known to inhibit the generation of free IP_3 (30, 31). U-73122 has been shown to act extracellularly within 15

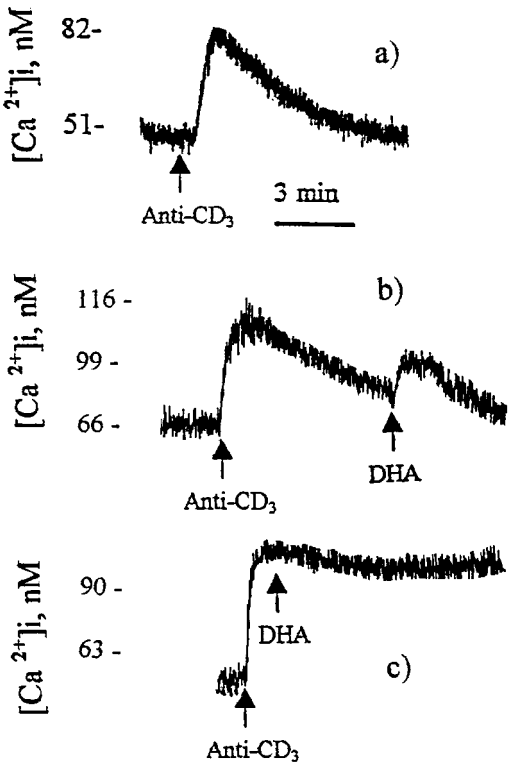

Fig 2. Effects of anti-CD3 and DHA on the increases in $[Ca^{2+}]i$ in Jurkat T-cells. Cells (4×10^6/assay) were loaded with the fluorescent probe, Fura-2/AM, as described in Materials and Methods. The arrow heads indicate the time when the test molecules, anti-CD3 (10 μg/ml) and DHA (20 μm), were added into the cuvette without interruption in the recordings. The figure shows the single traces of observations which were reproduced several times, independently.

sec (with the maximum action after 70 sec) in different cells, such as rat pancreatic GH_3 rat pituitary cells (30) and rat acinar cells (31). U-73122 exerts its action from 3 to 10 μm. **Figure 4b** suggests that prior addition of this agent into the cuvette did not influence the DHA-induced increases in $[Ca^{2+}]i$ in Jurkat T-cells. Hence, we further used this agent at various concentrations from 2 to 10 μm, but it failed to affect the DHA response at all the concentrations (results not shown). Interestingly, U-73122 abolished the anti-CD3-induced increases in $[Ca^{2+}]i$ in these cells (Fig. 4c).

DHA-induced rise in $[Ca^{2+}]i$ is also due to calcium influx by opening the CRAC channels

We were also tempted to assess whether the DHA-induced rise in $[Ca^{2+}]i$ is also due to calcium influx in Jurkat T-cells. We conducted experiments in the absence (0% Ca^{2+}) and presence (100% Ca^{2+}) of calcium. **Figure 5a** and **Fig. 6** show that the DHA-induced response in the increases in $[Ca^{2+}]i$ was significantly curtailed in 0% Ca^{2+} medium as compared to that in 100% Ca^{2+} medium (Fig. 6), indicating the influx of calcium from the extracellular medium.

In order to probe the role of calcium channels implicated in the influx of extracellular calcium, we used tyrphostin A9, an inhibitor of CRAC channels (32). Human Jurkat T-cells are known to express the CRAC channels and tyrphostin A9 at 10 μm has been used to inhibit the functioning of these channels in these cells (32). Figure 5b shows that preincubation of Jurkat T-cells in the presence of tyrphostin A9 significantly curtails the thapsigargin-induced increases in $[Ca^{2+}]i$. Similarly, DHA-induced increases in $[Ca^{2+}]i$ are significantly curtailed in the cells preincubated in the presence of tyrphostin A9 as compared to control cells (Figs. 5c and 6, Table 1). We also used diltiazem and ω-conotoxin which are inhibitors of L-type and N-type calcium channels. These agents did not diminish the DHA-induced increases in $[Ca^{2+}]i$ in these cells (Table 1).

DHA interacts extracellularly in Jurkat T-cells

In order to assess whether DHA acts extracellularly in calcium signalling, we used the fatty acid-free bovine serum albumin at a final concentration of 0.2% (BSA, 0.2% w/v) as this concentration of BSA has been shown to compete with PUFA, bound to the plasma membrane (33). **Figure 7** shows that addition of BSA during the peak response of DHA abruptly diminished the DHA-induced rise in $[Ca^{2+}]i$ in Jurkat T-cells. Addition of BSA alone exerted no significant perturbation in the Fura-2 fluorescence (results not shown).

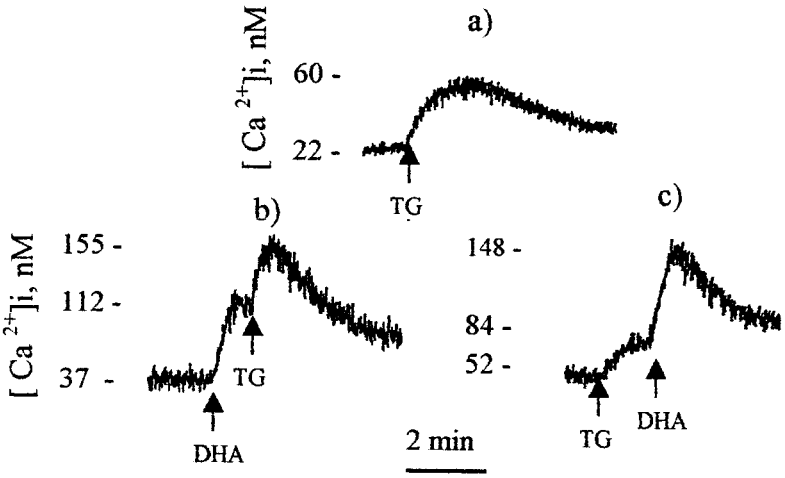

Fig. 3. Effects of thapsigargin and DHA on the increases in $[Ca^{2+}]i$ in Jurkat T-cells. Cells (4×10^6/assay) were loaded with the fluorescent probe, Fura-2/AM, as described in Materials and Methods. The arrow heads indicate the time when the test molecules, thapsigargin, TG (5 μm) and DHA (20 μm), were added into the cuvette without interruption in the recordings. The figure shows the single traces of observations which were reproduced several times, independently.

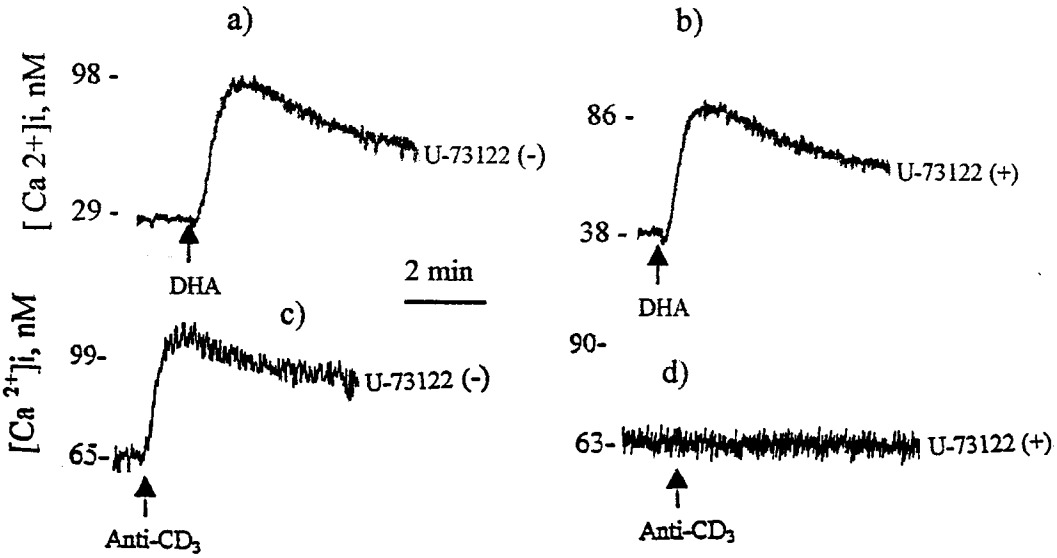

Fig. 4. Effects of U-73122 on DHA-evoked increases in $[Ca^{2+}]i$ in Jurkat T-cells. Cells (4×10^6/assay) were loaded with the fluorescent probe, Fura-2/AM, as described in Materials and Methods. The cells were pre-incubated in the presence of U-73122 (3 μm) for 10 min. The arrow heads indicate the time when the test molecules, anti-CD3 (10 μg/ml) and DHA (20 μm), were added into the cuvette without interruption in the recordings. The figure shows the single traces of observations which were reproduced several times independently.

DHA does not influence membrane potential, V_m, in Jurkat T-cells

In order to probe whether DHA acts by inducing membrane perturbations, we assessed its action on the modifications in membrane potential (V_m). **Figure 8** shows that DHA and anti-CD3 exert no influence on V_m in Jukat T-cells. Addition of gramacidin, a channel ion former, induces membrane depolarization in these cells (Fig. 8).

DHA inhibits T-cell proliferation

In order to shed light on the physiological importance of the DHA-induced response on calcium signalling, we stimulated Jurkat T-cells with Con-A or PMA plus ionomycin in the presence or absence of exogenous DHA. This PUFA exerted the growth inhibitory effects on both Con-A and PMA plus ionomycin stimulated T-cells (**Fig. 9**).

DISCUSSION

During the course of recent years, the n−3 PUFA have been considered as immunosuppressors (13). DHA is the end product of the metabolism of α-linolenic acid (18). The exact mechanism of action of DHA on T-cell activation is not well understood. In the present study, we elucidated the action of DHA in calcium signalling in Jurkat T-cells. We observed that DHA evoked a rapid transient increase in $[Ca^{2+}]i$, followed by a lower sustained level which eventually returned to the base line. DHA exerted a specific action as prior addition of other PUFA such as EPA (20:5 n−3) and AA (20:4 n−6) resulted in additive responses on the increases in $[Ca^{2+}]i$.

In order to trace the origin of calcium, recruited by DHA, we used anti-CD3 antibodies and thapsigargin. In human T-cells, an increase in $[Ca^{2+}]i$ can be brought about by using anti-CD3 which, by aggregating CD3/TCR, induces an increase from the endoplasmic reticulum (ER) pool via the production of inositol trisphophate, IP_3 (28). Thapsigargin, isolated from *Thapsia garganica*, without acting on IP_3 receptors, increases cytosolic Ca^{2+} by inhibiting the Ca^{2+}-ATPase present on the ER (29). We observed that anti-CD3 induced an increase in $[Ca^{2+}]i$. Addition of DHA after the anti-CD3 response further evoked an increase in $[Ca^{2+}]i$ in these cells. When DHA was added during the anti-CD3-induced calcium plateau response, the former failed to evoke an additive calcium peak, though a prolonged and sustained calcium response was apparently seen. These results suggest that DHA mobilizes intracellular Ca^{2+} by emptying the ER pool. However, the target of DHA does not seem to be the TCR as in the mutant TCR^- cells which do not express the functional TCR, the DHA-induced response was not curtailed as compared to that in wild-type TCR^+ cells. Furthermore, DHA does not seem to empty this pool via the production of IP_3 as an inhibitor of IP_3 production, U-73122, failed to curtail the DHA-induced rise in $[Ca^{2+}]i$. This notion is further supported by the observations of Chow and Jondal (25) who noticed that various PUFA including DHA evoked increases in $[Ca^{2+}]i$ without the production of IP_3 in Jurkat T-cells. In another study, these investigators have further demonstrated that another PUFA, α-linolenic acid, also induced increases in $[Ca^{2+}]i$ without generating IP_3 in Jurkat T-cells (33).

DHA also does not seem to act on Ca^{2+}-ATPase of the ER as the addition of thapsigargin after DHA and vice versa, during the plateau calcium responses, exerted the

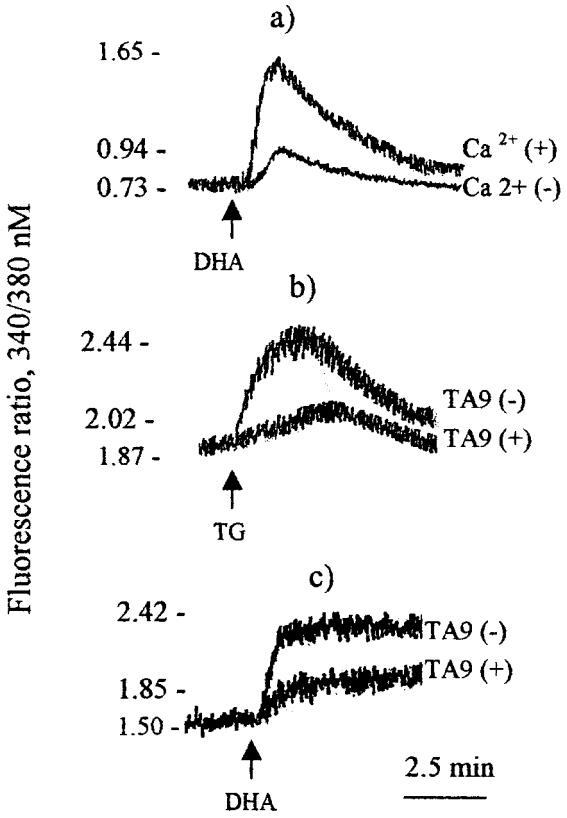

Fig. 5. Effects of extracellular calcium on DHA-evoked increases in $[Ca^{2+}]i$ in Jurkat T-cells. Cells (4×10^6/assay) were loaded with the fluorescent probe, Fura-2/AM, as described in Materials and Methods. Ca^{2+} (+) and Ca^{2+} (−) traces (a, b) represent the respective experiments, performed in Ca^{2+} containing (100% Ca^{2+}) and Ca^{2+} free buffer (0% Ca^{2+}, in the latter Ca^{2+} was replaced by EGTA, 1.3 mm). c) The cells were preincubated or not in the presence of tyrphostin A9 (TA9 at 10 μm). The arrow heads indicate the time when the test molecules, thapsigargin, TG (5 μm) and DHA (20 μm) were added into the cuvette without interruption in the recordings. The figure shows the single traces of observations which were reproduced several times independently.

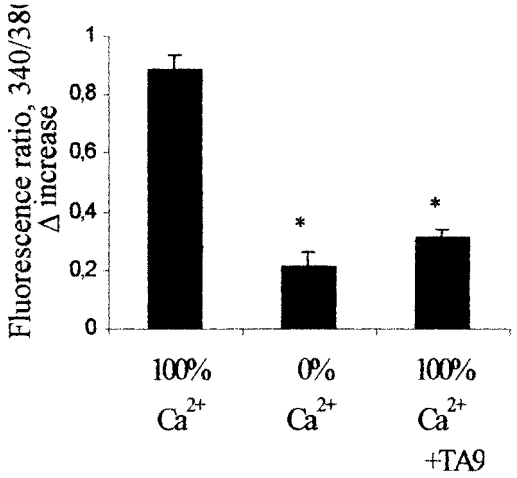

Fig. 6. DHA-evoked changes in fluorescent ratios (340/380) during Ca^{2+} influx in Jurkat T-cells. Cells (4×10^6/assay) were loaded with the fluorescent probe, Fura-2/AM, as described in Materials and Methods. The histograms represent the changes in fluorescent ratios (340/380) derived from the experiments performed in 100% Ca^{2+} (Ca^{2+} containing), 0% Ca^{2+} (Ca^{2+}-free) buffer and TA9-pretreated cells as mentioned in Fig. 5; * represents $P < 0.001$ as compared to control (100% Ca^{2+}). The values were evaluated using Student's *t*-test of significance.

additive effects. However, DHA does act extracellularly, as addition of fatty acid-free BSA abruptly diminished the Ca^{2+} rise evoked by the former. BSA is known to possess high affinity binding sites for free fatty acids. Hence, it seems that BSA detaches the plasma membrane bound-DHA and, thereby, contributes to the lowered response of this PUFA. How DHA delivers its signal, by acting extracellularly, to induce increases in $[Ca^{2+}]i$ is not well understood.

We were further interested in elucidating whether the DHA response to the increase in $[Ca^{2+}]i$ is also due to the influx of calcium from the extracellular medium. We conducted experiments in calcium-free buffer in which calcium was replaced by EGTA. We observed that the DHA-evoked Ca^{2+} response was significantly curtailed in 0% Ca^{2+} as compared to that in 100% Ca^{2+}, suggesting that calcium influx, indeed, takes place during the DHA-induced increases in $[Ca^{2+}]i$ in these cells. In human T-cells, calcium influx mainly takes place by opening of Ca^{2+} release–activated Ca^{+} (CRAC) channels. The CRAC channels were originally described in mast cells (34). Human Jurkat T-cells express nearly 10,000 CRAC channels per cell (8,35). The CRAC channels are not opened directly by depolarization, IP_3 or IP_4 (36). The depletion-activated CRAC channels underline mitogenic Ca^{2+} influx in T-cells. In order to ascertain the role of CRAC channels, we used tyrphostin A9 which has been recently shown to specifically inhibit the opening of these channels in Jurkat T-cells (32). These investigators have shown that tyrphostin A9 inhibits the calcium influx, induced by thapsigargin in a concentration-dependent manner (32). Similarly, we observed that a pre-treatment of Jurkat T-cells by tyrphostin A9 significantly curtailed the increases

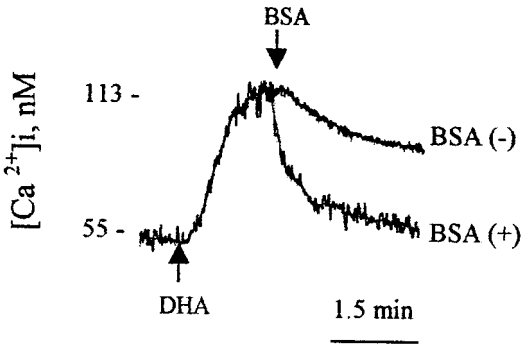

Fig. 7. Effects of addition of BSA on DHA-evoked increases in $[Ca^{2+}]i$ in Jurkat T-cells. Cells (4×10^6/assay) were loaded with the fluorescent probe, Fura-2/AM, as described in Materials and Methods. The arrow heads indicate the time when the test molecules, fatty acid-free bovine serum albumin, BSA (0.2% w/v) and DHA (20 μm) were added into the cuvette without interruption in the recordings. The figure shows the single traces of observations which were reproduced several times independently.

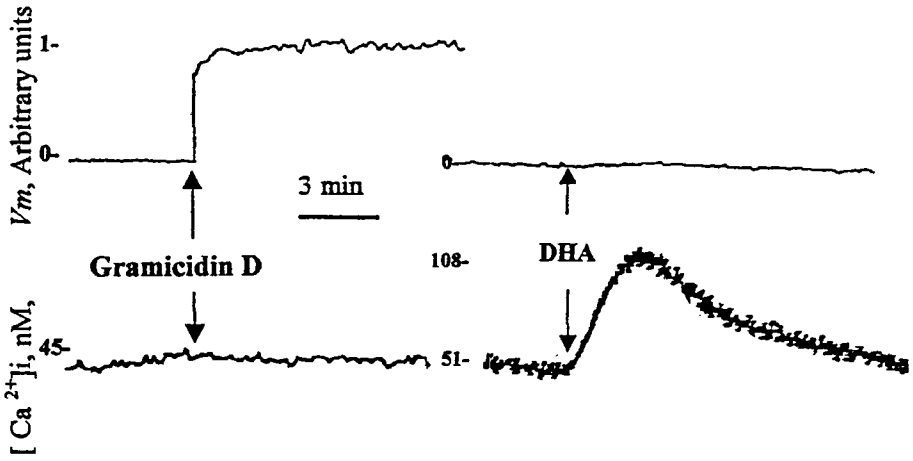

Fig. 8. Effects of DHA and gramicidin on membrane potential (V_m) in Jurkat T-cells. Cells (4×10^6/assay) were loaded with the fluorescent probe, bis-oxonol, as described in Materials and Methods. The arrow heads indicate the time when the test molecules, gramicidin (500 nm) and DHA (20 μm), were added into the cuvette.

in $[Ca^{2+}]$i evoked both by DHA and thapsigargin. We also used diltiazem and ω-conotoxin which are, respectively, the inhibitors of L-type and N-type calcium channels. These agents did not significantly curtail the DHA-induced increases in $[Ca^{2+}]$i Jurkat T-cells. Together these observations suggest that DHA contributes to the increases in $[Ca^{2+}]$i, in part, via opening of the CRAC channels.

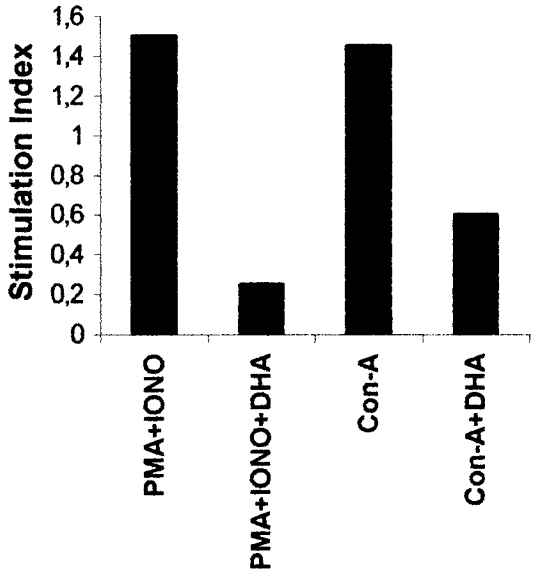

Fig. 9. Effects of DHA on Con-A and PMA plus ionomycin-stimulated proliferation of Jurkat T-cells. The human (Jurkat) T-cells (10×10^4) were cultured in 96-well flat-bottom tissue culture plates in the presence of Con-A (5 μg/ml) and/or PMA (10 nm) plus ionomycin, IONO (500 nm), containing or not DHA (20 μm) as described in Materials and Methods. After 36 h, 20 μl of [^{3}H]-thymidine (20 Ci/mmol, 0.8 μCi/well) was added and 12 h later, the cells were harvested using Dynatech cell harvester, trapping their DNA onto glass filtermats. The radioactivity was recorded in a scintillation counter (Packard). The index of stimulation was calculated as described in Materials and Methods. The figure shows the result of an identical experiment, reproduced several times with triplicate values.

In order to elucidate whether the effects of DHA on the increases in $[Ca^{2+}]$i may be merely due to the DHA-induced plasma membrane perturbation, we measured the modifications in the membrane potential (V_m) in these cells. We observed that DHA induced increases in $[Ca^{2+}]$i without affecting the V_m in these cells; whereas gramicidin, a channel ionophore, induced membrane depolarization without affecting the $[Ca^{2+}]$i. These observations suggest that the DHA exerts its specific action on the plasma membrane of Jurkat T-cells.

In order to evaluate the physiological importance of DHA on T-cell activation, we stimulated Jurkat T-cells. Con-A is a T-cell-specific mitogen and, therefore, stimulates T-cell proliferation, whereas PMA plus ionomycin induce T-cell proliferation via PKC activation (37). We observed that DHA exerted inhibitory effects on T-cell proliferation, as measured by the incorporation of [^{3}H] thymidine. These immunosuppressive effects of DHA on T-cell proliferation corroborate the observations of several investigators who have reported that DHA inhibits the lymphoproliferative responses in vitro and in vivo (38, 39).

The present study demonstrates that DHA induces increases in $[Ca^{2+}]$i, both from the ER pool and by opening of CRAC channels, and that this PUFA inhibits Jurkat T-cells proliferation. However, further studies are awaited to probe the mechanism of action of DHA in cell signalling. Whether Jurkat T-cells possess DHA receptors which may be implicated in T-cells activation and whether these receptors are coupled with an effector system such as protein kinase C and MAP kinases remain to be ascertained in future.

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