0026-895X/07/7206-1545–1556$20.00
MOLECULAR PHARMACOLOGY
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*Mol Pharmacol* 72:1545–1556, 2007

Vol. 72, No. 6
39792/3279399
*Printed in U.S.A.*



# Docosahexaenoic Acid Induces Increases in $[Ca^{2+}]_i$ via Inositol 1,4,5-Triphosphate Production and Activates Protein Kinase C$\gamma$ and -$\delta$ via Phosphatidylserine Binding Site: Implication in Apoptosis in U937 Cells

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Received July 9, 2007; accepted September 17, 2007

## ABSTRACT

We investigated, in monocytic leukemia U937 cells, the effects of docosahexaenoic acid (DHA; 22:6 n-3) on calcium signaling and determined the implication of phospholipase C (PLC) and protein kinase C (PKC) in this pathway. DHA induced dose-dependent increases in $[Ca^{2+}]_i$, which were contributed by intracellular pool, via the production of inositol-1,4,5-triphosphate ($IP_3$) and store-operated $Ca^{2+}$ (SOC) influx, via opening of $Ca^{2+}$ release-activated $Ca^{2+}$ (CRAC) channels. Chemical inhibition of PLC, PKC$\gamma$, and PKC$\delta$, but not of PKC$\beta$ I/II, PKC$\alpha$, or PKC$\beta$I, significantly diminished DHA-induced increases in $[Ca^{2+}]_i$. In vitro PKC assays revealed that DHA induced a ~2-fold increase in PKC$\gamma$ and -$\delta$ activities, which were temporally correlated with the DHA-induced increases in $[Ca^{2+}]_i$. In cell-free assays, DHA, but not other structural analogs of fatty acids, activated these PKC isoforms. Competition experiments revealed that DHA-induced activation of both the PKCs was dose-dependently inhibited by phosphatidylserine (PS). Furthermore, DHA induced apoptosis via reactive oxygen species (ROS) production, followed by caspase-3 activation. Chemical inhibition of PKC$\gamma/\delta$ and of SOC/CRAC channels significantly attenuated both DHA-stimulated ROS production and caspase-3 activity. Our study suggests that DHA-induced activation of PLC/$IP_3$ pathway and activation of PKC$\gamma/\delta$, via its action on PS binding site, may be involved in apoptosis in U937 cells.

Epidemiological, clinical, and experimental studies have established that ingestion of n-3 polyunsaturated fatty acids (n-3 PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), present in marine fish oils, exert beneficial effects in several autoimmune and inflammatory disorders (Calder, 2004). Although their beneficial effects can no longer be doubted, their molecular mechanisms of action are complex and involve a number of integrated signaling pathways. n-3 PUFAs have been shown to influence lipid microdomains such as lipid rafts and caveolae (Ma et al., 2004), Protein kinase C (PKC) activation (Madani et al., 2001), mitogen-activated protein kinase phosphorylation (Denys et al., 2002, 2004; Madani et al., 2004) and modulation of gene expression (Nakamura et al., 2004). In addition, in vitro studies have demonstrated that EPA and DHA may induce cell death or apoptosis via mitochondrial pathway (Kim et al., 2005).

Stimulation of plasma membrane receptors linked to phospholipase C and subsequent production of the second mes-

This work was supported by a contingent grant from Ministry of Higher Education and Research, France.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.039792.

**ABBREVIATIONS:** PUFA, polyunsaturated fatty acid; AA, arachidonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; CRAC, $Ca^{2+}$ release-activated $Ca^{2+}$ channels; DAG, diacylglycerol; DHA, docosahexaenoic acid; DHA-meth, DHA methyl ester; DHE, dihydroethidine; Dic8, 1,2-dioctanoyl-*sn*-glycerol; DPEA, *cis*-7,10,13,16,19-docosapentaenoic acid; DTT, dithiothreitol; EPA, eicosapentaenoic acid; GF 109203X, bisindolylmaleimide I; Gö-6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; HBDDE, 2,2′,3,3′,4,4′-hexahydroxy-1,1′-biphenyl-6,6′-dimethanol dimethyl ether; $IP_3$, inositol-1,4,5-trisphosphate; LSD, least-significant difference; MBP, myelin basic protein; MMP, mitochondrial membrane potential; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidyl-L-serine; ROS, reactive oxygen species; SKF-96365, 1-($\beta$-[3-(4-methoxyphenyl-)propoxy]-4-methoxyphenethyl)-1*H*-imidazole; SOC, store-operated $Ca^{2+}$ channels; TA9, tyrphostin A9; TG, thapsigargin; U-73122, 1-[6-[[17$\beta$-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione.

sengers, diacylglycerol (DAG), and inositol-1,4,5-trisphosphate ($IP_3$), lead to a rapid release of $Ca^{2+}$ from endoplasmic reticulum. Store depletion activates $Ca^{2+}$ influx across the plasma membrane, replenishing empty stores and providing a sustained increase in the concentration of cytoplasmic free $Ca^{2+}$ ($[Ca^{2+}]_i$) (Berridge et al., 2003). The calcium influx, termed capacitative or store-operated $Ca^{2+}$ (SOC), is essential for regulating diverse cellular responses to receptor-mediated stimuli, including enzyme activation, gene expression, secretion, cell division, and cell death (Berridge et al., 2003).

Several classes of protein kinases, especially protein kinases C (PKC), have been shown to regulate the activity of SOC channels in various cell types. PKCs have been found to inhibit (Vanden Abeele et al., 2003) or facilitate (Albert and Large, 2002) capacitative calcium entry. In fact, PKCs have been classified as conventional ($\alpha$, $\beta$I, bII, and $\gamma$), novel ($\delta$, $\varepsilon$, $\theta$, and $\eta$), and atypical ($\zeta$ and $\iota/\lambda$). The conventional PKCs are $Ca^{2+}$- and DAG-dependent, whereas the novel PKCs are $Ca^{2+}$-independent but are activated by DAG. The atypical PKCs are dependent on neither calcium nor DAG (Newton, 1995). Hence, the implication of the specific PKC isoforms in the regulation of SOC channels has not been thoroughly studied.

In the present study, we investigated the effects of DHA on calcium signaling and the implication of PKC isoforms in this pathway in U937 cell line. Owing to the capacity of n-3 PUFAs to induce cell death, we were also tempted to evaluate the impact of DHA-induced modulation of $Ca^{2+}$ homeostasis in apoptosis.

## Materials and Methods

**Materials.** The culture medium RPMI 1640, L-glutamine, HEPES buffer, streptomycin/penicillin were purchased from Lonza Verviers SPRL (Verviers, Belgium) and fetal calf serum was procured from Abcys S.A. (Paris, France). Fura-2/AM was obtained from Invitrogen (Carlsbad, CA) and [$\gamma$-$^{32}$P]ATP (7000 Ci/mmol) was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). The PKC inhibitors calphostin C, chelerythrine chloride, GF 109203X, SKF-96365, Gö-6976, HBDDE, and recombinant PKC isoforms ($\delta$ and $\gamma$) were purchased from Calbiochem (La Jolla, CA). Histone H1 as well as the calcium channel inhibitors [i.e., nifedipine, tyrphostin A9 (TA9), and $\omega$-conotoxin] were procured from Calbiochem (La Jolla, CA). PKC substrate for cell-free PKC assays and the myelin basic protein (MBP) were obtained from Invitrogen (Cergy Pontoise, France). Monoclonal anti-PKC$\gamma$ antibody was purchased from BD Biosciences Transduction Laboratories (Lexington, CA) and polyclonal anti-PKC$\delta$ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Proteins A and G Sepharose were purchased from Amersham. Docosahexaenoic acid (DHA), DHA methyl-ester (DHA-meth), arachidonic acid (AA), *cis*-7,10,13,16,19-Docosapentaenoic acid (DPEA), EPA, 1,2-dioctanoyl-*sn*-glycerol (Dic8), phosphatidyl-L-serine (PS), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated all other chemicals, were purchased from Sigma-Aldrich.

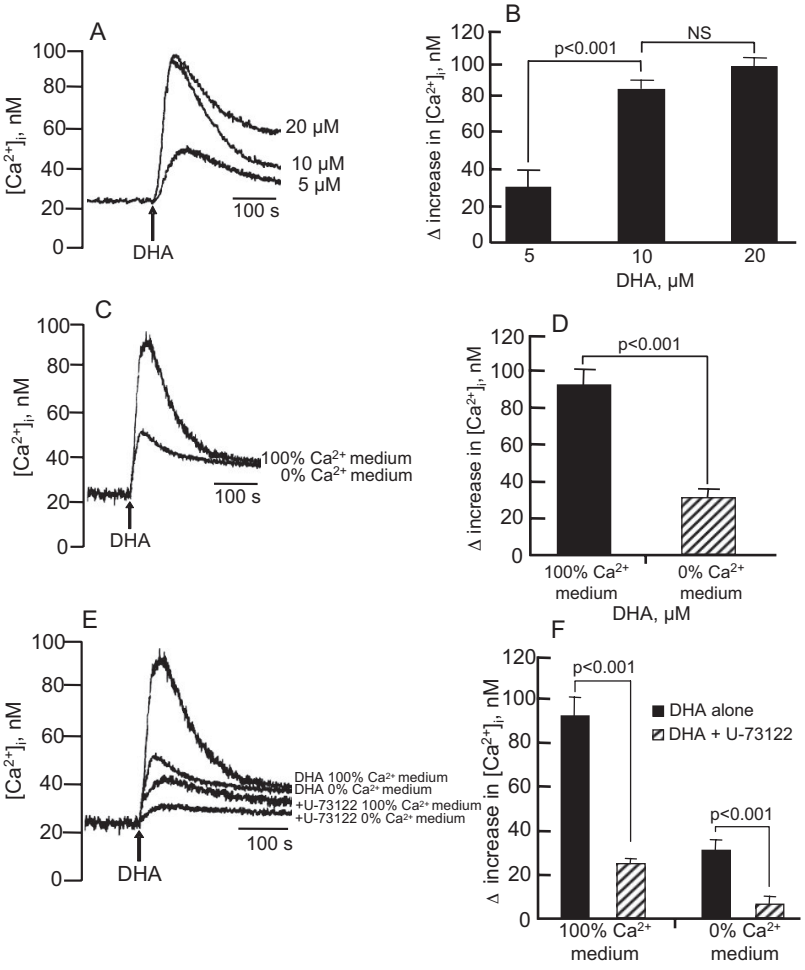


**Fig. 1.** DHA induces increases in $[Ca^{2+}]_i$ in U937 cells by mobilizing extra- and intracellular $Ca^{2+}$ pools. The arrowheads indicate the addition of DHA into the cuvette. Data in histograms (B, D, and F), represented as means ± S.D., were analyzed by employing LSD test of significance. A, increasing concentrations of DHA (5, 10, and 20 $\mu$M) were added to Fura-2 loaded cells. B, the Δ increases in $[Ca^{2+}]_i$ induced by different concentrations of DHA ($n$ = 6). NS, nonsignificant differences. C, traces represent the experiments performed in $Ca^{2+}$-containing (100%) or $Ca^{2+}$-free (0%) medium. The figure shows the single traces of the response, induced by DHA (10 $\mu$M). D, Δ increases in $[Ca^{2+}]_i$ evoked by DHA (10 $\mu$M) in 100% and 0% $Ca^{2+}$ medium ($n$ = 5). E, Fura-2-loaded cells were resuspended in 100% or 0% medium and preincubated in the presence or absence of U-73122 (3 $\mu$M) for 15 min before the addition of DHA (10 $\mu$M). F, Δ increases in $[Ca^{2+}]_i$ evoked by DHA (10 $\mu$M) in 100% and 0% $Ca^{2+}$ medium in the presence or absence of U-73122 ($n$ = 5).

**Cell Culture.** U937 human myeloid leukemic cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 $\mu$g/ml penicillin-streptomycin, and 20 mM HEPES. Cell viability was determined by trypan blue exclusion test. To ensure exponential growth, cells were resuspended at a density of $0.5 \times 10^6$ cells/ml in fresh medium for 24 h before each treatment.

**Measurement of Free Intracellular $Ca^{2+}$ Concentrations.** Cells ($2 \times 10^6$/ml) were washed with phosphate-buffered saline, pH 7.4, and then incubated with Fura-2/acetoxymethyl ester (1 $\mu$M) for 60 min at 37°C in $Ca^{2+}$ buffer containing the following: 110 mM NaCl, 5.5 mM KCl, 25 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.4 mM $KH_2PO_4$, 20 mM HEPES, and 1.2 mM $CaCl_2$, and pH was adjusted to 7.4. After loading, cells were washed three times (720$g$ × 10 min) and remained suspended in $Ca^{2+}$ buffer. The fluorescence intensities were measured in the ratio mode in a PTI spectrofluorometer at 340 and 380 nm (excitation filters) and 510 nm (emission filters). Cells were continuously stirred throughout the experiment. The concentrations of intracellular free $Ca^{2+}$ were calculated by using the following equation: $[Ca^{2+}]_i = K_d \times (R - R_{min})R_{min})/(F_{max}(F_{max} - F)(Sf2/Sb2)$. A value of 224 nM for $K_d$ was added into the calculations. $R_{max}$ value was obtained by addition of ionomycin (5 $\mu$M) and $R_{min}$ value was obtained by addition of $MnCl_2$ (2 mM), Triton X-100 (0.1%), and EGTA (24 mM).

For experiments conducted in absence of external calcium (0% $Ca^{2+}$), $CaCl_2$ was replaced by 1 mM EGTA in the buffer. Before addition of DHA in the free fatty acid form, the cells were incubated for 15 min with the inhibitors of calcium channels as well as PKC. All test molecules were added in small volumes with no interruption in recordings. DHA was dissolved in ethanol [0.1% (v/v)] and used immediately or kept at −20°C, tightly sealed under the stream of nitrogen. Control cells were treated only with 0.1% vehicle (ethanol or DMSO). At this concentration, ethanol or DMSO did not modify basal $[Ca^{2+}]_i$.

**Measurement of $IP_3$ Production.** U937 cells ($10 \times 10^6$) were treated, for 1 min, in $Ca^{2+}$ buffer with one of the following: 10 $\mu$M DHA, 10 $\mu$M DHA methyl ester, or 0.1% vehicle (ethanol). After stimulation, cells were washed twice with ice-cold PBS. Cell suspension was then lysed with 20% ice-cold perchloric acid (0.2:1; v/v). After centrifugation, the supernatant was assayed for $IP_3$ production, according to the manufacturer's instructions (D-*myo*-$IP_3$; [$^3$H] Biotrak Assay System; Amersham). In brief, the assay was based on competition between [$^3$H]$IP_3$ (tracer) and unlabeled (cellular) $IP_3$ for binding to D-*myo*-$IP_3$ bovine adrenal binding protein. The bound $IP_3$ was separated from the free $IP_3$ by centrifugation, and the resulting supernatant, containing free $IP_3$, was then collected. Bound and unbound $IP_3$ were assessed by determining the radioactivity in a $\beta$-scintillation counter.

**In Vitro Determination of PKC Kinase Activity.** Before measuring PKC activity, U937 cells ($10 \times 10^6$) were treated with test molecules in $Ca^{2+}$ buffer. After stimulation, cells were lysed for 1 h at 4°C with 800 $\mu$l of ice-cold lysis buffer A (20 mM HEPES, pH 7.4, 2 mM EDTA, 125 mM NaCl, 1 mM DTT, 1 mM sodium orthovanadate, 0.5 mg/ml benzamidine, and 1% Nonidet P-40) in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotinin, 2.5 $\mu$g/ml pepstatin, and 5 $\mu$g/ml leupeptin). The lysates were centrifuged at 13,500$g$ for 15 min at 4°C, and supernatants were further used for immunoprecipitation. One milligram of protein was immunoprecipitated with 1 $\mu$g of either anti-PKC$\delta$ or anti-PKC$\gamma$ antibodies overnight at 4°C, and then 25 $\mu$l of Sepharose-A beads (PKC$\delta$) or 25 $\mu$l of Sepharose-G beads (PKC$\gamma$) were added and further incubated for 3 h at 4°C. PKC$\delta$- or PKC$\gamma$-containing immunoprecipitates were then washed three times with buffer A and, later on, with buffer B (20 mM HEPES, pH 7.4, 25 mM NaCl, and 1 mM DTT). Immunoprecipitates were then incubated at 32°C for 15 min in a total volume of 40 $\mu$l with buffer C (0.5 mM EGTA, 12.5 mM $MgCl_2$, and 20 mM HEPES, pH 7.4) containing 30 $\mu$g of histone H1 as substrate and 10 $\mu$M [$\gamma$-$^{32}$P]ATP (1.5 $\mu$Ci/tube). Samples were mixed (v/v) with loading buffer (125 mM Tris-HCl, pH 6.8, 10% $\beta$-mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromphenol blue), boiled for 5 min, and separated by SDS-PAGE. Phosphorylated histone H1 was quantified by PhosphorImager (GE Healthcare).

**Preparation of Vesicles for Cell-Free PKC$\delta$ and $\gamma$ Assay.** The required amounts of PS, fatty acids, Dic8, and phorbol 12-myristate 13-acetate (PMA) in chloroform were dried under the stream of nitrogen in a glass tube and solubilized in 20 mM Tris-HCl, pH 7.5, by vortexing and sonication (3 × 5 min) at 30°C.

**PKC$\delta$ and -$\gamma$ Assays in a Cell-Free System.** PKC activity was assayed by measuring the incorporation of $^{32}$P from [$\gamma$-$^{32}$P]ATP into MBP as described previously (Madani et al., 2001). PKC$\delta$ was diluted with a buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 5 mM DTT, 0.05% Triton X-100, and 50% glycerol). PKC$\gamma$ was diluted in the same buffer except that it contained 250 mM NaCl. For PKC$\delta$ assay, the reaction mixture (40 $\mu$l) contained 20 mM Tris-HCl at pH 7.4, 20 mM $MgCl_2$, 200 $\mu$g/ml MBP, 5 $\mu$g/ml PS, and 50 ng of PKC (final concentrations). For PKC$\gamma$ assay, the reaction mixture was the same except that it contained 100 $\mu$g/ml PS, 100 ng of PKC, and 100 $\mu$M $CaCl_2$. The reaction was initiated by the addition of 100 $\mu$M [$\gamma$-$^{32}$P]ATP (20–25 $\mu$Ci/ml) for 10 min at 30°C and stopped by spotting 20 $\mu$l of samples on phosphocellulose filters that were then washed three times (10 min) with 1% $H_3PO_4$ and transferred to a scintillation counter to determine [$^{32}$P]MBP activity.

**Measurement of Reactive Oxygen Species Generation.** Levels of reactive oxygen species (ROS) were measured in U937 cells by flow cytometry as the fluorescence of ethidium, which is the oxidation product of dihydroethidine (DHE). DHE enters cells and is oxidized by ROS, with a relative selectivity for $O_2^-$ (Benov et al., 1998), to form ethidium, which intercalates with DNA and causes nucleus to exhibit a red shift in fluorescence. DHE and ethidium are retained within cells with minimal leakage. In brief, after stimulation in $Ca^{2+}$ medium, cells were incubated for 15 min at 37°C with 6.6 $\mu$M DHE (Sigma-Aldrich). Samples were cooled (4°C) to stop the reaction and were then immediately analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA). Signals were obtained using a 585-nm bandpass filter (FL-2 channel). We also conducted a few experiments by employing DHE in PTI spectrofluorometer, where we recorded the fluorescence intensities at 306 nm (excitation filters) and 609 nm (emission filters). We observed the same results by the two techniques.

**Measurement of Caspase-3 Activity.** Cells were incubated in the lysis buffer [20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate; antiprotease cocktail (2 $\mu$l in 1 ml of buffer)] for 30 min at 4°C and centrifuged (13,000$g$, 15 min, 4°C). The resulting supernatant (50 $\mu$g of protein) was incubated in assay buffer (100 mM HEPES, pH 7.0, 1 mM EDTA, 0.1% CHAPS, 10% glycerol, and 20 mM dithiothreitol) in the presence of 100 $\mu$M fluorogenic peptide substrate, Ac-DEVD-7-amino-4-methylcoumarin (France Biochem, Meudon, France). 7-Amino-4-methylcoumarin released from the substrate was excited at 380 nm to measure emission at 460 nm. Fluorescence was monitored continuously at 37°C for 30 min in a dual luminescence fluorimeter (MicroTek OS; Bio-Tek Kontron Instruments, Winooski, VT).

TABLE 1
Effect of DHA on the production of $IP_3$ in U937 cells

| Treatment | $IP_3$ Production | $n$ |
|---|---|---|
| | *pmol/min/10$^6$ cells* | |
| Control | 1.426 ± 0.06 | 4 |
| DHA (10 $\mu$M) | 3.41 ± 0.25*** | 4 |
| DHA-meth (10 $\mu$M) | 1.429 ± 0.02$^{NS}$ | 4 |

*N*, number of experiments performed; NS, insignificant difference compared with control (vehicle alone).
*** $P < 0.001$ compared with control (vehicle alone).

**Assay of Apoptosis.** Morphological changes in nuclear chromatin of cells undergoing apoptosis were monitored with a fluorescent microscope (Nikon, Champigny, France) after staining the cells with Hoechst 33342 dye (Sigma-Aldrich). In brief, untreated and treated cells ($1 \times 10^6$) were collected, washed with PBS, incubated with 1 $\mu$g/ml Hoechst 33342 for 15 min, at 37°C, and then mounted on glass slides and observed under a microscope. The percentage of apoptotic cells (chromatin condensation and nuclear fragmentation) was determined by counting at least 300 cells from randomly selected fields in each experiment.

**Statistical Analysis.** Statistical analysis of data were 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 (LSD) test.

## Results

**DHA Increases $[Ca^{2+}]_i$ in a Dose-Dependent Manner.** Figure 1A shows that DHA evoked a dose-dependent calcium increase in U937 monocytic cells. The calcium increase was maximal at a concentration of 10 $\mu$M, and there was no statistical difference in calcium peaks between 10 and 20 $\mu$M (Fig. 1B). We therefore used DHA at 10 $\mu$M in the subsequent experiments. In addition, calcium peak was transient, apparent between 40 s and 1 min. Cell viability, assessed by trypan blue exclusion test, was 98 ± 2% after DHA treatment.

**DHA Recruits Calcium via PLC-Mediated Mechanism from Intracellular Pool and via SOC Influx.** The absence of calcium in the extracellular medium considerably decreased the DHA-evoked amplitude of calcium peak compared with that in the presence of $Ca^{2+}$ in the extracellular medium (Fig. 1, C and D). To evaluate whether DHA evokes increases in $[Ca^{2+}]_i$ via the production of free $IP_3$, U937 cells were preincubated with U-73122, an inhibitor of phospholipase C, PLC (Bonin and Khan, 2000) in 100% $Ca^{2+}$ or 0% $Ca^{2+}$ medium. U-73122 significantly curtailed DHA-induced increases in $[Ca^{2+}]_i$ in 100% $Ca^{2+}$ buffer (Fig. 1, E and F). Likewise, the response evoked by this fatty acid in 0% $Ca^{2+}$ medium was significantly diminished in cells pretreated with U-73122 (Fig. 1, E and F). $IP_3$ release in cells stimulated with DHA was further assessed. As shown in Table 1, incubation of U937 cells with DHA for 1 min of stimulated significant production of $IP_3$. On the other hand, cells treated with DHA-meth, a DHA analog in which the −COOH end was replaced by a methyl ester group, did not show any significant production of $IP_3$. All together, these results suggest that DHA stimulates $IP_3$ production via a PLC-mediated mechanism.

To explore the mechanisms of $Ca^{2+}$ influx induced by DHA, different calcium channel blockers were tested. Pretreatment of U937 cells with SOC influx inhibitors (e.g., econazole and SKF-96365) significantly reduced DHA-induced $Ca^{2+}$ influx (Fig. 2, A and B). However, incubation of cells with either L-type calcium channel blockers (nifedipine and verapamil) or N-type calcium channel blockers ($\omega$-conotoxin), failed to influence DHA-induced increases in $[Ca^{2+}]_i$ (Fig. 2B). Pretreatment of U937 cells with TA9, an inhibitor of $Ca^{2+}$ release-activated $Ca^{2+}$ (CRAC) channels, reduced by 51.07 ± 7.37% the DHA-induced calcium peak (Fig. 2, A and B).

As a control, we employed thapsigargin (TG), known to increase $[Ca^{2+}]_i$ via the inhibition of the endoplasmic reticulum $Ca^{2+}$-ATPase (Thastrup et al., 1990). Figure 2 (C and D) shows that econazole, SKF-96365, and TA9, as expected, significantly blocked TG-induced rise in $[Ca^{2+}]_i$. L-type calcium channel blockers or N-type calcium channel blocker failed to influence TG-induced increases in $[Ca^{2+}]_i$ (Fig. 2D), which is in agreement with the study of Willmott et al. (1996), showing that TG-induced rises in $[Ca^{2+}]_i$ are not mediated by L-type or N-type calcium channels.

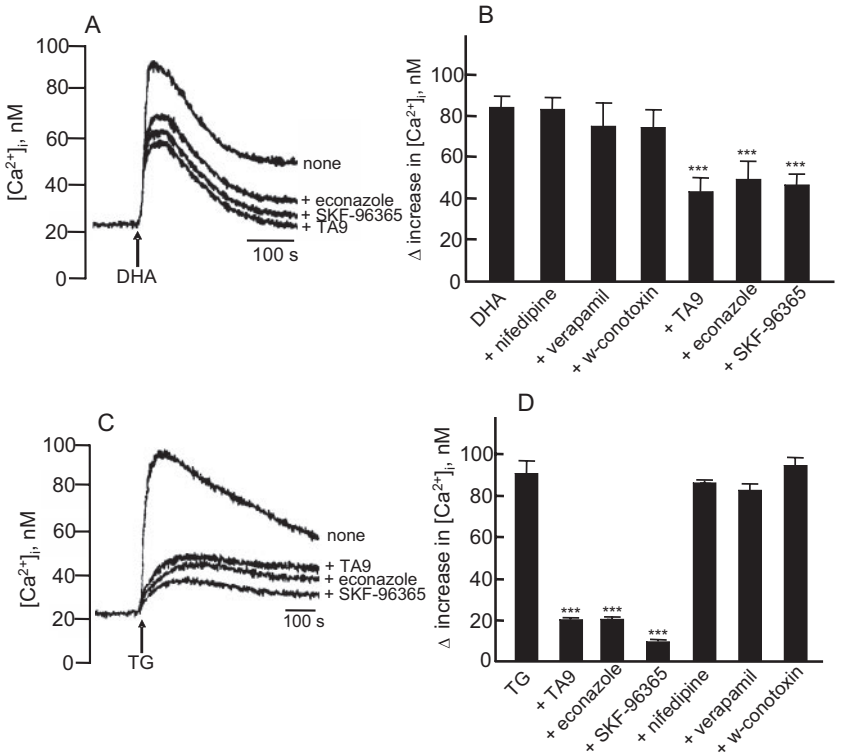


**Fig. 2.** Effects of calcium channel blockers on DHA- and TG-induced increases in $[Ca^{2+}]_i$. Fura-2-loaded cells were preincubated for 15 min at room temperature in the presence or absence of calcium channel blockers: nifedipine, 10 $\mu$M; verapamil, 10 $\mu$M; $\omega$-conotoxin, 1 $\mu$M; TA9, 10 $\mu$M; econazole, 15 $\mu$M; and SKF-96365, 30 $\mu$M. The arrowheads indicate the addition of DHA (10 $\mu$M) or TG (500 nM) into the cuvette. All experiments were performed in $Ca^{2+}$-containing medium. Data in histograms (B and D), represented as means ± S.D., were analyzed by employing LSD test of significance. Data (B and D) are significantly different compared with DHA-treated cells (***, $p < 0.001$). A, typical profile of the changes in DHA-induced calcium peak after preincubation or not with TA9, econazole, and SKF-96365. B, Δ increases in $[Ca^{2+}]_i$ evoked by DHA in the presence or absence of calcium channel inhibitors ($n = 3$). C, typical profile of the changes in TG-induced calcium peak after preincubation with TA9, econazole, and SKF-96365. D, Δ increases in $[Ca^{2+}]_i$ evoked by TG in the presence or absence of calcium channel blockers ($n = 6$).

**Effects of PKC Inhibitors on DHA-Induced Increases in $[Ca^{2+}]_i$.** U937 cells have been reported to possess $Ca^{2+}$-dependent PKCs ($\alpha$, $\beta$, and $\gamma$), $Ca^{2+}$-independent PKCs ($\delta$ and $\varepsilon$) and an atypical PKC isoform, $\zeta$ (Kiley et al., 1994). To investigate the role of PKC in the DHA-induced calcium response, we first employed three known inhibitors of PKC (i.e., GF109203X, calphostin C, and chelerythrine), which exhibit different specificity of actions. GF109203X acts as a competitive inhibitor for the ATP-binding site of PKC. Calphostin C is known to inhibit PKC by competing at the binding site of diacylglycerol and phorbol esters in the regulatory domain of PKC. Chelerythrine is a potent, selective, and cell-permeable inhibitor of PKC that acts on the catalytic domain of PKC. As shown in Fig. 3A and B, pretreatment of cells with calphostin C (Cal-C), GF109203X (GF109), or chelerythrine (CHE) (Fig. 3B) significantly diminished the DHA-induced calcium response.

HBDDE, a selective inhibitor of both PKC$\alpha$ and PKC$\gamma$ (Kashiwada et al., 1994), and rottlerin, a specific inhibitor of PKC$\delta$ (Gschwendt et al., 1994), significantly curtailed the DHA-induced $Ca^{2+}$ rise (Fig. 3, C and D), though preincubation of cells with Gö-6976, a selective inhibitor of both PKC$\alpha$ and PKC$\beta$ (Martiny-Baron et al., 1993) or with hispidin, a selective inhibitor of PKC$\beta$I and PKC$\beta$II (Gonindard et al., 1997), failed to do so (Fig. 3D).

**Effects of PKC Inhibitors on TG-Induced Increases in $[Ca^{2+}]_i$.** To gain insight into the specificity of the action of DHA, we determined whether TG-induced calcium influx required PKC activation. Preincubation of cells with calphostin C, GF109203X, or chelerythrine significantly decreased TG-induced calcium peak (Fig. 3, E and F). We further observed that HBDDE failed to curtail TG-evoked SOC influx in these cells (Fig. 3F).

**Effects of PKC Inhibitors on PMA-Induced Increases in $[Ca^{2+}]_i$.** PMA, an activator of $Ca^{2+}$- and DAG-dependent PKC (Kashiwada et al., 1994), was used to assess the specificity of DHA regarding the implication of PKC. PMA induced a slight increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$, 23,28 $\pm$ 7.48 nM) as shown in Fig. 3 (G and H). It was also observed that in presence of the PKC inhibitors (i.e., calphostin C, GF109203X, chelerythrine, or rottlerin), PMA-induced calcium peak was almost completely abolished. However, Fig. 3H shows that calcium influx was not influenced by preincubation of cells with Gö-6976, hispidin, or HBDDE.

**DHA Up-Regulates in Vitro PKC$\gamma$ and PKC$\delta$ Kinase Activities.** The effects of DHA on the kinase activities of PKC$\gamma$ and PKC$\delta$ were examined by an in vitro kinase assay. Using histone H1 and [$\gamma$-$^{32}$P]ATP as substrates for PKC, we observed that DHA induced a significant increase in PKC$\gamma$ and PKC$\delta$ activities (Fig. 4, A and B), at 40 s and 1 min, time

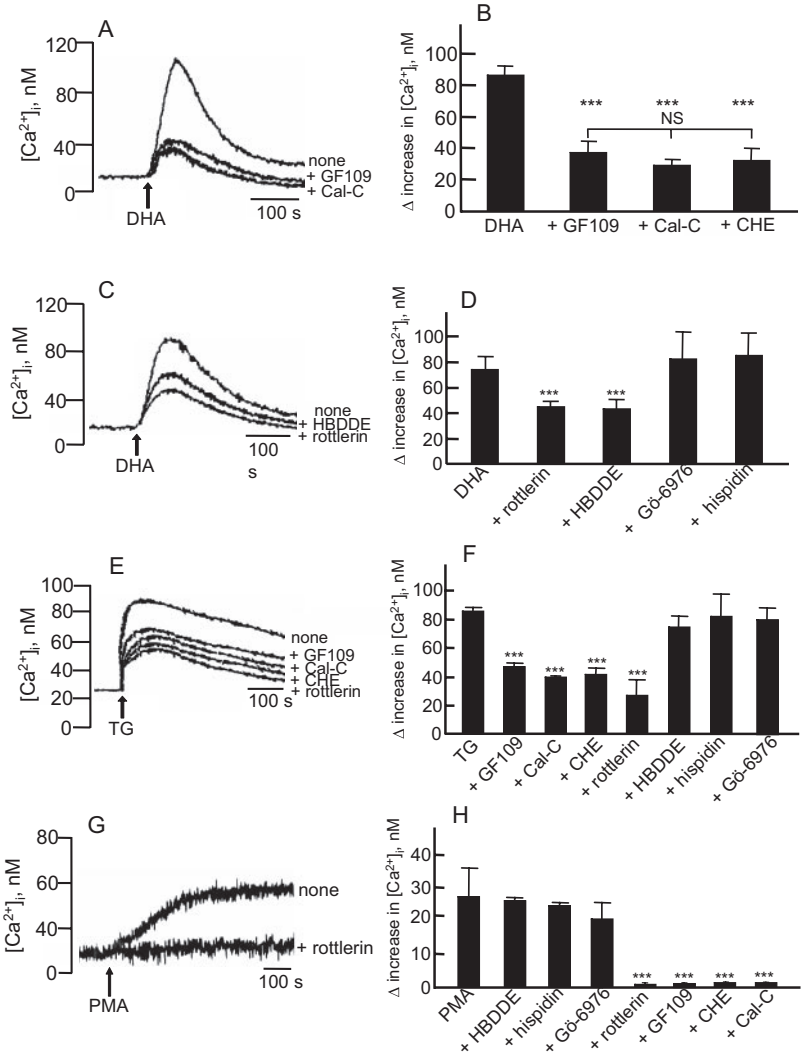


**Fig. 3.** Effects of PKC inhibitors on DHA-, TG-, and PMA-induced increases in $[Ca^{2+}]_i$. Fura-2 loaded U937 cells were preincubated in the presence or absence of various PKC inhibitors: GF109203X (GF109), 10 $\mu$M; calphostin C (Cal-C), 100 nM, chelerythrine (CHE), 10 $\mu$M; 2,2′,3,3′,4,4′-hexahydroxy-1–1′-biphenyl-6,6′-dimethanoldiethyl ether (HBDDE), 100 $\mu$M; hispidin, 5 $\mu$M; Gö-6976, 1 $\mu$M; and rottlerin, 3 $\mu$M. Cells were preincubated for 15 min at room temperature with the various inhibitors before the addition of DHA (10 $\mu$M), TG (500 nM), or PMA (1 $\mu$M), indicated by arrow heads. All experiments were performed in $Ca^{2+}$-containing medium. Data in histograms (B, D, F, and H), represented as means $\pm$ S.D., were analyzed by employing LSD test of significance. Data (***, $p < 0.001$) are significantly different as compared with DHA-treated cells (B and D), TG-treated cells (F) or PMA-treated cells (H). NS, non-significant differences. A, typical profile of the changes in DHA-induced calcium peak in presence or absence of PKC inhibitors (GF109 and Cal-C). B, $\Delta$ increases in $[Ca^{2+}]_i$ evoked by DHA in the presence or absence of GF109, Cal-C, or CHE ($n = 10$). C, original tracings show the influence of rottlerin and HBDDE on DHA-induced rises in $[Ca^{2+}]_i$. D, $\Delta$ increases in $[Ca^{2+}]_i$ evoked by DHA in the presence or absence of PKC isoform-specific inhibitors ($n = 12$). E, representative experimental curves showing inhibition of TG-induced calcium peak by the PKC inhibitors. F, $\Delta$ represents the amplitude of the calcium response triggered by TG in presence or absence of the various PKC inhibitors ($n = 15$). G, representative experimental curve showing inhibition of PMA-induced calcium peak by rottlerin. H, $\Delta$ represents the amplitude of the calcium response triggered by PMA in presence or absence of the various PKC inhibitors ($n = 15$).

periods corresponding to DHA-evoked calcium peak (Fig. 1A). TG-treated cells were also assessed for PKCγ and PKCδ kinase activity. As observed for DHA, TG at 2 and 3 min (time periods corresponding to TG-evoked calcium peak; see Fig. 2C), significantly increased PKCδ, but not PKCγ, activities (Fig. 4, C and D). However, PMA increased both PKCγ and PKCδ activities (Fig. 4, E and F).

**In a Cell-Free System, DHA Specifically Seems to Activate PKCγ and -δ by Interacting with the Phosphatidylserine Binding Site.** To determine whether DHA could directly activate PKCγ and -δ, we used the sonic dispersion of lipids/vesicles that have been previously shown suitable to determine the specificity and stoichiometry of PKC lipid interaction. Optimal concentration of PS to activate PKCγ and -δ were found to be 100 μg/ml for PKCγ, and 5 μg/ml for PKCδ (data not shown). These concentrations were thus used for further experiments. As shown in Figs. 5A and 6A (dashed lines), DHA activated directly both PKCγ and -δ in a dose-dependent fashion in the absence of PS. PKCγ and -δ activation was maximal for 10 μM DHA. On the other hand, DHA at high concentrations was found to decrease the activation of both PKCγ and -δ. In the presence of PS (Figs. 5A and 6A, solid lines), DHA-induced PKCγ and -δ activation was 2-fold higher than that in the absence of PS. PKC activity was also found maximal at 10 μM DHA under these experimental conditions. To validate our experimental approach, we used, as a control, Dic8, a cell-permeable analog of DAG that has been used to explore the cell signaling mechanisms involving DAG/PKC pathway (Khan and Hichami, 1999). As shown in Figs. 5A and 6A (*insets*), PKCγ and -δ activities were significantly enhanced in the presence of vesicles containing Dic8 compared with that in the presence of PS alone.

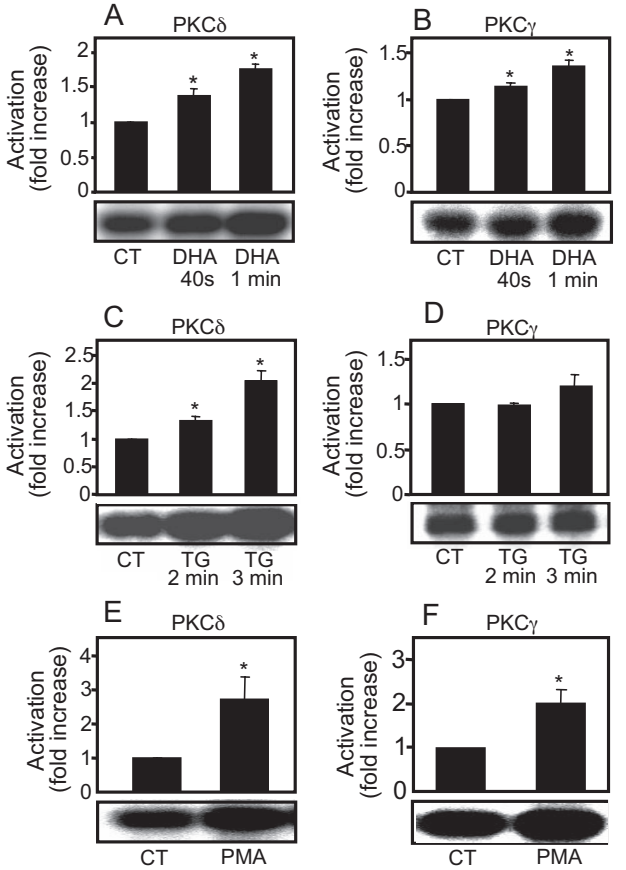


**Fig. 4.** Effects of DHA, TG, and PMA on PKCγ and PKCδ activities in U937 cells. Cells ($10 \times 10^6$) were treated with DHA (A and B) or TG (C and D) for the indicated times that corresponded to DHA- and TG-induced calcium peaks in Fura-2 loaded cells. Cells were also treated with PMA for 20 min (E and F). PKCγ and PKCδ activities were assessed as described under *Materials and Methods*. Histone H1 phosphorylation was visualized by Western blotting (representative experiments shown in bottom panels) and quantified by a PhosphorImager [upper panels, means ± S.D. (bars), $n = 6$]. *, $p < 0.05$ compared with controls (vehicles alone). Treatments of cells with test molecules were performed in $Ca^{2+}$-containing medium.

PKC activation by free fatty acids has been shown to be independent or dependent of the PS binding site (Kanno et al., 2006). To determine whether DHA interacts with the PS binding site to activate PKCγ or -δ, competition experiments were conducted with DHA and PS. In these experiments, we used DHA at 10 μM (optimal concentration for PKCγ and -δ activation) and increasing concentrations of PS. As shown in Figs. 5B and 6B, DHA-induced PKCγ and PKCδ activation was significantly reduced in the presence of 400 and 50 μg/ml PS, respectively, thus suggesting that DHA activates these PKC isoforms by binding to the PS binding site.

To further support the notion that DHA may act through the PS binding site, the ability of DHA to interact with phorbol ester/DAG binding site that is present in both conventional and novel PKCs was next evaluated. Hence, competition experiments with DHA and PMA were performed. In a first set of experiments, vesicles were prepared with increasing concentrations of DHA, in the presence of 3.2 μM PMA (for PKCγ) or 1.6 μM PMA (for PKCδ). DHA dose-dependently and significantly potentiated PMA-induced activation of PKCδ (Fig. 6C) and PKCγ (Fig. 5C). In both cases, DHA was found to be the most effective at 10 μM. In a second set of experiments, vesicles were prepared with increasing concentrations of PMA, in the presence of DHA. As shown in Fig. 5D, PMA dose-dependently and significantly potentiated DHA-induced PKCγ activation. Maximal potentiation was observed with PMA at 3.2 μM. Similar potentiation of DHA-induced PKCδ activation was observed; however, PMA at 1.6 μM was found to be the most effective for this PKC isoform (Fig. 6D).

To rule out the possibility that DHA could act via the ATP binding site of PKC, competition studies with ATP were performed. In these experiments, vesicles were prepared with PS in the presence or absence of DHA, and PKC activity was assayed as a function of increasing concentrations of ATP added to the reaction assay. As shown in Fig. 5E, PKCγ activity was higher (~2-fold) in vesicles containing DHA plus PS than that with PS alone, at each concentration of ATP used. Moreover, ATP at 100 μM was the optimal concentration for maximal PKC activation under these conditions. No statistical differences were observed between PS- or DHA+PS-induced PKCγ activation at 100 μM ATP and at 500 μM ATP. Similar results were obtained with regard to PKCδ activity under the same experimental conditions (Fig. 6E).

To evaluate the specificity of DHA-induced activation of PKCγ and -δ, a series of structurally related long-chain fatty acids were further used and examined for their efficacy as activators of both PKC isoforms. The details regarding the structure of the following compounds can be seen elsewhere (Mirnikjoo et al., 2001). DHA-meth was used to determine

whether the carboxylic end of DHA molecule is required for PKC activation (Fig. 7). DPEA is a 22-carbon n-3 fatty acid with five double bonds. EPA is a 21-carbon n-3 fatty acid with five double bonds. AA is a 20-carbon fatty acid that lacks the n-3 moiety and thus belongs to the n-6 PUFA family (Fig. 7). DPEA, EPA, and AA were used to determine whether the length of the carbon chain or degree of unsaturation could influence PKC activation. As shown in Fig. 5F, AA, DHA-meth and EPA (10 $\mu$M) failed to potentiate PS-induced PKC$\gamma$ activation. However, DPEA was found to decrease significantly PS- induced PKC$\gamma$ activation. No significant modification of PS-induced PKC$\delta$ activation was found in the presence of AA, DHA-meth, EPA, or DPEA (Fig. 6F).

**DHA Induces Apoptosis through ROS Generation and Caspase-3 Activation.** It has been shown that n-3 PUFAs can initiate apoptosis through different processes, including the modulation of free radical and ROS production (Larsson et al., 2004). To determine whether DHA induces apoptosis in U937 cells, we examined its effect on ROS production in the periods that correspond to DHA-induced calcium peak (Fig. 1A). A significant increase in ROS was observed after 1 min of treatment with DHA (DHA, 87 $\pm$ 4.57%; control, 17.85 $\pm$ 7.96%; $p < 0.001$), and the level of ROS was decreased as a function of time thereafter, up to 1 h of incubation (Fig. 8A). A consequence of mitochondrial damage during apoptosis is the activation of caspase-3. Thus, we investigated whether activity of caspase-3 was induced by DHA treatment. As represented in Fig. 8A, activity of caspase-3 was significantly enhanced in DHA-treated cells over untreated cells after 1 h of incubation. It is noteworthy that we noticed an inverse correlation between ROS production and caspase-3 activity; i.e., the peak of activated caspase-3 took place after decline of the ROS peak. As a positive control, we used etoposide (VP16), an important chemotherapeutic agent that has been shown to induce apoptosis independently of ROS production but via a caspase-3-dependent process (Abdelhaleem, 2002). Under our experimental conditions, after treatment with VP16 (50 $\mu$M, 4 h), caspase-3 activity was increased by 6-fold (Fig. 8A, inset).

To study a possible relationship between the DHA-induced $Ca^{2+}$ rise via activation of PKC$\gamma$ and PKC$\delta$ and their implication in ROS production and caspase-3 activation, cells were treated with DHA in the presence or absence of SOC channel blockers and inhibitors of these PKC isoforms in $Ca^{2+}$ medium. In Fig. 8B, cells were exposed for 1 min to DHA (10 $\mu$M) and then analyzed by flow cytometry to determine ROS production. This time-period was chosen because it corresponds to maximal induction of ROS by DHA (Fig. 8A), and to DHA-induced $Ca^{2+}$ peak in fura-2 loaded cells (Fig. 1A) and DHA-induced PKC$\gamma$ and -$\delta$ activation, as determined by in

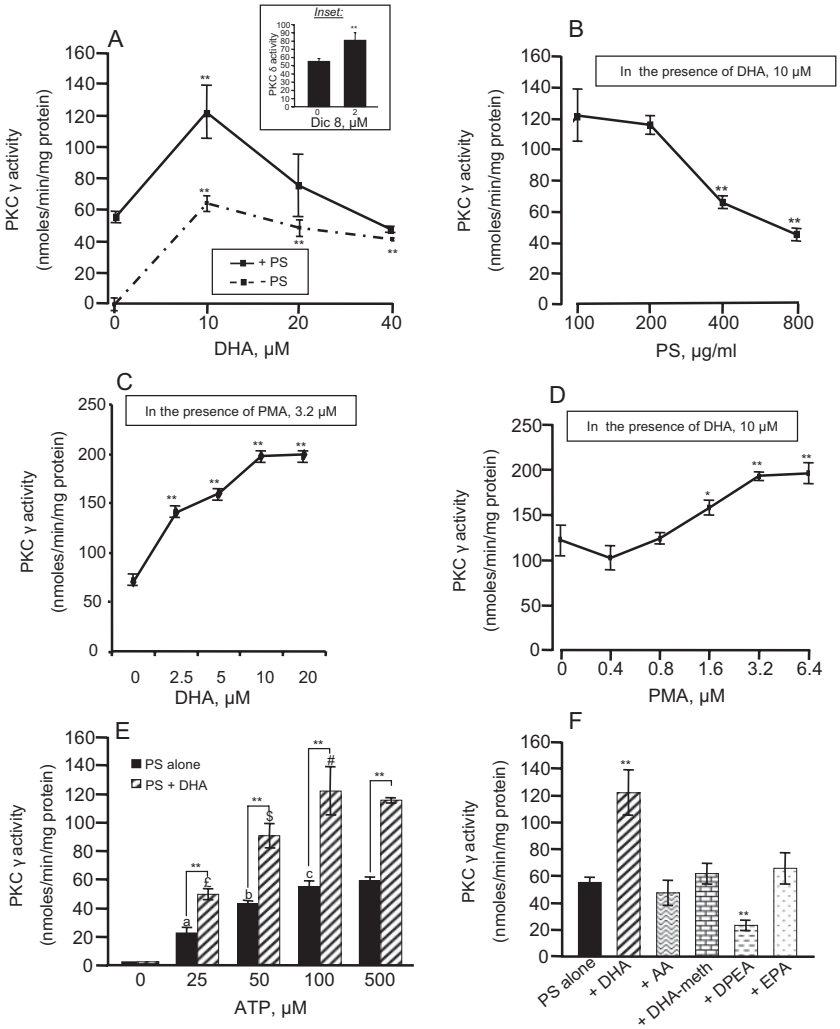


**Fig. 5.** Effects of DHA on PKC$\gamma$ activity in a cell-free system. Each point represents the mean $\pm$ S.E.M. of PKC activity (nanomoles per minute per milligram of protein) of at least two independent experiments, each performed in triplicate. A, PKC$\gamma$ activity was quantified in the presence of $CaCl_2$ (100 $\mu$M) and vesicles containing increasing concentrations of DHA alone (dashed line) or with PS (100 $\mu$g/ml; full line) as described under *Materials and Methods*. Data are significantly different between DHA-containing vesicles and vesicles without DHA (**, $p < 0.001$). Inset, effect of dioctanoyl-*sn*-glycerol (Dic8) on PKC$\gamma$ activity (nanomoles per minute per milligram of protein). Lipid vesicles were prepared with PS (100 $\mu$g/ml) alone or with Dic8 (2 $\mu$M), as described under *Materials and Methods*. Data are significantly different between Dic8-containing vesicles and vesicles containing PS alone (**, $p < 0.001$). B, PKC$\gamma$ activation induced by DHA (10 $\mu$M) in the presence of increasing concentrations of PS. Data are significantly different between vesicles containing DHA (10 $\mu$M) plus PS (100 $\mu$g/ml) versus vesicles containing DHA (10 $\mu$M) plus PS at 400 or 800 $\mu$g/ml (**, $p < 0.001$). C, lipid vesicles were prepared with increasing concentrations of DHA as indicated, in the presence of PMA (3.2 $\mu$M) and PS (100 $\mu$g/ml). **, $p < 0.001$; DHA-containing vesicles versus vesicles containing no DHA. D, lipid vesicles were prepared with increasing concentrations of PMA as indicated, in the presence of DHA (10 $\mu$M) and PS (100 $\mu$g/ml). *, $p < 0.05$; **, $p < 0.001$ compared with vesicles containing no PMA. E, lipid vesicles were prepared with DHA (10 $\mu$M) plus PS (100 $\mu$g/ml) or with PS (100 $\mu$g/ml) alone as described under *Materials and Methods*. PKC$\gamma$ activity was determined toward MBP in vesicles as a function of increasing concentrations of ATP added to the reaction assay. Data are significantly different compared with vesicles containing no DHA (**, $p < 0.001$), PS alone/ATP 0 $\mu$M (a, $p < 0.001$), PS alone/ATP 25 $\mu$M (b, $p < 0.001$), PS alone/ATP 50 $\mu$M (c, $p < 0.05$), PS + DHA/ATP 0 $\mu$M (£, $p < 0.001$), PS + DHA/ATP 25 $\mu$M ($, $p < 0.001$), and PS + DHA/ATP 50 $\mu$M (#, $p < 0.05$). F, lipid vesicles were prepared with different fatty acids (i.e., DHA, AA, DHA-meth, DPEA, and EPA) at 10 $\mu$M in the presence of PS (100 $\mu$g/ml) or with PS (100 $\mu$g/ml) alone. Data are significantly different compared with PS (100 $\mu$g/ml) alone (**, $p < 0.001$).

vitro kinase assay (Fig. 4, A and B). The Fig. 8B shows that blocking SOC channels or PKCγ and PKCδ resulted in a significant decrease in DHA-induced ROS production (percentage of cells producing ROS: DHA, 70.21 ± 9.11%; DHA + rottlerin, 46.13 ± 12.13%; DHA + HBDDE, 50.38 ± 5.55%; DHA + TA9, 35.46 ± 5.64%; DHA + econazole, 27.84 ± 5.55%). It is noteworthy that verapamil, hispidin, and Gö-6976 failed to diminish the ROS production. It is also noteworthy that in these cells, the DHA-induced ROS was significantly curtailed by CCCP, a mitochondrial uncoupler, suggesting that the ROS production is well controlled and produced by this organelle. The CCCP alone did not influence the ROS production (data not shown). Figure 8C shows that DHA-induced peak of activated caspase-3 was also significantly diminished by pretreatment with HBDDE, rottlerin, and TA9 (and also by econazole, data not shown). Furthermore, verapamil, hispidin, and Gö-6976 also failed to inhibit DHA-induced caspase-3 activation in these cells (Fig. 3C).

**DHA Induces Morphological Changes in Nuclear Chromatin.** To confirm that DHA, per se, does effectively trigger apoptosis, cells were treated with or without DHA in growth medium containing only 0.5% FCS to avoid incorporation of the fatty acid into membrane phospholipids and were then examined for typical apoptotic chromatin condensation after Hoechst 33342 staining. Hence, cells were treated for 72 h to assess the long-term effects of DHA-induced early events of apoptosis. As a positive control, cells were also treated with VP16 (0.5 μM) up to 72 h. Until 48 h of treatment with DHA or VP16, no significant increase in the percentage of apoptotic cells was observed (data not shown). However, at 48 h, a significant but discrete increase in the number of apoptotic cells was seen in DHA-treated cells, whereas in VP16-treated cells, more than 50% of them displayed nuclear chromatin condensation (Fig. 9A). At 72 h, 60% of DHA-treated cells showed typical nuclear chromatin condensation and in VP16-treated cells, 90% of cells were apoptotic (Fig. 9, A and B). In control cells, which were treated only with 0.1% vehicle (ethanol or DMSO), less than 10% of apoptotic cells were observed at 72 h of culture.

## Discussion

The present study shows that DHA, in U937 cells, evoked a rapid increase in $[Ca^{2+}]_i$ that was significantly curtailed in 0% $Ca^{2+}$ medium, suggesting that DHA, in part, mobilizes $Ca^{2+}$ from intracellular pool. U-73122, a pharmacological inhibitor of PLC, in 0% $Ca^{2+}$ medium, completely abolished the DHA-induced rise in $[Ca^{2+}]_i$. Besides, DHA, but not

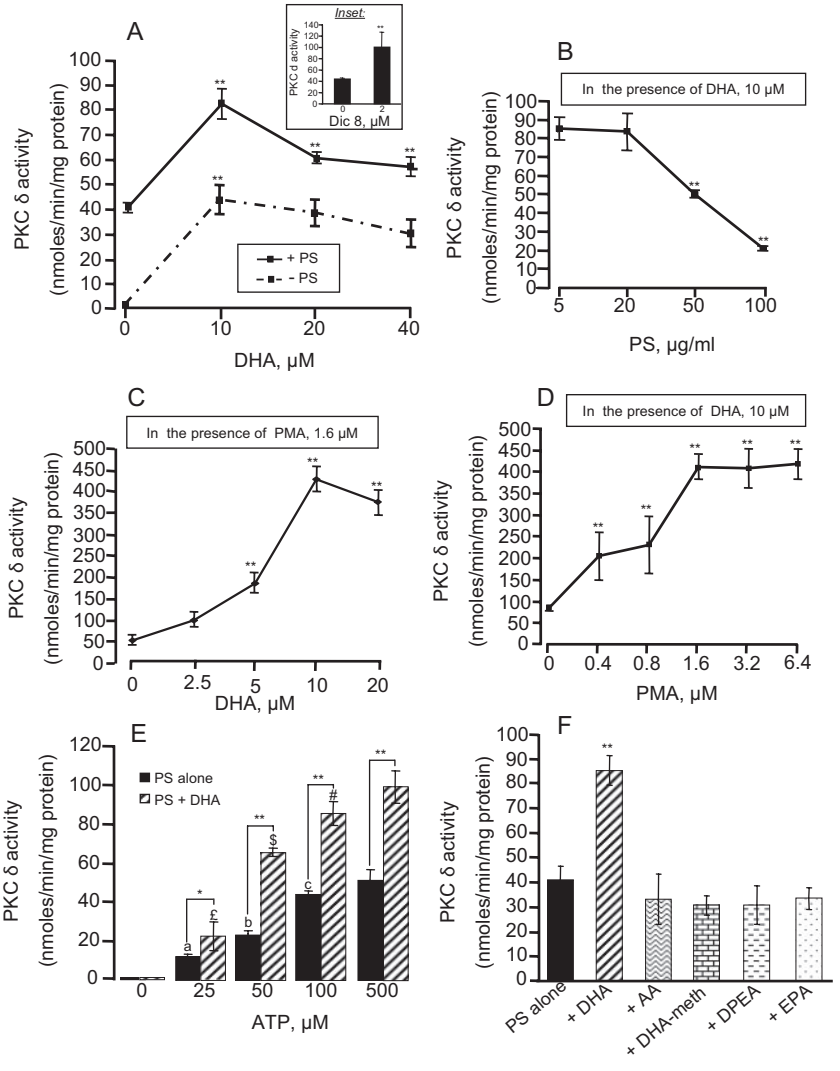


**Fig. 6.** Effects of DHA on PKCδ activity in a cell-free system. Each point represents the mean ± S.E.M. of PKC activity (nanomoles per minute per milligram of protein) of at least two independent experiments, each performed in triplicate. A, PKCδ activity was quantified in the absence of $CaCl_2$ and vesicles containing increasing concentrations of DHA alone (dashed line) or with PS (5 μg/ml; full line) as described under *Materials and Methods*. Data are significantly different between DHA-containing vesicles and vesicles containing no DHA (**, $p < 0.001$). Inset, effect of Dic8 on PKCδ activity (nanomoles per minute per milligram of protein). Lipid vesicles were prepared with PS (5 μg/ml) alone or with Dic8 (2 μM) as described under *Materials and Methods*. Data are significantly different between Dic8-containing vesicles and vesicles containing PS alone (**, $p < 0.001$). B, PKCδ activation induced by DHA (10 μM) in the presence of increasing concentrations of PS. Data are significantly different between vesicles containing DHA (10 μM) plus PS (5 μg/ml) versus vesicles containing DHA (10 μM) plus PS at 50 or 100 μg/ml (**, $p < 0.001$). C, lipid vesicles were prepared with increasing concentrations of DHA as indicated, in the presence of PMA (1.6 μM) and PS (5 μg/ml). **, $p < 0.001$, DHA-containing vesicles versus vesicles containing no DHA. D, lipid vesicles were prepared with increasing concentrations of PMA as indicated, in the presence of DHA (10 μM) and PS (5 μg/ml). **, $p < 0.001$ compared with vesicles containing no PMA. E, lipid vesicles were prepared with DHA (10 μM) plus PS (5 μg/ml) or with PS (5 μg/ml) alone as described under *Materials and Methods*. PKCδ activity was determined toward MBP in vesicles as a function of increasing concentrations of ATP added to the reaction assay. Data are significantly different compared with vesicles containing no DHA (**, $p < 0.001$), PS alone/ATP 0 μM (a, $p < 0.001$), PS alone/ATP 25 μM (b, $p < 0.001$), PS alone/ATP 50 μM (c, $p < 0.05$), PS + DHA/ATP 0 μM (£, $p < 0.05$), PS + DHA/ATP 25 μM ($, $p < 0.001$), and PS + DHA/ATP 50 μM (#, $p < 0.001$). F, lipid vesicles were prepared with different fatty acids (i.e., DHA, AA, DHA-meth, DPEA and EPA) at 10 μM in the presence of PS (5 μg/ml) or with PS (5 μg/ml) alone. Data are significantly different compared with PS (5 μg/ml) alone (**, $p < 0.001$).

DHA-methyl ester, induced $IP_3$ production in these cells. These observations demonstrate that DHA, at first hand, activates PLC, which hydrolyzes the phosphatidylinositol-4,5-bisphosphate and releases free $IP_3$. The free $IP_3$, produced by DHA, recruits calcium from the endoplasmic reticulum pool. However, the mechanism of action of DHA to activate PLC remains to be studied in future.

DHA-induced calcium response was also found to be significantly curtailed by the specific CRAC channel inhibitor TA9, as well as by SOC channel blockers, thus suggesting that DHA evokes an initial rise in $[Ca^{2+}]_i$ from intracellular pool followed by the opening of SOC channels in these cells. This is in close agreement with the findings of Gamberucci et al. (1997), who have shown that in vitro addition of EPA and DHA significantly mobilizes $Ca^{2+}$ from intracellular stores.

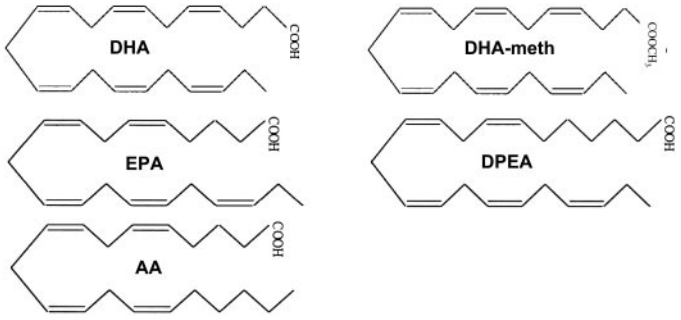


**Fig. 7.** Structure of DHA and control isomers. A graphic representation of fatty acids used in the experiments is shown. DHA (22:6 n-3), DHA-methyl (DHA analog in which the −COOH end has been replaced by a methyl ester group), EPA (20:5 n-3), DPEA (22:5 n-3), and AA (20:4 n-6).

We have also previously shown that DHA-evoked $Ca^{2+}$ mobilization from intracellular stores is followed by SOC influx through the opening of CRAC channels in Jurkat T cells (Bonin and Khan, 2000).

How DHA exactly controls the opening of CRAC channels is still not known. We hypothesized that this process could be mediated by PKC, shown to control the opening of various $Ca^{2+}$ channels, including SOC channels (Albert and Large, 2002; Vanden Abeele et al., 2003). Moreover, several studies have shown that PUFAs may modulate the activity of PKC (Madani et al., 2001). DHA-induced $Ca^{2+}$ influx was mediated through PKCγ- and PKCδ-dependent pathways because HBDDE, an inhibitor of PKCα, PKCγ, and rottlerin, an inhibitor of PKCδ, significantly curtailed DHA-induced increases in $[Ca^{2+}]_i$. Our results corroborate the observations of Heo et al. (2006), who have shown, in mouse embryonic stem cells, that PKCγ and PKCδ inhibition prevented EGF-induced increases in $[Ca^{2+}]_i$, thus suggesting a positive regulation of $Ca^{2+}$ channels by these PKC isoforms. It is noteworthy that TG-induced increases in $[Ca^{2+}]_i$ were also significantly curtailed by broad spectrum PKC inhibitors and rottlerin, but not by HBDDE, suggesting that only PKCδ mediated the TG-induced increases in $[Ca^{2+}]_i$ in these cells. This DHA-specific mode of action was further supported by the use of PMA which was found to evoke an increase in $[Ca^{2+}]_i$ in a PKCδ-dependent fashion.

In vitro kinase assays show that DHA, in 40-s to 1-min time periods coinciding with DHA-evoked calcium peaks in Fura-2 ratiometry experiments, induced significant PKCγ and PKCδ activities. Moreover, TG was found only to induce

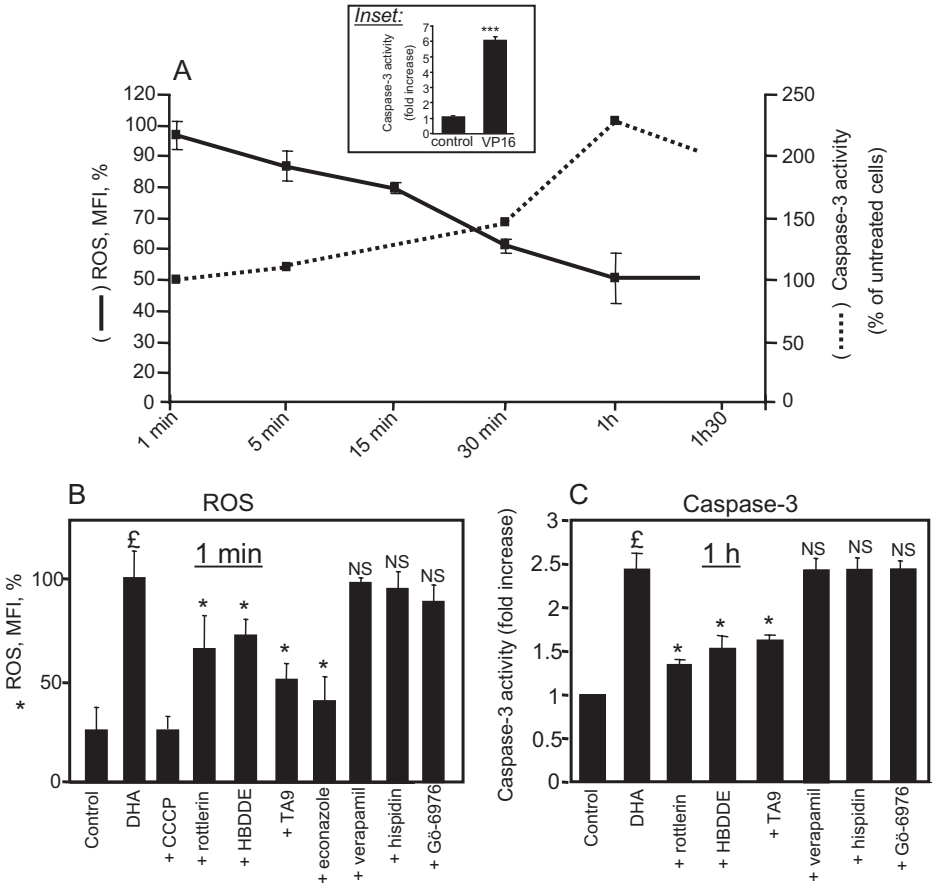


**Fig. 8.** Effects of DHA on ROS generation and caspase-3 activity. All the experiments were performed in $Ca^{2+}$-containing medium. A, U937 cells were cultured, at the indicated times, with 10 μM DHA or vehicle alone (untreated cells) and then assayed for ROS generation (MFI, mean fluorescence intensity) and caspase-3 activity as described under *Materials and Methods*. Results are expressed as means (%) ± S.D. of at least three independent experiments. Inset, cells were treated with vehicle alone or with etoposide (VP16, 50 μM) for 4 h and assayed for caspase-3 activity as described under *Materials and Methods*. Results are expressed as means ± S.D. ($n = 4$). *, $p < 0.05$ versus control cells. NS, nonsignificant data compared with DHA-treated cells. B, before addition of DHA (10 μM, 1 min), cells were allowed to incubate or not for 15 min with one of the following: TA9 (10 μM) and econazole (15 μM), HBDDE (100 μM), rottlerin (3 μM), verapamil (10 μM), hispidin (5 μM), Gö-6976 (1 μM), or CCCP (10 μM), and ROS production was then measured by flow cytometry. Results are expressed as means of mean fluorescence intensity (MFI %) ± S.D. of five independent experiments. £, $p < 0.05$ versus control cells; *, $p < 0.05$ versus DHA-treated cells. C, before addition of DHA (10 μM, 1 h), cells were incubated or not for 15 min with one of the following: TA9 (10 μM), HBDDE (100 μM), rottlerin (3 μM), verapamil (10 μM), hispidin (5 μM), or Gö-6976 (1 μM). Caspase-3 activity was measured as described under *Materials and Methods*. Results are expressed as means ± S.D. ($n = 4$). £, $p < 0.05$ versus control cells; *, $p < 0.05$ versus DHA-treated cells. NS, nonsignificant data compared with DHA-treated cells.

PKCδ activity, which also corroborated the data obtained in Fura-2-loaded U937 cells. Although DHA and TG both induce SOC influx, the mechanisms of PKC activation by DHA and TG are different. TG binds to endoplasmic reticulum $Ca^{2+}$-ATPase but not to PKC (Thastrup et al., 1990). Activation of PKC by TG thus seems to take place by an indirect mechanism. TG has also been shown, in GH4C1 cells, to induce selective redistribution of PKCε and to a lesser extent that of PKCδ, without influencing subcellular distribution of PKCα and PKCβ (Kiley et al., 1992). It is also possible that for detection of activated PKCγ, a longer preincubation time (>3 min) with TG is required. Nevertheless, a TG-induced calcium peak in U937 cells took place within 2 to 3 min; during this time, no PKCγ activation was observed. As far as PMA is concerned, it induced both PKCγ and PKCδ activities, although PMA induced increases in $[Ca^{2+}]_i$ only in a PKCδ-dependent manner. These results demonstrate the implication of PKCγ and PKCδ in the DHA-induced $Ca^{2+}$ influx.

We were further interested in assessing the mechanism of action of DHA in PKC activation. Cell-free PKC assays demonstrated that DHA, within the same concentration range used in intact cells, could by itself stimulate both PKCγ and PKCδ activities. Moreover, competition experiments revealed that DHA was most probably activating these PKC isoforms by binding to the PS binding site, because PKCγ and PKCδ activities induced by PS were enhanced in the presence of an optimal concentration of DHA (10 μM), which at high concentrations (20 and 40 μM) inhibited PKCγ and PKCδ activities. On the other hand, PS, by competing with DHA, diminished PKCγ and PKCδ activities as a function of its increasing concentrations. These findings are supported by the observations of Kanno et al. (2006), who have shown that activation of PKCε by a linoleic acid-derivative, 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid, was inhibited by dioleyl-PS. Taken together, these results strongly suggest that DHA may act via the PS binding site. The use of structurally related long-chain fatty acids allowed us to point out first that the carboxylic end of DHA molecule is critical for PKC activation, as evidenced by the fact that DHA methyl ester, an esterified form of DHA, failed to potentiate PKCγ and PKCδ activities in the presence of PS, thus corroborating the observations of Murakami et al. (1986), who have shown a requirement of the negative charge of lipid for PKC activation. The n-3 moiety was also found to play a critical role, in that arachidonic acid (n-6 PUFA), DPEA (same carbon-chain length as DHA but with only five double bonds), and EPA (20 carbons and five double bounds) were found unable to enhance PS-induced PKCγ and δ activities. These results show the specificity of the mechanism of action of DHA.

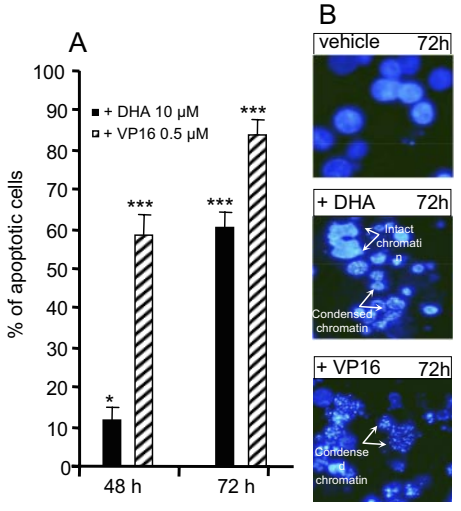


**Fig. 9.** Morphological changes in nuclear chromatin-induced by DHA. U937 cells ($2 \times 10^5$ cells/ml) were cultured in RPMI 1640 medium supplemented with 0.5% FCS and treated with vehicle alone (control cells), DHA (10 μM), or etoposide (VP16, 0.5 μM) for the indicated times. After two washes in ice-cold PBS, apoptotic cells were identified morphologically after nuclear chromatin staining with Hoechst 33342 dye as described under *Materials and Methods*. A, means % ± S.D. of four independent experiments. In control cells, the percentage of apoptotic cells was less than 10%. *, $p < 0.05$ versus control cells; ***, $p < 0.001$ versus control cells. B, representative images of nuclear chromatin condensation observed at 72 h after treatment with DHA or etoposide.

We set out to investigate further the physiological relevance of DHA-induced $Ca^{2+}$ signaling. In the apoptotic pathway, the collapse of the mitochondrial membrane potential (MMP) is a common event that leads to mitochondrial dysfunction and production of ROS (Arita et al., 2001). n-3 PUFAs are known to be able to disrupt the MMP, induce ROS, and sensitize tumor cells to ROS-inducing anticancer agents (Arita et al., 2001). Treatment of U937 cells with DHA was found to be associated with an increase in ROS production that took place as early as 1 min, and it decreased thereafter as a function of time. In addition, 1-h exposure of cells to DHA caused a significant increase in caspase-3 activity. This is in agreement with previous studies showing that DHA-induced apoptosis was mediated by an increase in intracellular ROS production and in caspase-3 activity (Diep et al., 2000; Kim et al., 2005). The action of DHA on ROS production is also further supported by an in vivo study conducted on colonic crypts isolated from rats fed with fish oil; hence, incorporation of EPA and DHA in the colonic mitochondrial phospholipids was correlated to altered MMP and increased caspase-3 activity (Chapkin et al., 2002). Besides, in our study, the ROS produced by DHA seems to be as a result of MMP, because the CCCP, a mitochondrial uncoupler, diminished the action of this fatty acid.

To establish a possible relationship between DHA-induced increases in $[Ca^{2+}]_i$ and PKC activation in apoptosis, cells were preincubated with either PKCγ and PKCδ inhibitors or SOC channel blockers and further assessed for ROS production and caspase-3 activation. Inhibition of PKCγ/δ and CRAC/SOC channels markedly reduced DHA-induced ROS generation and caspase-3 activation, thus raising the possibility that DHA-induced $Ca^{2+}$ influx and activation of PKCγ/δ play a pivotal role in ROS generation, which seems to act upstream of caspase-3 activation. Indeed, overexpression of PKCδ has been shown to reduce MMP and the release of cytochrome *c*, which subsequently leads to activation of caspases and apoptosis (Basu et al., 2001). The use of rottlerin to selectively block PKCδ has also provided evidence, in U937 cells, that this PKC isoform can activate caspase-3 (Jang et al., 2004). Phospholipase C-dependent elevation in $[Ca^{2+}]_i$ and activation of PKCδ has also been shown to positively regulate ROS production (Jeon et al., 2005). The implication of PKCγ in sodium butyrate-/PMA-induced apoptosis in U937 and HL-60 cells has also been described previously (Abdelhaleem, 2002). Although these data clearly describe a role for PKCγ and PKCδ in DHA-induced apoptosis, how PKC isoforms could stimulate ROS production and

subsequent activation of caspase-3 remains unresolved. As far as PKC$\delta$ is concerned, its translocation onto mitochondria has been correlated with cytochrome *c* release, caspase activation, and perturbation of MMP (Majumder et al., 2000). PKC$\delta$, once translocated onto mitochondria, may alter electron transport chain complexes or electron carriers, such as ubiquinone or cytochrome *c*, thus leading to the escape of electrons, which would be ultimately converted into ROS. Another possible mechanism is the activation of NADPH oxidase-like enzyme(s) by PKC$\delta$. It has been demonstrated that PKC$\delta$ induces the formation of active NADPH oxidase complex in human monocytes, possibly through phosphorylation of the regulatory subunit p47phox (Bey et al., 2004). In addition, oleic and linolenic acids have been found to induce ROS production via NADPH oxidase activation through PKC activation in Jurkat and Raji cells (Cury-Boaventura and Curi, 2005). Whatever the mechanism of action of DHA is, this fatty acid does trigger apoptosis in the U937 cell line, as evidenced by typical chromatin condensation observed after 72 h of treatment with this fatty acid.

To sum up, we can propose a schematic pathway of the mechanisms of action of DHA (Fig. 10). First, DHA mobilizes $Ca^{2+}$ from the intracellular pool via production of $IP_3$ that may activate the $Ca^{2+}$-dependent PKC$\gamma$ isoform. PKC$\delta$ will also be activated by the interaction of DHA, possibly at the PS-binding site. In turn, phosphorylation of CRAC channels by activated PKC$\gamma$ and PKC$\delta$ may lead to SOC influx. Increased $[Ca^{2+}]_i$ as well as activation of PKC$\gamma$ and PKC$\delta$ may stimulate ROS production from the mitochondria, followed by downstream signaling, which will involve the activation of caspase-3, leading to chromatin fragmentation and apoptosis in U937 cells. DHA might also exert its inhibitory action on ERK1/ERK2 or may give rise to the production of diacylglycerol-containing DHA (DAG-DHA) which will also evoke an increase in $[Ca^{2+}]_i$ and thus contribute to apoptosis.

The novelty of our findings is that DHA-induced activation of the PLC/$IP_3$ pathway increases in $[Ca^{2+}]_i$ from the endoplasmic reticulum pool, followed by the opening of CRAC channels, and DHA-induced activation of PKC$\gamma$ and PKC$\delta$ may be implicated in ROS generation and caspase-3 activation. Nevertheless, further study is needed to clarify the mechanism(s) involved in DHA-induced apoptosis.

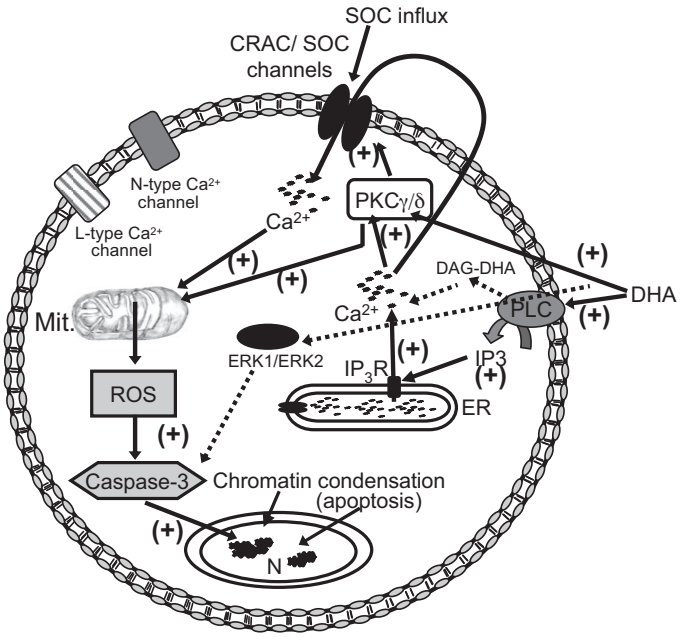


**Fig. 10.** Integrated schematic model of the DHA-induced $Ca^{2+}$ influx and subsequent induction of apoptosis in the U937 cell line. DHA will first mobilize $Ca^{2+}$ from the intracellular pool (i.e., endoplasmic reticulum) via generation of $IP_3$, thus leading to an initial rise in $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ would facilitate the activation of the $Ca^{2+}$-dependent PKC$\gamma$, where activation may occur possibly by the interaction of DHA to the PS binding-site. The $Ca^{2+}$-independent PKC$\delta$ would be directly activated by DHA, also possibly by its interaction with the PS-binding site. Therefore, activated PKC$\gamma/\delta$ would phosphorylate CRAC/SOC channels, allowing $Ca^{2+}$ entry from the extracellular environment. DHA-induced $Ca^{2+}$ influx and activated PKC$\gamma/\delta$ will trigger ROS production from mitochondria that subsequently will lead to activation of caspase-3, and ultimately to chromatin condensation and apoptosis. Hence, it is also possible that DHA may give rise to diacylglycerol-containing DAG (DAG-DHA), which, in turn, will induce increase in $[Ca^{2+}]_i$, as we have shown previously in guinea pig airway smooth muscles (Hichami et al., 2005). DHA may again exert an inhibitory direct action on ERK1/ERK2 phosphorylation (Denys et al., 2002, 2004) and consequently induce apoptosis as demonstrated by Goel et al. (2007). +, represents a stimulatory action. Dashed arrows show the other possible mechanisms of action of DHA as per other studies. ER, endoplasmic reticulum; Mit., mitochondria; N, nucleus.

**Acknowledgments**

We thank Anabelle Sequeira-Legrand and Arlette Hammann for technical assistance in the flow cytometric analyses.

**References**

Abdelhaleem M (2002) Differential effect of Bcl-xl over-expression on cell death of the monocytic leukemia cell line U937. *Anticancer Res* **22:**3911–3915.
Albert AP, and Large WA (2002) Activation of store-operated channels by noradrenaline via protein kinase C in rabbit portal vein myocytes. *J Physiol* **544:**113–125.
Arita K, Kobuchi H, Utsumi T, Takewara Y, Akiyama J, Horton AA, and Utsumi K (2001) Mechanism of apoptosis in HL-60 cells induced by n-3 and n-6 polyunsaturated fatty acids. *Biochem Pharmacol* **62:**821–828.
Basu A, Woolard MD, and Johnson CL (2001) Involvement of protein kinase C-delta in DNA damage-induced apoptosis. *Cell Death Differ* **8:**899–908.
Benov L, Sztejnberg L, and Fridovich I (1998) Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radic Biol Med* **25:**826–831.
Berridge MJ, Bootman MD, and Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* **4:**517–529.
Bey V, Xu EA, Bhattacharjee B, Oldfield A, Zhao CM, Li X, Subbulakshmi Q, Feldman GM, Wientjes FB, and Cathcart MK (2004) Protein kinase C delta is required for p47phox phosphorylation and translocation in activated human monocytes. *J Immunol* **173:**5730–5738.
Bonin A, and Khan NA (2000) Regulation of calcium signalling by docosahexaenoic acid in human T-cells: implication of CRAC channels. *J Lipid Res* **41:**277–284.
Calder PC (2004) n-3 Fatty acids, inflammation, and immunity—relevance to postsurgical and critically ill patients. *Lipids* **39:**1147–1161.
Chapkin RS, Hong MtendY, Fan YY, Davidson LA, Sanders LM, Henderson CE, Barhoumi R, Burghardt RC, Turner ND, and Lupton JR (2002) Dietary n-3 PUFA alter colonocyte mitochondrial membrane composition and function. *Lipids* **37:**193–199.
Cury-Boaventura MF, and Curi R (2005) Regulation of reactive oxygen species (ROS) production by $C_{18}$ fatty acids in Jurkat and Raji cells. *Clin Sci (Lond)* **108:**245–253.
Denys A, Aires V, Hichami A, and Khan NA (2004) Thapsigargin-stimulated MAP kinase phosphorylation via CRAC channels and PLD activation: inhibitory action of docosahexaenoic acid. *FEBS Lett* **564:**177–182.
Denys A, Hichami A, and Khan NA (2002) Eicosapentaenoic acid and docosahexaenoic acid modulate MAP kinase enzyme activity in human T-cells. *Mol Cell Biochem* **232:**143–148.
Diep QN, Touyz RM, and Schiffrin EL (2000) Docosahexaenoic acid, a peroxisome proliferator-activated receptor-$\alpha$ ligand, induces apoptosis in vascular smooth muscle cells by stimulation of p38 mitogen-activated protein kinase. *Hypertension* **36:**851–855.
Gamberucci A, Fulceri R, Bygrave FL, and Benedetti A (1997) Unsaturated fatty acids mobilize intracellular calcium independent of IP3 generation and via insertion at the plasma membrane. *Biochem Biophys Res Commun* **241:**312–316.
Goel A, Prasad AK, Parmar VS, Ghosh B and Saini N (2007) 7,8-Dihydroxy-4-methylcoumarin induces apoptosis of human lung adenocarcinoma cells by ROS-independent mitochondrial pathway through partial inhibition of ERK/MAPK signaling. FEBS Lett **581:**2447–2454.
Gonindard C, Bergonzi C, Denier C, Sergheraert C, Klaebe A, Chavant L, and Hollande E (1997) Synthetic hispidin, a PKC inhibitor, is more cytotoxic toward cancer cells than normal cells in vitro. *Cell Biol Toxicol* **13:**141–153.
Gschwendt M, Müller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, and Marks F (1994) Rottlerin, a novel protein kinase inhibitor. *Biochem Biophys Res Commun* **199:**93–98.
Heo JS, Lee YJ, and Han HJ (2006) EGF stimulates proliferation of mouse embry-

onic stem cells: involvement of $Ca^{2+}$ influx and p44/42 MAPKs. *Am J Physiol Cell Physiol* **290:**C123–C133.
Hichami A, Morin C, Rousseau E, and Khan NA (2005) Diacylglycerol-containing docosahexaenoic acid in acyl chain modulates airway smooth muscle tone. Am J Respir Cell Mol Biol **33:**378–386.
Jang BC, Lim KJ, Cho JW, Baek WK, Suh MH, Park JB, Kwon TK, Park JW, Kim SP, Shin DH, et al. (2004) Tetrandrine-induced apoptosis is mediated by activation of caspases and PKC-δ in U937 cells. *Biochem Pharmacol* **67:**1819–1829.
Jeon ES, Kang YJ, Song HY, Im DS, Kim HS, Ryu SH, Kim YK, and Kim JH (2005) Sphingosyl phosphorylcholine generates reactive oxygen species through calcium-, protein kinase Cδ-, and phospholipase D-dependent pathways. *Cell Signal* **17:**777–787.
Kanno T, Yamamoto H, Yaguchi T, Hi R, Mukasa T, Fujikawa H, Nagata T, Yamamoto S, Tanaka A, and Nishizaki T (2006) The linoleic acid derivative DCP-LA selectively activates PKC-epsilon, possibly binding to the phosphatidylserine binding site. *J Lipid Res* **47:**1146–1156.
Kashiwada Y, Huang L, Ballas LM, Jiang JB, Janzen WP, and Lee KH (1994) New hexahydroxybiphenyl derivatives as inhibitors of protein kinase C. *J Med Chem* **37:**195–200.
Khan NA and Hichami A (1999) Ionotrophic 5-hydroxytryptamine type 3 receptor activates the protein kinase C-dependent phospholipase D pathway in human T-cells. *Biochem J* **344:**199–204.
Kiley SC, Parker PJ, Fabbro D, and Jaken S (1992) Selective redistribution of PKC isozymes by thapsigargin and staurosporine. *Carcinogenesis* **13:**1997–2001.
Kim HJ, Vosseler CA, Weber PC, and Erl W (2005) Docosahexaenoic acid induces apoptosis in proliferating human endothelial cells. *J Cell Physiol* **204:**881–888.
Larsson SC, Kumlin M, Ingelman-Sundberg M, and Wolk A (2004) Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am J Clin Nutr* **79:**935–945.
Ma DWL, Seo J, Switzer KC, Fan YY, McMurray DN, Lupton JR, and Chapkin RS (2004) N-3 PUFA and membrane microdomains: a new frontier in bioactive lipid research. *J Nutr Biochem* **15:**700–706.
Madani S, Hichami A, Cherkaoui-Malki M, and Khan NA (2004) Diacylglycerols containing omega 3 and omega 6 fatty acids bind to RasGRP and modulate MAP kinase activation [published erratum appears in *J Biol Chem* **279:**23846, 2004]. *J Biol Chem* **279:**23846–23853.
Madani S, Hichami A, Legrand A, Belleville J, and Khan NA (2001) Implication of acyl chain of diacylglycerols in activation of different isoforms of protein kinase C. *FASEB J* **15:**2595–2601.
Majumder PK, Pandey P, Sun X, Cheng K, Datta R, Saxena S, Kharbanda S, and Kufe D (2000) Mitochondrial translocation of protein kinase C δ in phorbol ester-induced cytochrome *c* release and apoptosis. *J Biol Chem* **275:**21793–21796.
Martiny-Baron G, Kazanietz MG, Mischark H, Blumberg PM, Kochs G, Hug H, Marmé D and Schächtele C (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem* **268:**9194–9197.
Mirnikjoo B, Brown SE, Kim HF, Marangell LB, Sweatt JD, Weeber EJ (2001) Protein kinase inhibition by omega-3 fatty acids. *J Biol Chem* **276:**10888–10896.
Murakami K, Chan SY, and Routtenberg A (1986) Protein kinase C activation by cis-fatty acid in the absence of $Ca^{2+}$ and phospholipids. *J Biol Chem* **261:**15424–15429.
Nakamura MT, Cheon Y, Li Y, and Nara TY (2004) Mechanisms of regulation of gene expression by fatty acids. *Lipids* **39:**1077–1083.
Newton AC (1995) Protein kinase C: structure, function, and regulation. *J Biol Chem* **270:**28495–28498.
Thastrup O, Cullen PJ, Drobak BK, Hanley MR, and Dawson AP (1990) Thapsigargin, a tumor promoter, discharges intracellular $Ca^{2+}$ stores by specific inhibition of the endoplasmic reticulum $Ca^{2+}$ ATPase. *Proc Natl Acad Sci U S A* **87:**2466–2470.
Vanden Abeele F, Shuba Y, Roudbaraki M, Lemonnier L, Vanoverberghe K, Mariot P, Skryma R, and Prevarskawa N (2003) Store-operated $Ca^{2+}$ channels in prostate cancer epithelial cells: function, regulation, and role in carcinogenesis. *Cell Calcium* **33:**357–373.
Willmott NJ, Choudhury Q, and Flower RJ (1996) Functional importance of the dihydropyridine-sensitive, yet voltage-insensitive store-operated $Ca^{2+}$ influx of U937 cells. *FEBS Lett* **394:**159–164.

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