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ORIGINAL CONTRIBUTION

Oleic acid inhibits store-operated calcium entry in human colorectal adenocarcinoma cells

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Abstract
Aims Much evidence indicates the association between dietary fat and colorectal cancer risk. However, most of the studies focus on polyunsaturated fatty acids, and little is known about the role of monounsaturated ones and their precise mechanism of action. Being store-operated Ca^{2+} entry (SOCE) a Ca^{2+} influx pathway involved in the control of multiple cellular and physiological processes including cell proliferation, we studied the effect of oleic acid in Ca^{2+} signals of colorectal cancer cells, paying particular attention to SOCE.
Methods Carbachol was used to induce SOCE in Fura 2-loaded HT29 cells. We tested a saturated fatty acid to compare the physiological relevance of our results.
Results We show that oleic acid is a potent inhibitor of SOCE. By contrast, stearic acid failed to have a SOCE-inhibitory effect. The SOCE-inhibition induced by oleic acid was protein kinase C-independent and restored by albumin. We also demonstrated that oleic acid induced increases in $[Ca^{2+}]_i$. The novelty of our report is that little variability in the concentration could end in a large different physiological effect.
Conclusions In conclusion, we suggest a physiological pathway for the beneficial effect of oleic acid in colon carcinoma cells.

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Introduction

Colorectal cancer is one of the most common cancers in western countries. It has been estimated that a significant proportion of colorectal cancer cases may be explained by dietary habits and that they could be reduced through individual and social actions [1, 2].

Within the amount of dietary compounds that have been related to colon cancer, dietary lipids have been revealed as significant [3, 4] and the association of saturated and animal fat with colorectal cancer risk seems quite strong [5–8]. However, unsaturated fatty acids appear to have opposing effects. Thus, the type of dietary fatty acid (FA) would be even more determinant for colorectal cancer risk than the total amount of fat consumed [8–11].

While a wide range of studies focus on polyunsaturated fatty acids, there are few experimental studies addressing the role of oleic acid (OA) and olive oil in cancer. Olive oil, the main fat in the "Mediterranean Diet", has been linked to a potential role in lowering the risk of several types of cancer [12, 13].

Ecological and case–control studies suggest that olive oil may also have a protective effect on colorectal cancer development [14, 15]. Some animal studies have shown that dietary olive oil prevented the development of colon carcinomas in rats, suggesting that olive oil may have a chemo preventive effect against colon carcinogenesis [16, 17].

Several in vitro studies have examined the effect of OA, olive oil's main monounsaturated fatty acid, on cancer. OA can suppress the over expression of HER2 (erbB-2), a

well-characterized oncogene playing a key role in the etiology, invasive progression, and metastasis in several human cancers [18]. Llor et al. [19] conducted in vitro experiments on the effect of olive oil on colorectal neoplasia, concluding that olive oil induces apoptosis and cell differentiation.

Currently, there is an increasing understanding of the specific mechanisms by which dietary fat in general may exert their modulatory effects on cancer. Among them, the modulation of cell-signaling transduction pathways [20]. However, the precise mechanism by which olive oil could prevent colon cancer is still unclear.

There is much evidence indicating that Ca^{2+} signals are involved in the control of cell proliferation [21, 22], and particular attention has been paid to the role of Ca^{2+} entry through store-operated Ca^{2+} entry (SOCE), also termed capacitative Ca^{2+} entry [23]. SOCE is activated by the emptying of intracellular stores, either by inositol 1,4,5 triphosphate (IP_3) or by the pharmacological emptying of the Ca^{2+} stores with, for instance, thapsigargin [24–26]. The involvement of SOCE in cell proliferation has been deeply studied, and many observations relate SOCE to cell proliferation in several cell lines, thus showing that SOCE inhibition by different means abolishes tumor cell proliferation [27–29].

Assuming the emerging importance of Ca^{2+} signals in the regulation of cell growth and cell death, we decided to examine the effect of OA, as a preventive dietary fat, on Ca^{2+} signaling in colorectal cancer cells. Previous reports have shown the link between SOCE and proliferation in HT29 cells, and that is why this was the cell line chosen for the study. Altogether, our results demonstrate the potent inhibitory effect of OA on SOCE.

Materials and methods

Materials

HT29 cells were kindly donated by C. Villalobos (CSIC, Valladolid). Fura-2/AM was purchased from Fluka. Thapsigargin, Staurosporin, Carbachol, Phorbol 12-myristate 13-acetate (PMA), Oleic acid, Methyl oleate, and Bovine Serum Albumin (BSA) (free FA) from Sigma. U73122 and nifedipine from Calbiochem. Reagents for cell culture were purchased from GIBCO.

Cell culture

The human colorectal adenocarcinoma cell line HT29 was maintained in standard DMEM [complemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamine] and kept in an incubator (37 °C, 5% CO_2 humidified atmosphere).

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

Cells were propagated in 75-cm^2 culture flasks, detached by trypsin treatment, washed in culture medium, and loaded with the fluorescence dye Fura-2/AM (a ratiometric fluorescent dye that binds to free intracellular calcium) at 4 μM for 60 min at room temperature in an standard solution (Ca^{2+}-standard medium containing (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1, glucose, 10; Hepes, 10 (pH 7.4)).

Fluorescence was recorded from 1 mL aliquots of magnetically stirred HT29 cells (1.5×10^6 C/mL) at 37 °C using a Cary Eclipse Spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 510 nm. Baseline fluorescence was determined for 1 min before the stimulus was added. When experiments were performed in a Ca^{2+}-free medium, extracellular Ca^{2+} was chelated with EGTA-Tris 5 mM addition. Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2 340/380 fluorescence ratio. The $[Ca^{2+}]_i$ was calculated using the method of Grynkiewicz et al. [30]. Increases in $[Ca^{2+}]_i$ is expressed as nM (mean ± SD).

Mn^{2+} measurements

In the experiments on the determination of Mn^{2+} influx (1 mM $MnCl_2$ was added directly to the cuvette with cell suspension), Mn^{2+} uptake was monitored as a rate of quenching of Fura-2 fluorescence measured at the Ca^{2+}-insensitive wavelengths (excitation 360 nm and emission 510 nm). The rate of the fluorescence decrease provides a relative measure of the divalent cations permeability.

Fatty acids preparation

FA were dissolved in ethanol [0.1% (v/v)] and used immediately or kept at −20 °C, tightly sealed under the stream of nitrogen.

Statistical analysis

Analysis of statistical significance was performed using Statgraphics 5.1 Plus. The data are represented as mean ± SD for each group. One-way ANOVA was used to analyze the statistical significance between mean values followed by a least-significant difference (LSD) test. $P < 0.05$ was taken as the minimum level of significance.

Results

OA inhibits store-operated calcium entry in HT29 cells

In the present study, SOCE has been shown by the increase in $[Ca^{2+}]_i$ that follows the addition of Carbachol (Cch). Cch activates M_3 muscarinic receptors in HT29 cells provoking a biphasic $[Ca^{2+}]_i$ increase, a peak due to the transient release from intracellular stores followed by a plateau due to sustained Ca^{2+} entry via store-operated channels (SOC) [31]. Hence, we were tempted to assess whether OA inhibited SOCE induced by physiological stimulation with Cch in HT29 cells. Cells pretreated with several concentrations of OA for 3 min, differently modulate Cch response. Those below 1 μM did not modify Cch-induced increases in $[Ca^{2+}]_i$. However, in cells treated with the higher concentrations (10 and 100 μM), Cch induced a short-lasting increase in $[Ca^{2+}]_i$ immediately returning to near prestimulation levels, thus showing that the release of Ca^{2+} from the intracellular stores was not affected by pretreatment with the FA. By contrast, the subsequent plateau reflecting SOCE activation was totally abolished in cells treated with 10 and 100 μM OA (Fig. 1a).

We developed further experiments with Mn^{2+}, used as a tracer for Ca^{2+} entry [32], in order to establish whether the OA-induced decrease in $[Ca^{2+}]_i$ was actually due to an inhibition of Ca^{2+} influx. In addition, we used thapsigargin (Tg), to induce SOCE and to corroborate our previous results using Cch, excluding the possibility that non-SOC channels, would contribute to the overall Ca^{2+} response [28]. Tg without acting on IP_3 receptors induces SOCE by inhibiting the Ca^{2+}-ATPase present on the endoplasmic reticulum (ER) [33]. Figure 1 shows how OA inhibited Mn^{2+} influx in cells treated with both Cch (Fig. 1b) and Tg (Fig. 1c).

Mechanisms involved in the OA-induced Ca^{2+} inhibitory signal in HT29 cells

FAs have been shown to activate protein kinase C (PKC) in several cell types [34–36]. Since it has been previously demonstrated the SOCE-inhibitory properties of PMA, in several cell types [37, 38], we tested the effect of such a phorbol on HT29 cells. Activation of PKC inhibited Ca^{2+} entry in HT29 cells. However, inhibition of PKC with staurosporin (STA) did not affect the changes in $[Ca^{2+}]_i$ evoked by OA. Thus, the SOCE-inhibitory effect induced by OA was apparently not dependent on the activation of PKC (Fig. 2a).

On the basis of previous reports showing that the direct effect of FAs may result from these agents binding to membrane molecules at the outer side of the membrane [39], we performed experiments with cells treated with BSA, at a concentration shown to compete with unsaturated fatty acids bound to the plasma membrane [40]. The addition of BSA almost totally restored the Cch-induced increase in $[Ca^{2+}]_i$ in HT29 cells (Fig. 2b). These results suggest that the FA block Ca^{2+} entry by acting on the outside of the plasma membrane, and the addition of BSA reverses the effects of OA by binding the FA.

In addition, we demonstrated that the free carboxylate group is required. Methyl-OA failed to inhibit SOCE (data not shown).

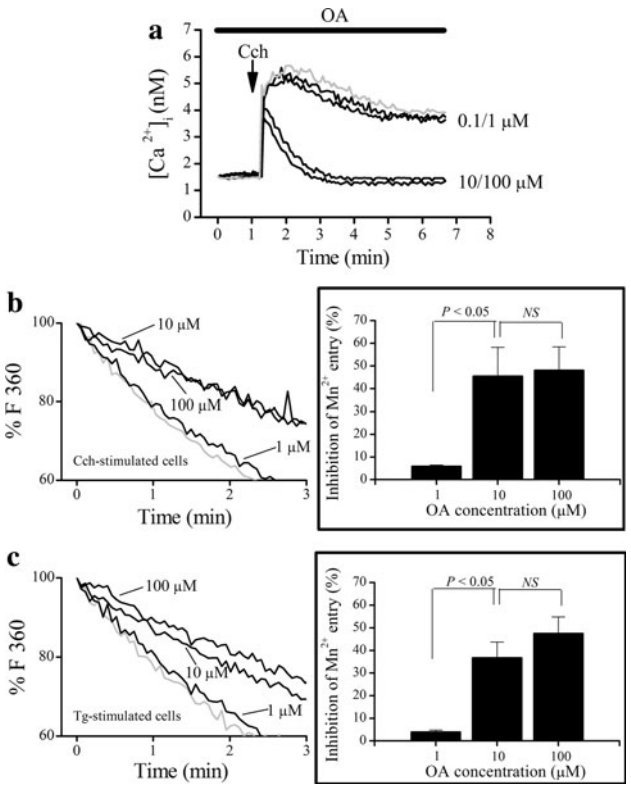

Fig. 1 Effect of oleic acid on SOCE. Effect of carbachol (Cch) on $[Ca^{2+}]_i$ in cells pretreated with oleic acid (OA) (**a**). Experiments were performed in a Ca^{2+}-containing medium. Cells were pretreated for 3 min with OA at the concentrations indicated (0.1, 1, 10, 100 μM). 100 μM Cch was added when indicated by the *arrow*. *Gray line* represents control cells (non-treated cells). The figure shows the single traces of observations, which were reproduced several times independently ($n \geq 6$). Effect of OA on Mn^{2+} entry in Cch (B)- and Thapsigargin (Tg) (C)-treated cells. The *gray line* represents control cells (cells treated with 100 μM Cch or 400 nM Tg). *Black solid line* represents cells in which SOCE has been induced by Cch or Tg, and immediately after OA (1-100 μM) was added to the cuvette. For Mn^{2+} entry measurements, 1 mM $MnCl_2$ was added and the decrease in the fluorescence excited at 360 nm was followed. Readings have been normalized to 100% at the time of Mn^{2+} addition. The *curves* shows the single traces of observations that were reproduced several times independently ($n \geq 6$). Data in *histograms* represented as mean (SD) values of inhibition of Mn^{2+} entry (%) were analyzed by employing LSD test of significance. NS, insignificant differences

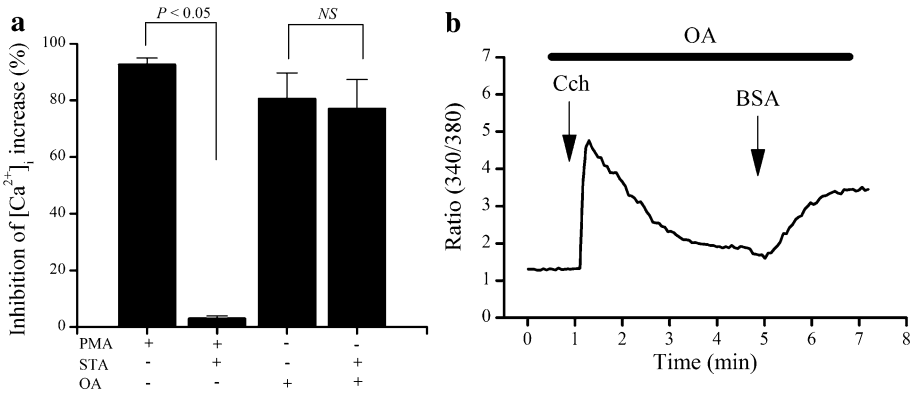

Fig. 2 Comparison of the effects of staurosporin (STA) on the inhibition of the SOCE induced either by PMA or by oleic acid (OA) in HT29 cells (**a**). Carbachol (Cch) (100 μM) was used to induce SOCE in a Ca^{2+}-containing medium. PMA and OA were added at 0.1 and 10 μM, respectively. Data in *histograms* represented as mean (SD) values of inhibition of the increases in $[Ca^{2+}]_i$ (%) were analyzed by employing LSD test of significance ($n \geq 6$). NS, insignificant differences. Effect of BSA on SOCE-inhibition induced by OA (**b**). Cells were pretreated for 3 min with OA (10 μM). Cch (100 μM) was used to induce SOCE. Cch and BSA (0.2%) were added at the time indicated by the *arrows*. The figure shows the single traces of observations that were reproduced several times independently ($n \geq 6$)

$[Ca^{2+}]_i$ evoked increases in HT29 cells

OA, per se, increased $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} in HT29 cells. The effects of different concentrations (0.1–100 μM) were tested. Those under 1 μM hardly induced any effect. Increasing the concentration above 1 μM clearly induced an $[Ca^{2+}]_i$ increase. However, when we compared concentrations above 1 μM maximum peak, no statistical differences ($P < 0.05$) were found. When stimulating the cells with the FA, a short-lasting $[Ca^{2+}]_i$ peak was observed. $[Ca^{2+}]_i$ returned immediately to resting values. Figure 3 gives the response curves for these experiments. It is noteworthy that 1 μM was a concentration lacking of SOCE-inhibitory effect; by contrast, this concentration induced the same increases in $[Ca^{2+}]_i$ as 10 μM (the same net value). Hence, we were tempted to investigate whether the mechanisms involved in the OA response (1–10 μM) were similar or not and if this could have something to do with their different SOCE-inhibitory properties.

Mechanisms involved in the increases in $[Ca^{2+}]_i$ induced by OA in HT29 cells

We conducted experiments in the absence of extracellular Ca^{2+}, so any increase in $[Ca^{2+}]_i$ must arised from Ca^{2+} release from the stores. Figure 4 shows the OA response in a Ca^{2+}-free medium. 10 μM OA failed to induce any rise in a Ca^{2+}-free medium, suggesting that extracellular Ca^{2+} influx was responsible for the whole effect. In contrast, 1 μM OA induced an initial release of intracellular stored Ca^{2+}, since under Ca^{2+}-free conditions in the medium, $[Ca^{2+}]_i$ transients were observed.

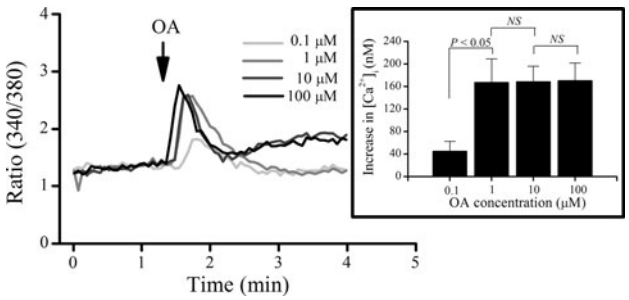

Fig. 3 Effect of oleic acid (OA) on $[Ca^{2+}]_i$ in HT29 cells. The experiments were performed in a Ca^{2+}-containing medium (1 mM). OA (0.1-100 μM) was added to the cells at the time indicated by the *arrow*. The *curves*, represented as Ratio (340/380), show the single traces of observations that were reproduced several times independently ($n \geq 6$). Data in *histograms* represented as means (SD) values of the increases in $[Ca^{2+}]_i$ (nM) were analyzed by employing LSD test of significance. The calibration of the experiment was performed as described in "Materials and methods". NS, insignificant differences

To further investigate the origin of the Ca^{2+} recruited by the FA (1 μM), we used Tg. The ER consists of physiologically distinct Ca^{2+} stores that have been classified by their sensitivity to pharmacological inhibition. The IP_3-sensitive ER compartment can be emptied by exposure to Tg [41]. Without acting on IP_3 receptors, Tg increases cytosolic Ca^{2+} by inhibiting the Ca^{2+}-ATPase present on the ER [33]. Application of 400 nM of Tg in a Ca^{2+}-free medium caused an $[Ca^{2+}]_i$ increase that comprised an initial rise and a gradual decay toward baseline; after depleting the ER Ca^{2+} store with Tg, addition of 1 μM OA did not induce any Ca^{2+} increase. This signal most likely reflected ER Ca^{2+}-release induced by this FA (Fig. 5a).

In addition, previous studies have shown that stored-Ca^{2+} can be released by pathways dependent or independent on phospholipase C (PLC)-associated IP_3 formation

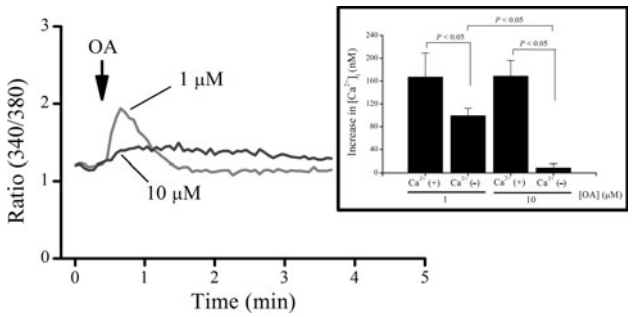

Fig. 4 Effect of oleic acid (OA) in a Ca^{2+}-free medium. OA (1 and 10 μM) was added at the time indicated by the *arrow*. The *curves*, represented as Ratio (340/380), show the single traces of observations that were reproduced several times independently ($n \geq 6$). Data in *histograms* represented as means (SD) values of the increases in $[Ca^{2+}]_i$ (nM) were analyzed by employing LSD test of significance. "Ca^{2+} (+)" represent Ca^{2+}-containing medium; "Ca^{2+} (−)" represent Ca^{2+}-free medium. The calibration of the experiment was performed as described in "Materials and methods". NS, insignificant differences

[42, 43]. Once shown that 1 μM OA recruited Ca^{2+} from intracellular stores, we aimed to evaluate whether OA evoked increases in $[Ca^{2+}]_i$ via production of IP_3. We used U73122, a PLC inhibitor, to block the formation of IP_3 [44–46]. In cells treated with U73122, 1 μM OA failed to induce an $[Ca^{2+}]_i$ increase (Fig. 5b).

Comparative SOCE-inhibitory effect of OA with saturated fatty acids

We were tempted to evaluate what happened if OA was applied after SOCE activation by Cch. While Cch-induced changes in $[Ca^{2+}]_i$ normally stabilized at a $[Ca^{2+}]_i$ well above normal, OA decreased the level of the $[Ca^{2+}]_i$ plateau reached by the agonist, consistent with the previous suggestion of a rapid inhibition of SOCE. However, OA was still able to induce increases in $[Ca^{2+}]_i$ before the SOCE-inhibitory effect. We also conducted experiments to compare the above-reported SOCE-inhibitory properties of OA, in order to establish the physiological relevance of the FA. Then, we used stearic acid (SA), a FA with the same carbon chain length (C:18), but without any double bounds. SA failed to inhibit Cch-induced SOCE in HT29 cells. Increasing the concentration of SA did not result in an inhibitory effect (data not shown). In addition, econazole, a well-known blocker of SOCE, was also used to confirm the results (Fig. 6).

Discussion

In the present study, we showed that cells exposed to OA resulted in changes in $[Ca^{2+}]_i$ homeostasis.

Our results suggest that OA is a potent inhibitor of Ca^{2+} fluxes. Previous reports in other cell lines suggest that FAs inhibit an entry pathway for Ca^{2+} [47]. OA inhibited agonist-induced SOCE reaching a maximal effect when 10 μM was added to the cells. These results are in agreement with those previously reported [48].

It has been proposed that the inhibition of Ca^{2+} influx by FAs may be due to increased Ca^{2+} extrusion [49–51]. In contrast, experiments with Mn^{2+} performed in our study showed a direct inhibitory effect on Ca^{2+} entry, which is corroborated by other investigators [37, 47, 48].

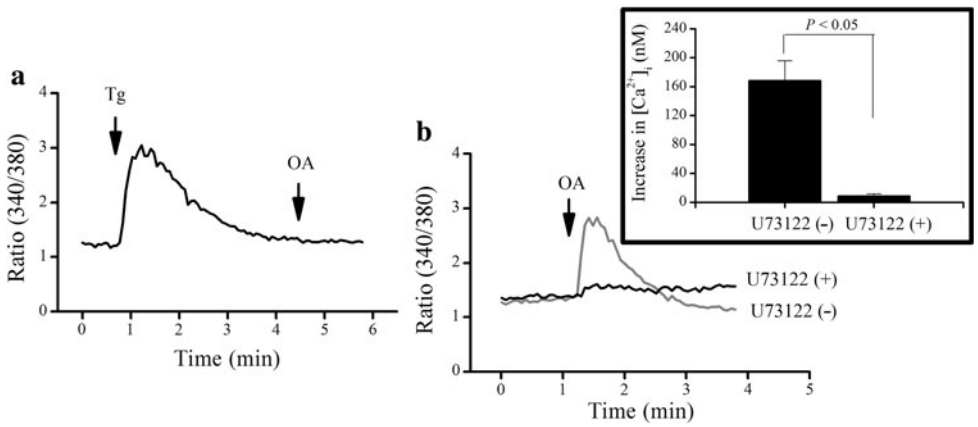

Fig. 5 Effect of oleic acid (OA) on thapsigargin-sensitive stores (**a**). The experiments were performed in a Ca^{2+}-free medium. 400 nM thapsigargin (Tg) and 1 μM OA were added at the time indicated by the *arrows*. The *curve* shows the single traces of observations that were reproduced several times independently ($n \geq 6$). Effect of OA on the increases in $[Ca^{2+}]_i$ in U73122 treated cells (**b**). Before starting the measure, cells were incubated during 5 min with 3 μM U73122. The *arrow heads* indicate the time when 1 μM OA was added into the cuvette. U73122 (−) represent non-treated cells. U73122 (+) represent U73122-treated cells. The curves, represented as Ratio (340/380), show the single traces of observations that were reproduced several times independently ($n \geq 6$). Data in *histograms* represented as means (SD) values of the increases in $[Ca^{2+}]_i$ (nM) were analyzed by employing LSD test of significance. The calibration of the experiment was performed as described in "Materials and methods"

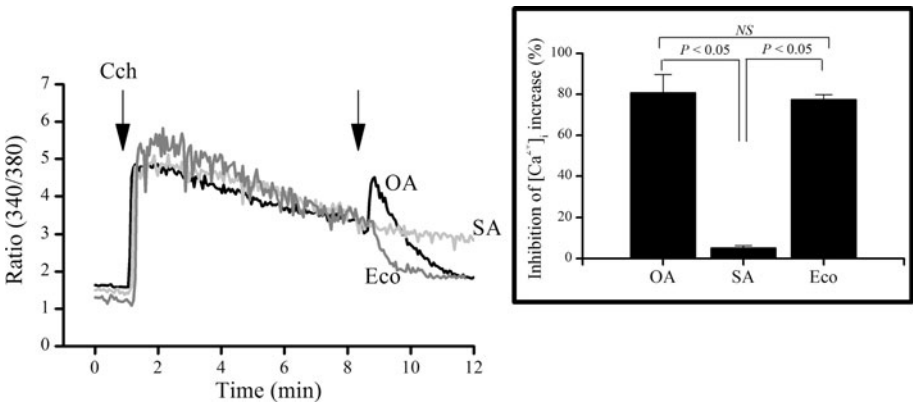

Fig. 6 Effect of oleic acid (OA) on carbachol-induced SOCE: comparison with stearic acid (SA) and econazole (Eco). Experiments were performed in a Ca^{2+}-containing medium. 100 μM carbachol (Cch) and 10 μM OA, SA, and Eco were added when indicated by the *arrows*. The *curves*, represented as Ratio (340/380), show the single traces of observations that were reproduced several times independently ($n \geq 6$). Data in histograms represented as means (SD) values of the inhibition of the increases in $[Ca^{2+}]_i$ (%) were analyzed by employing LSD test of significance. The calibration of the experiment was performed as described in "Materials and methods". NS, insignificant differences

Several reports have shown that unsaturated FAs are potent activators of PKC [34]. According to previous results [37, 38], we have demonstrated that activation of PKC by a phorbol ester in HT29 cells has a potent inhibitory effect on both Tg and Cch-induced SOCE. It could be proposed, therefore, that the SOCE-inhibitory effect of OA takes place by this mechanism. We found, however, that whereas the PKC inhibitor STA was able to prevent the inhibition of SOCE produced by the phorbol ester, it had no effect on the inhibition produced by OA. This suggests that inhibition of SOCE by OA do not take place via the activation of PKC.

We have demonstrated that the FA blocks Ca^{2+} entry by acting on the outside of the plasma membrane, as addition of BSA reversed the effect of OA. Similar results were obtained by several groups in other cell lines [37, 42, 48, 52, 53]. However, where OA is specifically acting is a very difficult question to answer that will need much more experiments.

Thus, assuming the above results suggested that OA exerts its effect by acting on the outside of the membrane, we were tempted to evaluate whether OA could induce any effect per se.

OA actually induced increases in $[Ca^{2+}]_i$ in HT29 cells. Similar results were obtained for different FAs in several cell lines [37, 52, 54–56]. Concentrations above 1 μM caused a monophasic rise in $[Ca^{2+}]_i$ followed by a rapid return to the basal level. The novelty of our study is that while 1 μM was not able to inhibit agonist-induced SOCE, such a concentration appeared to cause a similar $[Ca^{2+}]_i$ rise to the one evoked by 10 μM. Hence, 1 μM OA induced increases in $[Ca^{2+}]_i$ per se, without causing any disturbance in agonist-induced SOCE. Conversely, OA 10 μM suppressed Ca^{2+} influx, when this pathway was previously activated.

We further linked the different SOCE-inhibitory properties between concentrations to the mechanisms involved in the OA-induced increases in $[Ca^{2+}]_i$. In fact, although the $[Ca^{2+}]_i$ rises were similar when 1 and 10 μM OA were tested, we found that the sources contributing to such a rise proved to have a different origin. The low concentration induced increases in $[Ca^{2+}]_i$ apparently due to the mobilization of Ca^{2+} from Tg-sensitive internal stores, and results corroborated by those obtained by Ekokoski et al. [47] in FRTL-5 cells. Moreover, we found that there is a stimulation of the phosphoinositidase C pathway mediated by 1 μM OA. Thus, stored Ca^{2+} is released by pathways dependent on PLC-associated IP_3 formation in cells treated with 1 μM OA, which is consistent with previous results for other FAs [54]. In contrast, the increase in $[Ca^{2+}]_i$ induced by 10 μM OA proved to depend only on extracellular Ca^{2+}.

Different mechanisms may be involved depending on the concentration used. The higher concentration of OA seemed to activate store-independent channels, as corroborated by the fact that it is still able to induce a further increase in $[Ca^{2+}]_i$ after the Cch-induced SOCE activation, whereas the lower triggered Ca^{2+} release from intracellular stores. Our findings are in agreement with previous reports, showing different pathways when altering the concentration of agonist [57].

In addition, the free-carboxylate group is required because methyl-OA failed to induce any rise, as previously demonstrated in other cell types [37, 55].

Our results prove OA as a potent inhibitor of SOCE. Other investigators have shown that SOCE inhibition by different means abolishes tumor cell proliferation [27–29]. Our findings may have important implications as olive oil and oleic acid have been reported to have a protective

effect on colorectal cancer development [14, 15]. In addition, the inhibitory effect of OA on Ca^{2+} entry was observed at relatively low concentrations, which may be of therapeutic relevance. By contrast, we demonstrated that SA failed to inhibit SOCE in HT29 cells, which is in accordance with reports showing that diets containing high levels of saturated FAs promote colon carcinogenesis [8] or have little or no tumor-promoting effect [58].

All together, these results suggest that OA has a different effect on Ca^{2+} movements in HT29 cells depending on the concentration tested. It is noteworthy that both 1 and 10 μM induce a similar increase in $[Ca^{2+}]_i$, although different pathways contribute to such an increase. However, only the higher concentration proved to behave as an inhibitor of SOCE. This inhibition was PKC-independent and restored by BSA. If SOCE is essential for cell proliferation [43], OA could be considered nutritionally interesting, thus suggesting a physiological pathway for the preventive properties of such a FA in colon cancer cells. However, a little variability in the concentration could result in a large different physiological effect, reflecting a change in the mechanism involved. Nevertheless, definitive outcomes related to cell proliferation or apoptosis will be the subject of our future studies.

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