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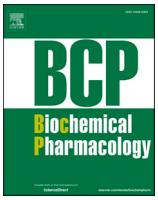

Complex FFA1 receptor (in)dependent modulation of calcium signaling by free fatty acids

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ABSTRACT

The expression of free fatty acid 1 receptors (FFA1R), activated by long chain fatty acids in human pancreatic β-cells and enhancing glucose-stimulated insulin secretion are an attractive target to treat type 2 diabetes. Yet several clinical studies with synthetic FFA1R agonists had to be discontinued due to cytotoxicity and/or so-called "liver concerns". It is not clear whether these obstructions are FFA1R dependent. In this context we used CHO-AEQ cells expressing the bioluminescent calcium-sensitive protein aequorin to investigate calcium signaling elicited by FFA1 receptor ligands α-linolenic acid (ALA), oleic acid (OLA) and myristic acid (MYA). This study revealed complex modulation of intracellular calcium signaling by these fatty acids. First these compounds elicited a typical transient increase of intracellular calcium via binding to FFA1 receptors. Secondly slightly higher concentrations of ALA substantially reduced ATP mediated calcium responses in CHO-AEQ cells and Angiotensin II responses in CHO-AEQ cells expressing human AT_1 receptors. This effect was less pronounced with MYA and OLA and was not linked to FFA1 receptor activation nor to acute cytotoxicity as a result of plasma membrane perturbation. Yet it can be hypothesized that, in line with previous studies, unsaturated long chain fatty acids such as ALA and OLA are capable of inactivating the G-proteins involved in purinergic and Angiotensin AT_1 receptor calcium signaling. Alternatively the ability of fatty acids to deplete intracellular calcium stores might underly the observed cross-inhibition of these receptor responses in the same cells.

1. Introduction

Particularly polyunsaturated long chain free fatty acids (FFAs) are essential dietary nutrients. While short chain fatty acids (SCFAs) are mostly obtained via the fermentation of carbohydrates by gut microbiota, the medium and long chain fatty acids (MCFAs, LCFAs) are ingested via food or are synthesized in the liver [48,60,72]. Importantly clinical studies have revealed a link between high levels of FFAs in the plasma and insulin resistance (i.e. type 2 diabetes), hypertension, and cardiovascular diseases, stroke and atrial fibrillation but also modulate inflammatory events in insulin target tissues like endothelial, liver and skeletal muscle cells [8,26;27;29;45,46,64,66]. Therefore, FFAs can be considered as physiologically important mediators that form a connection between insulin resistance, inflammation, obesity, T2DM and hypertension.

Regarding their molecular mechanism(s) it is well established that

Abbreviations: ALA, α-linolenic acid; OLA, oleic acid; MYA, myristic acid; LCFA, long chain fatty acids; SCFA, short chain fatty acids; MCFA, medium chain fatty acids; AUC, area under the curve; FFA, free fatty acid; GPCR, G-protein coupled receptor; CHO cells, Chinese hamster ovary cells; PI, propidium iodide; BSA, bovine serum albumin; CMC, critical micellar concentration; SD, standard deviation.

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FFAs are important as energy source, however it is becoming established that they modulate gene expression by their ability to inhibit histone deacetylation (FFAs are histone deacetylase inhibitors) as well as GPCR mediated signaling [70,79]. Relevant to these receptor signaling properties, a small cluster of four orphan GPCRs was identified on the human chromosome 19q13.1 in 1997 by Sawzdargo et al. [71]. Since they had no known ligands nor function they were considered orphan receptors. Almost two decades ago they were identified as being FFA binding receptors via ligand fishing strategies. The free fatty acid 1 receptor (FFA1R, formerly denoted as GPR40) was first deorphanized and is activated by medium, 7–12 carbons, to long, >12 carbons chain saturated and unsaturated fatty acids [11,43,49]. The FFA2 receptor (formerly denoted as GPR43) and FFA3 receptor (formerly denoted as GPR41) have short chain fatty acids, with a carbon chain under 6 carbon atoms, as their ligands [12,52]. The last member of this cluster located on human chromosome 19q13.1 is GPR42 and it turned out to be a pseudogene. It is most likely a result from a gene duplication of hFFA3 receptor [13,41]. The fourth member of the free fatty acid receptor family is the FFA4 receptor which, although located on a distinct chromosome (10q23.33), also responds to medium and long chain fatty acids and displays only 10 % homology with the FFA1 receptor [37]. When focusing on FFA1R, this receptor attracted a lot attention not only by its predominant expression in pancreatic beta-cells but essentially by the observation that its activation is found to potentiate glucose-stimulated insulin secretion [58]. These ground-breaking results led to the recognition of the FFA1 receptor as a promising target in the development of anti-diabetic drugs. Indeed several research groups and pharmaceutical companies initiated lines of research to synthesize and characterize non-lipid and synthetic FFA1 receptor agonists [14,59]. Initially such a success story started with the development of compounds such as fasiglifam (=TAK-875) by Takeda, a potent and selective FFA1 receptor agonist. Indeed this compound was found to improve glycemic control in type 2 diabetic patients without a risk for hypoglycemia [2,20,61]. In the same line FFA1 receptor agonists AMG-837 (from Amgen) and LY2881835 (from Eli Lilly) also went into clinical trials as anti-diabetic drugs [17,36,40,51,55,77]. Unfortunately the further development of these drugs had to be discontinued due to inadmissible liver toxicity reported in different clinical phases [53,82]. Strikingly a clear-cut explanation of these side-effects is not provided [34,59,73]. In other words it is not known whether this adverse effect is connected to intrinsic FFA1 receptor signaling and/or by distinct receptor-independent and yet unknown mechanism(s) [54,67]. In this background, we decided to investigate signaling mediated by FFA1 receptor ligands as well as their impact on cell viability. In particular we have used the polyunsaturated omega-3 fatty acid α -linolenic acid (ALA), the monounsaturated omega-9 fatty acid oleic acid (OLA) and the saturated C-16 fatty acid myristic acid (MYA) as probes to assess calcium signaling of the human FFA1 receptor expressed in CHO-AEQ cells. These are CHO-K1 cells stably transfected with the bioluminescent and calcium sensitive protein aequorin [22]. In parallel, we investigated the influence of bovine serum albumin (BSA) on the concentration response curves as several fatty acids (including α-linolenic acid) have been shown to bind to albumin which might influence their binding affinity, [25,43,50,80]. In order to assess the impact of fatty acids on the cell viability we have measured the uptake of propidium iodate in lysed/death cells [19,68]. Finally we have investigated whether the treatment of the cells with fatty acids can influence the calcium signaling mediated by the binding of ATP to purinergic receptors that are endogenously expressed in CHO cells as well as on the activation of recombinantly expressed angiotensin II AT_1 receptors in these cells [5,42]. The results in this paper reveal unanticipated complexity of the FFA1 receptor (in) dependent calcium signaling by FFAs and will shed light on the molecular mechanism(s) of these signaling molecules.

2. Material and methods

2.1. Materials

The cDNA for N-terminal 3xHA-tagged human Free Fatty Acid 1 (FFA1) receptor was obtained from the cDNA Resource Center (Bloomsburg University, Pennsylvania, US). Bovine serum albumin (BSA), α-linolenic acid (ALA), oleic acid (OLA) and myristic acid (MYA) (purity ≥ 99%) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Merck Life Science bv (Overijse, Belgium). Coelenterazine h was obtained from Nanolight Technology (Prolume Ltd., Pinetop, US). The FFA1 antagonist GW1100 was bought from Gentaur Molecular Products (Kampenhout, Belgium). Alexafluor488 anti-HA.11 Epitope Tag Antibody came from BioLegend (Amsterdam, The Netherlands). The phospholipase C inhibitor U73122 originated from Bio-Techne Ltd. (Abingdon, United Kingdom). [^{35}S]-GTPγS was bought from Perkinelmer (Zaventem, Belgium). All other reagents were of the highest available purity.

2.2. Cell culture and transfection

CHO cells that are stably transfected previously with the calcium sensitive bioluminescent protein aequorin and Gα16, were kindly donated by M. Detheux (Euroscreen s.a., Gosselies, Belgium). These cells were cultured as described previously [5] in 75 cm^2 flasks in Ham's F-12 Nutrient Mix supplemented with 10% FBS, 1.6 mM L-glutamine and 0.8 % of a stock solution containing 5000 units/ml of Penicillin and 5000 μg/ml of Streptomycin (ThermoFisher Scientific, Merelbeke, Belgium). All cells were cultured in a humidified incubator at 37 °C with 5% CO_2 until confluent.

Transient transfection with the FFA1 receptor was performed using the X-tremeGENE HP transfection reagent (Roche Diagnostics, Diegem, Belgium). Six million CHO cells were plated in a 75 cm^2 flask. The next day the transfection mixture was prepared in which the ratio between plasmid DNA (pDNA) and transfection reagent was 6 μg:20 μl. This transfection mixture contained 18 μg of pDNA, 60 μl of X-tremeGENE and was brought to a total volume of 1800 μl with pre-warmed OptiMEM medium (ThermoFisher Scientific, Merelbeke, Belgium) and incubated for 15 min at room temperature after which it was added to the cells. Two days after addition of the transfection mixture, the cells have optimal expression of the receptor and the functional experiments were carried out. Stable transfected cells were obtained by changing the culture medium to a culture medium containing 1 mg/ml geneticin (ThermoFisher Scientific, Merelbeke, Belgium) two days after transfection followed by further subculturing the cells in the same selection medium.

The efficiency of the transient transfection was determined by fluorescence-activated cell sorting (FACS). Cells were detached with Trypsin/EDTA two days after transfection (0.25%, ThermoFisher Scientific, Merelbeke, Belgium). Subsequently assay buffer (AB) i.e. DMEM-F12 supplemented with 0.1% (w/v %) BSA was added to the cells and after centrifugation cells were diluted to 1×10^6 cells/ml. Next 100 μl of cell suspension was placed in a FACS tube and kept on ice. To reduce non-specific binding the cells were first incubated with an anti-Fc antibody 10 μg/ml for 15 min. The presence of HA-tagged hFFA1 receptors on the membranes was detected using an AlexaFluor488 anti-HA antibody at 10 μg/ml. The cells were incubated with this antibody for 15 min after which the cells are centrifuged and resuspended in calcium-free PBS. The percentage of HA-positive cells was determined by flow cytometry based on the AlexaFluor488 label on a FACSCanto II analyzer.

2.3. Measurement of intracellular calcium (aequorin assay)

Cells were washed with calcium-free PBS before being detached by brief Trypsin/EDTA treatment. Assay buffer optionally supplemented with 0.1 % BSA, was then added to the detached cells after which they

were centrifuged (1400 rpm, 7 min at room temperature). The pellet was resuspended in assay buffer and cells were counted using Trypan Blue inside a Neubauer counting chamber. Cells were diluted in assay buffer to a cell density of 2.5 × 10^6 cells/ml and loaded with 5 μM of coelenterazine h overnight in the dark at room temperature while being gently shaken. After this first loading step, the cell suspension was centrifuged and the pellet was resuspended in assay buffer to a density of 5x10^5 cells/ml and loaded with 1 μM of coelenterazine h. The cells were then incubated for 1.5 h before the measurement.

Compounds were diluted in assay buffer either with or without BSA as indicated. EGTA (250 mM) was prepared in H_2O using 60% 1.25 M NaOH. Stock solutions of ALA, OLA and MYA (70 mM) were prepared in DMSO. Compound dilutions were made to obtain the indicated (final) concentrations during the measurements.

The luminescent measurements were executed using a TECAN M200 luminescence 96-well reader (Tecan Benelux, Mechelen, Belgium) equipped with two injectors to apply compounds or cells directly into the flat white 96-well plates (Greiner Bio-one, Vilvoorde, Belgium) after which the measurements can begin within less than 500 ms with an interval of 200 ms. All measurements were carried out in a final volume of 150 μl/well either by injecting of 50 μl of the cell suspension into each measured well containing the indicated compounds or by one or two subsequent injections of 50 μl of a compound into the measured wells.

2.4. Propidium iodide uptake

Cell viability was assessed in both adherent cells and cells in suspension to determine the possible cytotoxic effect after exposure of each compound at concentrations that were used in the calcium experiments and for different periods of time. For the adherent cells assay, 10^5 cells were plated in each well of a 96 well plate in a volume of either 100 or 200 μl of complete media and incubated overnight. The following day the cells were carefully washed twice (200 μl/well) with DMEM/F12. Subsequently the wells are filled with 100 μl of DMEM/F12 and 50 μl of a 20 μM Propidium Iodide solutions was added to each well giving a final concentration of 5 μM. Next the plate was incubated in the plate reader at room temperature for 10 min and the fluorescence measured each minute (wavelengths excitation/emission: 530 nm/620 nm). Finally 50 μl of the FFAs was added to the wells at the indicated final concentrations, shaken briefly (30 s) and the fluorescence was measured at the indicated time periods.

In the case of the assay with cells in suspension, 50 μl of a 2x10^6 cells per ml was injected into the 96-well plate which contains 50 μl of PI with or without the indicated concentrations of free fatty acids after which the fluorescence measurement started after a briefly shaking (30 s) as for the adherent cells. As a control cell lysis of the cells was instigated in the presence 0.2 % Triton X-100.

2.5. [^{35}S]-GTPγS binding assay in CHO-AEQ cell membranes

Cultured CHO-AEQ and CHO-AEQ-FFA1 cells were washed twice with PBS and harvested using Trypsin/EDTA. PBS was added to the detached cells and centrifuged for 7 min at 1400 rpm. The pellet was resuspended in ice-cold membrane buffer ($MgCl_2$ 10 mM, NaCl 100 mM, EDTA 1 mM and HEPES 20 mM, pH 7.4) and the cells were homogenized with four 5 s bursts using a T 10 basic Ultra-Turrax homogenizer (IKA, Staufen, Germany) with an interval of 30 s on ice between bursts. Subsequently the homogenate was centrifuged at 25000 rpm for 30 min at 4 °C and the pellet was resuspended in 1 ml ice-cold membrane buffer. Aliquots of 100 μl of these membranes were stored at −20 °C until needed. The cells were homogenized using membrane buffer containing 1% BSA during homogenization to capture endogenous fatty acids. Subsequently the obtained membranes are resuspended in membrane buffer without BSA and centrifuged for two additional times in order to remove all the leftover BSA. The protein content of the membranes was determined using the Pierce 660 reagent as outlined in the manufacturer's protocol. Membranes were diluted to a concentration of 100 μg/ml in membrane buffer and containing saponin to a final concentration of 10 μg/ml. In a deep 96-well plate, 50 μl of membrane (5 μg), 25 μl GDP (1 μM), 25 μl buffer and 50 μl compound were plated together with 50 μl of the non-hydrolyzable GTP analogue [^{35}S]-GTPγS to a final concentration of 0.3 nM). Non-specific binding was measured by plating 50 μl of membranes, 25 μl GDP (1 μM), 25 μl GppNHp (100 μM), 50 μl [^{35}S]-GTPγS and was subtracted from total binding to calculate specific binding. After one hour of incubation at room temperature, the reaction was stopped by rapid filtration through a GF/C glass filter plate (Unifilter, Perkinelmer, Zaventem, Belgium) using a 96-cell harvester. The unbound radioligand was washed from the filter by doing three washes with ice-cold wash buffer (Tris-HCl 50 mM, $MgCl_2$ 10 mM). After drying and adding 30 μl Scintillation liquid (MicroscintO, Perkinelmer, Zaventem, Belgium) the retained radioactivity was measured in a liquid scintillation counter (Microbeta Trilux, Perkinelmer, Zaventem, Belgium).

2.6. DPH incorporation in fatty acid solutions

A stock solution of DPH of 1 mM was obtained by dissolving it in DMSO. In each well of a 96-well plate 150 μl of a serial dilution of ALA, OLA or MYA in calcium assay buffer (see above) was incubated for 30 min in the dark after adding 2 μl of the DPH stock solution. Hereafter the fluorescence intensity was measured at 358 nm excitation and 430 nm emission wavelength in a TECAN M200 96-well reader (Tecan Benelux, Mechelen, Belgium). As a positive control the fluorescence was measured in a serial dilution of sodium dodecylsulphate (SDS).

2.7. Data analysis

The size of the calcium responses elicited by the receptor ligands was quantified by determining the area under the curves as described previously [63]. The EC_{50} and Emax values of the concentration response curves were calculated by non-linear regression analysis using the biostatistical software GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA. The data in the figures are presented as average ± SD values either from n independent experiments or from representative experiments as explained in the legends. Statistical significance was assessed by unpaired two-tailed t-tests and assigned significant when the corresponding p-values < 0.05.

3. Results

3.1. Functional expression of FFA1 receptors in CHO-AEQ cells.

The activation of FFA1 receptors was investigated in CHO cells that permanently express the calcium-sensitive bioluminescent protein aequorin (denoted as CHO-AEQ cells) after loading them with 5 μM coelenterazine h as described previously [5]. The first aequorin assays were done using cells that were transiently transfected with the human HA-tagged FFA1 receptor and compared with mock transfected cells. The transfection efficiency was determined by flow cytometric analysis using a AlexaFluor488 anti-HA antibody and ranged between 34 and 40 % of all the cells (data not shown). To assess FFA1 receptor activation the cells were suspended in assay buffer (i.e. DMEM-F12) containing 0.1 % BSA. 50 μl of this suspension containing 25,000 cells was injected in a 96-well plate containing the ALA, [11,43,49]. Immediately before and after this the intensity of luminescence as a relative measure of intracellular free calcium concentration was determined in a Tecan infinite M200 96-well reader. As shown in Fig. 1, ALA at a final concentration of 100 μM induced a rapid and transient rise of intracellular calcium concentration in FFA1 transfected cells, while no such response was seen in mock transfected cells. The size of these responses were quantified by calculation of the area under the curve after cell injection using GraphPad Prism [63]. Subsequent experiments revealed that these

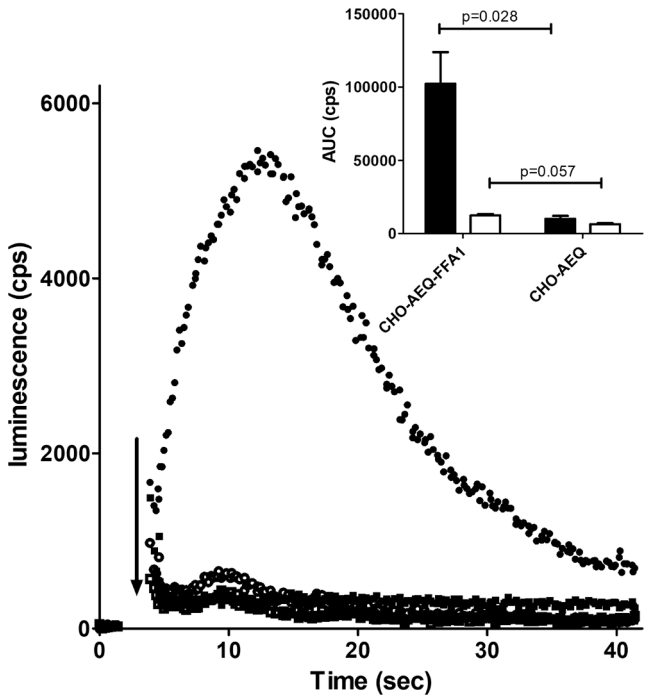

Fig. 1. ALA mediated calcium responses in cells expressing FFA1 receptors in the presence of 0.1 % BSA: Intensity of the luminescence before and after injection at the arrow of 100 μM ALA (●, ○) or assay buffer (■,□) to transient transfected CHO-AEQ cells (filled symbols) or control cells (open symbols). The values are from a representative experiment. Insert: The corresponding AUC values of the calcium responses after 100 μM ALA (filled bars) or assay buffer (open bars) which are the average ± SD of four measurements. Unpaired two-tailed t-tests (p-values) were carried out to compare the effects between control and FFA1 transfected cells.

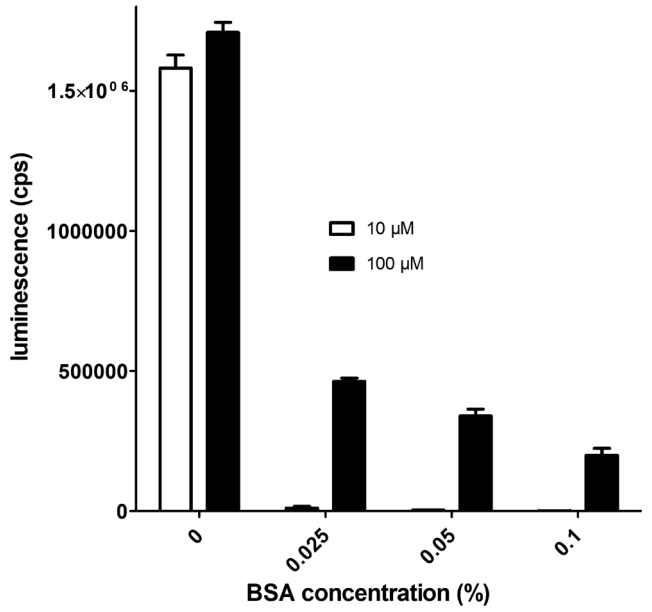

Fig. 2. ALA (10 or 100 μM) mediated calcium responses in CHO-AEQ-FFA1 cells given as AUC values in the absence or presence of different indicated concentrations of BSA. The values are the average ± SD of three measurements of one representative experiment. All the ALA responses are compared between those in the absence versus in the presence of the different concentrations BSA and yielded p-values < 0.0001 (unpaired two-tailed t-tests).

responses were substantially higher in the absence of BSA (Fig. 2) and therefore were carried out in assay buffer without BSA unless otherwise stated.

3.2. *Free fatty acids ALA, OLA and MYA mediate concentration-dependent transient calcium responses.*

By culturing transient FFA1 receptor transfected cells in the presence of 1 mg/ml geneticin we generated a mixture of stable receptor transfected cells denoted as CHO-AEQ-FFA1 for further pharmacological experiments. Using these cells we generated ALA response curves with concentrations between 0.1 μM and 3 mM. As illustrated in Fig. 3, clearly biphasic curves were obtained. In the first phase (between 0.1 and 100 μM) an expected sigmoid relation was seen, which is compatible with a classical concentration response curve when the activation of a receptor is measured. The corresponding EC_{50} values of ALA are given in Table 1. After reaching a plateau and at higher ligand concentrations > 100 μM a further increase of the luminescence is observed (second phase). While the first phase was completely absent in control non-FFA1 transfected cells (CHO-AEQ) the second phase responses were seen in both cells indicating that they are independent of FFA1 receptor activation. As depicted in Fig. 4 and investigating the first phase responses (ALA up to 100 μM), inclusion of 5 mM EGTA had no significant influence while the phospholipase C inhibitor U73122 (10 μM) completely blocked the responses. Furthermore the FFA1 receptor selective antagonist GW1100 clearly rightward shifted the response curves (Fig. 4C).

Subsequently we have investigated the effects of OLA and MYA at concentrations up to 100 μM, two other natural FFA1 receptor binding free fatty acids. To compare them we have normalized the size of the responses with those mediated by 100 μM ALA. As depicted in Table 1 the EC_{50} values of oleic acid and myristic acid were higher than that obtained for ALA. Moreover the maximal effect of both compounds was lower than that for ALA. In the same line as for ALA the calcium transient induced by 100 μM OLA or MYA were completely impeded in the presence of U73122 and not affected by EGTA (data not shown). Similar as for ALA up to 100 μM of both free fatty acids were devoid of any calcium response in control CHO-AEQ cells.

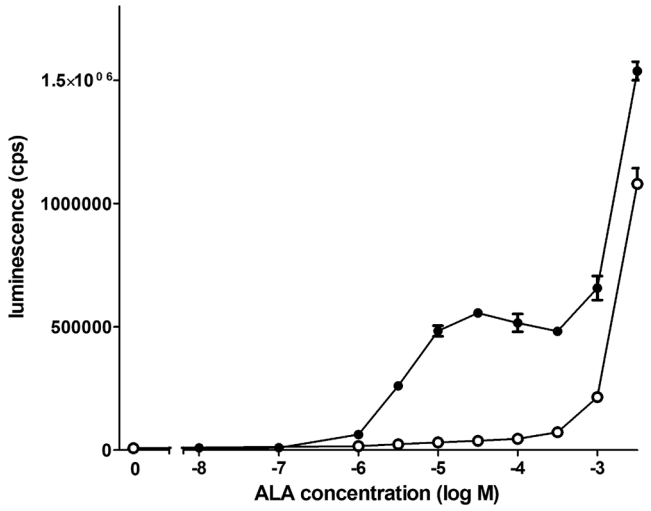

Fig. 3. Concentration-dependent increase of calcium responses mediated by ALA in CHO-AEQ-FFA1 cells (●) or CHO-AEQ cells (○) in the absence of BSA. The values are the average ± SD of three measurements of a representative experiment.

Table 1
Concentration-response parameters of three natural fatty acids mediating calcium responses in CHO-AEQ-FFA1 cells. The Emax values were normalized with the responses elicited by 100 μM ALA in experiments on the same day. nH refers to the Hill slope and n the number of independent experiments with minimally triplicate determinations. The EC_{50} values of OLA and MYA were significantly higher as compared with that of ALA with p-values < 0.0001 (unpaired two-tailed t-tests) and the corresponding fold increase was 2.25 and 4.27 respectively.

Fatty acid	EC_{50} ± SD (μM)	Emax ± SD (% of 100 μM ALA)	nH ± SD	n
α -linolenic acid	6.89 ± 4.27	100	1.76 ± 0.75	46
Oleic acid	15.50 ± 2.04	55 ± 8	1.83 ± 0.76	8
Myristic acid	29.43 ± 1.99	39 ± 3	0.83 ± 0.23	3

3.3. ATP and Angiotensin II mediated calcium responses in respectively CHO-AEQ-FFA1 and CHO-AEQ-AT_1 cells are reduced after pre-incubation of the cells with fatty acids.

ATP mediated calcium responses via endogenously expressed purinergic receptors were recorded after the completion of the transient FFA mediated calcium increase on the same cells. As outlined in Fig. 5 ATP responses were almost completely inhibited after pre-stimulation with 100 μM of ALA on CHO-AEQ-FFA1 cells with half-maximal inhibition occurring at 12 ± 2 μM. A similar inhibition was seen in CHO-AEQ cells (Fig. 5) in which up to 100 μM ALA had only a small effect indicating a FFA1 receptor independent underlying mechanism for this cross-inhibition.

In order to find out whether this inhibition was due to depletion of the coelenterazine h and/or the intracellular calcium stores, we have pre-stimulated these cells first with 10 μM ATP after which the ALA mediated calcium response was measured. As shown in Fig. 6 panel B, the ALA response was partially reduced. Statistically this reduction was marginally significant as the corresponding p-value was 0.05. Administration of OLA and MYA on both CHO-AEQ-FFA1 and CHO-AEQ cells also resulted in the cross-inhibition of ATP mediated calcium responses. However as compared to ALA they were less potent; and half-maximal inhibition of these fatty acids occurred at 249 ± 6 μM for OLA and was > 1000 μM for MYA.

In the former experiments illustrated in Fig. 5, the time between the administration of the fatty acid and ATP was only 0.66 min. To assess the time-dependency of the inhibitory effect of the fatty acids, subsequent experiments were carried out in which we increased the time that the cells were exposed to the fatty acids up to about 15 min (Fig. 7). As shown in Fig. 7, there is a time-dependent decline of the ATP responses. Subsequently we have compared the size of the ATP responses when applied simultaneously with the fatty acid (denoted as co-incubation) with those after pro-longed pre-incubation with the fatty acids (Table 2).

Subsequently, we found that the free fatty acids also affect subsequent calcium responses elicited by angiotensin AT_1 receptors which are established to be linked to calcium/IP_3 as their primary signaling pathway. For this purpose we have monitored the Angiotensin II mediated calcium responses in CHO-AEQ that were previously stably transfected with human AT_1 receptors, denoted as CHO-AEQ-AT_1 cells. Previously Angiotensin II concentration–response curves were carried out in these cells and yielded an EC_{50} value of 16.7 ± 2.7 nM [5]. To investigate the impact of the fatty acids on Angiotensin II responses, CHO-AEQ-AT_1 cells were pre-incubated with 20 or 100 μM of ALA for different periods of time followed by administration of 1 μM Angiotensin II. Similar as for ATP responses, the fatty acids also caused a time- and concentration-dependent inhibition of the Angiotensin II responses (Fig. 7 and Table 2).

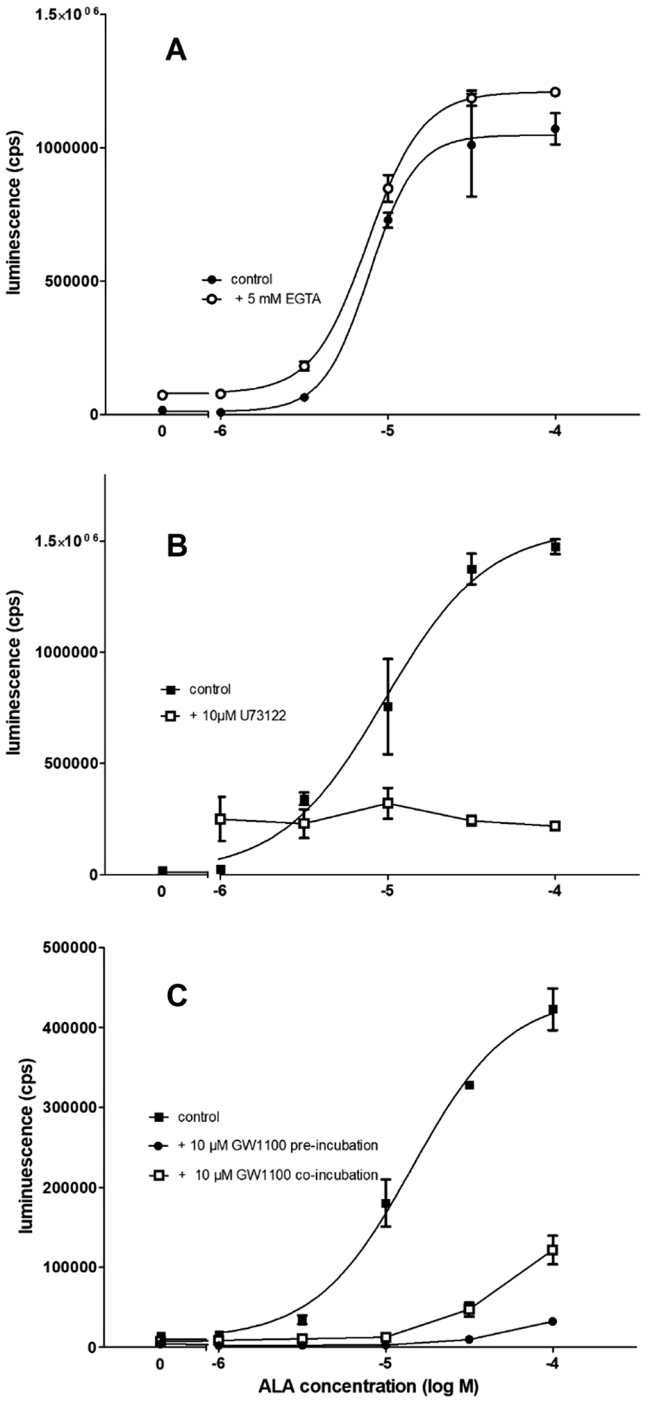

Fig. 4. Concentration calcium response (AUC) curves of ALA in CHO-AEQ-FFA1 cells in the absence of BSA. Panel A depicts the effect of including 5 mM EGTA, while the effect of the phospholipase C inhibitor U73122 (10 μM) is shown in panel B. Panel C describes the ability of the FFA1 receptor antagonist GW1100 to inhibit ALA responses either by co-incubation or after short (0.66 min) pre-incubation. The values are the average ± SD of three measurements of a representative experiment. To assess the statistical significance 95 % confidence intervals of the EC_{50} values (μM) were determined and were for control curve: 6.54–9.22, in the presence of EGTA: 7.18–7.94, GW1100 (co-incubation): 21–102 while they could not be determined in the presence of U73122 or after GW1100 pre-incubation.

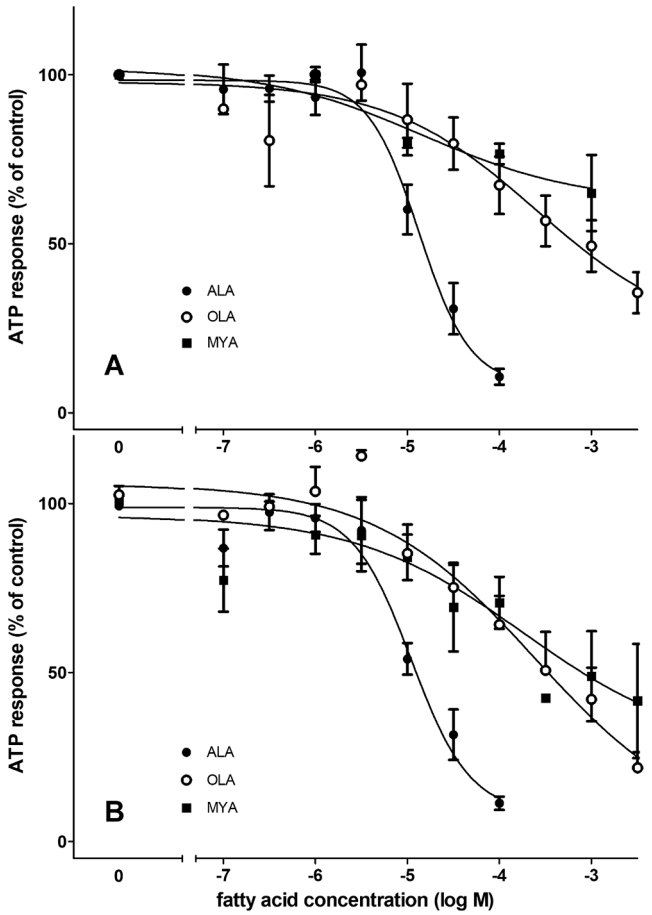

Fig. 5. Inhibition of ATP mediated calcium responses (AUC) after a short (0.66 min) pre-incubation with ALA (n = 4), OLA (n = 5) or MYA (n = 6) in CHO-AEQ cells (panel A) or CHO-AEQ-FFA1 cells (panel B) in the absence of BSA. The values are the average ± SD and the number of n independent experiments each performed in triplicate.

3.4. Fatty acid pretreatment of CHO-AEQ-AT$_1$ cells inhibits calcium depletion by thapsigargin.

To investigate possible calcium store depletion we quantified the effect of fatty acid pre-treatment on the effect of 1 μM thapsigargin. Thapsigargin inhibits the ATP dependent calcium re-uptake carrier in intracellular calcium stores, resulting in a large and slow rise in the intracellular calcium level [78,81]. Calcium responses on CHO-AEQ-AT$_1$ cells were monitored during pre-treatment for 2 min. with 10, 100 or 1000 μM of the fatty acids, and as control with assay buffer, after which 1 μM thapsigargin responses were measured during another 2 min. The area under the curves of the resulting calcium responses (both after fatty acid treatment and after thapsigargin) are shown in Fig. 8. Although the fatty acids induced calcium responses were small (similar as also in CHO-AEQ cells), pre-treatment with 100 μM ALA completely prevented the thapsigargin mediated calcium store depletion. A similar but partial inhibition of the thapsigargin effect was seen with 100 μM OLA and MYA.

3.5. ALA inhibits [^{35}S]-GTPγS binding in CHO-AEQ cell membranes.

To further examine a possible mechanism underlying the cross-inhibition of both ATP and Angiotensin II mediated calcium signaling we have measured the binding of [^{35}S]-GTPγS in permeabilized membranes of CHO-AEQ-FFA1 and CHO-AEQ cells in the absence or presence of increasing concentrations of ALA. Although ALA did not increased

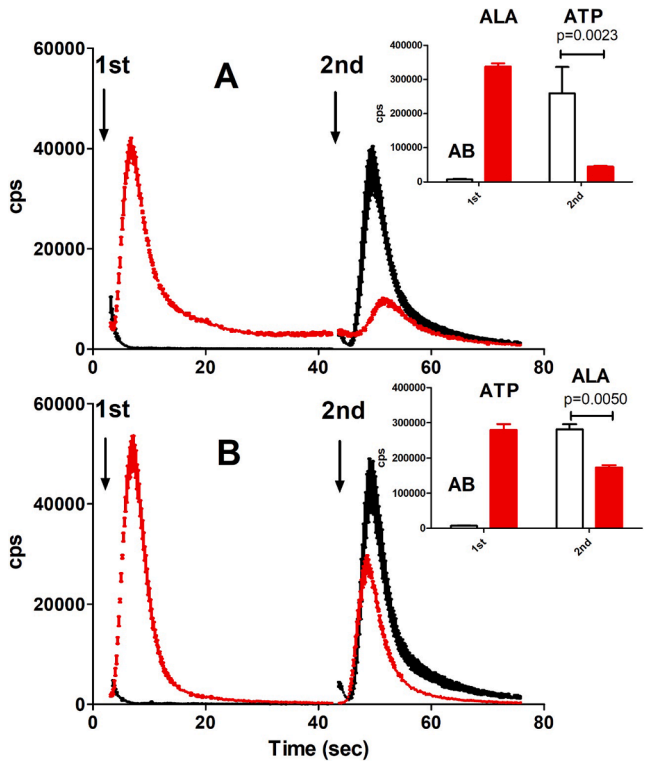

Fig. 6. Calcium traces of two subsequent compound administrations on CHO-AEQ-FFA1 cells in the absence of BSA. The sizes of the corresponding responses are given in the inserts and are the average ± SD of the three replicates of this representative experiment. The interval between both administrations was 0.66 min. Panel A: First administration of assay buffer (black symbols) or 100 μM ALA (red symbols) both followed by 10 μM ATP. Insert: assay buffer (AB) followed by ATP (white bars) and 100 μM ALA followed by 10 μM ATP (red bars). Panel B : First administration of assay buffer (black symbols) or 10 μM ATP (red symbols) both followed by 100 μM ALA. Insert: assay buffer (AB) followed by 100 μM ALA (white bars) and 10 μM ATP followed by 100 μM ALA (red bars). Unpaired two-tailed t-tests (p-values) were carried out to compare the effects between ALA and assay buffer pre-stimulation on ATP responses (insert panel A) and between ATP and assay buffer pre-stimulation on ALA responses (insert panel B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[^{35}S]-GTPγS binding in membranes of CHO-AEQ-FFA1 cells, a substantial and concentration-dependent inhibition was seen with a maximal effect at 100 μM in both cell types (Fig. 9). In the absence of ALA a significantly higher level of [^{35}S]-GTPγS binding was seen in CHO-AEQ-FFA1 versus CHO-AEQ cell membranes which might be indicative for constitutive activity of the FFA1 receptor and/or the binding of endogenous free fatty acids, which were released from the cell during the membrane preparation and are able to bind the receptor [74,75]. This would also be consistent with the observation that a higher concentration of ALA is needed to inhibit [^{35}S]-GTPγS binding in CHO-AEQ-FFA1 as compared to CHO-AEQ cell membranes (Fig. 9). In the same line the (partial) inverse FFA1 receptor agonist GW1100 reduced [^{35}S]-GTPγS binding to CHO-AEQ-FFA1 but not CHO-AEQ cell membranes [74].

3.6. Free fatty acids do not affect cell viability at concentrations up to 100 μM.

A possible explanation of the cross-inhibitory effects of free fatty acids on intact CHO cells could be related to their influence on cell viability i.e. necrosis and/or lysis of the cells. For this purpose we have measured the uptake of propidium iodide (PI) that is able to bind to double-stranded nuclear DNA and resulting in a strong fluorescent

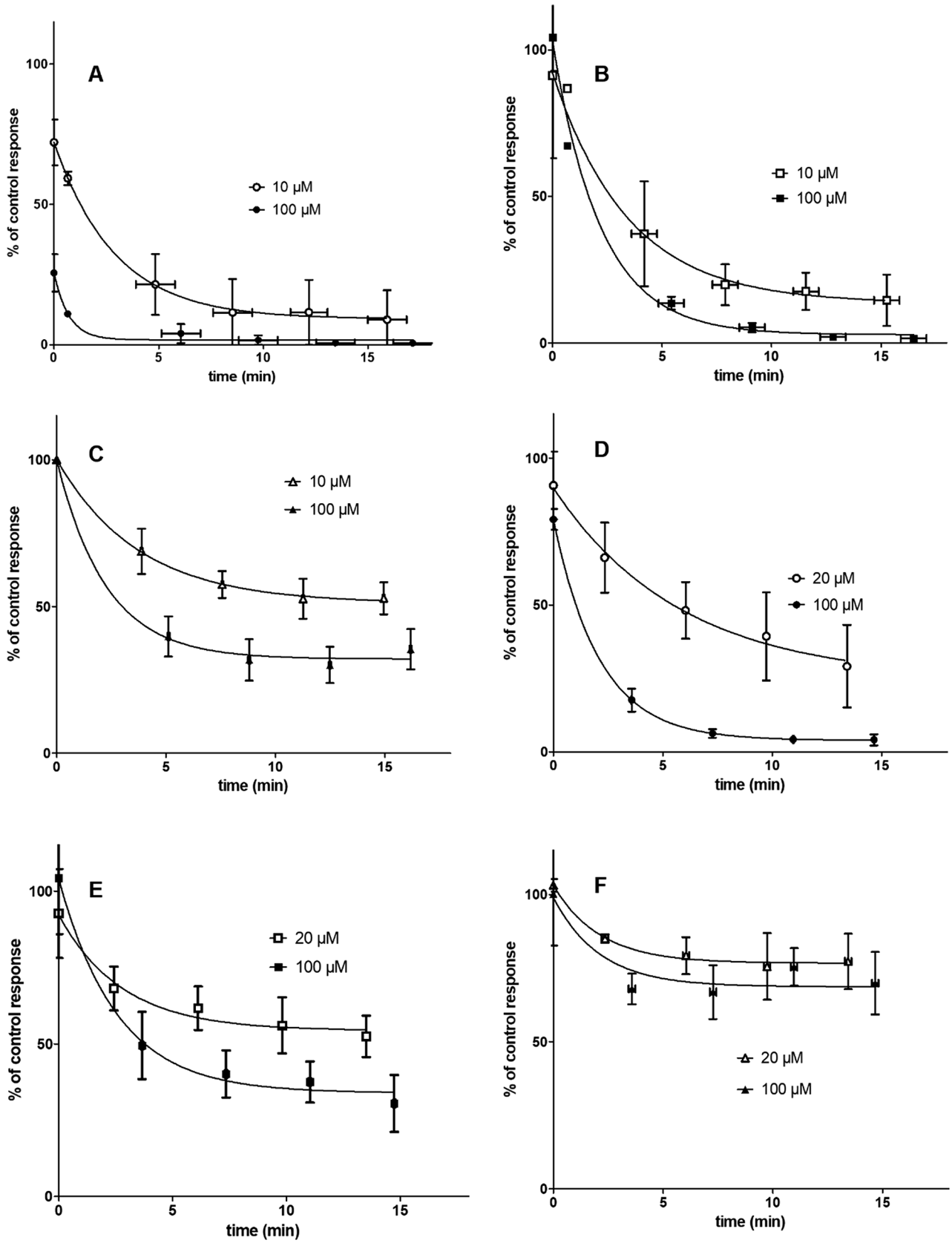

Fig. 7. Time- and concentration-dependent inhibition of 10 μM ATP mediated calcium responses (panels A, B and C) or of 1 μM Angiotensin II mediated calcium responses (panels D, E and F) after pre-incubation for the indicated time periods with ALA (panels A and D), OLA (panels B and E) or MYA (panels C and F) at the indicated concentrations. These assays are done in CHO-AEQ-AT_1 cells in the absence of BSA and the values are given as the percentage of the responses in the absence of fatty acids (denoted at % control response) and are the average ± SD of two independent experiments each performed as 3–4 replicates.

Table 2
Time- and concentration dependent reduction of ATP/Angiotensin II calcium responses in CHO-AEQ-AT_1 cells by fatty acid pre-stimulation. The remaining calcium responses induced by ATP or Angiotensin II in the presence of the indicated fatty acids either in co-incubation i.e. when applied simultaneously to the cells, or after prolonged pre-incubation corresponding to the plateau obtained by non-linear regression analysis with a one-phase exponential decay function. The values correspond to the data shown in Fig. 7, given as percentage of the responses in the absence of the fatty acids and are the average ± SD of two independent experiments each carried out as 3–4 replicates. Unpaired two-tailed t-tests of the remaining responses between the co– versus prolonged pre-incubation all yielded p-values < 0.0001. The control calcium responses (AUC and average ± SD) by ATP and Angiotensin II are respectively $1.88 \times 10^6 \pm 0.85 \times 10^6$ and $1.78 \times 10^6 \pm 1.43 \times 10^6$ cps.

Remaining ATP calcium responses in the presence of fatty acids (% of control)		
Fatty acid	co-incubation	prolonged pre-incubation
10 μM ALA	72.6 ± 4.3	9.25 ± 5.3
100 μM ALA	25.6 ± 1.5	1.7 ± 1.54
10 μM OLA	91.9 ± 5.3	13.6 ± 4.9
100 μM OLA	102.7 ± 4.8	2.8 ± 3.2
10 μM MYA	100.2 ± 5.7	51.1 ± 2.9
100 μM MYA	100.1 ± 6.7	32.1 ± 2.3

Remaining Angiotensin II responses in the presence of fatty acids (% of control)		
Fatty acid	co-incubation	prolonged pre-incubation
20 μM ALA	89.8 ± 4.2	24.6 ± 8.9
100 μM ALA	79.1 ± 2.6	3.9 ± 1.8
20 μM OLA	92.2 ± 8.9	54.4 ± 7.4
100 μM OLA	104.1 ± 5.5	33.9 ± 4.1
20 μM MYA	103.0 ± 3.7	76.7 ± 1.9
100 μM MYA	98.9 ± 5.3	68.6 ± 2.8

signal, only when it is taken up by permeabilized necrotic cells. We have measured PI uptake both in CHO-AEQ-FFA1 and CHO-AEQ cells either in adherent cells after overnight incubation in a cell incubator or cells in suspension. As shown in Fig. 10, 100 μM ALA did not affected PI uptake in both cell types either adherent or as cell suspension while 1 mM caused a slow and partial PI uptake (compared to the uptake in the presence of 0.2 % Triton X-100). The latter might be compatible with the non-receptor mediated calcium uptake observed in the calcium aequorin experiments at this high concentration (see above, in Fig. 3). In the same line 100 μM of OLA or MYA did not induced PI uptake (data not shown).

3.7. DPH incorporation in serial dilutions of the investigated fatty acids.

The amphipathic nature of fatty acids reveals their capability to form micelles in a water environment. As such the formation of these micelles might result in the disruption of the plasma membrane integrity and lead to cell necrosis. To investigate this we have measured the fluorescence intensity in the presence of (DPH) [16,31]. When incorporated in the hydrophobic core of micelles an abrupt increase of DPH fluorescence indicates the formation of micelles. For this purpose fluorescence was measured in serial dilutions of the ALA, OLA and MYA that were made in the calcium aequorin assay buffer at a volume of 150 μl, after which 2 μl of 1 mM DPH was added and pre-incubated for 30 min at room temperature protected from light. As a positive control DPH incorporation was determined in a serial dilution of sodium dodecylsulphate (SDS) to validate the results with the free fatty acids. As shown in Fig. 11 only concentrations ≥ 1 mM of ALA produced a noticeable increase of DPH fluorescence while only minimal increase was seen with OLA or MYA.

4. Discussion

A large number of clinical studies established a correlation between high levels free fatty acid in the plasma and insulin resistance (i.e. type 2 diabetes) and hypertension, cardiovascular disease, stroke and atrial

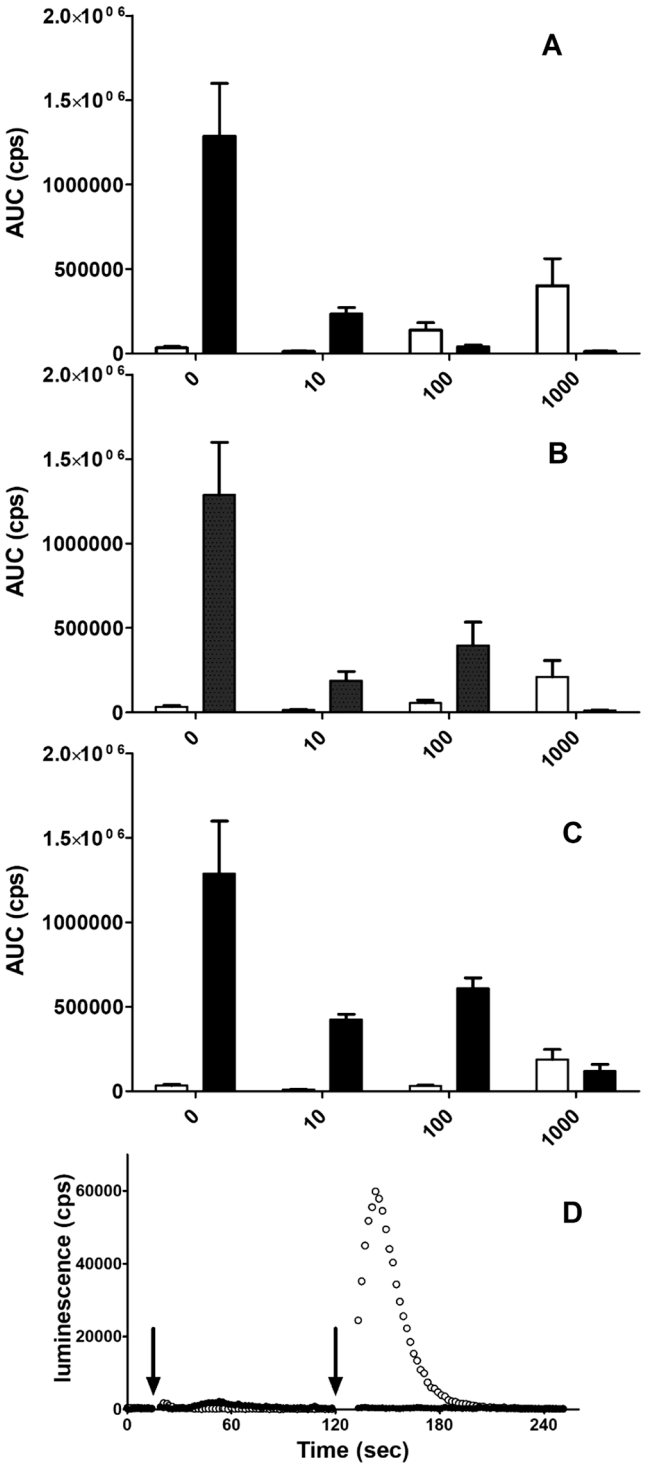

Fig. 8. Calcium responses (AUC) mediated after FFA and subsequent thapsigargin administration on CHO-AEQ-AT_1 cells in the absence of BSA. The interval between both administrations was 1.9 min. White unfilled bars represent the responses by the first administration of ALA (panel A), OLA (panel B) and MYA (panel C) at the indicated concentrations (μM) followed by a subsequent administration of 1 μM thapsigargin (filled bars). Values are the average ± SD of a tripliqate determinations in a representative experiment. Unpaired two-tailed t-tests between the thapsigargin responses when preceded by assay buffer and fatty acid administration yielded all p-values < 0.0001. Panel D depicts a typical calcium trace of this experiment in which assay buffer (○) or 100 μM ALA (●) was first applied (left arrow) after which 1 μM thapsigargin was administrated (right arrow).

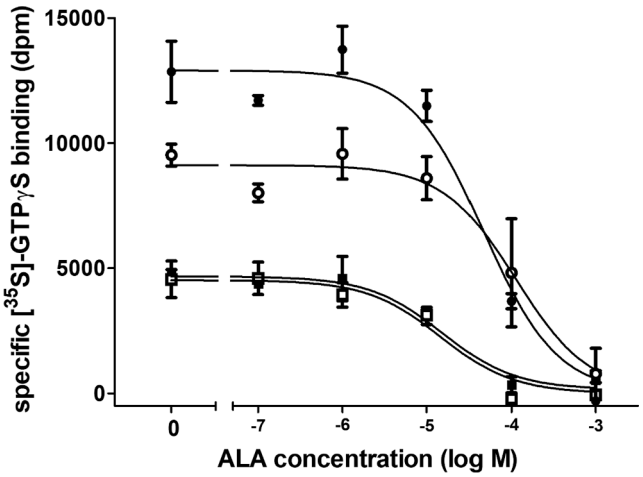

Fig. 9. ALA concentration-dependent inhibition of specific [^{35}S]-GTPγS binding to membranes of CHO-AEQ cells in the absence (■) or presence (□) of 10 μM GW1100 or to membranes of CHO-AEQ-FFA1 cells in the absence (●) or presence (○) of GW1100. The values are the average ± SD of a representative experiment with triplicate determination. The specific binding of [^{35}S]-GTPγS in the absence of ALA, was compared between CHO-AEQ and CHO-AEQ-FFA1 membranes ($p = 0.0003$) as well as between CHO-AEQ and CHO-AEQ-FFA1 membranes in the absence and presence of GW1100 which gave $p = 0.720$ and 0.011 respectively. The average ± SD of the pIC_{50} values of ALA inhibition in CHO-AEQ and CHO-AEQ-FFA1 membranes were respectively 4.82 ± 0.16 and 4.33 ± 0.12 with $p = 0.013$ from a unpaired two-tailed *t*-test .

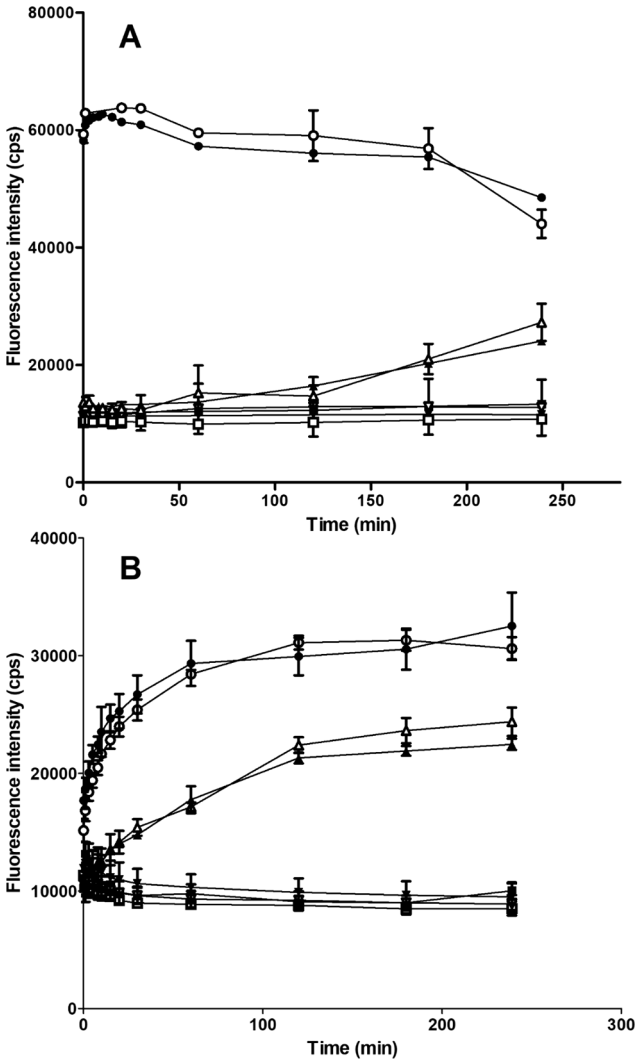

Fig. 10. Time-dependent uptake of PI in cells in suspension (panel A) or adherent cells (panel B) after treatment with ALA. Open symbols refer to control CHO-AEQ-AT$_1$ cells in panel A and to CHO-AEQ cells in panel B. Closed symbols refer to CHO-AEQ-FFA1 cells in both panels. Cells are treated with buffer (▽,▼), 100 μM ALA (□, ■), 1 mM ALA (△,▲) or 0.2 % Triton X-100 (○,●). Values are the average ± SD of quadruplicate determinations of a representative experiment.

fibrillation [26,27,45,46,64,66]. In addition FFAs are also found to modulate inflammatory events in insulin target tissues like endothelial, liver and skeletal muscle cells [29]. Some of which have toxic effects on these cells. The cytotoxic effects of free fatty acids and in particular saturated fatty acids are mediated via distinct mechanisms [7,9,18,24,30,32,34,35,54,56,57,62,65,67].

On the other hand protective/beneficial effects of medium to long chain fatty acids have been established in pancreatic endocrine cells (beta cells) [21,76,83]. Furthermore the binding of these free fatty acids to GPR40/FFA1 receptors that are expressed in these cells, have been shown to potentiate both *in vitro* as well as *in vivo* glucose stimulated insulin secretion and, as such expected to be effective as a treatment strategy in type 2 diabetes [11,14,20,23,28,43,48]. Yet synthetic FFA1 receptor ligands dampened this optimism since their further development had to be discontinued due to the occurrence of liver toxicity [53]. Here we report complex intracellular calcium signaling mediated by ALA, OLA and MYA in CHO cells expressing the calcium-sensitive bioluminescent protein aequorin [22]. In agreement with previous ground-breaking results [11,28,43,49], transfection of these cells with human FFA1-receptors displayed a transient increase of intracellular calcium is observed after administration of 100 μM of these free fatty acids while no such response was observed in mock-transfected cells. These initial assays were carried out as described previously in the presence of 0.1 % BSA [5,63]. Omitting the BSA in the assay buffer resulted in much higher calcium responses because of its ability to bind long chain fatty acids such as palmitic acid, oleic acid and linoleic acid [50]. In the same line previous studies [11,74] described a similar reduction of FFA responses in the presence of BSA and therefore, subsequent experiments were carried out in the absence of BSA.

As outlined in the results section the concentration response curves of the free fatty acids were clearly biphasic. In the first phase (up to and including 100 μM) appeared as a typical sigmoid shaped concentration–response curve and was completely FFA1 receptor mediated as it is absent in mock-transfected CHO cells. The obtained EC_{50} values for ALA, OLA and MYA and given in Table 1, were in agreement with those reported in previous studies [10]. Furthermore a clear-cut inhibition of these responses is seen in the presence of the FFA1 receptor antagonist GW1100 [74]. Additionally the ALA mediated responses were mediated via IP_3 release of intracellular calcium since they were completely prevented in the presence of the phospholipase C inhibitor U73122 while chelating extracellular calcium by EGTA had no significant influence on FFA1 receptor responses.

Interestingly at concentrations higher than 100 μM (as illustrated for ALA in Fig. 3) a further increase of intracellular calcium is observed (denoted as a second phase) in both FFA1 and mock transfected cells indicating a clearly distinct and non-FFA1 receptor mechanism. A possible explanation for this effect might be found in the membrane-deteriorating effects of fatty acids. Due to the amphipathic nature of fatty acids numerous biophysical studies in synthetic lipid bilayers have extensively shown that incorporation of fatty acids destabilizes the lipid bilayer resulting in the disruption of its function as permeability barrier [3,4]. Although not yet fully understood, the addition of exogenous fatty acids to pre-formed membranes is more disruptive than when premixed with lysolipids before the formation of a lipid membrane. Moreover the permeability disruption (read pore formation) generally depends on the chain length and the degree of unsaturation of the fatty acids. In

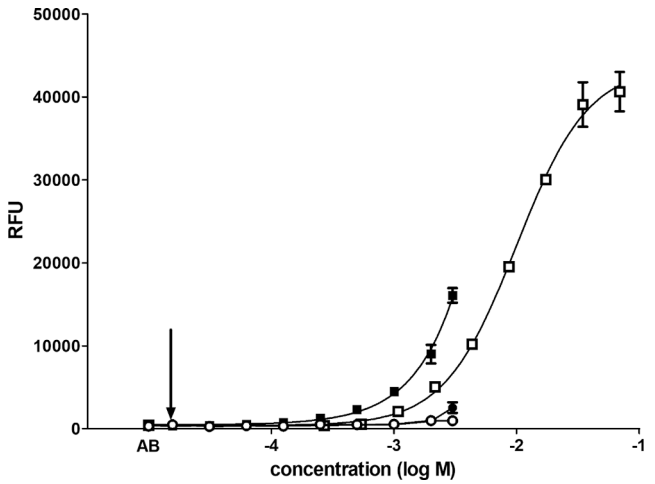

Fig. 11. DPH fluorescence in the presences of increasing concentration of SDS (□), ALA (■), OLA (●) or MYA (○). The arrow points to the ALA concentration at which 50 % of the ATP mediated calcium response was inhibited in CHO-AEQ cells (see also Fig. 5A). AB refers to assay buffer. Values are the average ± SD of quadruplicate determinations of a representative experiment.

agreement with this we found that treatment of CHO cells with 1 mM but not 0.1 mM ALA caused an uptake of propidium iodide, a well-established method to assess plasma membrane integrity [19,68]. This effects was seen in both FFA1 and mock transfected CHO-AEQ cells both in suspension as well as after adherence to the 96-well plates. Yet this effect appeared to be substantially slower as compared with the treatment of the cells with 0.2 % Triton-X-100 (see Fig. 10). It was also slower as compared to the rate by which 1 mM ALA increases intracellular calcium in our aequorin assays (data not shown). A possible explanation for this discrepancy could be that fatty acids initially have a pronounced membrane disruption after which the lipid molecules rearrange and protect for this effect [6,69].

Another fascinating effect of the investigated FFAs was revealed when measuring two subsequent calcium responses in the same cells. As explained in the results section we first measured the response of the fatty acids and secondly that of ATP. ATP was found to produce substantial and transient calcium responses that were completely inhibited by the PLC inhibitor U73122 and not affected by including EGTA in the assay buffer. This was in full agreement with the expression of endogenous G-protein coupled P2U-purinoceptors which are associated to an increase of intracellular calcium by IP_3 generated by phospholipase C in CHO-K1 cells [42]. In similar experiments CHO-AEQ-AT_1 cells were first treated with fatty acids after which the calcium response mediated by activation of the recombinantly expressed AT_1 receptors by Angiotensin II was recorded. In both experimental setups we found that pretreatment of the cells with ALA, OLA or MYA produced a concentration- and time-dependent reduction of the ATP and Angiotensin II mediated calcium transients which we denoted as a cross-inhibitory effect (Fig. 8 and Table 2).

A possible explanation for this cross-inhibition could be a depletion of intracellular calcium stores when cells were pre-stimulated with a polyunsaturated fatty acid such as ALA and to a lesser extent with saturated fatty acids as was seen in rat thymocytes [15].

In line with this hypothesis pre-stimulation of CHO-AEQ-FFA1 cells with ATP, which results in calcium transients due to activation of endogenously expressed purinergic receptors in CHO cells, also caused a reduction of subsequently measured ALA responses. However as illustrated in Fig. 6, this reduction was only partial in the case of ATP pre-stimulation while this effect was clearly stronger when these cells were pre-stimulated by 100 μM ALA and the subsequent ATP responses were monitored. Notwithstanding this we found that 100 μM ALA pre-stimulation produced complete inhibition of the large rise of cytosolic calcium by 1 μM thapsigargin (Fig. 8). This large rise in intracellular calcium caused by thapsigargin is linked to its inhibition of the endoplasmic re-uptake calcium/ATP transporter leading to depletion of the intracellular calcium stores [78]. Furthermore a similar though partial inhibition of the thapsigargin response was seen with OLA and MYA. Yet the underlying mechanism of this inhibition is not clear nor whether it is linked to the cross-inhibition of the purinergic and angiotensin AT_1 receptor mediated calcium responses by pre-treatment with the fatty acids. As shown in Figs. 5 and 6, pre-stimulation with investigated fatty acids produced a similar inhibition of ATP mediated calcium responses in CHO-AEQ and CHO-AEQ-AT_1 cells as well as Angiotensin II responses in CHO-AEQ-AT_1 cells, indicating that FFA1 receptor signaling is not involved in this cross-inhibition.

The observed cross-inhibition can also not be explained by the cellular necrosis as none of the investigated fatty acids affect the uptake of propidium iodide in both adherent and suspension CHO cells (Fig. 10). Finally it cannot be ruled out that the investigated fatty acids might modulate G-protein activation. In this respect it has been reported that the unsaturated fatty acids linoleic acid, linolenic acid and oleic acid selectively impair GTP binding to the Gz-protein a-subunit, while other G-protein are less sensitive [31]. The Gz protein investigated in this study is a member of the G-protein family involved in pertussis toxin-insensitive signal transduction and is expressed in peripheral as well as brain areas [38]. In line with this proposed mechanism we found that ALA is indeed inhibiting [^{35}S]-GTPγS binding in membranes from both CHO-AEQ and CHO-AEQ-FFA1 cells in agreement with a FFA1 receptor independent mechanism of cross-inhibition. On the hand it is not clear whether the Gz protein is involved in both ATP and Angiotensin II calcium responses in aequorin expressing CHO cells. Interestingly the Gz protein is found to be expressed in pancreatic beta cells and its selective gene knock-out results in a higher glucose-stimulated insulin secretion when compared to wild-type mice and is suggested to be a potential molecular target to improve beta-cell dysfunction in type 2 diabetes [47]. As such the ability of unsaturated fatty acids to inactivate this Gz protein would therefore represent an additional molecular mechanism beneficial in diabetes treatment. Yet it cannot be excluded that the investigated fatty acids might also be able to inactivate nucleotide binding to Gα16, a G-protein that is also co-expressed in the aequorin expressing CHO cells. In previous studies it was suggested that Gα16 is able to couple in a nonselective manner to most GPCRs to the calcium/IP_3 signaling pathway rendering the aequorin CHO cells as an excellent read out system for known receptors as well as to screen potential ligands for orphan GPCR's [1].

The cross-inhibition of Angiotensin II mediated calcium responses by fatty acids might also be explained by blocking Angiotensin II binding to AT_1 receptors. In this respect both saturated and unsaturated fatty acids have previously been shown to inhibit specific [^{125}I]-Angiotensin II binding to membranes prepared from bovine adrenal glomerulosa and fasciculata cells as well as bovine adrenal and renal arteries, cells and tissues that are known to express AT_1 receptors [33]. The IC_{50} values of ALA, OLA and MYA reported in this study were 12.1, 4.1 and 18 μM indicating that OLA was the most potent inhibitor. In contrast to this and as shown in Fig. 6 preincubation of only ALA at 100 μM completely inhibited ATP as well as Ang II mediated calcium responses while only partial inhibition was observed with OLA or MYA which does not lends support to the hypothesis of these compounds being competitive Angiotensin II receptor ligands.

Finally another explanation for the cross-inhibition might be sought in the ability of fatty acids to stimulate the activity of PKC [39,44]. PKC is a group of kinases that is involved in the phosphorylation of hydroxyl groups of serine and threonine amino acids. In respect to cell signaling PKC is known to be involved in the heterologous desensitization of GPCR's and therefore could contribute in reducing and/or impairing the calcium signaling of purinergic and angiotensin II receptors.

The question remains whether the observed cross-inhibition requires the formation of fatty acid micelles. As for all amphiphilic substances

fatty acids in aqueous solution are capable in the formation of micelles. Subsequently such micelles are able to intercalate into the plasma membranes resulting in some destruction of the membrane barrier leading to cell permeabilization and/or necrosis. By diluting in the calcium assay buffer ALA is indeed increasing the fluorescence produced by DPH as a result of micelle formation. As illustrated in Fig. 11 this is found at concentrations $\geq$ 1 mM clearly beyond the concentrations of ALA that impair ATP and Angiotensin II mediated calcium responses. Therefore the FFA mediated cross-inhibition can't be linked to micelle formation. On the other hand, it can be linked to the ability of ALA at $\geq$ 1 mM to provoke cell necrosis and the resulting propidium iodate uptake as well as the large increase of intracellular calcium.

In conclusion we found that the natural free fatty acids ALA, OLA and MYA are ligands that activate FFA1 receptors linked with the classical IP_3/calcium signaling pathway as measured in CHO cells expressing the calcium sensitive bioluminescent protein aequorin. Interestingly these free fatty acids also exerted two other FFA1 receptor independent effects. At concentrations between 10 and 100 μM these molecules inhibited calcium responses mediated by purinergic receptors endogenously expressed in the CHO-AEQ cells as well as recombinantly expressed angiotensin AT_1 receptors. A possible mechanism explaining this cross-inhibition include a fatty acid mediated depletion of intracellular calcium stores as was reported in rat thymocytes [15]. Alternatively and in particular unsaturated fatty acids, such as ALA, might impair guanine nucleotide binding to certain G-proteins involved in the calcium signaling. Finally at concentrations at or above 1 mM ALA caused a massive influx of calcium in these cells, likely caused by cell lysis/permeabilization.

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