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Free fatty acids and protein kinase C activation induce GPR120 (free fatty acid receptor 4) phosphorylation

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GPR120, free fatty acid receptor 4, is a recently deorphanized G protein-coupled receptor that seems to play cardinal roles in the regulation of metabolism and in the pathophysiology of inflammatory and metabolic disorders. In the present work a GPR120-Venus fusion protein was expressed in HEK293 Flp-In T-REx cells and its function (increase in intracellular calcium) and phosphorylation were studied. It was observed that the fusion protein migrated in sodium dodecyl sulfate-polyacrylamide gels as a band with a mass of $\approx$ 70–75 kDa, although other bands of higher apparent weight (> 130 kDa) were also detected. Cell stimulation with docosahexaenoic acid or α-linolenic acid induced concentration-dependent increases in intracellular calcium and GPR120 phosphorylation. Activation of protein kinase C with phorbol esters also induced a marked receptor phosphorylation but did not alter the ability of 1 μM docosahexaenoic acid to increase the intracellular calcium concentration. Phorbol ester-induced GPR120 phosphorylation, but not that induced with docosahexaenoic acid, was blocked by protein kinase C inhibitors (bis-indolyl-maleimide I and Gö 6976) suggesting that conventional kinase isoforms mediate this action. The absence of effect of protein kinase C inhibitors on agonist-induced GPR120 phosphorylation indicates that this kinase does not play a major role in agonist-induced receptor phosphorylation. Docosahexaenoic acid action was associated with marked GPR120 internalization whereas that induced with phorbol esters was smaller at early times.

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1. Introduction

Free fatty acids (FFAs) are important metabolic fuels and those that are polyunsaturated are essential components of the human diet. In addition to these metabolic roles, it is now clear that they are bioactive ligands for several types of receptors, including nuclear receptors, such as peroxisome proliferator-activated receptors, and a family of G protein-coupled receptors (GPCRs) (Chawla et al., 2001; Hirasawa et al., 2008; Stoddart et al., 2008). Four GPCRs have been deorphanized/identified as FFA receptors: GPR40, GPR41, GPR43 (Stoddart et al., 2008) and, recently, GPR120 (Hirasawa et al., 2005). GPR40 (FFA receptor 1) and GPR120 (FFA receptor 4) are considered of particular interest because they appear to be involved in physiological and pathophysiological processes related with metabolic disorders such as the so-called “metabolic syndrome” and type 2 diabetes mellitus (Hara et al., 2011). It has been observed that GPR120-deficient mice develop obesity, glucose intolerance, and fatty liver, and that a dysfunctional variant of GPR120 (R270H) is associated with obesity and other metabolic disturbances in humans (Ichimura et al., 2012). Additionally, this receptor mediates insulin-sensitizing and anti-inflammatory effects in cells (adipocytes and macrophages) and in whole organisms (mice) (Oh et al., 2010).

Two splice variants of GPR120 exist in humans, a long form of 361 residues and a short one (lacking 16 residues at the third intracellular loop) (Moore et al., 2009); the short receptor (but not the long one), couples to Gαq/11 and activates calcium signaling, whereas both receptors interact with β-arrestin and internalize in response to agonists (Watson et al., 2012). It has also been reported that both variants forms are phosphorylated in response to agonists (Burns and Moniri, 2010).

In the present work we show that both, receptor stimulation with agonists, and activation of protein kinase C (PKC) resulted in strong receptor phosphorylation. The magnitude and time-course

Abbreviations: FFA, free fatty acid; GPCR, G protein-coupled receptor; PKC, protein kinase C; DHA, docosahexaenoic acid; α-LA, α-linolenic acid; TIRF, total internal reflection fluorescence

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of these effects were similar, but showed some differences. PKC activation with phorbol esters, did not induce receptor desensitization but increased GPR120 internalization. PKC inhibitors block phorbol ester-induced GPR120 phosphorylation but not that due to agonist action; some of these data were presented in an international meeting (Sánchez-Reyes et al., 2013).

2. Materials and methods

2.1. Materials and cell lines

The sources of materials were the same published previously (Avendaño-Vázquez et al., 2005; Castillo-Badillo et al., 2012; Hara et al., 2009; Hirasawa et al., 2005). An anti-GFP rabbit antiserum generated in our laboratory (Avendaño-Vázquez et al., 2005; Castillo-Badillo et al., 2012) (Venus is a variant of yellow fluorescent protein that cross-reacts with this antiserum) was used for receptor immunoprecipitation and immunoblotting; similar results were obtained using a commercial anti-GFP polyclonal antibody obtained from Clontech. Plasmids for the expression of a carboxyl-terminus Venus-tagged short variant of human GPR120 and the free Venus protein were prepared as described previously (Hara et al., 2009; Hirasawa et al., 2005). Flp-In T-REx 293 cells were utilized to develop stable cell lines, which were subjected to selection in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 10 μg/ml blasticidin S and 100 μg/ml hygromycin B. Cells were cultured in this medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B at 37 °C under a 95% air/5% CO_2 atmosphere. Venus or GPR120-Venus protein expression was induced by adding doxycycline hyclate 1 μg/ml, 24–36 h prior to performance of the experiments and was confirmed by epifluorescence microscopy. Uninduced GPR120-Venus cells and cells expressing the free Venus protein were used as controls (Hara et al., 2009; Hirasawa et al., 2005) (Supplementary Fig. S1).

2.2. Intracellular calcium determinations

Intracellular calcium was determined as previously described (Castillo-Badillo et al., 2012). Briefly, cells were serum-starved for 2 h, loaded with 2.5 μM Fura-2/AM for 1 h at 37 °C, and immediately washed to eliminate unincorporated dye. Fluorescence measurements were carried out at 340- and 380-nm excitation, and at 510-nm emission wavelengths; chopper interval was set at 0.5 s. Intracellular calcium ($[Ca^{2+}]_i$) was calculated according to Grynkiewicz et al. (1985).

2.3. Receptor phosphorylation

Receptor phosphorylation was studied employing a procedure described to study other receptors fused to the green fluorescent protein (GFP) (Avendaño-Vázquez et al., 2005; Castillo-Badillo et al., 2012). Briefly, cells were incubated in 1 ml of phosphate-free Dulbecco's modified Eagle's medium containing $[^{32}P]Pi$ (100 μCi/ml) for 3 h at 37 °C. Labeled cells were stimulated as indicated, washed with ice-cold phosphate-buffered saline, and solubilized in detergent-containing buffer (Avendaño-Vázquez et al., 2005; Castillo-Badillo et al., 2012). Samples were centrifuged at 12,700 × g for 15 min at 4 °C and supernatants were incubated overnight at 4 °C

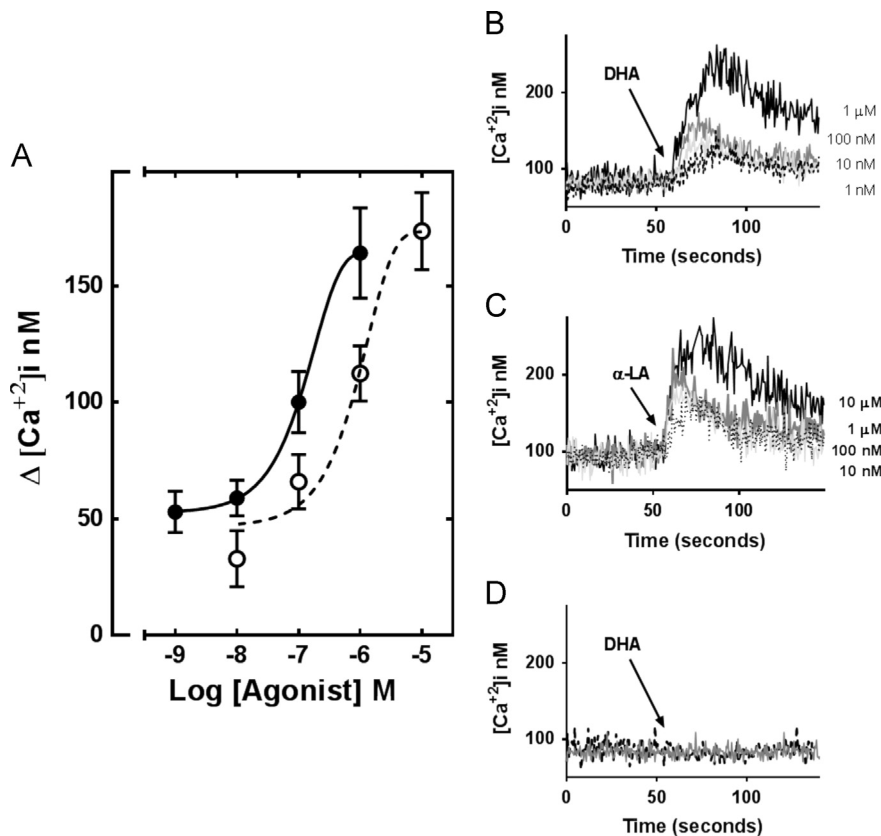

Fig. 1. Effect of DHA and α-LA on intracellular calcium concentration. Panel A: increases in intracellular calcium concentration ($\Delta[Ca^{2+}]_i$) in cells challenged with the indicated concentration of DHA (solid circles) or α-LA (open circles). Means are plotted with vertical lines representing the S.E.M. of 5 to 6 determinations using different cells preparations. Panel B: Representative tracings of DHA-triggered intracellular calcium increases. Panel C: α-LA-triggered intracellular calcium increases. Arrow indicates the time of addition of the concentration of these fatty acids indicated at the right. Panel D: absence of effect of 1 μM DHA on intracellular calcium in cells expressing the free Venus protein (solid line) and uninduced GPR120-Venus cells (dotted line).

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