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Polyunsaturated fatty acids mobilize intracellular Ca^{2+} in NT2 human teratocarcinoma cells by causing release of Ca^{2+} from mitochondria

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arachidonic acid; mitochondrial Ca^{2+} uniporter; G protein-coupled receptor; IP_3 receptor

IN A VARIETY OF DISORDERS, overaccumulation of lipid in nonadipose tissues, including the heart, skeletal muscle, kidney, and liver, is associated with deterioration of normal organ function and is accompanied by excessive plasma and cellular levels of free fatty acids (FA) (61). Elevated concentrations of FA are observed in Types 1 and 2 diabetes and have been causally linked to impaired insulin secretion and insulin action (see Ref. 61 and the references therein). Moreover, high-serum FA detected in obese individuals are believed to play a critical role in the "metabolic syndrome" (5). Excessive concentrations of FA during myocardial and brain ischemia have also been implicated in ischemia-reperfusion injury (31). Diastolic dysfunction observed in diabetic rats may result from FA overload and FA-induced mitochondria damage in cardiac myocytes (18).

At the cellular level, excessive FA can increase oxidative stress, influence ion channel activities, activate multiple kinase pathways, and induce apoptosis. All of these cellular effects of FA may contribute to the altered physiological functions of different tissues observed in association with lipid overload. Production of reactive oxygen species by polyunsaturated FA (PUFA) has been reported in HL-60 and HeLa cells (23, 24, 26, 59), neutrophils (11), and human breast carcinoma cells (6, 13, 70). PUFA-stimulated reactive oxygen species production in some cells has been shown to be mediated by NADPH oxidase activation (1).

The induction of apoptosis by FA has been reported in a variety of cells. In human retinoblastoma Y79 cells apoptosis is induced by arachidonic acid (AA) through the action of its oxidative metabolites (73). In contrast, AA-induced apoptosis in chronic myeloid leukemia cells does not require AA metabolism (55). AA is also partially responsible for the apoptotic effect of oxidized LDL in the macrophage cell line Chinese hamster ovary-K1 (48). Saturated FA, such as palmitic acid (PA) and stearic acid, induce apoptosis in human granulosa cells and cause downregulation of Bcl-2 and upregulation of Bax proteins (47). It has been shown in LLCPKc14 cells that AA causes apoptosis through increased production of ceramide (9). Docosahexaenoic acid (DHA) induces apoptosis in Jurkat cells by a protein phosphatase 1- and 2B-sensitive mechanism (67). In mitochondria isolated from rat liver, both AA and PA cause the opening of the mitochondrial permeability transition pore (MPT), a process leading to apoptosis (51, 64). These studies suggest that FA induce apoptosis by diverse mechanisms.

Growing evidence indicates that excessive concentrations of FA affect cell functions by altering the activities of various ion transporters and channels, including proton, K^+, Na^+, Cl^-, and Ca^{2+} currents, as well as nonselective cation channels and the cardiac Na^+/Ca^{2+} exchanger (17, 25, 28, 36, 44, 63, 74). PUFA inhibit voltage-dependent Ca^{2+} channels in cardiac myocytes (17), retinal glial cells (4), and sympathetic neurons (39). The mechanisms by which PUFA modulate ion channels

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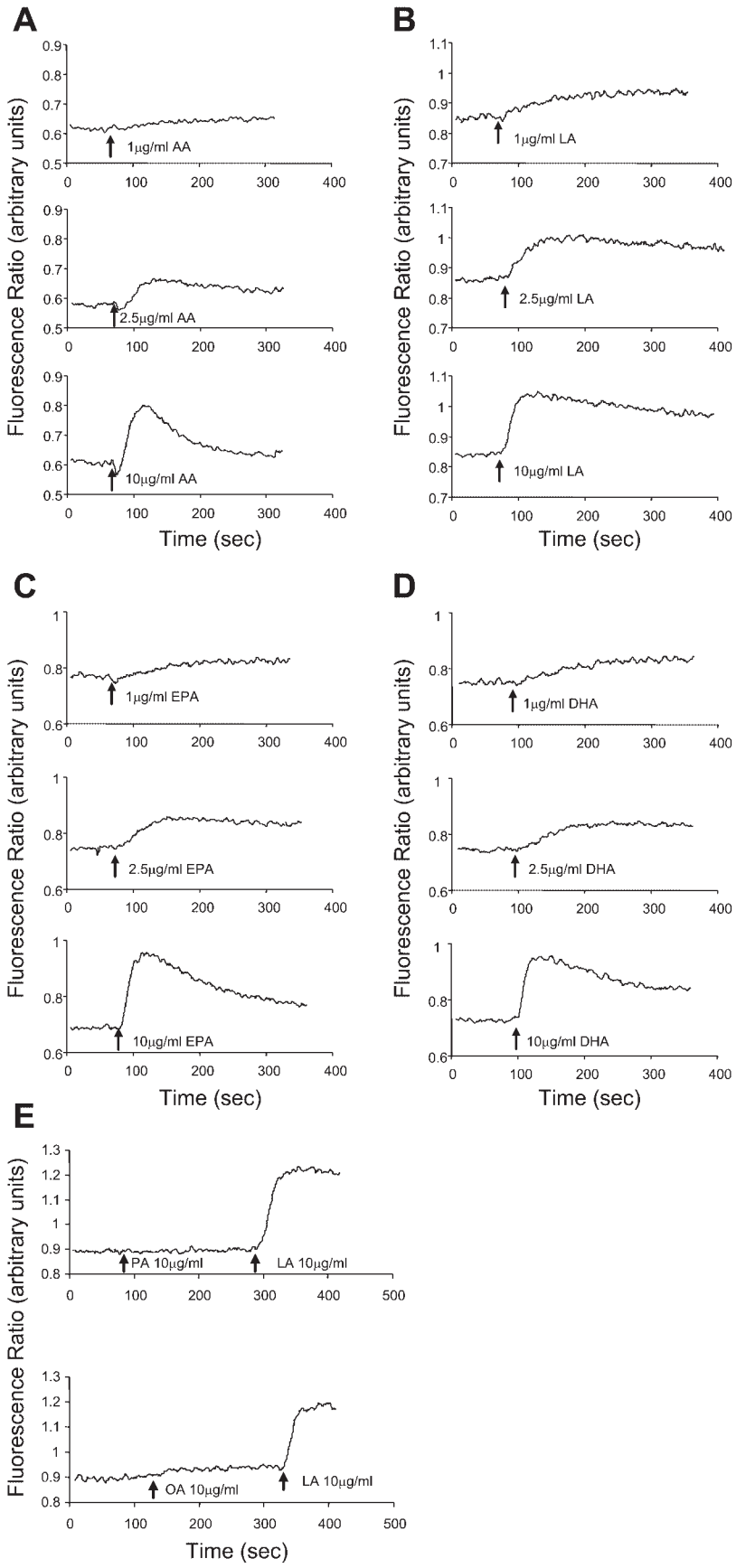


Fig. 1. Mobilization of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by polyunsaturated fatty acids (PUFA) in suspensions of NT2 cells. NT2 cells labeled with fura-2 (1.2 μM; incubated at 37°C for 30 min) in suspensions were treated with different FA as indicated, and changes in $[Ca^{2+}]_i$ were measured as described in MATERIALS AND METHODS. Representative $[Ca^{2+}]_i$ responses to increasing concentrations of arachidonic acid (AA; *A*), linoleic acid (LA; *B*), docosahexaenoic acid (DHA; *C*), and eicosapentanoic acid (EPA; *D*) are demonstrated by the traces. The arrows indicate the time of addition of different concentrations of FA as labeled. The *y*-axis is the fluorescence ratio of 340/380 in arbitrary units and the *x*-axis is time in seconds. *E*: traces are representative $[Ca^{2+}]_i$ responses to the saturated FA palmitic acid (PA; 10 μg/ml) and the mono-unsaturated FA oleic acid (OA; 10 μg/ml).

vary among different cell types and different channels. PUFA may directly bind and modulate the activity of some channels, whereas in other cases, PUFA regulate channel activities indirectly through metabolites and protein kinases (58). FA exert diverse effects on the transient receptor potential (TRP) or TRP-like families of Ca^{2+} channels (75). PUFA have been demonstrated to be reversible agonists for TRP and TRP-like channels (TRPC) in both *Drosophila* photoreceptors and *Drosophila* S2 cells (10). TRP channels in mammalian cells are composed of seven subfamilies, including TRPC, TRP vanilloid, and TRP melastatin (45). Some TRPC channels may be involved in the capacitative or store-operated Ca^{2+} entry during classic phospholipase C (PLC)-inositol-1,4,5-trisphosphate (IP_3)-mediated cytosolic, or intracellular, Ca^{2+} ($[Ca^{2+}]_i$) signaling (75, 78). AA and other PUFA either inhibit or stimulate the store-operated Ca^{2+} influx during G protein-coupled receptor (GPCR)-mediated $[Ca^{2+}]_i$ mobilization depending on the cell type and the nature of the channel (58).

PUFA-induced $[Ca^{2+}]_i$ mobilization has also been observed in vascular endothelial and human embryonic kidney (HEK)-293 cells (33, 40, 65). In HEK-293 cells, $[Ca^{2+}]_i$ mobilization induced by AA and other PUFA involves activation of the AA-specific Ca^{2+} influx pathway I_{avc} (40, 65). In the presence of 10 μM Gd^{3+} AA- but not carbachol- or thapsigargin-mediated Ca^{2+} release was completely inhibited in HEK-293 cells, suggesting that AA-induced Ca^{2+} release in these cells may be mediated by a process distinct from the traditional PLC-IP_3 pathways (40). However, the mechanism of PUFA-induced $[Ca^{2+}]_i$ mobilization remains unclear.

In this study, experiments have been designed to clarify the role of the classic PLC-IP_3 pathway and other Ca^{2+} transport pathways in FA-mediated $[Ca^{2+}]_i$ mobilization. We have found that PUFA but not monounsaturated or saturated FA cause $[Ca^{2+}]_i$ mobilization in NT2 human teratocarcinoma cells. Unlike the $[Ca^{2+}]_i$ response to the muscarinic agonist carbachol, PUFA-mediated $[Ca^{2+}]_i$ mobilization in NT2 cells is independent of PLC and IP_3 receptor activation, as well as IP_3-sensitive internal Ca^{2+} stores. Furthermore, PUFA-mediated $[Ca^{2+}]_i$ mobilization is inhibited by the mitochondrial uncoupler carboxyl cyanide *m*-chlorophenylhydrozone (CCCP). Direct measurements of mitochondrial Ca^{2+} with X-rhod-1 and $^{45}Ca^{2+}$ indicate that PUFA induce Ca^{2+} efflux from mitochondria. These experiments suggest that PUFA-gated Ca^{2+} release from mitochondria is the underlying mechanism for PUFA-induced $[Ca^{2+}]_i$ mobilization in NT2 cells.

MATERIALS AND METHODS

Materials. Fura-2 AM, Fluo-3 AM, BAPTA-AM, and X-rhod-1 AM were purchased from Molecular Probes (Eugene, OR). Thapsigargin was purchased from RBI (Natick, MA). Dulbecco's modified Eagle's medium, EGTA, trypsin-EDTA, Opti-MEM, and PBS [containing (in mM) 1 KH_2PO_4, 3 Na_2HPO_4, and 154 NaCl, pH 7.2] powders were obtained from Life Technologies (Gaithersburg, MD). Arachidonic acid and other fatty acids were purchased from Cayman (Ann Arbor, MI). The NT2 cell line and primary cultured human aortic endothelial cells (HAEC) were purchased from American Type Culture Collection (Manassas, VA) and Cambrex (Walkersville, MD), respectively. MDCB-131 medium, carbachol, CCCP, oligomycin, and other chemicals were from Sigma (St. Louis, MO).

Cell culture. NT2 cells were plated at a density of 10^4 cells/cm^2 and cultured in 100 mm dishes in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and penicillin (10 U/ml)/streptomycin (10 μg/ml), at 37°C in a humidified 5% CO_2-atmosphere incubator. Cells grown to near confluence were harvested with trypsin (0.05%)-EDTA (0.02%) and suspended in PBS solutions for use in experiments described below.

Human aortic endothelial cells (HAEC) were plated at a density of 6×10^3/cm^2 in 75-ml flasks in MDCB-131, supplemented with 15 mM HEPES, 14 mM $NaHCO_3$, EGF (10 ng/ml), fibroblast growth factor (2.5 ng/ml), hydrocortisone (1 μg/ml), 10% fetal calf serum, and penicillin (10 U/ml)/streptomycin (10 μg/ml), and cultured at 37°C in a humidified 5% CO_2-atmosphere incubator. After 1 wk of culture, when the cells reached confluence, HAEC were harvested with trypsin (0.05%)-EDTA (0.02%) and labeled with fura-2 AM for measurement of $[Ca^{2+}]_i$ in cell suspensions or used to isolate mitochondria to measure $[Ca^{2+}]_m$ with X-rhod-1.

Measurement of $[Ca^{2+}]_i$ in suspensions of NT2 cells. NT2 cells suspended in a buffer containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 1.2 KH_2PO_4, 10 glucose, and 10 HEPES, pH 7.4, were loaded with fura-2 AM (2 μM) at 37°C for 30 min with gentle shaking. Loaded cells were washed with 5 volumes of PBS supple-

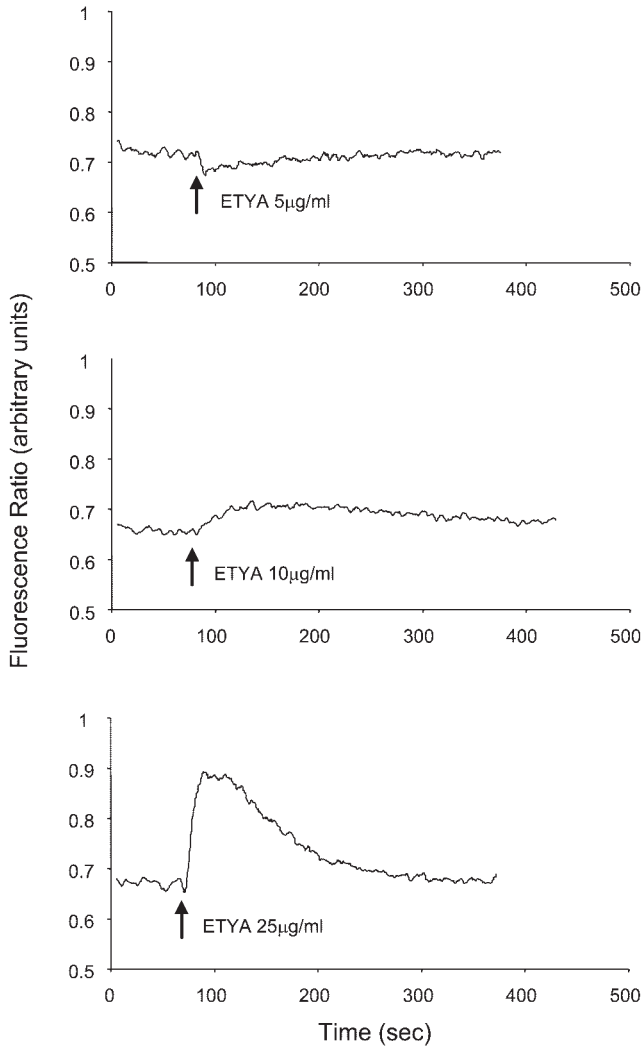


Fig. 2. Eicosatetraynoic acid (ETYA)-mediated $[Ca^{2+}]_i$ mobilization in NT2 cells. NT2 cells were treated with increasing concentrations of ETYA, and changes in $[Ca^{2+}]_i$ were measured as described in MATERIALS AND METHODS.

mented with 1 mM $CaCl_2$ and 1 mM $MgSO_4$ (PBS1Ca). Alterations in $[Ca^{2+}]_i$ were measured by changes in fluorescence ratio with emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm. Fura-2-loaded cells in suspension were incubated at 37°C in a cuvette with a magnetic stirrer, and changes of fluorescence ratio were monitored in a fluorometer manufactured by Photon Technology International (PTI; Lawrenceville, NJ). For measurement of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, fura-2-loaded NT2 cells were washed with 5 volumes of PBS supplemented with 200 μM EGTA (pH 7.4) and 1 mM $MgSO_4$ (PBS0Ca) immediately before experiments; $[Ca^{2+}]_i$ measurements were performed in PBS0Ca solutions (76).

Measurement of mitochondrial Ca^{2+} in suspensions of NT2 cells. NT2 cells suspended in PBS were double labeled with X-rhod-1 AM (2 μM) and BAPTA-AM (50 μM), or labeled with X-rhod-1 AM alone, at 37°C for 30 min with gentle shaking. The loaded cells were washed with 5 volumes of PBS1Ca. Alterations in mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) were measured by changes in fluorescence intensity with emission wavelength of 602 nm and excitation wavelength of 578 nm. X-rhod-1-AM- and BAPTA-AM-loaded cells were incubated in PBS supplemented with 2 mM EGTA (pH 7.4) and 1 mM $MgSO_4$ at 37°C in a cuvette in the presence of constant magnetic stirring; cells loaded with X-rhod-1 alone were incubated in PBS supplemented with 2 mM EGTA/1 mM $MgSO_4$ or in PBS1Ca. Changes of X-rhod-1 fluorescence intensity were monitored in a PTI fluorometer.

Measurement of $[Ca^{2+}]_m$ in isolated mitochondria from NT2 cells and HAEC. Mitochondria from NT2 cells were isolated as described previously (30). Briefly, NT2 cells from seven to ten 100-mm dishes were suspended in PBS, incubated on ice, and centrifuged at 1,000 rpm for 15 min at 4°C in an Eppendorf 5804R centrifuge. Cells were resuspended in 2 ml ice-cold mitochondria isolation buffer containing 250 mM mannitol, 75 mM succinic acid, 100 μM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 7.5 (MB1). The cell suspensions were homogenized manually with 15–20 strokes in a glass-glass homogenizer. The homogenates were centrifuged at 1,000 rpm for 15 min at 4°C. The supernatants were collected and centrifuged at 10,000 *g* for 20 min at 4°C. The pellets were resuspended in 500 μl of MB1 and diluted to a final volume of 3 ml with PBS1Ca. The mitochondria suspensions were labeled with the mitochondrial Ca^{2+} fluorescent indicator X-rhod-1 AM (2 μM) at 37°C for 30 min, after which the mixtures were centrifuged at 10,000 *g* at room temperature in a tabletop high-speed centrifuge (Savant Instruments, Farmingdale, NY) for 10 min. The labeled mitochondria were resuspended in PBS1Ca and used for measurement of $[Ca^{2+}]_m$. Alterations of $[Ca^{2+}]_m$ in suspensions of isolated mitochondria were measured by changes in fluorescence intensity with emission wavelength of 602 nm and excitation wavelength of 578 nm. X-rhod-1 loaded mitochondria were incubated in PBS0Ca at 37°C in a cuvette in the presence of continuous magnetic stirring, and changes of X-rhod-1 fluorescence intensity were monitored in a PTI fluorometer. The same procedure was adopted to isolate mitochondria and measure $[Ca^{2+}]_m$ from HAEC, except that the cells were grown in 75-ml flasks.

Measurement of PUFA-induced Ca^{2+} efflux in isolated mitochondria using $^{45}Ca^{2+}$. Loading of $^{45}Ca^{2+}$ into mitochondria was performed according to a previously reported procedure (8) with modification. Briefly, isolated mitochondria (0.4–1.2 mg protein) were resuspended in 500 μl of MB1, diluted to a final volume of 3 ml with PBS1Ca, and loaded with $^{45}Ca^{2+}$ at 37°C for 30 min with gentle agitation. The $^{45}Ca^{2+}$-loaded mitochondria were then diluted in 10 volumes of PBS0Ca-containing vehicle or 10 μg/ml PUFA or palmitic acid (PA). The mixtures were incubated at 37°C for 5 min and the $^{45}Ca^{2+}$ contents remaining in the mitochondria were counted in a liquid scintillation counter.

Data analysis. Individual figures shown in RESULTS are representative of at least three experiments. Statistical analysis of $^{45}Ca^{2+}$ measurements was performed using the Wilcoxon scores for variable assay and Monte Carlo estimate for significance.

RESULTS

Polyunsaturated but not saturated or monounsaturated FA induce $[Ca^{2+}]_i$ mobilization in NT2 cells. In suspensions of NT2 cells PUFA, including AA, DHA, eicosapentanoic acid, and linoleic acid (LA), increased $[Ca^{2+}]_i$ (Fig. 1, *A–D*). The

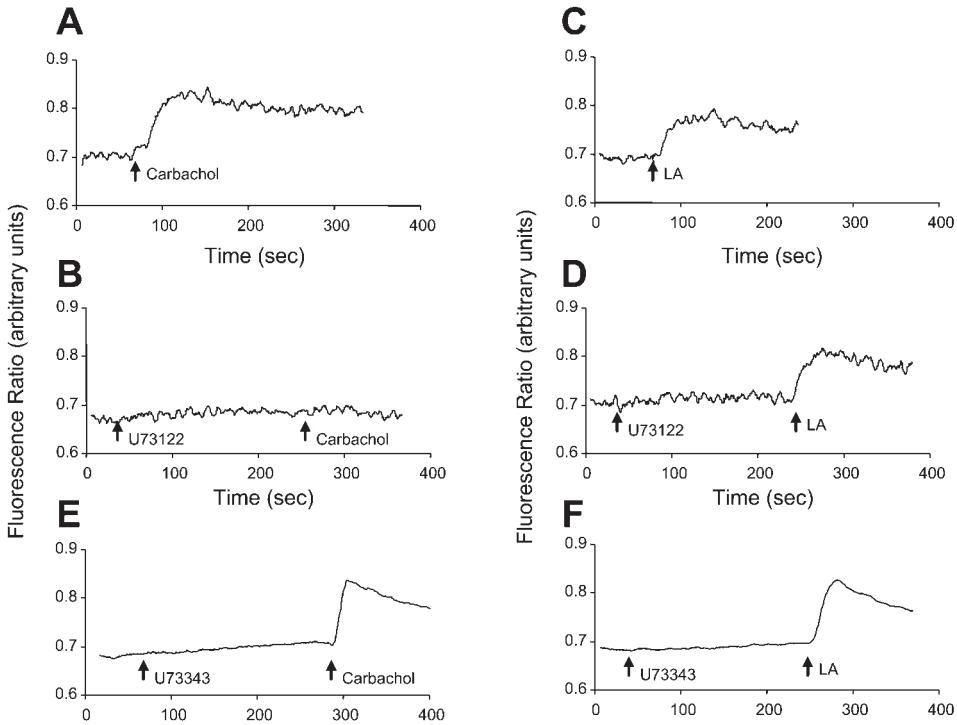


Fig. 3. Effect of U73122 and U73343 on carbachol- and PUFA-mediated $[Ca^{2+}]_i$ mobilization in NT2 cells. NT2 cells were either untreated (*A* and *C*) or treated with U73122 (10 μM) (*B* and *D*) or U73343 (10 μM) (*E* and *F*), followed by measurement of carbachol (100 μM)- and linoleic acid (LA; 10 μg/ml)-mediated $[Ca^{2+}]_i$ mobilization, as described in MATERIALS AND METHODS.

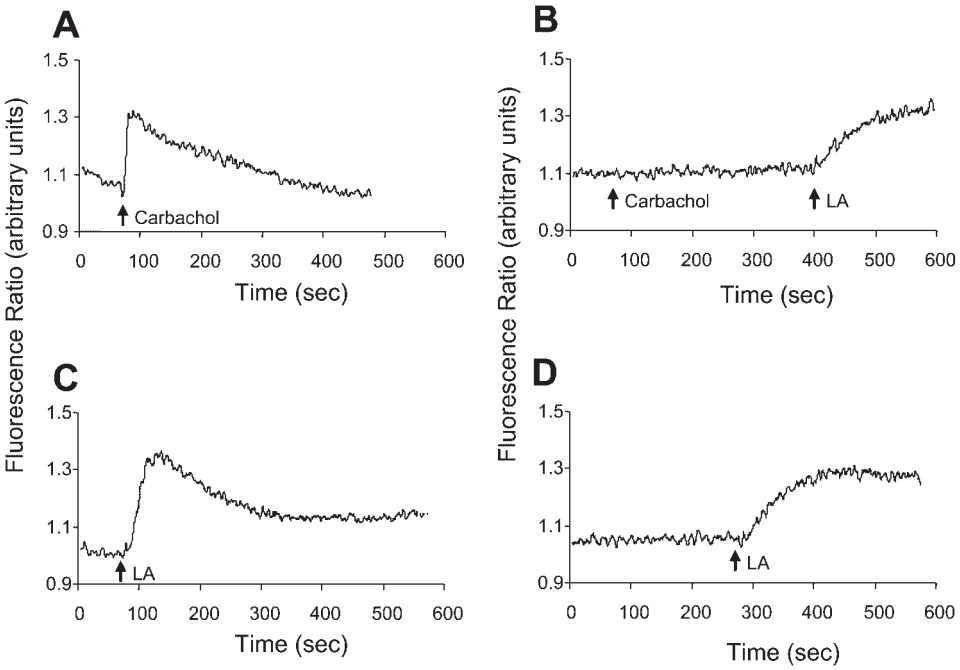


Fig. 4. Effect of 2-APB on carbachol- and PUFA-mediated $[Ca^{2+}]_i$ mobilization. NT2 cells in suspensions were either untreated (*A* and *C*) or treated with 2-APB (25 μM) for 15–30 min at room temperature (*B* and *D*). $[Ca^{2+}]_i$ mobilization in response to carbachol (100 μM) and LA (10 μg/ml) was measured as described in MATERIALS AND METHODS.

initial rate of $[Ca^{2+}]_i$ increase and the amplitude of the $[Ca^{2+}]_i$ signal were dependent on the concentration of PUFA used, with half-maximal $[Ca^{2+}]_i$ response observed at 2.46 ± 0.05 μg/ml LA and AA. The monounsaturated FA oleic acid and the saturated FA PA did not induce $[Ca^{2+}]_i$ mobilization (Fig. 1*E*) even at a concentration (10 μg/ml) at which PUFA produced maximal $[Ca^{2+}]_i$ response. These results indicate that the PUFA-induced $[Ca^{2+}]_i$ responses in NT2 cells do not result from nonspecific detergent effects of FA on cell membranes.

It is well known that PUFA are readily oxidized in various cell types and that the oxidative products of PUFA may activate specific GPCRs and cause $[Ca^{2+}]_i$ mobilization. To examine whether PUFA-induced $[Ca^{2+}]_i$ responses in NT2 cells involve the action of FA oxidative metabolites, we tested the effect of the nonmetabolizable AA analog 5,8,11,14-eicosatetraynoic acid (ETYA) on $[Ca^{2+}]_i$ mobilization. As shown in Fig. 2, ETYA caused a concentration-dependent increase of $[Ca^{2+}]_i$ in NT2 cells. Compared with AA, a higher concentration of ETYA (25 μg/ml) was required to elicit a maximal $[Ca^{2+}]_i$ response. These findings suggest that PUFA-mediated $[Ca^{2+}]_i$ mobilization in NT2 cells does not require or involve FA metabolites of lipid oxidation.

PUFA-induced $[Ca^{2+}]_i$ mobilization is independent of the classic PLC-IP$_3$ signaling pathways. It has recently been shown that FA are the native ligands of several newly discovered orphan GPCRs, such as the GPR40–43 subfamily of receptors, and thus may regulate cellular functions by activation of these receptors; GPR43 has been linked to classic PLC-IP$_3$-mediated $[Ca^{2+}]_i$ signaling (16). Accordingly, the PUFA-induced $[Ca^{2+}]_i$ mobilization in NT2 cells shown in Fig. 1 could result from PUFA activation of GPR40–43 and the PLC-IP$_3$ pathway. To determine whether PUFA-induced $[Ca^{2+}]_i$ mobilization in NT2 cells is mediated by PLC activation, we have tested the effect of U73122, a widely used PLC inhibitor, on the PUFA-induced $[Ca^{2+}]_i$ response. As demonstrated in Fig. 3, *A* and *B*, pretreatment of NT2 cells with

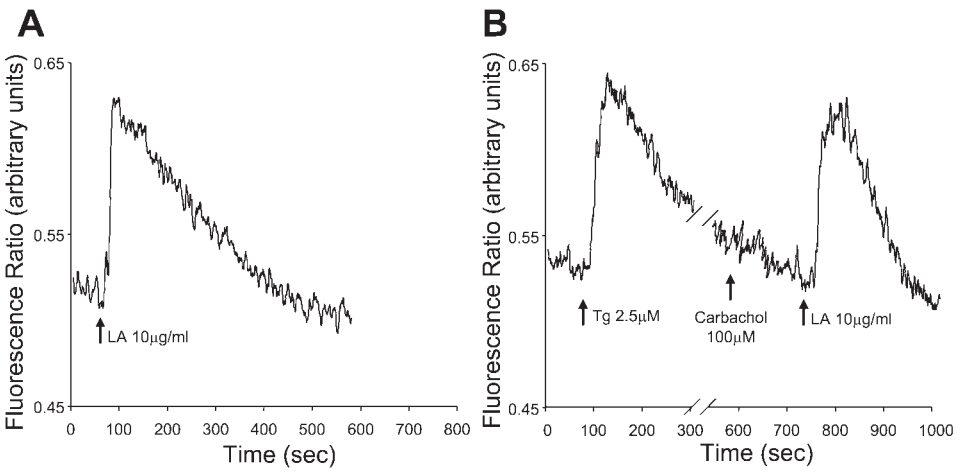
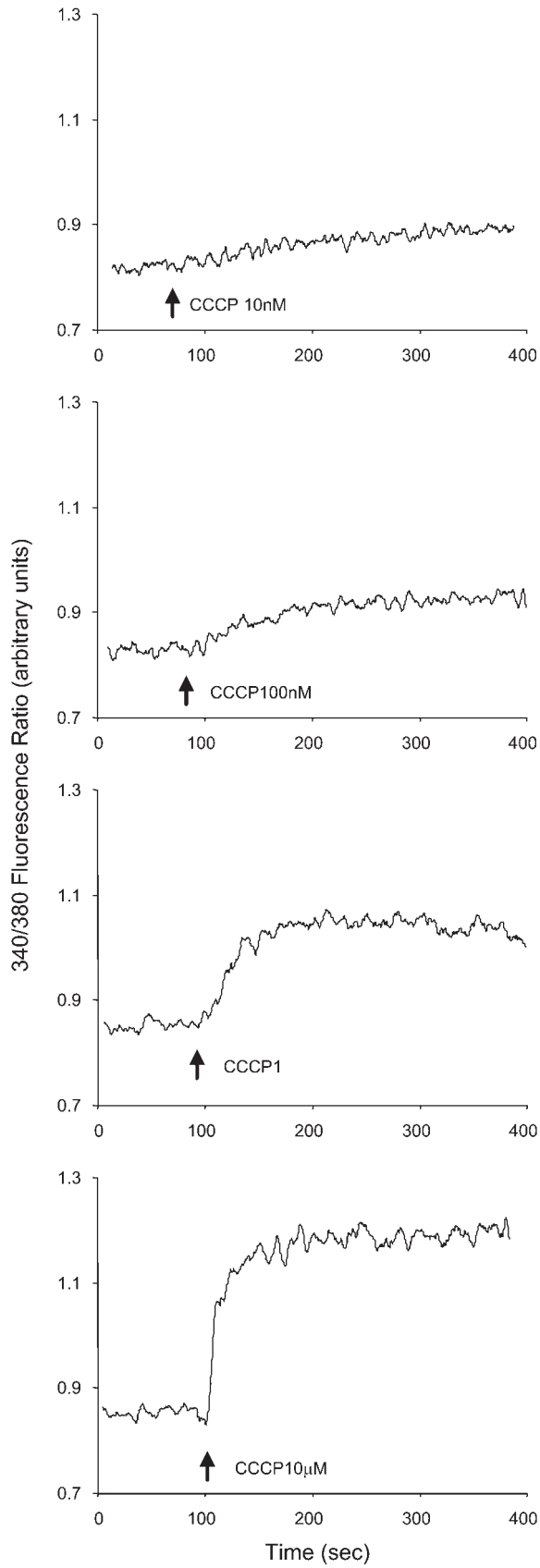


Fig. 5. Effect of thapsigargin (Tg) on carbachol- and PUFA-mediated $[Ca^{2+}]_i$ mobilization in the absence of extracellular Ca^{2+}. NT2 cells (2.5–5 × 10^5) were washed with 10 ml of PBS0Ca buffer immediately before $[Ca^{2+}]_i$ measurements. *A*: LA-induced $[Ca^{2+}]_i$ mobilization in the absence of extracellular Ca^{2+}. *B*: $[Ca^{2+}]_i$ responses of NT2 cells to sequential treatment with Tg, carbachol, and LA in the absence of extracellular Ca^{2+}.



U73122 (10 μM) completely blocked the $[Ca^{2+}]_i$ signal induced by the muscarinic agonist carbachol, which is known to cause $[Ca^{2+}]_i$ mobilization by the GPCR-PLC-IP_3 pathway. In contrast, the inhibitor had no effect on PUFA-mediated $[Ca^{2+}]_i$ mobilization (Fig. 3, *C* and *D*). Preincubation of the cells with U73343, the nonactive analog of U73122, had no effect on either carbachol- or LA-induced $[Ca^{2+}]_i$ mobilization (Fig. 3, *E* and *F*). Furthermore, treatment of NT2 cells with 2-APB (25 μM), a cell-permeable IP_3 receptor antagonist, abolished carbachol-induced $[Ca^{2+}]_i$ mobilization (Fig. 4, *A* and *B*). However, in 2-APB-treated cells that did not respond to carbachol stimulation, subsequent application of PUFA (LA; 10 μg/ml) induced $[Ca^{2+}]_i$ mobilization (Fig. 4*B*). Compared with untreated cells, 2-APB pretreatment also influenced the rate of LA-induced $[Ca^{2+}]_i$ mobilization (Fig. 4, *C* and *D*). Nonetheless, LA clearly caused $[Ca^{2+}]_i$ mobilization in NT2 cells under conditions in which IP_3-mediated Ca^{2+} release was inhibited by 2-APB (Fig. 4*B*).

In the absence of extracellular Ca^{2+}, PUFA caused a transient increase of $[Ca^{2+}]_i$, followed by a rapid decay toward the baseline (Fig. 5*A*), indicating that the PUFA-induced $[Ca^{2+}]_i$ signal involved Ca^{2+} release. We also examined whether PUFA induced Ca^{2+} release from the IP_3-sensitive internal Ca^{2+} stores. As demonstrated in Fig. 5*B*, depletion of the IP_3-releasable internal Ca^{2+} stores by treatment with thapsigargin (2.5 μM) in the absence of extracellular Ca^{2+} completely blocked the carbachol-mediated $[Ca^{2+}]_i$ response. However, in the same thapsigargin-treated cells, which had no response to carbachol stimulation, PUFA still induced a $[Ca^{2+}]_i$ signal in the absence of extracellular Ca^{2+} (Fig. 5*B*), indicating that PUFA may induce Ca^{2+} release from a different cellular store than the IP_3-releasable internal Ca^{2+} stores. The results in Figs. 3–5 suggest that PUFA-induced $[Ca^{2+}]_i$ mobilization in NT2 cells is not mediated by the traditional PLC-IP_3 signaling pathways.

PUFA-induced $[Ca^{2+}]_i$ mobilization may involve Ca^{2+} release from mitochondria. Recent studies (14) show that mitochondria play a critical role in $[Ca^{2+}]_i$ signaling. Because PUFA-induced $[Ca^{2+}]_i$ mobilization is independent of classic GPCR-PLC-IP_3 mechanisms (Figs. 3–5), we tested the involvement of mitochondrial Ca^{2+} handling in the PUFA response. In several cell types, the mitochondrial uncoupler CCCP induces $[Ca^{2+}]_i$ mobilization by inhibition of mitochondrial Ca^{2+} uptake and efflux of Ca^{2+} from mitochondria (7, 49, 60). We also observed CCCP-mediated $[Ca^{2+}]_i$ mobilization in resting NT2 cells (Fig. 6). Both the initial rate of the signal and the amplitude of the $[Ca^{2+}]_i$ response were dependent on the concentration of CCCP (Fig. 6). Furthermore, pretreatment with CCCP (10 μM for 15 min at room temperature) completely blocked PUFA (LA and AA)-induced $[Ca^{2+}]_i$ mobilization in NT2 cells (Fig. 7, *A–D*). $[Ca^{2+}]_i$ mobilization by other PUFA, such as DHA and eicosapentanoic acid, was similarly inhibited by the mitochondrial blocker (not shown). In contrast, CCCP had no effect on the amplitude of the initial peak of $[Ca^{2+}]_i$ mobilization induced by carbachol (Fig. 7, *E*

Fig. 6. Effect of carboxyl cyanide *m*-chlorophenylhydrazone (CCCP) on $[Ca^{2+}]_i$ in NT2 cells. Fura-2-labeled NT2 cells in suspensions were treated with increasing concentrations of CCCP as indicated, and changes in $[Ca^{2+}]_i$ were measured as described in MATERIALS AND METHODS.

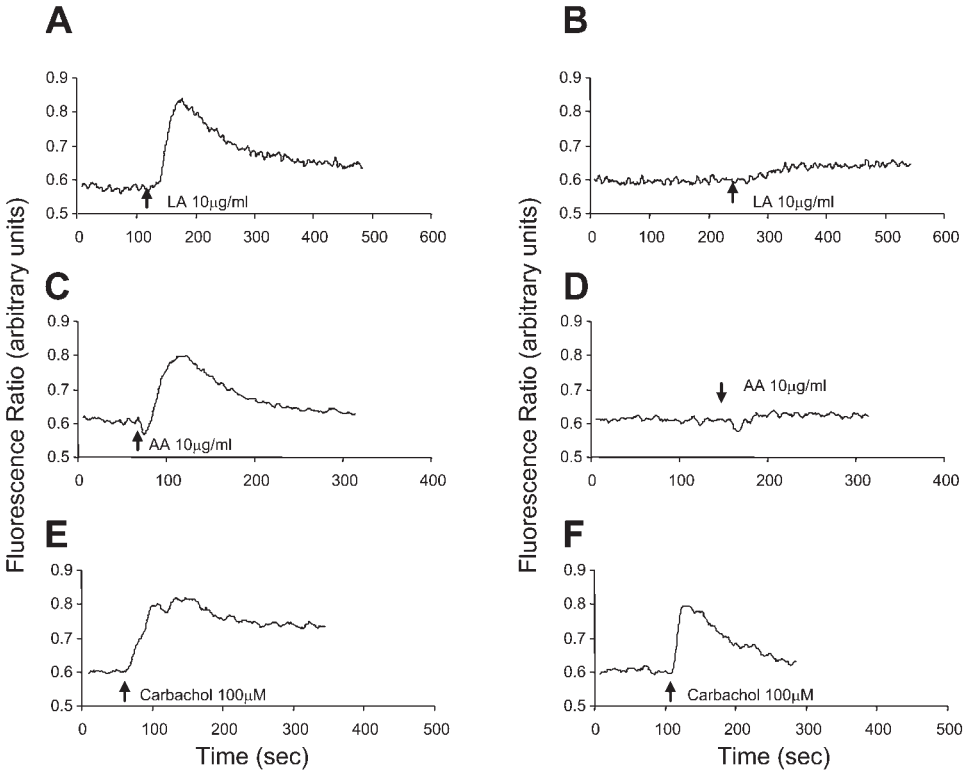


Fig. 7. Effect of CCCP on carbachol- and PUFA-mediated $[Ca^{2+}]_i$ mobilization. NT2 cells in suspensions were either untreated (*A*, *C*, and *E*) or treated with CCCP (10 μM) for 15 min at room temperature (*B*, *D*, and *F*). After treatment, carbachol- (100 μM) and PUFA (AA and LA; 10 μg/ml)-mediated $[Ca^{2+}]_i$ mobilization was measured as described in MATERIALS AND METHODS. The results of a typical experiment are presented in *A–F*.

and *F*). However, CCCP pretreatment did block the sustained plateau phase of the carbachol-induced $[Ca^{2+}]_i$ signal (Fig. 7, *E* and *F*). The plateau phase of the carbachol-induced $[Ca^{2+}]_i$ signal is sustained by capacitative Ca^{2+} influx, which is dependent on continuous mitochondrial Ca^{2+} uptake (42). Blockade of the plateau phase of the carbachol response after CCCP treatment probably reflects the attenuation of capacitative Ca^{2+} influx after CCCP inhibition of mitochondrial Ca^{2+} uptake.

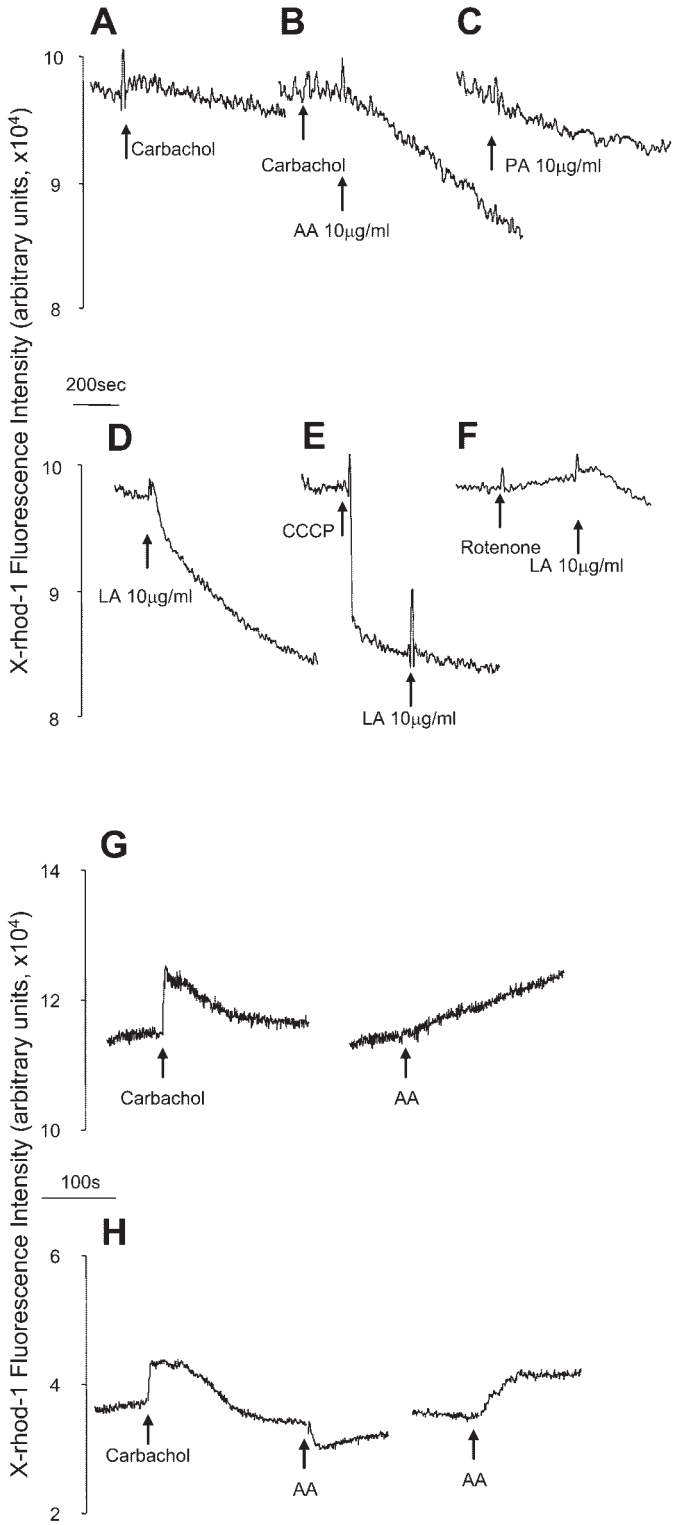
In other experiments, incubation of the cells with CCCP (10 μM) in combination with oligomycin (0.5 μg/ml) to inhibit F_0F_1-ATPase-mediated rapid ATP hydrolysis and thus a rapid decrease of cytosolic ATP did not prevent the inhibitory effect of CCCP on LA-induced $[Ca^{2+}]_i$ mobilization (data not shown). This finding indicates that inhibition of the PUFA response by CCCP is not caused by loss of ATP mediated by F_0F_1-ATPase.

The effects of CCCP observed in NT2 cells suggest that PUFA-induced $[Ca^{2+}]_i$ mobilization requires functional mitochondria. To determine how mitochondria are involved in the PUFA response, we compared the change of $[Ca^{2+}]_m$ during carbachol and PUFA treatment of NT2 cells. In these experiments, intact NT2 cells were labeled with the mitochondrial Ca^{2+} indicator X-rhod-1 and the fluctuation in cytosolic Ca^{2+} was buffered with BAPTA. In BAPTA and X-rhod-1 double-labeled cells, stimulation of the cells with carbachol had no effect on X-rhod-1 fluorescence in the absence of extracellular Ca^{2+} (Fig. 8*A*), indicating that activation of the traditional GPCR-PLC-IP_3 pathway under the experimental conditions used had no effect on $[Ca^{2+}]_m$. In contrast, in the same cells in which carbachol had no effect on $[Ca^{2+}]_m$ subsequent treatment with AA (10 μg/ml) caused a reduction in X-rhod-1 fluorescence (Fig. 8*B*), suggesting a reduction in $[Ca^{2+}]_m$ in intact NT2 cells by AA. Under the same conditions, the saturated FA PA (10 μg/ml) showed no effect on X-rhod-1 fluorescence (Fig. 8*C*), whereas the PUFA LA also caused a reduction in X-rhod-1 fluorescence (Fig. 8*D*). These results suggest that PUFA but not carbachol or saturated FA cause Ca^{2+} release from mitochondria in intact NT2 cells.

To clarify further that PUFA cause Ca^{2+} release indeed from mitochondria but not other organelles, we examined the effects of the two mitochondrial blockers CCCP and rotenone on LA responses in NT2 cells doubled labeled with X-rhod-1 and BAPTA. The mitochondrial uncoupler CCCP (10 μM) induced a rapid decrease in X-rhod-1 fluorescence, after which LA did not alter the X-rhod-1 signal, indicating that both LA and CCCP released Ca^{2+} from the same mitochondrial pool. In addition, the mitochondrial complex I blocker rotenone (10 μM), which alone did not alter X-rhod-1 fluorescence, reduced the slope of the subsequent LA response by 81.7 ± 7.3% (Fig. 8, *D–F*). These experiments demonstrate that in the presence of BAPTA, the LA-induced decrease in X-rhod-1 fluorescence is an indication of a decrease in mitochondrial Ca^{2+}, i.e., PUFA-induced mitochondrial Ca^{2+} efflux.

Additional experiments were conducted to compare the effects of carbachol and PUFA on X-rhod-1 signals in NT2 cells in the absence of BAPTA. In the presence of extracellular Ca^{2+}, carbachol caused a transient increase in $[Ca^{2+}]_i$, followed by an elevated plateau, indicating a typical GPCR response, whereas AA caused a more gradual increase of $[Ca^{2+}]_i$ (Fig. 8*G*). The AA response observed may represent the sum of the change in cytosolic and mitochondrial Ca^{2+}. In Ca^{2+}-free media, both carbachol and AA caused increases in $[Ca^{2+}]_i$ without BAPTA (Fig. 8*H*). However, after carbachol-induced $[Ca^{2+}]_i$ decayed to basal levels, addition of AA caused

a reduction in X-rhod-1 signal (Fig. 8*H*). This observation is consistent with a decrease of $[Ca^{2+}]_m$ under conditions of enhanced removal of $[Ca^{2+}]_i$ by the endoplamic reticulum and plasma membrane Ca^{2+}-ATPase, which has been reported to be activated during GPCR stimulation in other cell types (62, 77).



To confirm that PUFA may induce $[Ca^{2+}]_i$ mobilization by causing Ca^{2+} release from mitochondria, we examined the effect of PUFA in isolated mitochondria using both the mitochondrial Ca^{2+} indicator X-rhod-1 and $^{45}Ca^{2+}$. In these experiments, mitochondria were prepared from NT2 cells and labeled with X-rhod-1 or $^{45}Ca^{2+}$ in the presence of high concentration of Ca^{2+} (PBS1Ca buffer). The labeled mitochondria were then treated with PUFA and alterations in $[Ca^{2+}]_m$ were measured by X-rhod-1 fluorescence or by the remaining $^{45}Ca^{2+}$ content in the mitochondria. As demonstrated in Fig. 9*A*, DHA caused a concentration-dependent release of Ca^{2+} from mitochondria, as measured by X-rhod-1 fluorescence; LA and AA had similar effects (data not shown). Measurement of $^{45}Ca^{2+}$ indicated that AA and LA but not PA also caused a significant reduction in mitochondrial $^{45}Ca^{2+}$ content (Fig. 9*B*). Compared with control, AA and LA reduced the $^{45}Ca^{2+}$ content by 36.3 ± 12.4% ($P = 0.0079$, $n = 5$ measurements) and 35.9 ± 10.6% ($P = 0.0039$, $n = 6$), respectively, whereas PA had no significant effect on mitochondrial $^{45}Ca^{2+}$ content ($P = 0.1256$ vs. control, $n = 5$).

We also investigated whether PUFA-induced Ca^{2+} release from mitochondria occurs in other cell types, e.g., primary cultured HAEC. The results shown in Fig. 10*A* demonstrate that AA caused concentration-dependent $[Ca^{2+}]_i$ mobilization in HAEC, with maximal $[Ca^{2+}]_i$ response observed at 5–10 μg/ml AA; other PUFA also increased $[Ca^{2+}]_i$ in HAEC (not shown). In mitochondria isolated from HAEC, AA and LA (10 μg/ml) caused Ca^{2+} release from X-rhod-1 labeled mitochondria, as evidenced by decreased X-rhod-1 fluorescence intensity (Fig. 10*B*). Thus, in HAEC as in NT2 cells, PUFA may induce $[Ca^{2+}]_i$ mobilization by causing release of Ca^{2+} from mitochondria. Interestingly, the amplitude of the $[Ca^{2+}]_i$ response to AA in HAEC was greater than that observed in NT2 cells (cf. Figs. 1*A* and 10*A*), despite comparable release of mitochondrial Ca^{2+} in the two types (cf. Figs. 9*B* and 11). This finding raises the possibility that the response in HAEC may involve more than Ca^{2+} efflux from mitochondria.

To determine whether PUFA-induced Ca^{2+} release from mitochondria of NT2 cells is mediated by known mitochondrial Ca^{2+} transport pathways or a novel mechanism, we studied the effects of cyclosporin A (CsA) and bongkrekic acid (BA), two known MPT inhibitors, and ruthenium red, which inhibits the mitochondrial Ca^{2+} uniporter. As demonstrated in Fig. 11, pretreatment of isolated mitochondria with 10 μM CsA or BA had no effect on LA-induced reduction of X-rhod-1 fluorescence, whereas the addition of ruthenium red (10 μM) before LA completely blocked the LA response. Thus the mitochondrial Ca^{2+} uniporter but not the MPT may be involved in PUFA-induced mitochondrial Ca^{2+} efflux.

Fig. 8. Effect of carbachol and PUFA on $[Ca^{2+}]_m$ measured in intact NT2 cells. NT2 cells were loaded either sequentially with X-rhod-1 AM (2 μM) and BAPTA-AM (50 μM) (*A–F*) or with X-rhod-1 AM (2 μM) alone (*G* and *H*) at 37°C for 30 min. Loaded cells were resuspended in PBS supplemented with high EGTA (2 mM) (*A–F*, and *H*) or PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgSO_4$ (PBS1Ca) (*G*), and immediately used to measure carbachol-, PUFA-, or palmitic acid (PA)-induced changes in $[Ca^{2+}]_m$ following the procedure described in MATERIALS AND METHODS. Mitochondrial blockers CCCP (10 μM) and rotenone (10 μM) were tested in *panels E* and *F*, respectively. Changes in $[Ca^{2+}]_m$ following carbachol and FA treatment were indexed by alterations in X-rhod-1 fluorescence intensity (*y*-axis).

DISCUSSION

We have shown in the current study that PUFA, but not saturated or monounsaturated FA, induce $[Ca^{2+}]_i$ mobilization in NT2 human teratocarcinoma cells. Unlike the response to carbachol, which mobilizes $[Ca^{2+}]_i$ by activation of the GPCR-PLC-IP_3 pathway, PUFA-induced $[Ca^{2+}]_i$ mobilization was not inhibited by the PLC inhibitor U73122 and the IP_3 receptor antagonist 2-APB. Moreover, PUFA-mediated $[Ca^{2+}]_i$ mobilization was observed after thapsigargin-induced Ca^{2+} depletion of the IP_3-sensitive internal Ca^{2+} stores. These results suggest that PUFA-induced $[Ca^{2+}]_i$ mobilization occurs through a mechanism independent of the traditional PLC-IP_3 signaling pathway. Further studies demonstrating inhibition of PUFA-induced $[Ca^{2+}]_i$ mobilization by the mitochondrial uncoupler CCCP suggested a role for mitochondria in the response to PUFA. Direct measurement of $[Ca^{2+}]_m$ in intact NT2 cells and in isolated mitochondria using X-rhod-1 and $^{45}Ca^{2+}$ indicated that PUFA but not saturated FA or carbachol induced Ca^{2+} release from mitochondria. Our results thus provide evidence that PUFA mobilize $[Ca^{2+}]_i$ in NT2 cells by causing release of Ca^{2+} from mitochondria. These observations further imply that mitochondrial dysfunction observed under pathophysiological conditions associated with lipid overload and/or elevated concentrations of FA may occur via alteration of mitochondrial and/or cellular Ca^{2+} homeostasis.

In several cell types, CCCP increases $[Ca^{2+}]_i$ by causing Ca^{2+} release from mitochondria (7, 12, 20, 29, 34, 49, 60, 67, 72). CCCP is a mitochondrial uncoupler that collapses the proton gradient across the mitochondrial inner membrane and thus eliminates the driving force for mitochondrial Ca^{2+} uptake. In addition, Ca^{2+} efflux from mitochondria requires the opening of a conducting pathway for Ca^{2+} exit. During GPCR-stimulated $[Ca^{2+}]_i$ mobilization CCCP prevents mitochondrial Ca^{2+} uptake, which leads to inhibition of the store-operated or capacitative Ca^{2+} influx. Consistently in NT2 cells CCCP eliminated the plateau phase of carbachol-mediated $[Ca^{2+}]_i$ mobilization, indicating the inhibition of store-operated Ca^{2+} influx (Fig. 7*F*). The effect of CCCP on $[Ca^{2+}]_i$ in resting cells has been found to vary among different cell types. For example, in studies (20) of pancreatic acinar cells, CCCP did not induce $[Ca^{2+}]_i$ signals in resting cells but did prevent mitochondrial Ca^{2+} uptake and cause $[Ca^{2+}]_i$ mobilization in agonist-stimulated cells. In chromaffin cells, CCCP triggered $[Ca^{2+}]_i$ mobilization and reduced $[Ca^{2+}]_m$ under resting conditions (46), indicating that in some cell types, energized mitochondria retain higher concentrations of ionic Ca^{2+} than the cytosol even in the resting state. Direct measurement of $[Ca^{2+}]$ in mitochondria with low-affinity aequorin yields a value of 5.8 μM (46), which is >50-fold higher than the normal resting $[Ca^{2+}]_i$. Thus mitochondria may be an independent intracellular Ca^{2+} store. It is known that the two intracellular Ca^{2+} stores, i.e., the mitochondrial Ca^{2+} pool and the

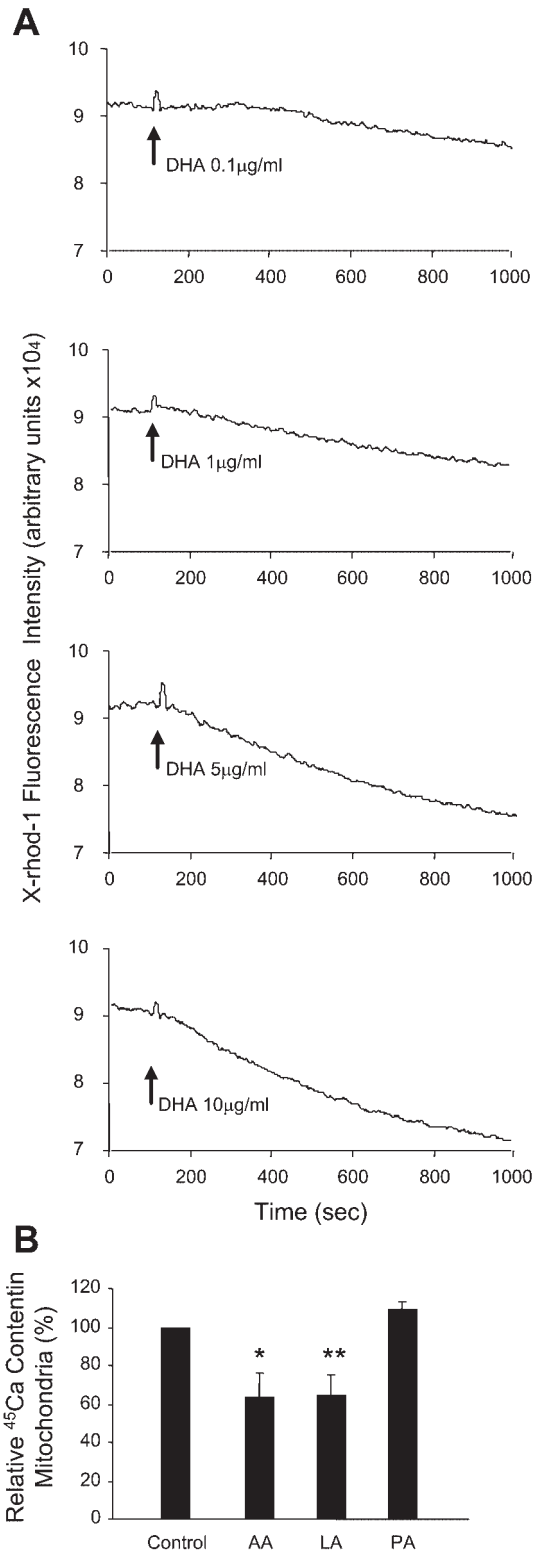


Fig. 9. Effect of PUFA and PA on $[Ca^{2+}]_m$ in isolated mitochondria from NT2 cells. *A*: mitochondria were isolated and labeled with X-rhod-1 (2 μM) at 37°C for 30 min in PBS1Ca. Loaded mitochondria were resuspended in PBS supplemented with 200 μM EGTA and 1 mM $MgSO_4$ (PBS0Ca) and Ca^{2+} release from mitochondria in response to increasing concentrations of DHA was measured immediately, as described in MATERIALS AND METHODS. *B*: isolated mitochondria were labeled with $^{45}Ca^{2+}$ at 37°C for 30 min in PBS1Ca. $^{45}Ca^{2+}$-loaded mitochondria were then transferred and incubated with PUFA (AA or LA; 10 μg/ml) or the saturated FA PA (10 μg/ml) in 10 vol of PBS0Ca at 37°C for 5 min. The mixtures were then filtered and washed three times with PBS0Ca and the $^{45}Ca^{2+}$ content in mitochondria was counted. Values in the figure are means ± SE from 5–6 measurements. *$P = 0.0079$ vs. untreated (control); **$P = 0.0039$ vs. control.

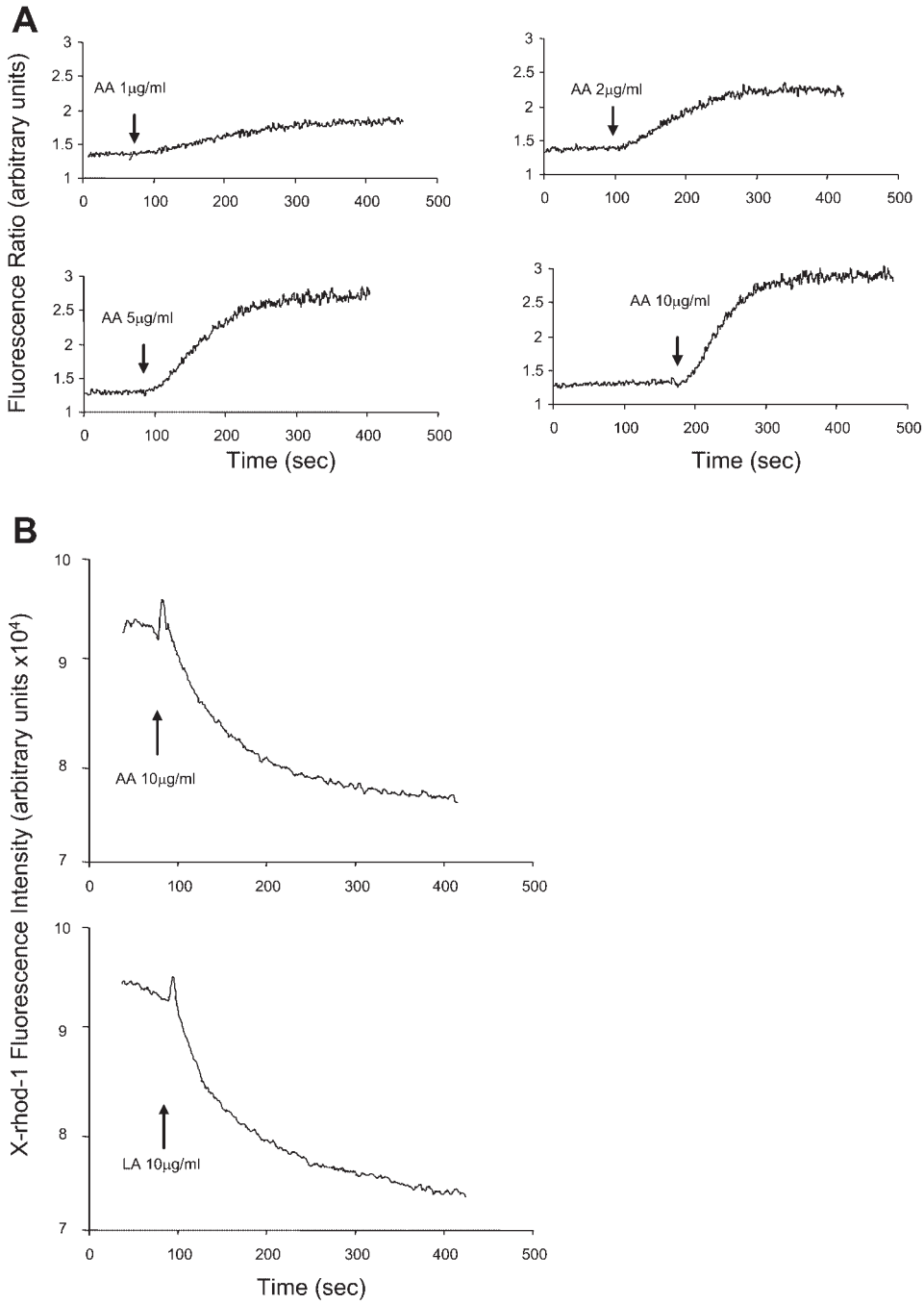


Fig. 10. PUFA-induced $[Ca^{2+}]_i$ mobilization in human aortic endothelial cells (HAEC) and mitochondrial Ca^{2+} efflux in isolated mitochondria of HAEC cells. *A*: confluent HAEC grown in 75-ml flasks were trypsinized and labeled with fura-2 AM (2 μM) at 37°C for 30 min. $[Ca^{2+}]_i$ mobilization in response to AA (1–10 μg/ml as indicated) was measured in labeled cells, as described in MATERIALS AND METHODS. Typical AA concentration-dependent $[Ca^{2+}]_i$ responses are shown. *B*: mitochondria were isolated from confluent HAEC and labeled with X-rhod-1, as described in MATERIALS AND METHODS. PUFA (AA or LA)-induced Ca^{2+} release from mitochondria was measured by the decrease in X-rhod-1 fluorescence intensity (*y*-axis).

IP_3-sensitive endoplasmic reticulum pool, are dynamically linked by their structural proximity during $[Ca^{2+}]_i$ mobilization (57). Part of the Ca^{2+} released from the endoplasmic reticulum by the IP_3-gated channels is taken up by mitochondria (68), leading to increased $[Ca^{2+}]_m$. The increase in $[Ca^{2+}]_m$ upregulates the activities of multiple enzymes involved in energy production, as indicated by changes in mitochondrial reduction-oxidation substrates (21, 68). On the other hand increased Ca^{2+} uptake into mitochondria and/or $[Ca^{2+}]_m$ also sensitize cells for the induction of apoptosis by proapoptotic factors (69). Mitochondrial Ca^{2+} uptake and subsequent efflux also modify the amplitude, duration, localization, and propagation of cytosolic Ca^{2+} transients (15, 19, 27, 71). In this study, we provide evidence that mitochondria may serve as an independent intracellular source for PUFA-responsive $[Ca^{2+}]_i$ mobilization in NT2 cells. CCCP-induced $[Ca^{2+}]_i$ mobilization was observed in resting NT2 cells (Fig. 6). The CCCP-mediated $[Ca^{2+}]_i$ signal in these cells may occur by elimination of the driving force for Ca^{2+} uptake, i.e., the proton gradient, and opening of a conducting pathway such as the mitochondrial Ca^{2+}-induced Ca^{2+} release process (46) or Ca^{2+} leak along the Ca^{2+} gradient between mitochondria and cytosol. PUFA-induced mitochondrial Ca^{2+} release and $[Ca^{2+}]_i$ mobilization in NT2 cells may occur by depolarization

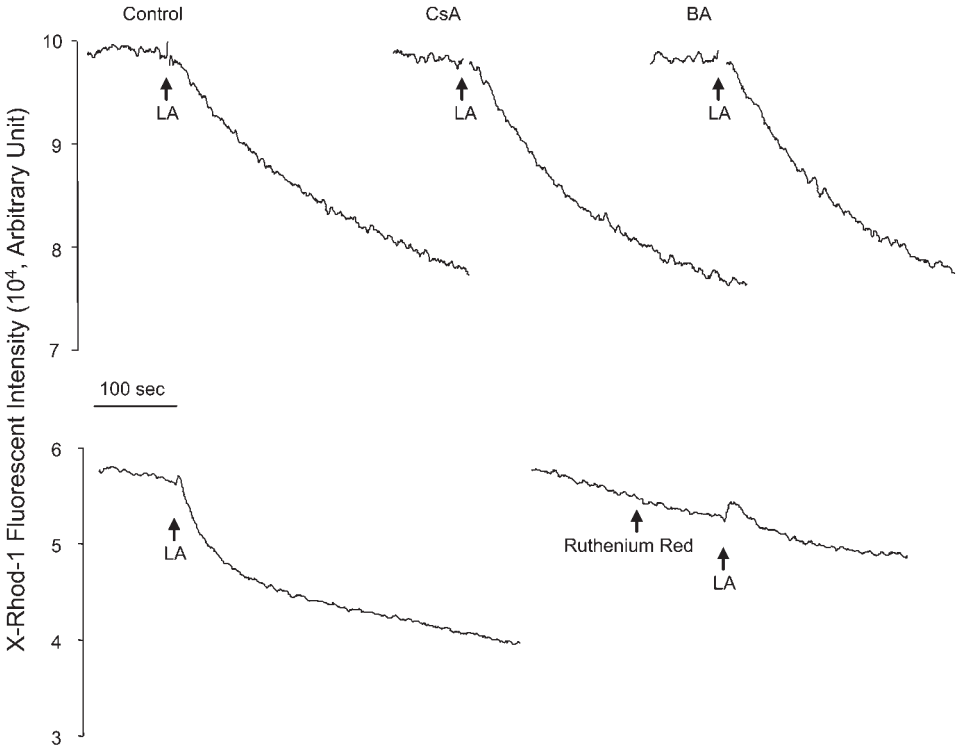


Fig. 11. Effect of cyclosporin A (CsA), bongkrekic acid (BA), and ruthenium red on LA-induced Ca^{2+} efflux in isolated mitochondria from NT2 cells. Mitochondria were isolated and labeled with X-rhod-1 (2 μM) at 37°C for 30 min in PBS1Ca. Loaded mitochondria were resuspended in PBS0Ca and Ca^{2+} release from mitochondria in response to LA (10 μg/ml) was measured as described in MATERIALS AND METHODS. In experiments with CsA and BA (*top*), the reagents (each at 10 μM) were preincubated with loaded mitochondria for 15 min at room temperature before the addition of LA; ruthenium red (10 μM) was added immediately before LA (*bottom*). Changes in $[Ca^{2+}]_m$ after LA treatment were indexed by alterations in X-rhod-1 fluorescence intensity (*y*-axis).

of the mitochondrial proton gradient through an uncoupling protein-dependent mechanism (32) and/or opening of the MPT for Ca^{2+} exit (41, 50, 64). Pretreatment of NT2 cells with CCCP inhibits subsequent PUFA-induced $[Ca^{2+}]_i$ mobilization probably by causing Ca^{2+} efflux from mitochondria and depletion of the mitochondrial Ca^{2+} pool (Fig. 7).

In this study, we used U73122 and 2-APB to examine the involvement of the classic PLC-IP_3 signaling pathway in PUFA-induced $[Ca^{2+}]_i$ mobilization. U73122 is a synthetic aminosteroid PLC inhibitor. The specificity of the inhibitor toward PIP_2 specific PLCs has been validated by numerous studies (22, 38, 53, 54, 75) from different laboratories that had used various cell types. We observed that treatment of NT2 cells with U73122 completely blocked carbachol-induced $[Ca^{2+}]_i$ mobilization but had no effect on the PUFA response (Fig. 3). The results confirm that as in other cell types, U73122 specifically targets the GPCR-PLC-IP_3 signaling pathway in NT2 cells; $[Ca^{2+}]_i$ mobilization mediated by other mechanisms, such as PUFA-induced Ca^{2+} efflux from mitochondria, is unaffected (Fig. 3). Although 2-APB was originally reported as a cell permeable IP_3 receptor antagonist (43), recent studies (3, 37) demonstrated effects of this agent on other pathways that may or may not be related to PLC-IP_3-mediated $[Ca^{2+}]_i$ mobilization. Moreover, inhibition of IP_3 receptor-mediated Ca^{2+} release by 2-APB has been shown to be variable among different cell types (3, 37). The inhibitory effect of 2-APB may depend on the isoforms of IP_3 receptors expressed in the cell and the cytosolic concentrations of IP_3 during agonist stimulation (3). In NT2 cells 2-APB effectively blocked $[Ca^{2+}]_i$ mobilization in response to carbachol but not PUFA (Fig. 4). 2-APB was found to reduce the rate of the initial rise of PUFA-induced $[Ca^{2+}]_i$ mobilization by 80% (Fig. 4). This action of 2-APB could result from a nonspecific effect on mitochondria, insofar as 2-APB has been suggested to inhibit mitochondrial Ca^{2+} efflux in Jurkat T cells (52a).

Multiple mitochondrial pathways, including the Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers, the Ca^{2+} uniporter, as well as the MPT, are capable of transporting Ca^{2+} out of mitochondria (14, 52, 56). Opening of MPT by PUFA and other FA has been reported in isolated mitochondria (2). The MPT is theoretically permeable to Ca^{2+} and other small molecules, which makes the MPT a possible candidate for mediating PUFA-induced Ca^{2+} efflux. In addition, the opening of MPT by PUFA may collapse the mitochondrial membrane potential and proton gradients and thus indirectly activate the reversal mode of the Ca^{2+} uniporter, or affect the activities of the Na^+/Ca^{2+} and/or H^+/Ca^{2+} exchangers, to release mitochondrial Ca^{2+}. We have found that PUFA depolarize mitochondrial membrane potential in intact NT2 cells (data not shown). However, preincubation of NT2 mitochondria with CsA and BA had no effect on PUFA-induced Ca^{2+} efflux (Fig. 11), suggesting that the MPT and other pathways indirectly linked with MPT through mitochondrial membrane potential may not be involved in PUFA-mediated mitochondrial Ca^{2+} efflux. This does not exclude the possibility that PUFA might directly activate mitochondrial Ca^{2+} transporters to release Ca^{2+} in Ca^{2+}-loaded mitochondria. Indeed, as we have demonstrated, the addition of ruthenium red to block the mitochondrial Ca^{2+} uniporter inhibited LA-induced mitochondrial Ca^{2+} efflux, implicating the involvement of the Ca^{2+} uniporter in PUFA-mediated mitochondrial Ca^{2+} efflux (Fig. 11). The Na^+/Ca^{2+} exchanger blocker CGP37157 did not affect LA-induced $[Ca^{2+}]_i$ mobilization in NT2 cells (data not shown). PUFA and other FA may also depolarize the mitochondrial membrane potential by uncou-

pling protein 2 (UCP-2)-dependent mechanisms. However, the UCP-2 pathways are unlikely to be the underlying mechanism for PUFA-induced mitochondrial Ca^{2+} efflux because saturated and monounsaturated FA activate the UCP-2 pathway in other systems (35) but had no effect on $[Ca^{2+}]_i$ in NT2 cells and $[Ca^{2+}]_m$ in isolated mitochondria (Figs. 1 and 8). Additional studies are ongoing to define the role of the mitochondrial Ca^{2+} uniporter and possibly other transporters in PUFA-mediated mitochondrial Ca^{2+} efflux.

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