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Possible mechanisms underlying the biphasic regulatory effects of arachidonic acid on Ca^{2+} signaling in HEK293 cells

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ABSTRACT

Arachidonic acid (AA), an endogenous lipid signal molecule released from membrane upon cell activation, modulates intracellular Ca^{2+} ($[Ca^{2+}]_i$) signaling positively and negatively. However, the mechanisms underlying the biphasic effects of AA are rather obscure. Using probes for measurements of $[Ca^{2+}]_i$ and fluidity of plasma membrane (PM)/endoplasmic reticulum (ER), immunostaining, immunoblotting and shRNA interference approaches, we found that AA at low concentration, 3 μM, reduced the PM fluidity by activating PKCα and PKCβII translocation to PM and also the ER fluidity directly. In accordance, 3 μM AA did not impact the basal $[Ca^{2+}]_i$ but significantly suppressed the thapsigargin-induced Ca^{2+} release and Ca^{2+} influx. Inhibition of PKC with Gö6983 or knockdown of PKCα or PKCβ using shRNA significantly attenuated the inhibitory effects of 3 μM AA on PM fluidity and agonist-induced Ca^{2+} signal. However, AA at high concentration, 30 μM, caused robust release and entry of Ca^{2+} accompanied by a facilitated PM fluidity but decreased ER fluidity and dramatic PKCβI and PKCβII redistribution in the ER. Compared with ursodeoxycholate acid, a membrane stabilizing agent that only inhibited the 30 μM AA-induced Ca^{2+} influx by 45%, Gd^{3+} at concentration of 10 μM could completely abolish both release and entry of Ca^{2+} induced by AA, suggesting that the potentiated PM fluidity is not the only reason for AA eliciting Ca^{2+} signal. Therefore, the study herein demonstrates that a lowered PM fluidity by PKC activation and a direct ER stabilization contribute significantly for AA downregulation of $[Ca^{2+}]_i$ response, while Gd^{3+}-sensitive 'pores' in PM/ER play an important role in AA-induced Ca^{2+} signal in HEK293 cells.

1. Introduction

Arachidonic acid (AA), esterified in the *sn*-2 position of membrane phopholipids, is released upon activation of phospholipase A_2 and at small extent upon other phospholipases such as phospholipases C and D in response to various extracellular stimuli [1,2]. The freed AA, independent of its metabolism, has been found to regulate multiple cellular activities including Ca^{2+} signaling in a variety of cells [1–6]. For example, exogenous [6,9–13] as well as endogenous AA [7,8] cause an intracellular Ca^{2+} signaling through Ca^{2+} release and Ca^{2+} entry processes in many types of cells. At the same time, however, AA also tremendously inhibits other agonists-induced Ca^{2+} responses, in particular the capacitative Ca^{2+} entry (CCE), in our previous studies [14,15] and also others [3,8,16]. Thus, AA exerts biphasic roles in the regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), i.e. producing its own Ca^{2+} signal and suppressing other $[Ca^{2+}]_i$ responses to stimuli. Yet, little is known about the underlying mechanisms involved for either effect of AA.

Activation of protein kinase C (PKC) has been recognized as a main intracellular downstream signal transduction to AA stimulation [1,2,4,17–19]. Additionally, our recent finding demonstrates an obvious downregulation of $[Ca^{2+}]_i$ response because of the lowered-membrane fluidity by PKC activation in non-excitable cells [20]. Moreover, studies in multiple cell types have found that AA beyond physiological concentration causes an increase in membrane penetration in a dose-dependent way [1,21–23]. Therefore, a question is raised that AA may negatively regulate $[Ca^{2+}]_i$ response to stimuli by activation of PKC and positively enhance $[Ca^{2+}]_i$ activities because of the facilitated membrane permeability/fluidity.

The present study aimed to answer the question by determining the effects of AA on PKC translocation, a typical manifestation of PKC activation, and plasma membrane (PM) and endoplasmic reticulum (ER) fluidities in HEK293 cells, a well defined cell line for AA and PKC effects [9,14,15,18–20]. The results show that AA at low concentration activates PKC, mainly PKCα and PKCβII, and significantly downregulates the PM and ER membrane fluidities as well as the $[Ca^{2+}]_i$ response to agonist. In another hand, AA at high concentration

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increases the PM fluidity, which partially contributes to its increased extracellular Ca^{2+} influx, but other unknown pathway sensitive to Gd^{3+} should be an important player involved in AA-mediated Ca^{2+} mobilization from internal stores and Ca^{2+} entry from extracellular space.

2. Materials and methods

2.1. Materials

Fura-2/AM, $DiIC_{16}$ (3) and Concanavalin A conjugates labeled with Alexa Fluor 594 (Con A) were obtained from Molecular Probes (Invitrogen Inc, Carlsbad, California, USA). All polyclonal antibodies including PKCα, PKCβI, PKCβII, β-actin, calnexin and shRNA plasmids targeting PKCα and PKCβ were purchased from Santa Cruz Biotechnology, Inc (CA, USA). Arachidonic acid, ursodeoxycholate acid (UDCA) and Animal Cell High-purity ER Divide Kit were from Calbiochem (Darmstadt, Germany) and Genmed Scientifics Inc. (Arlington, MA, USA), respectively. Besides, all other reagents were gotten from Sigma-Aldrich (St Louis, MO, USA).

2.2. Cell culture

HEK293 cells, obtained from the American Type Culture Collection, were cultured at 37 °C in Dulbecco's modified Eagles medium supplemented with 10% inactivated fetal bovine serum and 2 mM glutamine in a humidified incubator with 5% CO_2 and 95% air. For Ca^{2+} measurements, HEK293 cells were detached with 0.1% trypsin when grown to 80% confluence and centrifuged for 5 min at 700 × *g*. HEK293 cells were washed with HEPES buffered physiological saline solution (HBSS) containing (in mM): 120 NaCl, 5.4 KCl, 0.8 Mg_2SO_4, 20 HEPES, 1.8 $CaCl_2$ and 10 glucose, adjusted to pH 7.4 with NaOH. Nominally Ca^{2+}-free buffer was the HBSS without Ca^{2+}.

2.3. Ca^{2+} fluorescence measurements

Fluorescence measurements of $[Ca^{2+}]_i$ in HEK293 cells were carried out as previously described [9,14,15,20]. In brief, the cells were loaded with 1 μM Fura-2/AM for 25 min at 37 °C and then washed three times with Ca^{2+}-containing HBSS. The Fura-2 fluorescence changes in cell suspension were observed by a fluorescence spectrophotometer (Hitachi, F7000) under constant stirring at 37 °C with 340 nm and 380 nm excitation wavelengths and 510 nm for the emission wavelength. The ration of 340/380-nm and sometime the concentration of Ca^{2+} were expressed as the changes of the Fura-2 fluorescence.

2.4. Membrane fluidity—FRAP method

As previously described [20], HEK293 cells were exposed to 1 μg/ml dye $DiIC_{16}(3)$ for 5 min at room temperature, and fluorescence recovery after photobleaching (FRAP) was measured with a 63× oil-immersion objective (NA 1.4) in a laser-scanning microscope (Leica SP5). The excitation wavelength and emission wavelength were 561 nm and 590 nm, respectively. After background subtracted, the fluorescence emissions among cells was recorded to generate fluorescence recovery curves. Analysis of the curves using equations described previously [22] resulted in two values, the mobile fraction (M_f) and the diffusion constant (D).

2.5. Isolation of the endoplasmic reticulum and fluorescence polarization measurements

After the cells were treated with DMSO (0.1%, Con), AA (3 μM) or AA (30 μM) for 10 min, respectively, ER was isolated using the Animal Cell High-purity ER Divide Kit at 4 °C according to the manufacturer's instructions. The identification and ER membrane fluidity were performed exactly as previously described [20].

The fluorescence polarization of the labeled ER was measured at 25 °C with emission and excitation wavelength at 430 nm and 360 nm, respectively, and the P value was calculated according to the following formula: $P = (I_{VV} - G \times I_{VH}) / (I_{VV} + G \times I_{VH})$ [24].

2.6. Preparation and transfection of shRNA plasmids targeting isoforms of PKC

For transfection, the HEK293 cells were cultured for 24 h with 3 ml fresh culture medium without Penicillin–Streptomycin. Plasmids containing shRNA targeting PKCα or PKCβ were incubated with DMEM prior to transfection for 30 min, and then equal amounts of the luciferase (shCon), PKCα, and PKCβ shRNA plasmids were transfected into the cells with transfection reagents as the manufacturer's instructions described. All experimental measurements were performed at 48 h after transfection. At least 80% of HEK293 cells were transfected under our experimental conditions, as evaluated using the copGFP Control plasmid.

2.7. Western blotting

The isolated total ER membranes and HEK 293 cells lysates were blotted for detection of isotypes of PKC translocation as previously described [20]. The primary antibodies used were rabbit polyclonal anti-PKCα antibody (1:1000, sc-208), rabbit polyclonal anti-PKCβI antibody (1:1000, sc-209), rabbit polyclonal anti-PKCβII antibody

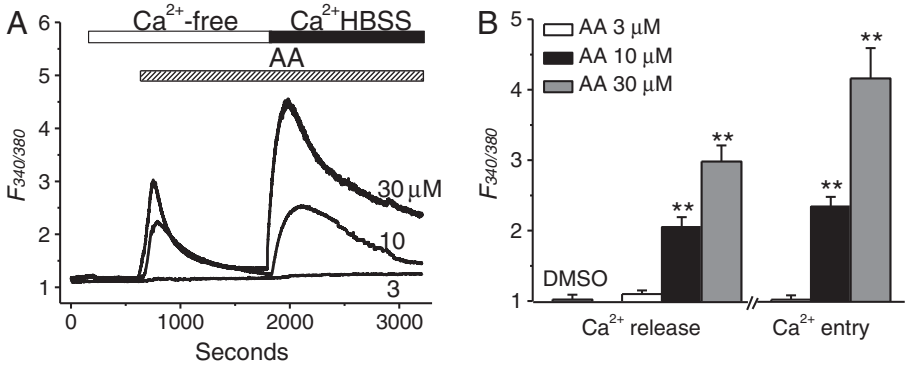

Fig. 1. Concentration-dependent inducible effect of AA on Ca^{2+} signaling. (A and B), HEK293 cells, loaded with Fura-2 as described in Materials and methods section, were treated with 3, 10 or 30 μM AA as indicated in the absence of extracellular Ca^{2+} followed by addition of Ca^{2+}. A transient rise in $[Ca^{2+}]_i$ in Ca^{2+}-free medium is indicative of intracellular Ca^{2+} release, while the robust $[Ca^{2+}]_i$ increase following re-addition of extracellular Ca^{2+} indicates a Ca^{2+} entry through the plasma membrane (A). The summarized data were obtained from 6 to 8 independent determinations for each bar (B). ** represents $p<0.01$ vs. DMSO.