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ORIGINAL PAPER

Eicosapentaenoic acid triggers Ca^{2+} release and Ca^{2+} influx in mouse cerebral cortex endothelial bEND.3 cells

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Abstract Eicosapentaenoic acid (EPA), an omega-3 fatty acid abundant in fish oil, protects endothelial cells (EC) from lipotoxicity and triggers EC NO release. The latter is related to an elevation of cytosolic Ca^{2+}. Although EPA has been shown to cause human EC cytosolic Ca^{2+} elevation, the mechanism is unclear. Microfluorimetric imaging was used here to measure free cytosolic Ca^{2+} concentration. EPA was shown to cause intracellular Ca^{2+} release in mouse cerebral cortex endothelial bEND.3 cells; interestingly, the EPA-sensitive intracellular Ca^{2+} pool(s) appeared to encompass and was larger than the Ca^{2+} pool mobilized by sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase inhibition by cyclopiazonic acid. EPA also opened a Ca^{2+} influx pathway pharmacologically distinct from store-operated Ca^{2+} influx. Surprisingly, EPA-triggered Ca^{2+} influx was Ni^{2+}-insensitive; and EPA did not trigger Mn^{2+} influx. Further, EPA-triggered Ca^{2+} influx did not involve Na^{+}–Ca^{2+} exchangers. Thus, our results suggest EPA triggered unusual mechanisms of Ca^{2+} release and Ca^{2+} influx in EC.

Keywords Eicosapentaenoic acid · Endothelial cells · Ca^{2+} release · Ca^{2+} influx

Introduction

Eicosapentaenoic acid (EPA), an omega-3 fatty acid, is a polyunsaturated fatty acid abundant in fish oil and microalgae. In addition to the latter sources, EPA can be synthesized from dietary α-linolenic acid. A number of beneficial health effects of EPA have been reported. For instance, EPA has been demonstrated to have beneficial effects on schizophrenia [1] and depression [2]. Recent evidence suggests that EPA is an antidepressant possibly because of inhibition of inflammatory cytokine production in microglia [3]. EPA can also ameliorate health status in cancer patients by inhibiting pro-inflammation cytokine production, increasing insulin sensitivity and improving calorie and protein uptake [4]. Chronic heart failure patients with dyslipidemia, have reduced inflammation, better cardiac performance and improved endothelial functions after EPA treatment [5]. EPA causes relaxation of agonist-contracted aortic rings and reduces agonist-triggered Ca^{2+} transients in aortic vascular smooth muscle of spontaneous hypertensive rats [6], in part accounting for the blood pressure-lowering effect of EPA.

Beneficial effects of EPA on endothelial cell (EC) functions have been studied. In human umbilical vein EC (HUVEC), EPA, by activating AMP-activated protein

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kinase, can protect palmitic acid-induced apoptotic death, and also alleviate palmitic acid-induced inhibition of eNOS and Akt [7]. EPA enhances neovasculogenesis and cell migration of human endothelial progenitor cells in vitro by up-regulating c-kit proteins and causing phosphorylation of ERK1/2, Akt and eNOS [8]. EPA reduces basal and insulin-stimulated endothelin-1 production in HUVEC [9]. EPA activates AMP-activated protein kinase, leading to enhanced eNOS phosphorylation and NO release in bovine aortic EC [10]. EPA has been shown to cause Ca^{2+} elevation in human EC, but the mechanisms for such a signal is unknown [11]. EPA directly stimulates human EC NO release [12].

In this work we investigated the effects of EPA on mouse cerebral cortical endothelial cells (bEND.3 cells). We observed that whilst EPA caused Ca^{2+} release from internal stores, it also triggered Ca^{2+} influx by opening a pathway unrelated to the store-operated Ca^{2+} channel. Interestingly, the EPA-triggered Ca^{2+} influx pathway is Mn^{2+}-impermeable and Ni^{2+}-insensitive.

Methods

Chemicals and cell culture

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA). EPA and cyclopiazonic acid (CPA) were from Sigma-Aldrich. Fura-2AM was purchased from Calbiochem-Millipore. Brain microvascular bEND.3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen).

Assay of cell viability

Cell viability was examined by the 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) method. Cells were cultured in a 96-well plate at a density of 1.5×10^4/well, and were then treated with drugs for 24 h. MTT (final concentration at 0.5 mg/ml) was subsequently added to each well and then further incubated for 4 h. The culture medium was then removed and 100 µl of DMSO was added to each well for 15 min (with shaking) to dissolve the cells. The absorbance at 595 nm was measured using an ELISA reader and was used as an indicator of cell viability.

Microfluorimetric measurement of cytosolic Ca^{2+} and Mn^{2+} influx

Microfluorimetric measurement of cytosolic Ca^{2+} concentration was performed using Fura-2 as the Ca^{2+}-sensitive fluorescent dye as described previously [13]. Briefly, cells were incubated with 5 µM Fura-2 AM (Invitrogen, Carlsbad, CA) for 1 h at 37 °C and then washed in extracellular bath solution which contained (mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (pH 7.4 adjusted with NaOH). When intracellular Ca^{2+} release was assayed, Ca^{2+}-free solution was used. This Ca^{2+}-free solution was the same as the extracellular bath solution mentioned above except that Ca^{2+} was omitted and 300 µM EGTA was supplemented. Cells were alternately excited with 340 and 380 nm using an optical filter changer (Lambda 10-2, Sutter Instruments). Emission was collected at 500 nm and images were captured using a CCD camera (CoolSnap HQ2, Photometrics, Tucson, AZ) linked to an inverted Nikon TE 2000-U microscope. For detection of Mn^{2+} influx, excitation wavelength was set at 360 nm (isosbestic point at which Fura-2 is insensitive to Ca^{2+}) and emission was collected at 500 nm. In each experiment, 4–11 cells were imaged and the data from at least three separate experiments were averaged to yield mean ± SEM. Images were analyzed with MAG Biosystems Software (Sante Fe, MN). All imaging experiments were performed at room temperature (25 °C).

Statistical analysis

Data are presented as mean ± SEM. ANOVA was used to compare multiple groups, followed by Tukey's HSD post hoc test. The unpaired or paired Student's *t* test was used where appropriate to compare two groups. A value of $P < 0.05$ was considered to represent a significant difference.

Results

EPA opened a Ca^{2+} influx pathway dissimilar to store-operated Ca^{2+} entry

An experiment was performed to examine whether EPA caused cell death. As shown in Fig. 1, treatment of cells with EPA at 30 µM for 24 h did not cause cell death. As a positive control, staurosporine (1 µM) caused 58% cytotoxicity.

In experiments with extracellular Ca^{2+}, exposure of bEND.3 cells to increasing concentrations of EPA caused a concentration-dependent rise in $[Ca^{2+}]_i$ (Fig. 2a). The basal $[Ca^{2+}]_i$ was stable with time (black line). EPA-elicited elevation in $[Ca^{2+}]_i$ did not appear to be cell membrane damage, as revealed by negative trypan blue exclusion test (not shown). This finding is also consistent with results in Fig. 1 that EPA as high as

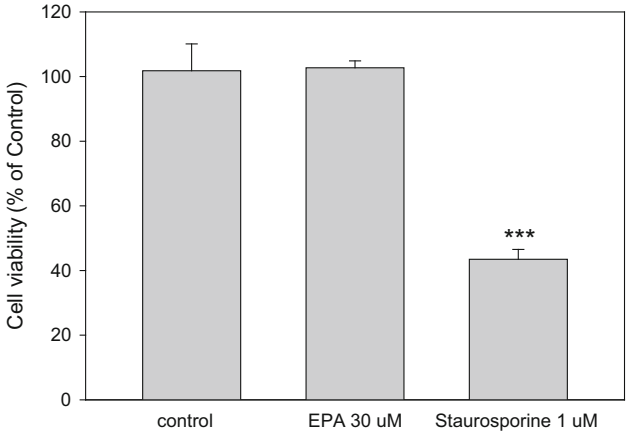

Fig. 1 EPA did not cause cell death. bEND.3 cells were incubated in the absence or presence of 30 μM EPA or 1 μM staurosporine for 24 h and MTT assay was performed to measure cell viability. Staurosporine was used as a positive control to inflict cell death. Results are mean ± SEM from 4 separate experiments. ***Significantly different ($P < 0.001$) from control

30 μM was not cytotoxic. EPA at 30 μM was chosen for further studies since it has been shown to offer cytoprotection and trigger NO release and vasodilation at 10–50 μM [14–16]. The EPA-triggered Ca^{2+} signal was smaller in Ca^{2+}-free solution (Fig. 2b, black). This suggests EPA-triggered Ca^{2+} release from internal stores. Thus, the larger EPA-triggered Ca^{2+} signal in Ca^{2+}-containing solution (Fig. 2b, red) suggests the later part of the signal was Ca^{2+} influx. To examine the reversibility of EPA-triggered Ca^{2+} signal, EPA-treated cells were rigorously washed for 6 min, but $[Ca^{2+}]_i$ remained elevated, suggesting that the EPA effect was not readily reversible (Fig. 2c).

In the absence of bath Ca^{2+}, exposure to 30 μM EPA triggered a $[Ca^{2+}]_i$ elevation (of smaller magnitude), suggesting release of intracellular Ca^{2+} (Fig. 3a). Addition of Ca^{2+} resulted in a much larger $[Ca^{2+}]_i$ elevation suggesting influx of extracellular Ca^{2+}. Since Ca^{2+} store depletion could trigger store-operated Ca^{2+} entry (SOCE), we examined whether the EPA-triggered Ca^{2+} influx was SOCE by deploying tetrandrine (TET), an SOCE blocker. We found that, rather surprisingly, TET did not inhibit the Ca^{2+} influx. We performed another set of experiments using CPA to trigger Ca^{2+} store depletion and SOCE (Fig. 3b, black line). Addition of TET substantially blocked SOCE (red line). When experiments were performed in the presence of extracellular Ca^{2+} (Fig. 3c), TET did not suppress EPA-triggered $[Ca^{2+}]_i$ elevation. By contrast, TET strongly suppressed CPA-triggered $[Ca^{2+}]_i$ elevation (Fig. 3d). Taken together, the data suggest the EPA-triggered Ca^{2+} influx was dissimilar to SOCE (but see Discussion).

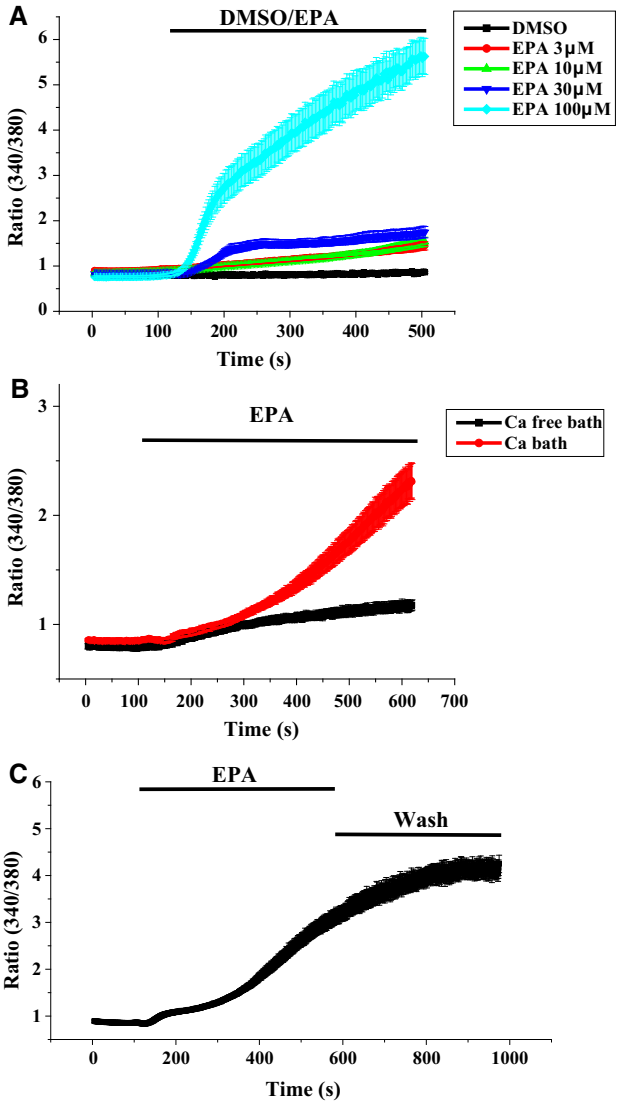

Fig. 2 EPA-triggered Ca^{2+} release and influx. **a** $[Ca^{2+}]_i$ in bEND.3 cells was monitored in Ca^{2+}-containing bath solution. The cells were challenged with DMSO (*black line*) or different concentrations of EPA. **b** $[Ca^{2+}]_i$ in bEND.3 cells was monitored in Ca^{2+}-containing or Ca^{2+}-free bath solution. The cells were challenged with 30 μM EPA. There is significant difference ($P < 0.05$) between the two groups beginning at 300 s and afterwards. **c** bEND.3 cells in Ca^{2+}-containing bath solution were treated with 30 μM EPA and then washed. Results are mean ± SEM of 19–46 cells from 3 to 5 separate experiments

EPA-triggered Ca^{2+} influx pathway was Ni^{2+}-insensitive and Mn^{2+}-impermeable

During EPA-triggered $[Ca^{2+}]_i$ elevation, addition of 10 mM EGTA to chelate extracellular Ca^{2+} caused a decline in $[Ca^{2+}]_i$, possibly as a result of Ca^{2+} extrusion (Fig. 4a). This is supported by the observation of the reciprocal changes in fluorescence at 340 and 380 nm after EGTA addition (Fig. 4b). It is noted from the latter figure that emission intensity at both excitation wavelengths

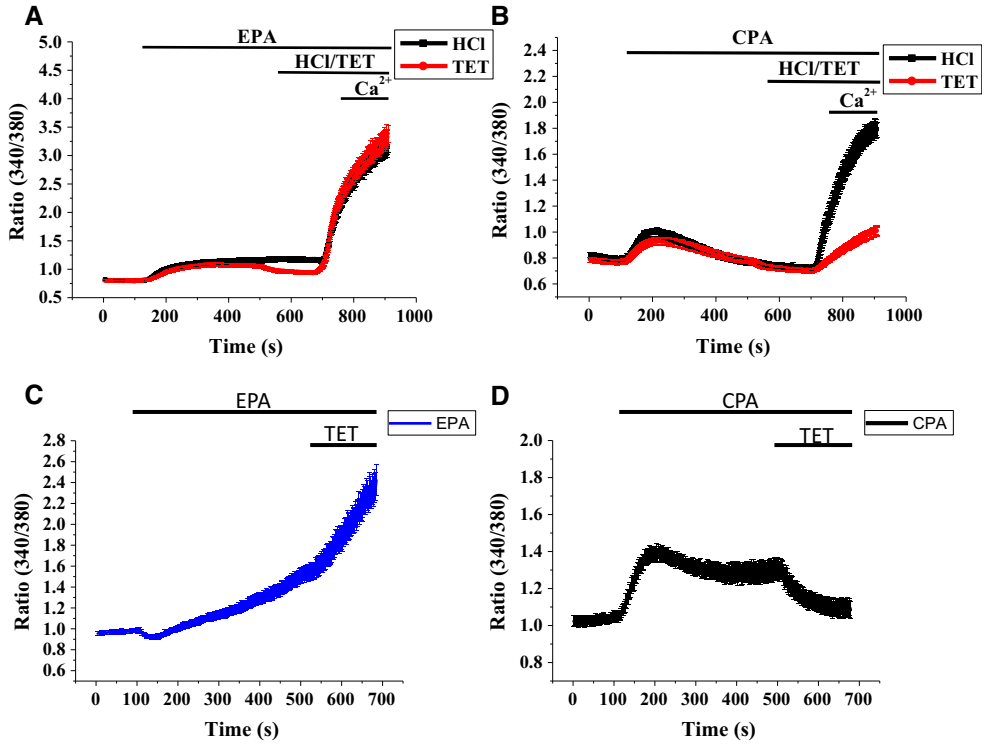

Fig. 3 EPA-triggered Ca^{2+} influx was insensitive to tetrandrine. **a** and **b**: $[Ca^{2+}]_i$ in bEND.3 cells was monitored in Ca^{2+}-free bath solution. The cells were treated with 30 μM EPA (**a**) or 30 μM CPA (**b**), followed by HCl (solvent) or tetrandrine (100 μM) addition and finally replenishment of 2 mM $CaCl_2$. The final concentration of HCl was 0.33 mM, which did not significantly affect the pH of the bath solution. There is a significant difference ($P < 0.05$) between the two groups beginning at 719 s and afterwards. **c** and **d**: $[Ca^{2+}]_i$ in bEND.3 cells was monitored in Ca^{2+}-containing bath solution. The cells were treated with 30 μM EPA (**c**) or 30 μM CPA (**d**), followed by tetrandrine (100 μM) addition. Results are mean ± SEM of 26–44 cells from 4 to 6 separate experiments

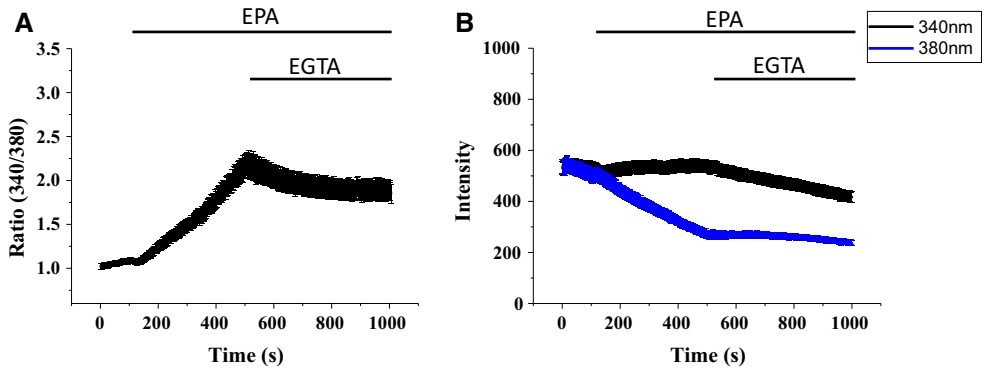

Fig. 4 Effects of EGTA on EPA-triggered Ca^{2+} influx. bEND.3 cells were bathed in Ca^{2+}-containing solution and were treated with 30 μM EPA followed by 10 mM EGTA. **a** Fura 2 ratio. **b** Fluorescence intensity (arbitrary units) at 340 and 380 nm excitation. Results are mean ± SEM of 28–35 cells from 4 to 7 separate experiments

declined slowly right from the beginning, most likely due to inevitable photobleaching. If DMSO was added instead of EPA (Figs. 5d, 6d), emission intensity at both wavelengths continued to fade slowly. Thus, when compared to these DMSO controls, it becomes obvious that EPA addition caused an increase and decrease in fluorescence emission intensity at 340 and 380 nm excitation, respectively (Figs. 4b, 5b, 6b).

We examined the pharmacological properties of this EPA-triggered Ca^{2+} influx pathway (Fig. 5). Addition of 10 mM Ni^{2+} did not significantly affect EPA-triggered $[Ca^{2+}]_i$ elevation (Fig. 5a), suggesting Ni^{2+} did not block Ca^{2+} entry. This notion is supported by the observation that there was no reciprocal change in fluorescence at 340 and 380 nm after Ni^{2+} addition (Fig. 5b). Was there an influx of Ni^{2+} instead? If Ni^{2+} did enter the cell and since

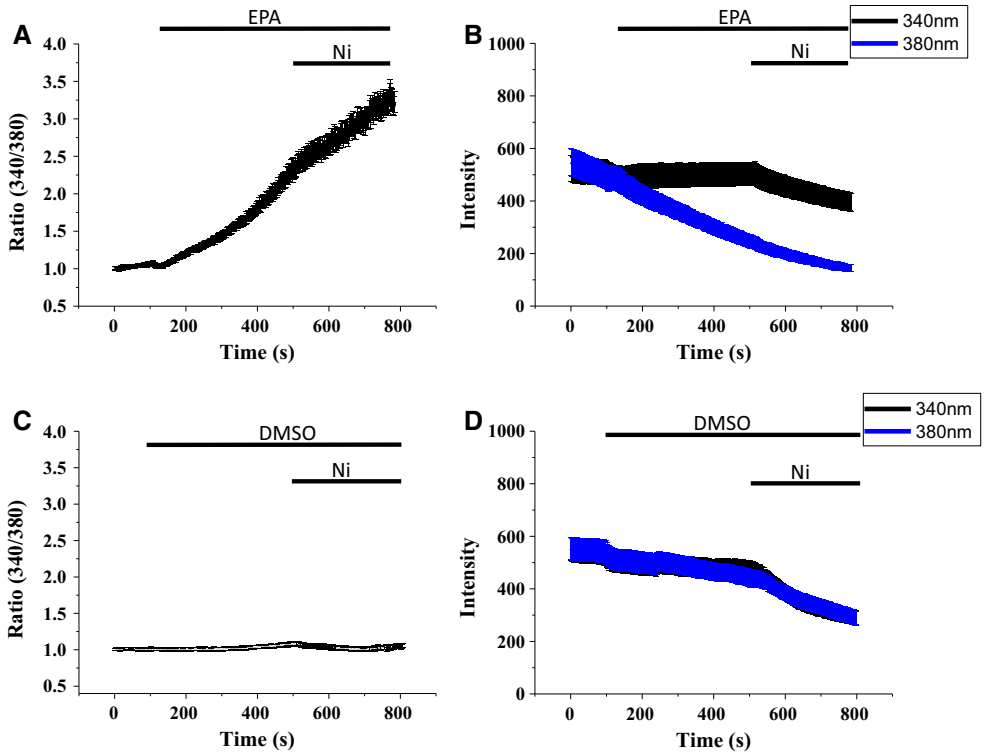

Fig. 5 Ni^{2+} did not block EPA-triggered Ca^{2+} influx. bEND.3 cells were bathed in Ca^{2+}-containing solution and were treated with DMSO (**a**) or 30 μM EPA (**c**) followed by 10 mM Ni^{2+}. Fura 2 ratios are shown in (**a**) and (**c**), while the corresponding fluorescence intensities (arbitrary units) at 340 and 380 nm excitations are shown in (**b**) and (**d**). Results are mean ± SEM of 23–32 cells from 4 to 5 separate experiments

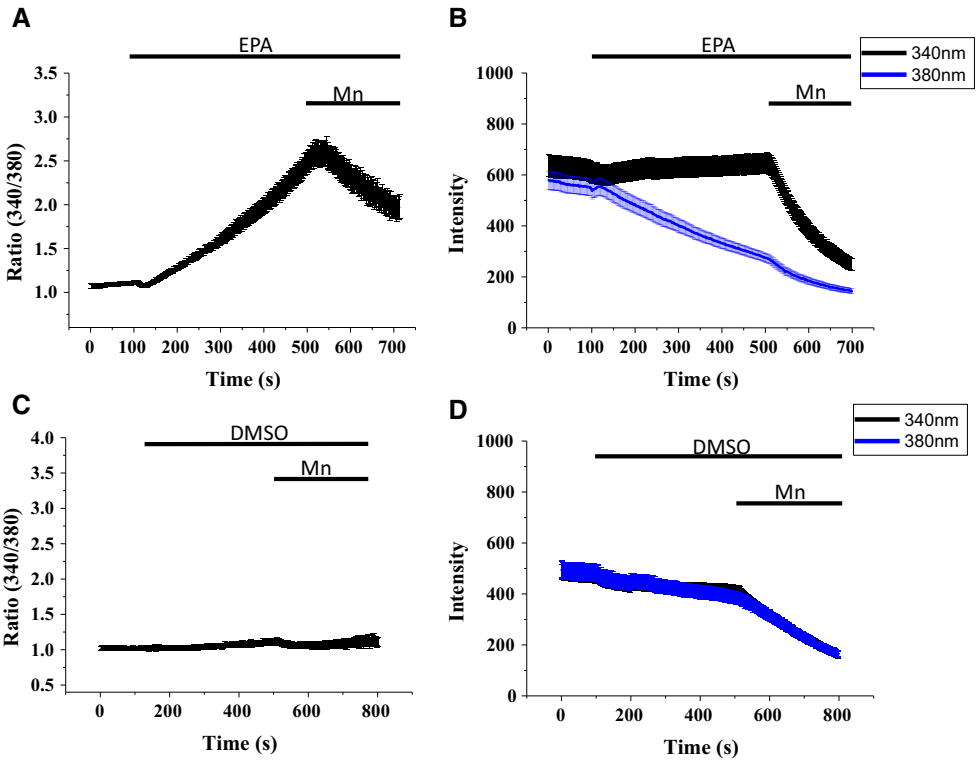

Fig. 6 Mn^{2+} did not permeate the EPA-triggered Ca^{2+} influx pathway. bEND.3 cells were bathed in Ca^{2+}-containing solution and were treated with 30 μM EPA (**a**) or DMSO (**c**) followed by 1 mM Mn^{2+}. Fura 2 ratios are shown in (**a**) and (**c**), while the corresponding fluorescence intensities (arbitrary units) at 340 and 380 nm excitations are shown in (**b**) and (**d**). Results are mean ± SEM of 29–43 cells from 4 separate experiments

this metal ion quenches Fura-2 [17], a drop at 340 and 380 nm is expected. However, an examination revealed that the drop in fluorescence at these two wavelengths (after Ni^{2+}) was similar in the absence and presence of EPA (Fig. 5d vs Fig. 5b), suggesting that Ni^{2+} influx was a leakage but not EPA-elicited.

We then examined how this EPA-triggered Ca^{2+} influx pathway was affected by Mn^{2+} (Fig. 6). Addition of 1 mM Mn^{2+} appeared to suppress EPA-triggered $[Ca^{2+}]_i$ elevation (Fig. 6a). However, the expected reciprocal change in fluorescence at 340 and 380 nm (if $[Ca^{2+}]_i$ was lowered, as in the case of EGTA; Fig. 4) was not observed after Mn^{2+} addition (Fig. 6b). Instead, fluorescence at both 340 and 380 nm dropped. Since Mn^{2+} quenches Fura-2 fluorescence [18], the result suggests Mn^{2+} entry. The stronger quench at 340 than 380 nm resulted in an apparent drop in ratio. However, the drop in fluorescence at these two wavelengths (after Mn^{2+}) was similar in the absence and presence of EPA (Fig. 6d vs Fig. 6b), suggesting that Mn^{2+} influx was a leakage but not EPA-elicited. Therefore, the EPA-triggered Ca^{2+} influx pathway was not Mn^{2+}-permeable. To verify this, we performed the Mn^{2+} influx experiment using a single excitation wavelength of 360 nm, the isosbestic point at which Fura-2 is insensitive to Ca^{2+} (Fig. 7). EPA at 30 μM did not promote but instead slightly inhibited Mn^{2+} influx; ionomycin was used as a positive control to promote entry of Mn^{2+} (Fig. 7a). EPA, even at 100 μM, did not promote but instead slightly inhibited Mn^{2+} influx (Fig. 7b).

EPA-sensitive intracellular Ca^{2+} store

The relationship between the EPA-sensitive and CPA-sensitive Ca^{2+} store was investigated (Fig. 8). In these experiments, we used a high concentration of EPA (100 μM) to cause more substantial Ca^{2+} release. When EPA was added to the cells in Ca^{2+}-free bath, Ca^{2+} release was substantial and when CPA was added later, there was no more Ca^{2+} release. In another experiment, when CPA was added first, a subsequent EPA treatment still caused further Ca^{2+} release. These results suggest the EPA-sensitive Ca^{2+} store was larger and encompassed the CPA-sensitive one.

EPA-triggered Ca^{2+} signal was not affected in the absence of extracellular Na^+

Next we examined whether the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) was involved in the EPA-triggered Ca^{2+} influx. To test this, EPA effects were investigated in normal Na^+-containing extracellular bath solution or in an extracellular bath solution with NaCl completely substituted by choline chloride. The latter Na^+-free solution was expected to favor the reverse mode of NCX. Results in Fig. 9 show that the EPA-triggered Ca^{2+} signal was not significantly affected in the absence of extracellular Na^+, arguing against NCX involvement in EPA actions.

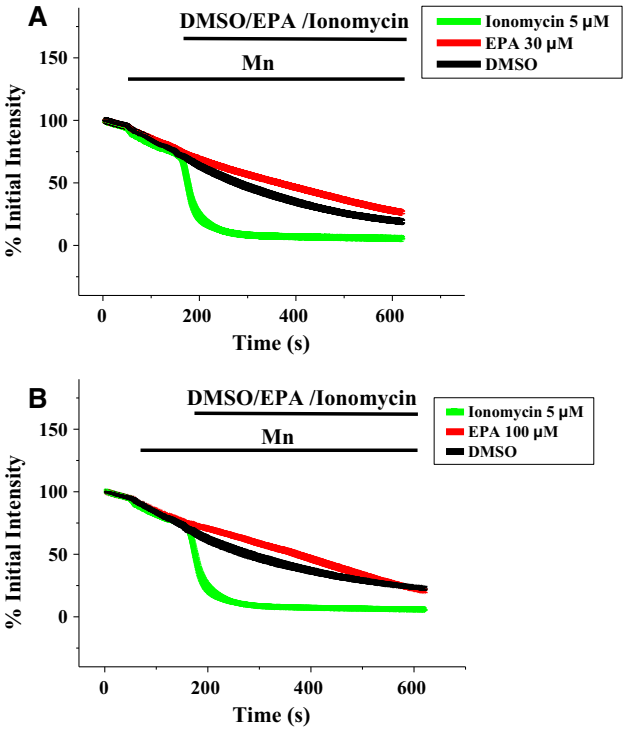

Fig. 7 EPA did not stimulate Mn^{2+} entry. **a** bEND.3 cells in Ca^{2+}-containing solution were excited with 360 nm, treated with 1 mM Mn^{2+} and subsequently DMSO, 30 μM EPA or 5 μM ionomycin. There are significant differences ($P < 0.05$) between DMSO-EPA beginning at 180 s and afterwards, and between DMSO-ionomycin beginning at 164 s and afterwards. **b** The same protocol as in (**a**) in which 100 μM EPA and respective DMSO control were added. There are significant differences ($P < 0.05$) between DMSO-EPA between 159 and 569 s, and between DMSO-ionomycin beginning at 164 s and afterwards. The data are normalized with the initial fluorescence reading. Results are mean ± SEM of 28–51 cells from 3 to 5 separate experiments

Discussion

Since the report by Okuda et al. [11] showing EPA could raise Ca^{2+} in human EC, very few works have been published showing the effect of EPA on EC Ca^{2+} signaling. Since a rise in EC cytosolic Ca^{2+} is essential in triggering NO release and hence vasodilatory effects, understanding the mechanism by which EPA raises EC Ca^{2+} is of great physiological and pharmacological interest. In this work we characterized the EPA-triggered Ca^{2+} influx pathway. Given that, in general, Mn^{2+} is a Ca^{2+} surrogate and Ni^{2+} is a Ca^{2+} channel blocker, the observation that the EPA-triggered Ca^{2+} influx pathway was Ni^{2+}-insensitive and

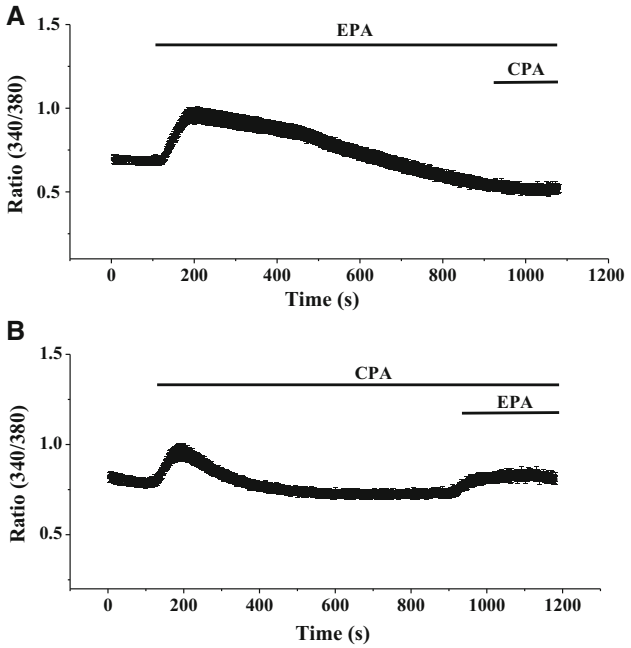

Fig. 8 EPA-sensitive Ca^{2+} pool encompassed the CPA-sensitive Ca^{2+} pool. bEND.3 cells were bathed in Ca^{2+}-free solution and were treated with 100 μM EPA and then 30 μM CPA (**a**) or 30 μM CPA and then 100 μM EPA (**b**). Results are mean ± SEM of 18–26 cells from 3 separate experiments

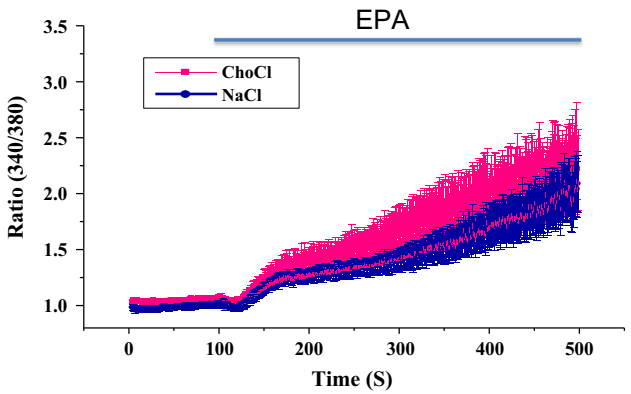

Fig. 9 EPA-triggered Ca^{2+} signal was not affected in the absence of extracellular Na^{+}. bEND.3 cells were bathed in normal Na^{+}-containing extracellular bath solution or in an extracellular bath solution with NaCl completely substituted by choline chloride. The cells were treated with 30 μM EPA. There is no significant difference ($P > 0.05$) between the two groups. Results are mean ± SEM of 21–23 cells from 5 to 6 separate experiments

Mn^{2+}-impermeable was unusual. It is noteworthy, however, that in human EC, EPA ethyl ester-triggered Ca^{2+} influx was blocked by Ni^{2+} [12], suggesting that EPA and its ester form might have subtly different pharmacological effects. EPA-triggered Ca^{2+} influx was not due to membrane damage, as suggested by our negative trypan blue exclusion test result and cell viability assay. In fact, if EPA did cause plasma membrane damage, Ni^{2+} and Mn^{2+} might be able to enter the cells, but they did not in the presence of EPA. We did consider whether the reverse mode of NCX provided a pathway for Ca^{2+} influx in EPA-treated cells. However, the data in Fig. 9 are inconsistent with NCX being a route for EPA-triggered Ca^{2+} influx. Indeed, EPA has been shown to inhibit both the forward and reverse activities of NCX1 expressed in HEK293 cells [19, 20].

A possible mechanism of EPA-stimulated Ca^{2+} influx is by its incorporation into the plasma membrane, increasing membrane fluidity and modulating ion channel activities. The fact that EPA effect could not be washed out (Fig. 2c) appears to be supportive of incorporation of EPA into the plasma membrane or organelles. Animals fed with EPA-rich diets have a much higher EPA percentage in the membrane phospholipids in neural tissues when compared to controls [21]. In EPA-treated aortic EC, plasma membrane fluidity, measured by 1,6-diphenyl-1,3,5-hexatriene polarization, was enhanced whilst total plasma membrane cholesterol content decreased [22]. The change in membrane fluidity, and/or EPA on its own, may modify channel activities. For instance, acute EPA treatment modulates Kv7.1 current amplitude and gating [23]. EPA also modulates L-type Ca^{2+} channel activities in rat cardiac myocytes [24]. Which ion channel(s) are modified by EPA to facilitate Ca^{2+} influx warrants further investigation (also see below).

What is also surprising is that the EPA-sensitive Ca^{2+} pool was larger and encompassed the CPA-sensitive pool. Did EPA inhibit sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase (SERCA)? The ability of EPA to further release Ca^{2+} after maximal inhibition of SERCA by CPA (Fig. 7) is not in concordance with this proposal. Further, in view of the reported enhancing effects of EPA on brain and cardiac microsomal Ca^{2+} ATPase activity [25, 26; also see below), we consider inhibition of SERCA not a likely mechanism of EPA-induced Ca^{2+} release. It is remarkable that, in contrast to our observation that EPA caused emptying of the Ca^{2+} store in EC, it increased SR Ca^{2+} content in rat ventricular myocytes [27]. Thus, other Ca^{2+} pools such as mitochondria and Golgi apparatus may be worth examination. Dietary EPA supplementation has been shown to enhance the level of EPA in canine myocardial microsomes [26]. It is possible that extracellular addition of EPA (as in our case) caused incorporation of EPA into organelle membranes; this may eventually facilitate "Ca^{2+} leak" into the cytosol. Intriguingly, since EPA emptied the CPA-sensitive Ca^{2+} pool, it should have activated store-operated Ca^{2+} entry; however, EPA-triggered Ca^{2+} entry was insensitive to tetrandrine, which blocked CPA-triggered store-operated Ca^{2+} entry (Fig. 3). This discrepancy is difficult to explain but it is tempting to suggest that store-operated Ca^{2+} entry could have been activated in the presence of EPA, but channel pharmacological properties might have been altered due to EPA incorporation into the plasmalemma. This notion, of course, will need further verification.

While EPA did not affect basal Ca^{2+} level in polymorphonuclear leukocytes, pretreatment reduced agonist-induced Ca^{2+} rise [28]. It is interesting to note that EPA effect on vascular smooth muscle Ca^{2+} signaling is suppressive. In cultured rat vascular smooth muscle cells, a 24-day exposure to EPA has been demonstrated to lower basal Ca^{2+} and blunt agonist-triggered Ca^{2+} signaling [29]. A similar finding was obtained by Asano et al. [30] in which 7-day treatment with EPA caused membrane hyperpolarization, lowered resting Ca^{2+} levels and reduced agonist-triggered Ca^{2+} signaling (both release and influx) in A7r5 rat smooth muscle cells. In the same work, it could be demonstrated that EPA is slowly incorporated into the phosphospholipid fraction with time of EPA treatment. EPA has also been demonstrated to suppress L-type Ca^{2+} channel activities and inhibit vasopressin-triggered Ca^{2+} entry and proliferation in rat vascular smooth muscle cells [30]. Through all these actions, EPA could exert hypotensive and antiatherosclerotic effects.

Not only is EPA beneficial because of reciprocal changes in smooth muscle and EC Ca^{2+} signaling, it is also antiarrhythmic as it suppresses L-type currents in adult and neonatal rat ventricular myocytes [31]. EPA, by increasing activities of cardiac microsomal Ca^{2+} ATPase and hence lowering intracellular Ca^{2+} concentration, produces antiarrhythmic effects during myocardia infarction [26].

EPA offers protection in brain too. In rabbits fed with a high cholesterol-containing diet for 45 days, brain cortical microsomal Ca^{2+} ATPase activities were reduced; such reduction could be alleviated by co-feeding with EPA [25].

In conclusion, EPA caused Ca^{2+} release and Ca^{2+} influx via unusual mechanisms in bEND.3 cells. Given the versatile health effects of EPA, it is interesting and important to study the Ca^{2+} signaling triggered by this fatty acid in EC and other cell types, whose Ca^{2+} responses to EPA are very different from those in EC.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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