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Evidence against Calcium as a Mediator of Mitochondrial Dysfunction during Apoptosis Induced by Arachidonic Acid and Other Free Fatty Acids[1]

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Apoptosis is often accompanied by activation of phospholipase A_2, causing release of free fatty acids (FFAs), which in turn are thought to contribute to the loss of mitochondrial transmembrane potential ($\Delta\psi_m$). In these experiments, we asked whether calcium plays a role as an intermediate in this process. A total of 14 FFAs were compared for their ability to cause loss of $\Delta\psi_m$ and for their ability to affect levels of intracellular calcium. Among the FFAs, unsaturated FFAs tended to induce apoptosis while saturated FFAs did not. Arachidonic acid (AA) was most damaging, causing loss of $\Delta\psi_m$ and cell death in 8–10 h while linoleic acid, γ-linolenic acid, and docosapentaenoic also strongly induced apoptosis. Effects of the FFAs on levels of intracellular calcium were very different. Many caused strong calcium responses; however, the ability to induce a strong calcium response was not predictive of ability to induce apoptosis, and overall, we did not find a correlation between apoptosis and calcium induction. Also, verapamil and TMB-8 were able to block the calcium response, but these inhibitors did not prevent loss of $\Delta\psi_m$, indicating that the calcium response is not necessary for FFA-induced loss of $\Delta\psi_m$. In contrast, we found that cyclosporine A could inhibit the AA-induced loss of $\Delta\psi_m$ with both whole cells and isolated mitochondria, confirming that the antimitochondrial effects of FFA can stem from direct effects on the mitochondrial permeability transition pore. Finally, we show that the strong apoptosis-inducing activity of AA may stem from its ability to selectively induce its own release. ***The Journal of Immunology,*** **2006, 177: 6398–6404.**

Apoptosis involves activation of many intracellular molecules including a number of different phospholipase A_2 (PLA_2)[3] enzymes. Several isozymes of cytosolic PLA_2 ($cPLA_2$) (1–6), the calcium-independent PLA_2 (7, 8), and secretory PLA_2 (9, 10) have been linked to apoptosis in different model systems. A number of hypotheses have been put forward to explain the role that these enzymes and their free fatty acid (FFA) products might play in apoptosis. Several studies have suggested that the PLA_2s are important during apoptosis, for the release of arachidonic acid (AA), which is in turn converted to proapoptotic prostaglandins (11) or leukotrienes (12). Alternatively, AA has also been linked to sphingomyelinase activation resulting in the production of proapoptotic ceramide (13). Most recently, research on the proapoptotic effects of FFA has focused on the mitochondria. AA has been shown to exert a direct effect on mitochondria, causing the mitochondrial permeability transition pore (MPTP) to open irreversibly leading to loss of mitochondrial transmembrane potential ($\Delta\psi_m$), release of cytochrome *c*, and inhibition of mitochondrial respiration (14–17). This effect has also been noted with several other long-chain fatty acids (FAs) (15) clearly indicating that the activity of both arachidonyl-selective and arachidonyl-nonselective PLA_2s can contribute to mitochondrial dysfunction during apoptosis.

Calcium is an important intracellular mediator during apoptosis (18). Originally, calcium was linked to activation of endonucleases during glucocorticoid-induced apoptosis in thymocytes (19). More recently, calcium has been linked to calcineurin activation in lymphocytes (20) and $cPLA_2$ activation in fibroblasts (21). Calcium has also been linked to the loss of $\Delta\psi_m$ that accompanies apoptosis. Experiments with paclitaxel (22), neurotoxins (23), ceramide (24), and thapsigargin (25) all suggest calcium plays a role in the irreversible opening of the MPTP that can accompany apoptosis. Apparently, the MPTP opens in response to abnormally high levels of calcium that accumulate in mitochondria during apoptosis. Cyclosporine A (CSA) can inhibit this process (26) suggesting that calcium is working through cyclophilin D, a component of the MPTP.

AA and other FFAs have also been shown to trigger elevated levels of intracellular calcium (21, 27–30). We hypothesized, therefore, that in addition to the direct effects of FFAs on mitochondria during apoptosis, an indirect effect might also be occurring via a calcium intermediate. To address this hypothesis, we tested a panel of FFAs both for their affects on intracellular calcium and loss of $\Delta\psi_m$. We also tested the effects of calcium channel blockers on the loss of $\Delta\psi_m$ during FFA-induced apoptosis. The results of our experiments do not support a role for calcium as the mediator of mitochondrial dysfunction during FFA-induced apoptosis.

Materials and Methods

Cell culture and reagents

C3HA cells were cultured in DMEM supplemented with 10% FBS and maintained at 37°C and 8% CO_2. The concentration of FBS was reduced

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[3] Abbreviations used in this paper: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; FA, fatty acid; FFA; free FA; AA, arachidonic acid; MPTP, mitochondrial permeability transition pore; $\Delta\Psi_m$, mitochondrial transmembrane potential; CSA, cyclosporine A; MYA, myristic acid; PA, palmitic acid; SA, stearic acid; AIA, arachidic acid; POA, palmitoleic acid; OA, oleic acid; LA, linoleic acid; EDA, eicosadienoic acid; GLA, γ-linolenic acid; MA, mead acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester.

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to 5% for experiments with added FFAs. Media and reagents, unless otherwise indicated, were obtained from Sigma-Aldrich. The calcium inhibitors 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) and verapamil were obtained from Calbiochem. Fluo-4 AM, FITC-annexin V, and rhodamine 123 (Rho^{123}) were purchased from Molecular Probes.

Microscopy

Cells were plated in 8-well glass chamber-slides (Nalge Nunc International). Microscopy was performed using a Axioskop 2 plus (Carl Zeiss), and images were captured and processed by a Spot charge-coupled device camera and software (Diagnostic Instruments). Intracellular calcium was detected using Fluo-4 AM as described by the manufacturer. Briefly, for a working solution of Fluo-4 AM, an equal volume of Fluo-4 AM stock (2 mM in anhydrous DMSO) was added to a 20% pluronic solution. Then, 15 min before the end of treatments, Fluo-4 AM was added to a final concentration of 5 μM. Cells were then washed two times with PBS, fixed with formalin, mounted for microscopic observation, and digital images were recorded. To quantifying the amount of Fluo-4 AM staining, each cell was outlined and mean fluorescence intensity in the outlined area was determined using Adobe Photoshop 5.5 (Adobe Systems).

$\Delta\psi_m$ was monitored using the fluorescent dye Rho^{123}, which selectively accumulates in live polarized mitochondria without causing cytotoxic effects (31). For direct observation, the cells were incubated with Rho^{123} (5 μM final concentration) during the last 30 min of each treatment, washed two times with PBS, and mounted for observation. To quantify Rho^{123} staining, ×40 images were converted to gray scale and mean fluorescence intensity per field was determined using the Spot Software. For FITC-annexin V staining, cells were treated then washed two times with binding buffer, incubated with FITC-annexin V for 15 min, washed again, and mounted for microscopy.

Fluorometry

Fluorometric analysis of staining with Rho^{123} was performed using a PolarStar Galaxy microplate reader (BMG LabTechnologies), with the excitation and emission filters set at 492 and 538 nm, respectively. Either 2×10^4 C3HA cells, or 50 μl of the standard mitochondrial preparation were added per well in 96-well plates (Corning). Specific treatments were added for the indicated times and Rho^{123} was added as described above. The cells were subsequently washed two times with PBS and fluorescence intensity was recorded. Washes were not performed in experiments with isolated mitochondria.

Mitochondria isolation

The mitochondria isolation protocol was adapted from Zhou et al. (32). C3HA cells (2×10^6/well) were plated overnight, washed two times with PBS, and once with washing buffer (250 mM sucrose, 200 mM mannitol). Lysis was initiated with a 1-min incubation in homogenization buffer (200 mM mannitol, 50 mM sucrose, 1 mM EDTA, 20 mM HEPES-KOH (pH 7.4), and protease inhibitors). The cells were then scraped and homogenized by 20 passes through a 26-gauge needle. The nuclear pellet and debris was discarded following centrifugation at $1,000 \times g$ for 10 min and the heavy mitochondrial pellet discarded following a second centrifugation at $3,000 \times g$ for 10 min. The supernatant from this step was then centrifuged at $17,000 \times g$ for 45 min and the resulting pellet (light mitochondrial fraction) was purified further using an Optiprep (Sigma-Aldrich) discontinuous iodixanol gradient (40:10%). The gradient was centrifuged at $50,000 \times g$ for 4 h at 4°C using an Optima TL ultracentrifuge (Beckman Instruments). Purified mitochondria were harvested from the interface and used in apoptosis experiments. Protein assays were performed using the BCA protein assay kit (Pierce) to standardize each mitochondrial preparation.

[^{3}H]AA release assays

A total of 1×10^5 cells was plated into 12-well flat-bottom tissue culture plates (Fisher Scientific) and labeled overnight with 0.1 μCi/ml [^{3}H]AA. The following morning, the cells were washed two times with HBSS, allowed to recover for an additional 2 h, and washed again before treatment with FFAs. At indicated time points after treatment, 300-μl aliquots of medium were removed from the wells and centrifuged to remove debris. A total of 200 μl of the supernatant was removed for scintillation counting (Beckman model LS 5801) and total [^{3}H]AA release was calculated by multiplying by a factor of 3. Each point was performed in triplicate and maximum radiolabel incorporation was determined by lysing untreated controls with 0. + 0.1% SDS and counting the total volume.

Statistical analysis

Prism software (GraphPad) was used for statistical analysis of data and figure preparation. A nonparametric, two-tailed Mann-Whitney *U* test was used to compare means in Figs. 2 and 5.

Results

The effects of AA and other FFAs on mitochondria in C3HA fibroblasts

These experiments were performed using the murine cell line C3HA. C3HA cells are large, thin fibroblasts (2 μM thick) (33), that are well-suited for microscopic investigations. In addition, we have characterized many of the biochemical changes that occur in these cells as they undergo apoptosis (1, 21, 33–35). Rho^{123} was used to monitor $\Delta\psi_m$ in these cells they underwent FFA-induced apoptosis. Rho^{123} accumulates in healthy mitochondria and is lost from mitochondria during apoptosis (31). With C3HA cells, we found that staining with Rho^{123} became notably decreased 2–4 h after treatment with the AA was initiated (Fig. 1, *A* and *B*) and by 6 h many cells were seen which stained only very faintly with Rho^{123} (Fig. 1*C*). After this, the cells began to bleb and detach from the substrate at which point they bound FITC-annexin V (Fig. 1*D*). Flattened adherent cells, as shown in Fig. 1, *C* or *D*, consistently failed to bind FITC-annexin V. Ten hours after treatment with 50 μM AA, extensive apoptosis had occurred and the culture was filled with debris. We found that washing with PBS removed the debris and produced images which dramatically illustrated the toxic effects of AA (compare Fig. 1, *E* and *F*).

Changes in Rho^{123} staining could also be measured by fluorometer and we used this technique to characterize the effects of FFAs

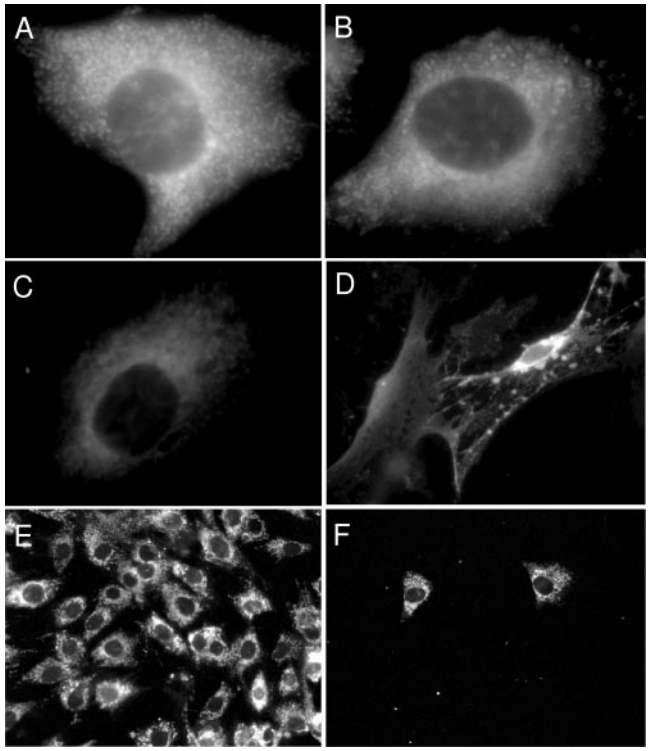

FIGURE 1. Loss of $\Delta\psi_m$ during AA-induced apoptosis. C3HA fibroblasts were either left untreated (*A*) or treated with 50 μM AA in culture medium with 5% FBS for 2 (*B*) or 6 (*C*) h. Staining with FITC-annexin V was also performed after a 6-h treatment with 50 μM AA (*D*). Lower magnification images of C3HA cells either untreated (*E*) or following a 10-h treatment with 50 μM AA (*F*). Microscopy was performed as indicated in *Materials and Methods*.

on C3HA cells and mitochondria. As shown in Fig. 2*A*, this technique typically revealed a 50% decrease in staining after 6 h and after 10 h treatment with 50 μM AA staining was reduced by 75%. In contrast, significant reduction in staining did not occur following treatment with palmitic acid (PA) (Fig. 2*A*). The ability of AA to cause loss of Rho^{123} staining was blocked by treatment with CSA ($p < 0.05$) (Fig. 2*B*). CSA acts on cyclophilin D, one of the components of the MPTP, preventing opening of the MPTP (16, 17). As shown in Fig. 2*C*, we found that treating mitochondria isolated from C3HA cells also caused loss of staining with Rho^{123}. Typically, treatment of mitochondria with 50 μM AA for 2 h caused a 50–60% loss in Rho^{123} staining, and again PA did not cause this effect (Fig. 2*C*). This effect could also be strongly inhibited by CSA (75%, $p < 0.05$) (Fig. 2*D*) confirming (15, 16) that AA can mediate an antimitochondrial effect directly through the MPTP.

The proapoptotic effects of AA were not unique and were seen with other FFAs. In this study, a total of 14 FFAs were examined for effects on $\Delta\psi_m$ (Table I). Included were several saturated FAs (myristic acid (MYA), PA, stearic acid (SA), arachidic acid (AIA)) and a number of unsaturated FAs with 16–22 carbons (palmitoleic acid (POA), oleic acid (OA), linoleic acid (LA), γ-linolenic acid (GLA), mead acid (MA), AA, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA)). MYA, OA, PA, POA, SA, AA, and LA were selected because these comprise 99.9% of the sn-2 FAs in C3HA membranes (2) while the others were included because of characteristics similar to the natural FAs. As shown in Fig. 2*E*, we found that these FFAs exerted a wide range of effects on staining with Rho^{123}. The saturated FAs did not generally cause substantial loss of staining and in longer experiments staining recovered and the cells appeared to

Table 1. *FFA tested in this report*

Name[a]	No. of Carbons	No. of Unsaturated Bonds
Myristic acid (MYA)	14	0
Palmitic acid (PA)	16	0
Stearic acid (SA)	18	0
Arachidic acid (AIA)	20	0
Palmitoleic acid (POA)	16	1
Oleic acid (OA)	18	1
Linoleic acid (LA)	18	2
Eicosadienoic acid (EDA)	20	2
γ-Linolenic acid (GLA)	18	3
Mead acid (MA)	20	3
Arachidonic acid (AA)	20	4
Eicosapentaenoic acid (EPA)	20	5
Docosapentaenoic acid (DPA)	22	5
Docosahexaenoic acid (DHA)	22	6

[a] FFA abbreviations shown in parentheses.

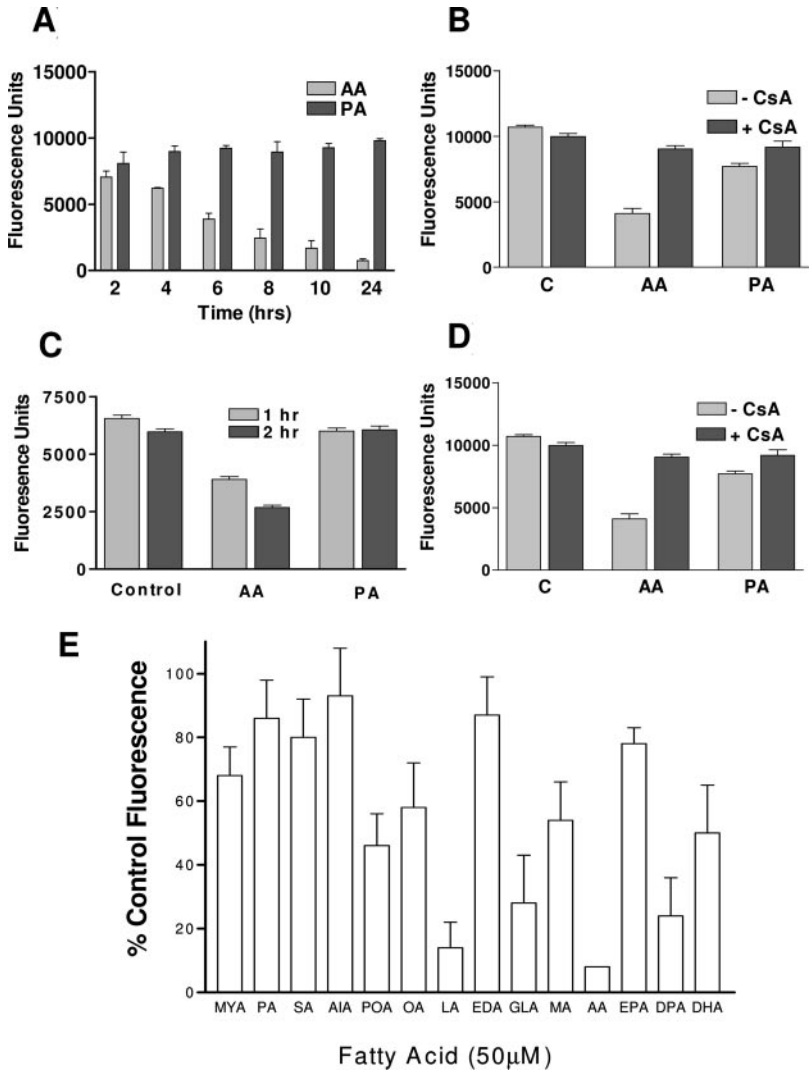

FIGURE 2. AA causes loss of $\Delta\psi_m$ in situ and with isolated mitochondria. C3HA fibroblasts were plated overnight in 96-well plates (10^4/well) (*A*) or isolated mitochondria (*C*), were treated with 50 μM AA or PA for the indicated times, stained with Rho^{123} and fluorescence monitored using a Polarstar microplate reader as described in *Materials and Methods*. CSA (10 μM) inhibits the AA-induced loss of $\Delta\psi_m$ with whole cells (*B*), and CSA (2 μM) inhibits loss of $\Delta\psi_m$ with isolated mitochondria (*D*). The data shown are means ± SEM of triplicate treatments from representative experiments. In *E*, 14 FFAs were tested for effects on $\Delta\psi_m$ with whole cells. Values shown are means ± SEM from three experiments following a 10-h treatment with each FA.

grow normally (data not shown). In contrast, the unsaturated FAs were generally toxic although we did note wide variation and that variation did not correlate with chain length or number of double bonds. AA was clearly the most toxic, although several other FAs, including LA, GLA, and DPA caused substantial levels of cell death and loss of staining with Rho^{123} (Fig. 2*E*).

The AA-induced calcium response

The effects of AA and other FFAs on levels of intracellular calcium were tested using the indicator Fluo-4 AM. As shown in Fig. 3, *A–D*, we found that treatment with 50 μM AA could cause a robust calcium response. Initially, 5–10 min after treatment was initiated; punctate cytosol staining was observed in ~40–50% of the cells (Fig. 3*B*). Later, along with the punctate cytosolic staining, bright staining was seen in the nuclear region of the cell (Fig. 3*C*). Eventually, by 30 min, many cells took on a "fried egg" appearance with bright nuclei and cytosol, while the punctate cytosolic staining was no longer visible (Fig. 3*D*). As shown in Fig. 3*E*, we found that the response reached a maximum 30 min after treatment was initiated and returned to pretreatment levels after 1 h. The ability of AA to induce elevated intracellular calcium was not unique to this FA. As shown in Fig. 3*E*, we found that PA induced a response with virtually identical kinetic parameters.

The ability of FFAs to induce apoptosis does not correlate with levels of intracellular calcium

As shown in Fig. 4*A*, we found that the ability of AA to cause elevated intracellular calcium could be blocked by either verapamil or TMB-8, suggesting that both extra- and intracellular calcium are required for this response. These inhibitors also blocked the increase in calcium observed following treatment with PA. The inhibitors were then tested for their effects on staining with Rho^{123}. Interestingly, with control cells, we found that both verapamil and TMB-8 enhanced staining with Rho^{123} × 20–25% and 40–50%, respectively. In contrast, neither verapamil nor TMB-8 inhibited the loss of Rho^{123} staining seen following treatment with AA, indicating that increased calcium is not required for AA-mediated loss of $\Delta\psi_m$. Calcium's role in this response was also addressed with the broader panel of FFAs. As shown in Fig. 5*A*, we found that all FFAs, except for AIA, caused significant ($p < 0.05$) increases in levels of intracellular calcium. Several FFAs induced strong responses, comparable to AA, including: PA, SA, and eicosadienoic acid (EDA) (Fig. 5*A*), while many FFAs induced intermediate level responses. We noted that those FFAs that caused the highest levels of intracellular calcium displayed a wide range of apoptosis-inducing activity and that strong induction of apoptosis was not predictive of a strong calcium response. The lack of correlation between the ability of FFAs to induce apoptosis and the

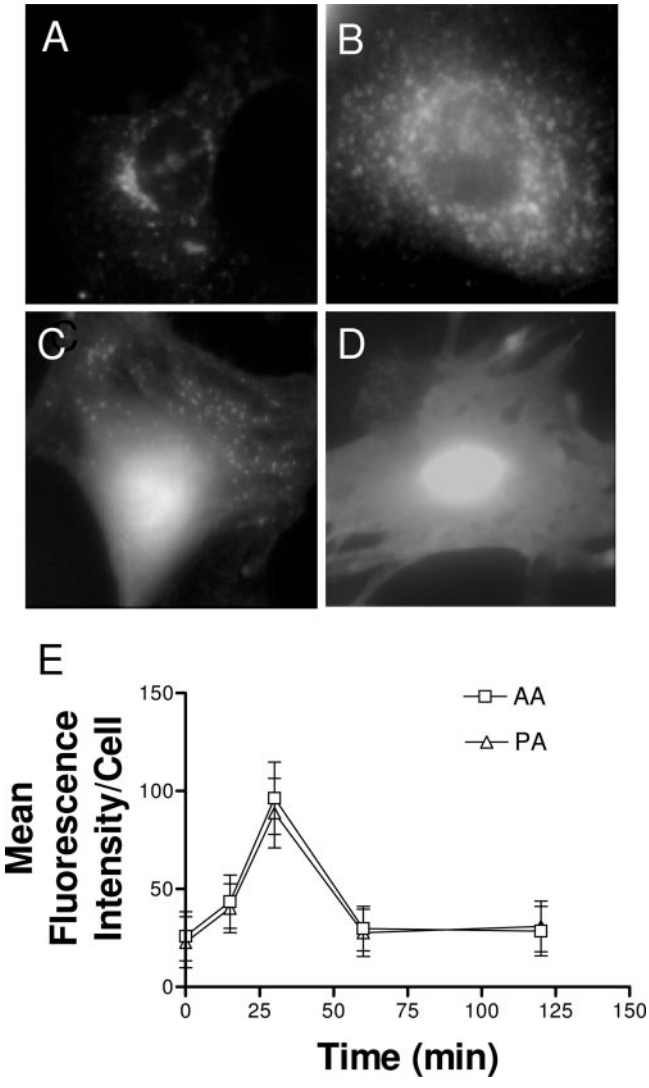

FIGURE 3. FFAs cause elevated intracellular calcium. C3HA cells were either left untreated (*A*) or treated with 50 μM AA for 10 (*B*), 20 (*C*), or 30 (*D*) min and stained with Fluo-4, AM as described in *Materials and Methods*. Values shown in *E* are mean fluorescent intensity measurements ± SEM from three experiments, with calcium levels analyzed in 40 cells, at each time point in each experiment.

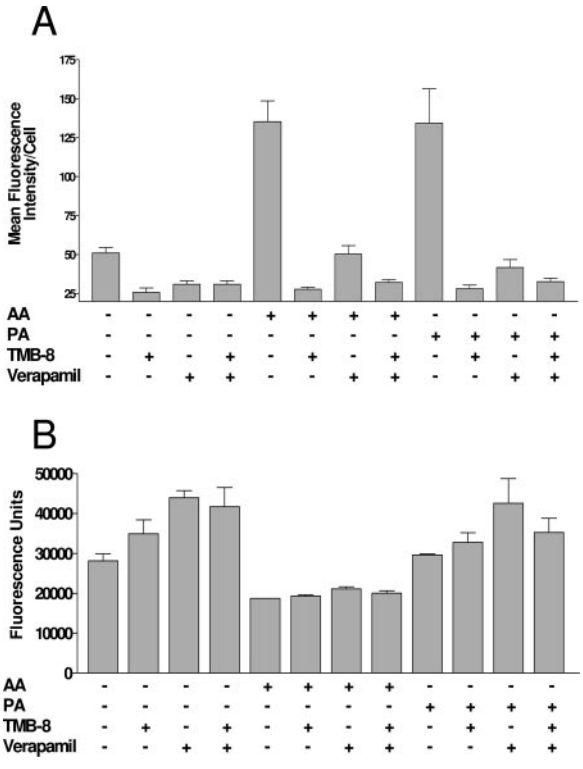

FIGURE 4. Verapamil and TMB-8 prevent FA-induced changes in calcium concentration, but fail to inhibit loss of $\Delta\psi_m$. C3HA fibroblasts were treated with 50 μM concentrations of AA or PA in the absence/presence of verapamil (10 μM) and/or TMB-8 (50 μM). Following treatments, cells were stained either with Fluo-4, AM, or Rho^{123} and fluorescence quantified by image analysis (*A*) or fluorometry (*B*). Values shown are mean ± SEM of triplicate treatments from a representative experiment.

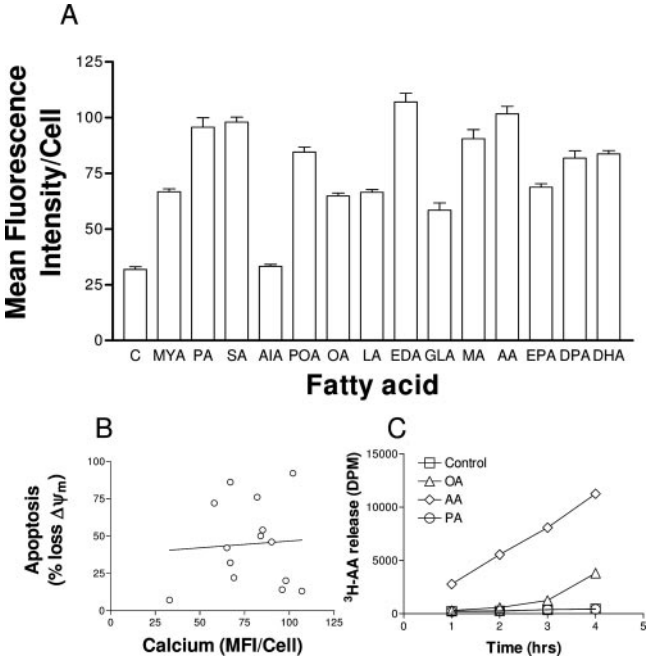

FIGURE 5. Most FAs elevate levels of intracellular calcium. C3HA fibroblasts were plated in 8-well chamber slides, treated with 50 μM concentrations of the indicated FAs for 30 min, stained with Fluo-4, AM and images analyzed as described in *Materials and Methods* (*A*). Percentage of increases for means above control values for the FAs shown in *A* were 108, 200, 207, 165, 103, 108, 83, 4, 235, 184, 219, 116, 156, and 162, respectively. *B*, A linear regression analysis comparing the ability of FFA to induce apoptosis (as determined by loss of Rho^{123} staining as shown in Fig. 2*E*) with the ability of FFA to cause elevated intracellular calcium (as determined by mean fluorescence intensity as shown in *A*). In *C*, cells were labeled overnight with [^{3}H]AA as described in *Materials and Methods*, treated with 50 μM concentrations of unlabeled FFAs, and supernatants were collected for liquid scintillation counting. Values shown are means ± SEM from a representative experiment. All points were performed in triplicate. Where not shown, error bars are less than symbol size.

ability to cause elevated levels of intracellular calcium is shown graphically in Fig. 5*B*. Overall, we did not observe a correlation between the two activities ($r = 0.06$).

The autocrine AA response

Although the data we have presented tend to rule out calcium as an indirect mediator of FA-induced loss of $\Delta\psi_m$, it is possible that other indirect mediators may be involved. As shown in Fig. 5*C*, we find that AA itself can induce the release of AA, an effect that was not seen with PA and to a much lesser extent with OA. This response was rapid and preceded the onset of reduced staining with Rho^{123} by several hours. It is possible, therefore, that the AA induced through autoinduction by AA contributes to the total AA-induced loss of $\Delta\psi_m$ and may explain why AA was most toxic of all the FAs tested.

Discussion

Mitochondria can play a pivotal role in apoptosis (36). Active mitochondrial respiration is necessary for the production of oxygen radicals which can function as important intracellular signals (37). Additional proapoptotic signals such as cytochrome *c* (38) and Smac/DIABLO (39) are also released from the mitochondria as the mitochondria incur damage. Several mechanisms have been suggested as the cause of mitochondrial damage and release of proapoptotic signals including high levels of intracellular calcium that trigger the MPTP to open irreversibly (18) and polymerization of proapoptotic BCL-2 family members, such as Bax, on mitochondrial membranes to form additional pores (40). Scorrano et al. (16) showed originally that AA caused mitochondrial dysfunction with isolated hepatocyte mitochondria. These observations were extended by Penzo et al. (15) who reported that many, but not all, long-chain FAs could have negative effects on isolated mitochondria. These authors also showed that a number of FFA (PA, OA, LA, GLA, AA, and EDA) could induce apoptosis with MH1C1 hepatoma cells. The results of our experiments with C3HA fibroblasts produced very similar results. Apparently, the ability of specific FFA to induce apoptosis by directly acting on the MPTP is a characteristic of the FA itself and is cell type independent. Inhibitors of this process, i.e., CSA, would be predicted to have broad biological activity in situations where cellular pathology is FFA dependent.

FFAs, including AA, are known to trigger elevations in intracellular calcium in a variety of cell types (29, 41–47). Recent investigations into this phenomenon have lead to the characterization of a novel calcium channel in HEK293 cells that has been reported to display specificity for AA over other FAs (42, 43). These results, coupled with the well-established role of calcium in apoptosis and loss of the $\Delta\psi_m$, made it reasonable to hypothesize that a component of the antimitochondrial effect of FFA in situ could be caused by increased intracellular calcium. Our data did not support this hypothesis. We found that many of the FFAs we tested triggered significant ($p < 0.05$) increases in intracellular calcium, with many cells displaying 4- to 5-fold increases. Based on previous quantitative determinations with fura-2 in C3HA fibroblasts (21), the peak levels of calcium reported here can be estimated to be 500-1000 nm, well into the range necessary to activate intracellular processes. The time course of the calcium response and the intracellular location of the calcium also appeared identical for both apoptotic and nonapoptotic FAs.

We found that the rise in calcium triggered by FAs could be blocked by an inhibitor of plasma membrane L-type channels (verapamil) or an inhibitor of calcium release from the endoplasmic reticulum (TMB-8), suggesting a store-operated channel requiring both intra and extracellular calcium that is typical of nonexcitable cell types (48). Although not typical, the ability of verapamil to block a store-operated or capacitative channel has been reported previously (49). Regardless, we used these inhibitors to test whether FA-induced changes in calcium concentration are necessary for apoptosis and found that neither verapamil nor TMB-8 could inhibit the loss of Rho^{123} staining from C3HA fibroblasts. We conclude, therefore, that the elevations in intracellular calcium triggered by AA are neither necessary nor sufficient to induce apoptosis. This contrasts with the apoptotic response of C3HA fibroblasts following treatment with TNF and cycloheximide, where a calcium response occurs 2–4 h after treatment is initiated and is necessary for the activation of $cPLA_2$ (21). During TNF-induced apoptosis, our results suggest that once $cPLA_2$ has been activated and AA released, the apoptotic process continues in a calcium-independent manner, at least for the pathological effects on mitochondria. We should also note that the AA-induced loss of staining with Rho^{123} is caspase independent. We could not inhibit this effect with the broad specificity caspase inhibitor Z-VAD, while Z-VAD worked effectively to block loss of staining with Rho^{123} following treatment with TNF and cycloheximide (data not shown).

Of the PLA_2s linked to apoptosis, $cPLA_2$ is unique in that it displays cleavage selectivity toward AA. AA is clearly a strong inducer of apoptosis, and therefore, the activation of $cPLA_2$ has the potential for causing more severe pathology than does activation of

the other PLA_2s linked to apoptosis. AA displays several unique proapoptotic characteristics such as the ability to be converted to proapoptotic eicosanoids (50, 51) or regulate the expression of genes involved in susceptibility and resistance to apoptosis (52). In this study, we showed that treatment with AA rapidly induces release of additional AA. Any number of different mechanisms may be responsible for this effect including activation of $cPLA_2$ itself by elevated intracellular calcium (29), or by activation of protein kinase C_α (53) or p42/44 MAPK (54). Calcium is probably not the mediator in this case, because PA and AA had similar effects on intracellular calcium, but PA did not cause release of AA. Regardless of the mechanism, it is possible that this "autocrine" response also contributes to the highly proapoptotic nature of AA. In contrast, AA should not be the only FA considered during the apoptotic activation of $cPLA_2$. Several of the other FFA we tested displayed strong apoptosis-inducing activity and their release during apoptosis by $cPLA_2$ and other nonselective PLA_2s must be considered. In addition, the selectivity of $cPLA_2$ for AA is muted in many cells by the relative lack of sn-2 AA relative to other FAs. For example, in C3HA cells, AA constitutes only 6% of sn-2 FA and activation of $cPLA_2$ actually causes similar amounts of AA and PA to be released (2). Although PA was found not to be apoptosis inducing, OA and POA were moderately toxic in these experiments and are found in high concentration in C3HA membranes. These FAs would, therefore, be expected to contribute to cellular pathology when released during apoptosis by $cPLA_2$ and other PLA_2s. Loss of $\Delta\psi_m$ following PLA_2 activation should, therefore, be viewed as a complex, cumulative effect caused by several proapoptotic FFAs.

In summary, the results of our experiments did not indicate that calcium plays a role in FFA-induced loss of $\Delta\psi_m$. Instead, our experiments support the hypothesis that FFA-induced apoptosis is dependent on a direct effect of FFAs on the MPTP. In general, the apoptosis-inducing activity of FFAs was greater for those with longer chain length and multiple unsaturations; although these characteristics were not entirely predictive of strong apoptosis-inducing activity. EDA (20:2) and EPA (20:5), for example, were relatively poor inducers of apoptosis in these experiments. Potentially useful inhibitors of apoptosis might come from understanding the mechanism by which certain FFA interact with the MPTP and mitochondria to cause loss of $\Delta\psi_m$ and cell death.

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Disclosures

The authors have no financial conflict of interest.

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