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RESEARCH PAPER

The cytoprotective effects of oleoylethanolamide in insulin-secreting cells do not require activation of GPR119

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BACKGROUND AND PURPOSE

β-cells express a range of fatty acid-responsive G protein-coupled receptors, including GPR119, which regulates insulin secretion and is seen as a potential therapeutic target in type 2 diabetes. The long-chain unsaturated fatty acid derivative oleoylethanolamide (OEA) is an endogenous agonist of GPR119 and, under certain conditions, some long-chain unsaturated fatty acids can promote β-cell cytoprotection. It is not known, however, if OEA is cytoprotective in β-cells. The present study has examined this and determined whether GPR119 is involved.

METHODS

Clonal rat insulin-secreting cell lines, BRIN-BD11 or INS-1E, were exposed to fatty acids complexed with BSA. cAMP levels, insulin release and cell viability were measured. Protein expression was studied by Western blotting and receptor expression by RT-PCR.

KEY RESULTS

GPR119 was expressed in both BRIN-BD11 and INS-1E cells and OEA was cytoprotective in these cells. However, cytoprotection was not reproduced by any of a range of selective, synthetic ligands of GPR119. The cytoprotective response to OEA was lost during exposure to inhibitors of fatty acid amide hydrolase (FAAH) suggesting that OEA *per se* is not the cytoprotective species but that release of free oleate is required. Similar data were obtained with anandamide, which was cytoprotective only under conditions favouring release of free arachidonate.

CONCLUSIONS AND IMPLICATIONS

Activation of GPR119 is not required to mediate the cytoprotective actions of OEA in BRIN-BD11 or INS-1E cells. Rather, OEA is internalised and subjected to hydrolysis by FAAH to release free oleate, which then mediates the cytoprotection.

Abbreviations

AEA, arachidonylethanolamide; FAAH, fatty acid amide hydrolase; GLP-1, glucagon-like peptide-1; OEA, oleoylethanolamide; PEA, palmitoylethanolamide

Introduction

Various long-chain unsaturated fatty acids exert, under certain conditions, multiple effects in pancreatic β-cells, including the ability to stimulate insulin secretion and to protect β-cells against a range of cytotoxic stimuli (Eitel *et al.*, 2002; Welters *et al.*, 2004; Newsholme *et al.*, 2007; Dhayal *et al.*, 2008; Gehrmann *et al.*, 2010; Thorn and Bergsten, 2010). Both of these actions are potentially important therapeutically because in patients with type 2 diabetes, defects in insulin secretion develop early in the course of the disease and are followed by a later decline in β-cell mass. Thus, the

capacity to minimise the impact of these responses could lead to improved glucose homeostasis.

The mechanisms by which long-chain unsaturated fatty acids regulate insulin secretion and β-cell viability are still under investigation but a considerable body of evidence has implicated a family of lipid-responsive G protein-coupled receptors as potential mediators of fatty acid responses. Among these, the orphan receptor GPR119 (receptor nomenclature follows Alexander *et al.*, 2011) has attracted considerable attention as ligands at this receptor stimulate insulin secretion in a glucose-dependent manner (Overton *et al.*, 2008; Dhayal and Morgan, 2010; Shah and Kowalski, 2010; Talukdar *et al.*, 2011). GPR119 displays a relatively restricted tissue distribution, being found mainly in the endocrine cells of the pancreas and in incretin-secreting enteroendocrine cells of the intestine (Overton *et al.*, 2006; Chu *et al.*, 2007; Lauffer *et al.*, 2009). Therefore, selective agonists of GPR119 could exert their effects on insulin secretion via two possible mechanisms, a direct action at the level of the β-cell and a more indirect response mediated by increased circulating levels of glucagon-like peptide-1 (GLP-1) released from intestinal L-cells (Overton *et al.*, 2006; 2008; Madiraju and Poitout, 2007; Chu *et al.*, 2008). A range of synthetic molecules have been developed as ligands of GPR119 and these are effective as enhancers of glucose-induced insulin secretion (Overton *et al.*, 2006; Chu *et al.*, 2007; Ning *et al.*, 2008; Lauffer *et al.*, 2009; Yoshida *et al.*, 2010; Szewczyk *et al.*, 2011; Xia *et al.*, 2011). Additionally, it has also been established that endogenous GPR119 agonists exist and that these include derivatives of long-chain mono-unsaturated fatty acids such as oleoylethanolamide (OEA), the ethanolamide derivative of the C18:1 fatty acid, oleate (Overton *et al.*, 2006). This observation is of particular interest in view of the ability of some mono-unsaturated fatty acids (including oleate) to promote β-cell viability under conditions that would otherwise lead to lipotoxicity, but it is not known whether OEA has cytoprotective activity in β-cells. OEA lacks the free carboxyl group present in oleate but derivatisation of the carboxyl group of unsaturated fatty acids does not necessarily preclude cytoprotection because methyl esters of saturated fatty acids are fully cytoprotective in β-cells (Diakogiannaki *et al.*, 2007; Dhayal *et al.*, 2008). On this basis, it seems possible that OEA might also mediate such effects but this hypothesis has not previously been investigated.

Therefore, the focus of the present study was to establish whether OEA exerts a cytoprotective response in pancreatic β-cells and to assess the extent to which this may reflect the activation of GPR119. Because OEA has also been found to interact with a range of additional molecular targets (Ryberg *et al.*, 2007; Ho *et al.*, 2008; Swaminath, 2008; Borrelli and Izzo, 2009; Cluny *et al.*, 2009; Martinez de Ubago *et al.*, 2009; Ropero *et al.*, 2009; Eder and Ringseis, 2010) the involvement of these targets was also assessed.

Methods

Cell culture

The rat insulinoma derived β-cell lines BRIN-BD11 and INS-1E (Asfari *et al.*, 1992; McClenaghan *et al.*, 1996) were used in the present study. They were cultured in RPMI-1640 media containing 11 mM glucose supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U.mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The medium used to culture INS-1 cells was also supplemented with 50 uM β-mercaptoethanol. Both cell lines were grown in monolayers at 37°C and 5% CO2. For the individual cell viability experiments, cells were seeded into six-well plates at a concentration of 1×10^5 cells per well in complete RPMI-1640 medium for 24 h. After the 24 h incubation, this media was removed and replaced by serum-free RPMI-1640 containing the appropriate fatty acid–BSA complexes. The control cells received BSA and ethanol (vehicle) only.

Preparation of fatty acid–BSA complexes

Stock solutions of palmitate were prepared in 50% ethanol by heating to 70°C and stock solutions of oleate and OEA were prepared in 90% ethanol at room temperature. The fatty acids were bound to 10% fatty-acid-free BSA (in PBS) by incubation at 37°C for 1 h. The BSA final concentration was maintained at 1% and that of ethanol was maintained at 0.5%. The control cells received the vehicle alone.

Quantification of cell death

Cell viability was determined by the ability of cells to exclude trypan blue. After fatty acid incubation periods (18 h for palmitate-induced cell death and 30 h for serum-starvation), cells were harvested (both adherent and detached), centrifuged for 5 min at 200 g and resuspended in complete RPMI-1640 containing trypan blue (0.4% in PBS) in a 1:1 ratio. Numbers of viable and dead cells were then counted using a haemocytometer and the dead cells were expressed as the percentage of total cells for each of the experimental conditions.

Agonists

Agonists and antagonists were made up into 10 mM stock solutions in DMSO except for URB597 and AM404 (which were prepared in100% ethanol) and added to the fatty acid–BSA complex prior to incubation. Final DMSO and ethanol concentrations did not exceed 0.1 and 0.5%, respectively.

Western blotting

Whole cell protein was extracted from BRIN-BD11 and INS-1 cells and quantified using the bicinchoninic acid method (Fisher Scientific, Loughborough, UK) with BSA as standard. Equal concentrations of protein were denatured and loaded, alongside markers and a positive control (recombinant rat FAAH with a His-tag; Cayman Chemicals, Ann Arbor, MI, USA), onto pre-cast Tris-HCl buffered 12.5% polyacrylamide gels (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). Proteins were subsequently transferred onto a PVDF membrane by electotransfer. The membrane was blocked for 1 h in tris-buffered saline containing 0.05% Tween and 5% dried milk powder. Membranes were then probed for 5 h at room temperature with either anti-FAAH (Cayman Chemicals) or β-actin (Sigma) antibodies (FAAH; 1:1000, β-actin; 1:10 000). The membrane was then washed and probed for 1 h with an anti-rabbit (FAAH) or anti-mouse (β-actin) alkaline phosphatase-conjugated secondary antibody (1:30 000). The membrane was incubated with CDP-*Star*® chemilumi-

nescent substrate, disodium 2-chloro-5-[4-methoxyspiro [1,2-dioxetane-3,2′-(5 chlorotricyclo(3.3.1.1^{3,7})decan)]-4-yl]-1-phenyl phosphate, for 5 min at room temperature and then bands were detected by exposure to X-ray film.

cAMP ELISA assay

INS-1 cells were plated at a concentration of 1×10^5 cells per well (96-well plate) and cultured for 24 h. Cells were then washed and incubated in bicarbonate buffered physiological saline solution containing 0.1% BSA and 0.2 mM glucose at 37°C for 1.5 h. The cells were then stimulated with 20 mM glucose in the absence or presence of OEA, forskolin or GPR119 agonists in the same buffer for a further 30 min. Cells were lysed and stored at –80°C prior to assay of cAMP which was measured using the GE Healthcare Amersham cAMP Biotrak enzymeimmunoassay (EIA) system, according to the manufacturer's instructions.

Insulin release

INS-1E cells were seeded into 24-well plates at a concentration of 1×10^5 cells per well and cultured for 24 h prior to the start of the experiment. They were then washed and incubated in bicarbonate buffered physiological saline solution containing 0.1% BSA and 0.2 mM glucose at 37°C for 1.5 h. Cells were then incubated with 0.2 mM glucose, 20 mM glucose or 20 mM glucose plus relevant agonists in the presence of 10 μM isobutylmethylxanthine for a further 1 h. After this period of time, the supernatant was removed and insulin release was determined by radioimmunoassay.

PCR

Using Trizol reagent (Invitrogen), total RNA was extracted from BRIN-BD11 and INS-1E cell lines and then was used to generate cDNA, which was amplified in single tube reactions (Abgene, Epsom, Surrey, UK) using primers designed for rat GPR119, TRPV1, PPARα, GPR55 or FAAH (GPR119 F gctgtgt ttcacccaaggtt, R cagggtgaagctcccaataa; TRPV1 F agtaactgccag gagctgga, R ttctccaccaagagggtcac; GPR55 F tcttccccctggaga tcttt, R cgaggcagcagttgatgtta; PPAR-alpha F gtgtgatcgaagctg caaga, R gtcttctcagccatgcacaa, FAAH F ggaagtgaacaaagggacca, R taacatggactggggcacat). PCR was performed for 35 cycles and products were separated by agarose gel electrophoresis and viewed under long-wave UV illumination. Products were extracted from the gel and their identity confirmed by direct sequencing.

Statistical analysis

All experiments were repeated a minimum of three times with two or three replicates for each experimental condition within each experiment. The results are expressed as the mean values ± SEM and the levels of significance were calculated using Student's *t*-test or ANOVA with Tukey's *post hoc* test.

Materials

Glutamine, penicillin/streptomycin and RPMI-1640 medium were purchased from Invitrogen (Paisley, Scotland). Fetal calf serum was purchased from PAA laboratories (Yeovil, England). Fatty acid free BSA was purchased from MP Biomedicals (Thame, UK). Palmitate, oleate, OEA, AM404 and anandamide were from Sigma (Poole, England). URB597 and URB532 were purchased from Calbiochem (Darmstadt, Germany) and JNJ-1661010 from Tocris (Bristol, UK). The polyclonal anti-fatty acid amide hydrolase (FAAH) antibody, FAAH blocking peptide and the FAAH positive control of recombinant rat FAAH were all purchased from IDS-Ltd (Newcastle, England). Rat FAAH primers were designed in house and supplied by Invitrogen. The cAMP Direct Biotrak EIA came from GE Healthcare (Little Chalfont, UK).

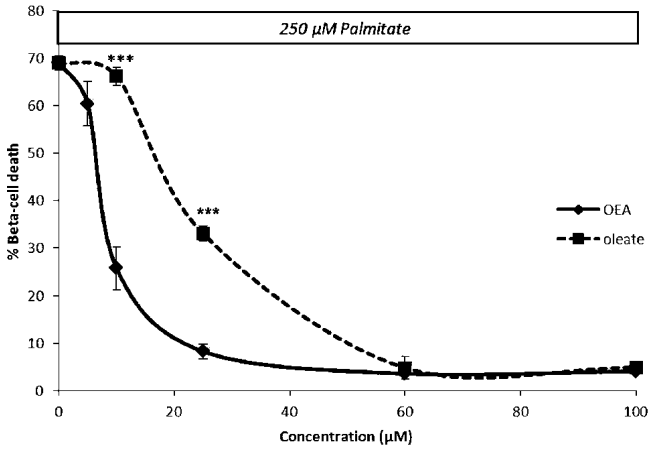

Figure 1

Effects of the mono-unsaturated fatty acid oleate and its ethanolamide derivative, OEA, on the loss of viability caused by exposure of BRIN-BD11 cells to palmitate. Cells were treated with 250 μM palmitate in the presence of increasing concentrations of oleate or OEA as shown. Cell viability was assessed after culture for 18 h. ***$P < 0.001$ compared with the equivalent concentration of OEA.

Results

Effects of OEA on the viability of BRIN-BD11 and INS-1 cells

Initial studies revealed that treatment of either of two rat β-cell lines (BRIN-BD11 or INS-1) with OEA at concentrations up to 250 μM did not lead to any loss of viability during culture periods of at least 24 h (not shown). By contrast, and as expected from previous studies (Newsholme *et al.*, 2007; Dhayal *et al.*, 2008; Diakogiannaki *et al.*, 2008), culture of these cells in the presence of 250 μM palmitate for a period of 18–24 h resulted in approximately 70% death amongst the cell population (Figure 1). When the two agents were used in combination, the loss of viability caused by palmitate was reduced (Figure 1) thereby revealing a cytoprotective action of OEA. This response was seen in both BRIN-BD11 (Figure 1) and INS-1 (untreated: 3.9 ± 1.1% dead cells; 250 μM palmitate: 50.3 ± 3.8%; palmitate + 150 μM OEA: 4.1 ± 0.5%; $P < 0.001$) cells and was dose- dependent over the concentration range 5–100 μM OEA. Essentially, complete protection of cell viability was achieved with 60 μM OEA in BRIN-BD11 cells. When the cytoprotective actions of OEA were compared with those of its parent free fatty acid, oleate, the ethanolamide derivative appeared to somewhat more potent than oleate under the conditions used, although formal EC_{50} values were not established due to uncertainties about the absolute binding affinity of each fatty acid to BSA.

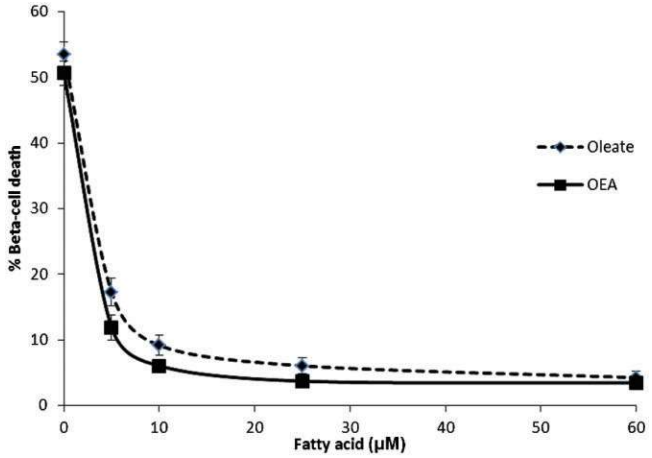

Figure 2

Effects of oleate and OEA against the loss of viability of BRIN-BD11 cells caused by removal of serum from the culture medium. BRIN-BD11 cells were incubated with increasing concentrations of oleate or OEA in serum free medium for 30 h. Cell viability was assessed at the end of this culture period.

In addition to its ability to attenuate the cytotoxic effects of palmitate, OEA also provided dose-dependent protection against the loss of viability arising from withdrawal of serum from the cell culture medium over a period of 30 h in BRIN-BD11 cells (Figure 2). Again, OEA appeared to be marginally more potent than oleate under these conditions (Figure 1).

Effects of GPR119 agonists on cell viability, cAMP generation and insulin secretion in β-cells

Because OEA has been suggested to act as an endogenous agonist of the lipid responsive receptor GPR119 in mammalian cells (Overton *et al.*, 2006; Swaminath, 2008; Lan *et al.*, 2009), it was possible that activation of this receptor might underlie OEA cytoprotective responses in β-cells. Accordingly, experiments were performed to ascertain whether any of a number of synthetic GPR119 agonists could reproduce the cytoprotective actions of OEA in these cells. The ligands selected were AR231453, PSN-375963, PSN-632408 and PSN119-1, all of which have been reported to influence β-cell function via the activation of GPR119 (Overton *et al.*, 2006; Chu *et al.*, 2007; Ning *et al.*, 2008; Lan *et al.*, 2009; Lauffer *et al.*, 2009). However, none of these reagents was able to replicate the cytoprotection achieved with OEA during exposure of either BRIN-BD11 or INS-1 cells to 250 μM palmitate (Figure 3).

These results led us to re-evaluate the actions of this range of GPR119 agonists in rat β-cells because the majority of previously published functional studies have been conducted in HIT-T15 cells, a hamster β-cell line. Initially, RT-PCR was used to amplify GPR119 transcripts from BRIN-BD11 and INS-1 cells and it was confirmed that this receptor is expressed at the RNA level (Figure 4 inset). Direct sequencing of the extracted DNA confirmed that the amplified products were derived from transcripts encoding GPR119 in both cases. However, when the cells were treated with either increasing concentrations of OEA or with 0.1 μM AR231453, PSN-375963 or PSN-632408 and cAMP levels monitored 2 h later, no changes were seen (Figure 4). As a positive control, it was noted that the adenylate cyclase activator forskolin induced a marked increase in cAMP and, in support of the earlier work, it was verified in separate studies, that GPR119 agonists do raise cAMP in HIT-T15 cells (data not shown). Thus, we were not able to obtain evidence that GPR119 is functionally competent in clonal rat β-cells despite detecting the expression of GPR119 mRNA. In support of this observation, it was also found that none of the synthetic GPR119 agonists caused any enhancement of glucose-induced insulin secretion from INS-1 cells (Table 1). Furthermore, OEA was similarly ineffective and, if anything, it attenuated glucose-induced insulin secretion at concentrations where it was effective as a cytoprotective agent (Figure 5).

TRPV1, PPAR-alpha and GPR55 are unlikely to be involved in mediating the cytoprotection achieved with OEA

Our data suggested that the cytoprotection achieved with OEA may not derive from the activation of GPR119 in rat β-cells, and so further studies were undertaken to evaluate the possible contributions of additional molecules with which OEA has been reported to interact. These include TRPV1, PPARα and a second G protein-coupled receptor, GPR55 (Ryberg *et al.*, 2007; Ho *et al.*, 2008; Swaminath, 2008; Borrelli and Izzo, 2009; Cluny *et al.*, 2009; Martinez de Ubago *et al.*, 2009; Ropero *et al.*, 2009; Eder and Ringseis, 2010). mRNA species encoding each of these three molecules has been detected by RT-PCR in both BRIN-BD11 and INS-1 cells and DNA sequencing confirmed the identity of the relevant amplicons (data not presented). The functional effects of a range of selective agonists and antagonists of these various receptors were therefore tested. The TRPV1 agonist olvanil (Di Marzo *et al.*, 1998; Ralevia *et al.*, 2001; Glaser *et al.*, 2003), the PPARα agonist WY-14643(Izzo *et al.*, 2009; Martinez de Ubago *et al.*, 2009; Ropero *et al.*, 2009; Eder and Ringseis, 2010) and the GPR55 agonist palmitoylethanolamide (PEA) (Borrelli and Izzo, 2009; Cluny *et al.*, 2009) did not attenuate palmitate-induced cell death and also, they had no effect on the ability of OEA to mediate cytoprotection in cells treated with 250 μM palmitate. SB366791, a TRPV1 antagonist (Ho *et al.*, 2008), was similarly ineffective (data not shown).

Effects of AM404, an inhibitor of fatty acid ethanolamide transport, on cell viability in cells exposed to palmitate and OEA

In view of the finding that the cytoprotective actions of OEA are achieved at an equivalent or higher potency than oleate and that the response is not apparently mediated by GPR119 (or the other receptors discussed above) it was important to try to establish whether OEA acted at an extracellular site or whether it must enter cells to exert cytoprotection. This hypothesis was evaluated using a putative inhibitor of fatty acylethanolamide transport, AM404 [*N*-(4-hydroxyphenyl) arachidonoyl-ethanolamide] (Figure 6A). This compound is structurally related to arachidonylethanolamide (AEA) (anandamide) and contains a phenylamide substituent in place of the ethanolamide moiety; it is reported to block the entry of

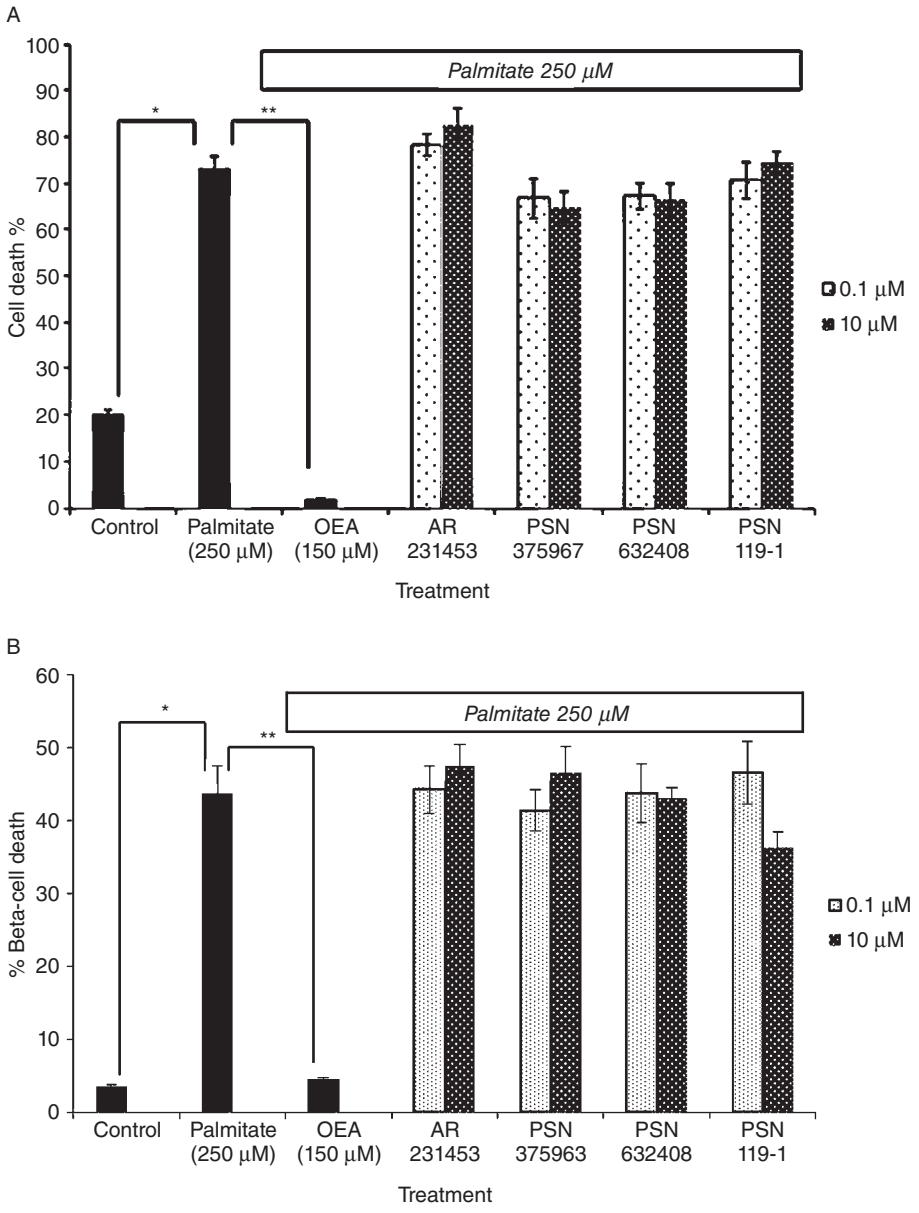

Figure 3

Effects of synthetic GPR119 agonists on the viability of clonal β-cells during exposure to palmitate. BRIN-BD11 (A) or INS-1 cells (B) were treated with vehicle, 250 μM palmitate alone, 250 μM palmitate plus 150 μM OEA or 250 μM palmitate plus either 0.1 μM or 10 μM of each of AR231453, PSN375963, PSN632408 or PSN119-1. Cell viability was assessed following culture periods of 18 h (BRIN-BD11) or 48 h (INS-1). *$P < 0.001$ compared with control; **$P < 0.001$ compared with palmitate alone.

fatty acyl ethanolamides into cells (Hillard and Jarrahian, 2000; Fowler and Jacobsson, 2002; Glaser *et al.*, 2003; Hogestatt *et al.*, 2005). Therefore, the actions of AM404 were examined in β-cells and the compound was found to be well tolerated. Moreover, it failed to alter the cytoprotection achieved with OEA in cells exposed to palmitate (Figure 6B). However, the interpretation of these results was confounded by the observation that AM404 unexpectedly prevented the cytotoxic actions of palmitate in the absence of OEA (Figure 6B). Thus, rather than attenuating the effects of OEA, AM404 reproduced the cytoprotection achieved with the fatty acyl ethanolamide.

AM404 may be susceptible to hydrolysis by fatty acid amide hydrolase (FAAH) yielding the free fatty acid, arachidonate (Hillard and Jarrahian, 2003; Hogestatt *et al.*, 2005). Because arachidonate has been shown to protect β-cells from palmitate toxicity (Dhayal and Morgan, 2011), it seemed possible that hydrolysis of AM404 might account for its unexpected cytoprotective activity. Initially, therefore, the expression of FAAH was investigated and FAAH mRNA was detected by RT-PCR and DNA sequencing in BRIN-BD11 and INS-1 cells [Figure 6C (i)]. Western blotting of cell extracts confirmed the presence of appropriately sized immunoreactive bands and a protein doublet corresponding to species with

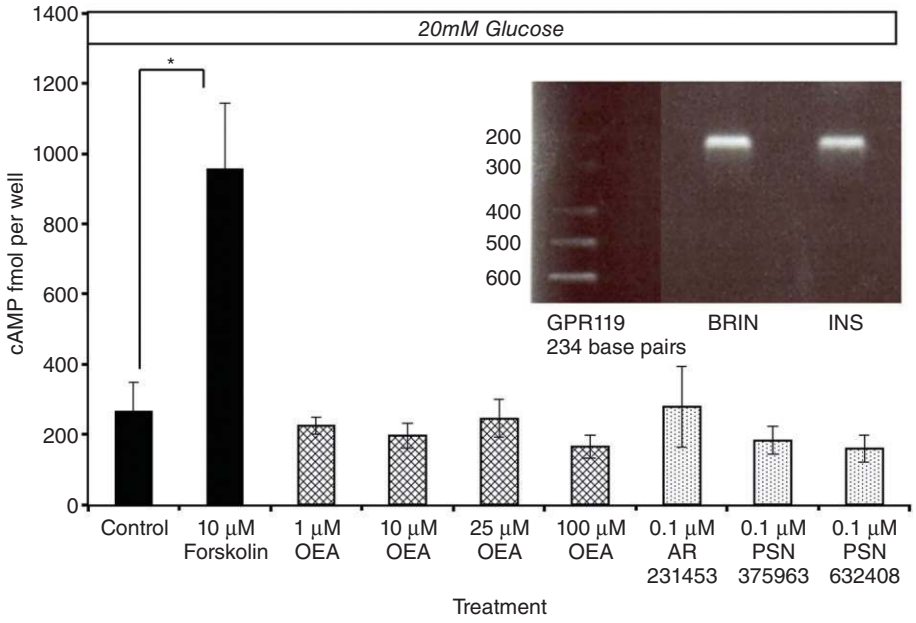

Figure 4

Expression and functional competence of GPR119 in rodent β-cells. GPR119 mRNA expression was confirmed by RT-PCR in INS-1 and BRIN-BD11 cells (inset). INS-1 cells were also cultured with a variety of GPR119 agonists for 30 min before extraction and measurement of cAMP by ELISA (main panel). *$P < 0.01$ compared with control.

Table 1

Effects of GPR119 agonists on insulin secretion in INS-1 cells

Treatment	Insulin secretion (ng per well)
0.2 mM glucose	1.40 ± 0.19
20 mM glucose	2.52 ± 0.23*
20 mM glucose plus:	
0.1 μM AR231453	2.52 ± 0.09^{NS}
10 μM AR231453	2.53 ± 0.05^{NS}
0.1 μM PSN 375963	2.60 ± 0.12^{NS}
10 μM PSN 375963	2.68 ± 0.01^{NS}
0.1 μM PSN 632408	2.53 ± 0.11^{NS}
10 μM PSN 632408	2.50 ± 0.04^{NS}
0.1 μM PSN 119	2.50 ± 0.08^{NS}
10 μM PSN 119	2.42 ± 0.09^{NS}

*$P < 0.001$ relative to 0.2 mM glucose; ^{NS}not significantly different from 20 mM glucose alone.
INS-1 cells were incubated with 0.2 mM glucose, 20 mM glucose alone, or 20 mM glucose plus various GPR119 agonists for 1.5 h. The supernatant was then removed and insulin content determined by radioimmunoassay. Values are presented as mean (±SEM) rates of insulin secretion.

apparent molecular weights of approximately 52 and 57 kDa was obtained consistently on Western blots [Figure 6C (ii)]. Both bands were displaced by co-incubation of the primary antibody with a blocking peptide corresponding to the relevant FAAH epitope. Given this evidence that FAAH is present in β-cells, the effects of an inhibitor of FAAH, URB597 were tested (Fegley *et al.*, 2005). URB597 did not, itself, cause toxicity and it failed to alter the response to palmitate. Significantly, however, the ability of AM404 to protect the viability of cells exposed to palmitate was lost in the presence of URB597 (Figure 7).

Inhibition of FAAH prevents OEA-mediated cytoprotection against palmitate toxicity

Because the results obtained with AM404 imply that its susceptibility to the catalytic activity of FAAH might account for cytoprotection (via release of arachidonate) it was important to evaluate whether a similar effect could also underlie the actions of OEA (where release of oleate would result). Three different selective inhibitors were used, two of which (URB597 and URB532) act non-competitively and bind covalently to the enzyme (Patel *et al.*, 2005; Astarita *et al.*, 2006; Tang *et al.*, 2006; Labar and Michaux, 2007; Murillo-Rodriguez *et al.*, 2007; Keith *et al.*, 2008), whilst the third (JNJ-1661010) is a competitive inhibitor (Keith *et al.*, 2008; Karbarz *et al.*, 2009). Each of these agents attenuated the cytoprotective effects of OEA in palmitate-treated BRIN-BD11cells (Figure 8). None of the compounds altered cell viability when added to control cells in the absence of fatty acids nor did they directly influence the loss of viability caused by palmitate in the absence of OEA.

In order to verify the conclusions arising from use of the FAAH inhibitors, their effects on the response to a second ethanolamide fatty acid derivative, PEA, were also studied. Unlike OEA, PEA contains a saturated species (palmitate) coupled to the ethanolamide constituent and hydrolysis of

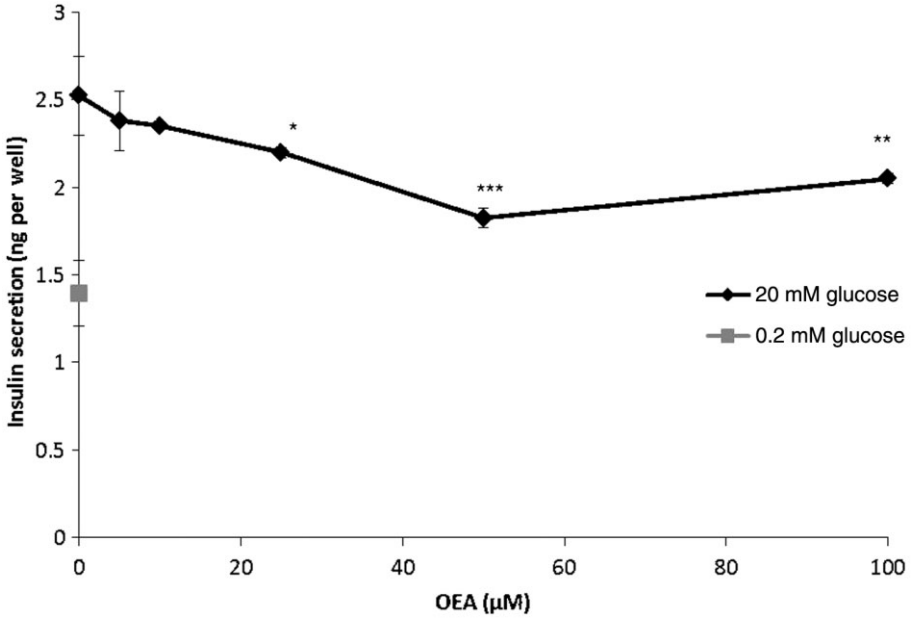

Figure 5

Effects of OEA on glucose-induced insulin secretion from cultured rat β-cells. INS-1 cells were incubated with 0.2 mM glucose, 20 mM glucose or 20 mM glucose plus increasing concentrations of OEA for 1.5 h. The media was then sampled and insulin secretion determined by radioimmunoassay. *$P < 0.05$ compared with 20 mM glucose; **$P < 0.01$ compared with 20 mM glucose; ***$P < 0.005$ compared with 20 mM glucose.

this molecule would therefore be expected to yield a potentially toxic fatty acid. In support of this, exposure of cells to 100 μM PEA resulted in a significant loss of viability, which was attenuated by co-incubation with URB597, URB532 or JNJ-1661010 (Figure 8A, B, C). Taken together, it appears from these results that the cytoprotective species responsible for maintenance of cell viability during incubation of β-cells with OEA is not the fatty acid ethanolamide *per se*, but rather this function arises via the release of free oleate derived from the hydrolysis of OEA.

In view of these findings, we conducted a further series of studies to examine whether the endocannabinoid, anandamide (AEA) was cytoprotective in β-cells and, if so, to determine the sensitivity of this response to an inhibitor of FAAH. Exposure of BRIN-BD11 cells to AEA in the co-presence of palmitate resulted in a marked improvement in viability compared with cells treated with palmitate alone (Figure 7) suggesting that AEA was cytoprotective. However, as in the case of OEA, this improved viability was reversed by the FAAH inhibitor, URB597 (Figure 7, right panels), suggesting that AEA is not the cytoprotective species but, rather, that free arachidonate is required to mediate this response. Additionally, it is interesting to note that the protective response to AEA was not attenuated by AM404 despite the fact that this agent is purported to inhibit the transport of AEA into the cells.

Discussion

In the present work, we show that the mono-unsaturated fatty acid derivative, OEA, exerts a powerful cytoprotective effect under conditions where β-cells are exposed either to elevated concentrations of the lipotoxic saturated fatty acid, palmitate, or when they are deprived of the survival factors present in serum. Because OEA has been shown to activate GPR119 in β-cells, these results raise the important possibility that activation of GPR119 may propagate a cytoprotective signal in these cells. The present data do not preclude this possibility but, rather, they reveal that the cytoprotective actions of OEA can be exerted independently of GPR119. There are several lines of evidence which support this conclusion.

Firstly, although it was confirmed at the mRNA level that GPR119 is expressed in the rat β-cell lines employed (BRIN-BD11 and INS-1) we were not able to reproduce the cytoprotection achieved by OEA with any of several, selective, synthetic GPR119 agonists. This observation led us to test whether these agents elicited a direct rise in insulin secretion which should arise from direct GPR119 activation (Chu *et al.*, 2007; 2008; Lan *et al.*, 2009; Lauffer *et al.*, 2009). Surprisingly, none of the synthetic ligands tested potentiated glucose-induced insulin secretion. Furthermore, OEA also failed to enhance glucose-induced insulin secretion from the cells under similar conditions to those which allowed a clear demonstration of its cytoprotective activity. Indeed, if anything, OEA tended to inhibit glucose-induced insulin secretion; although this response was only seen at the higher concentrations examined.

Secondly, cAMP levels were monitored in cells during exposure to a range of selective GPR119 ligands, since this receptor is known to couple to Gαs, thereby causing the activation of adenylate cyclase (Madiraju and Poitout, 2007; Ning *et al.*, 2008). However, in the two cell lines studied, cAMP levels were unchanged during treatment with syn-

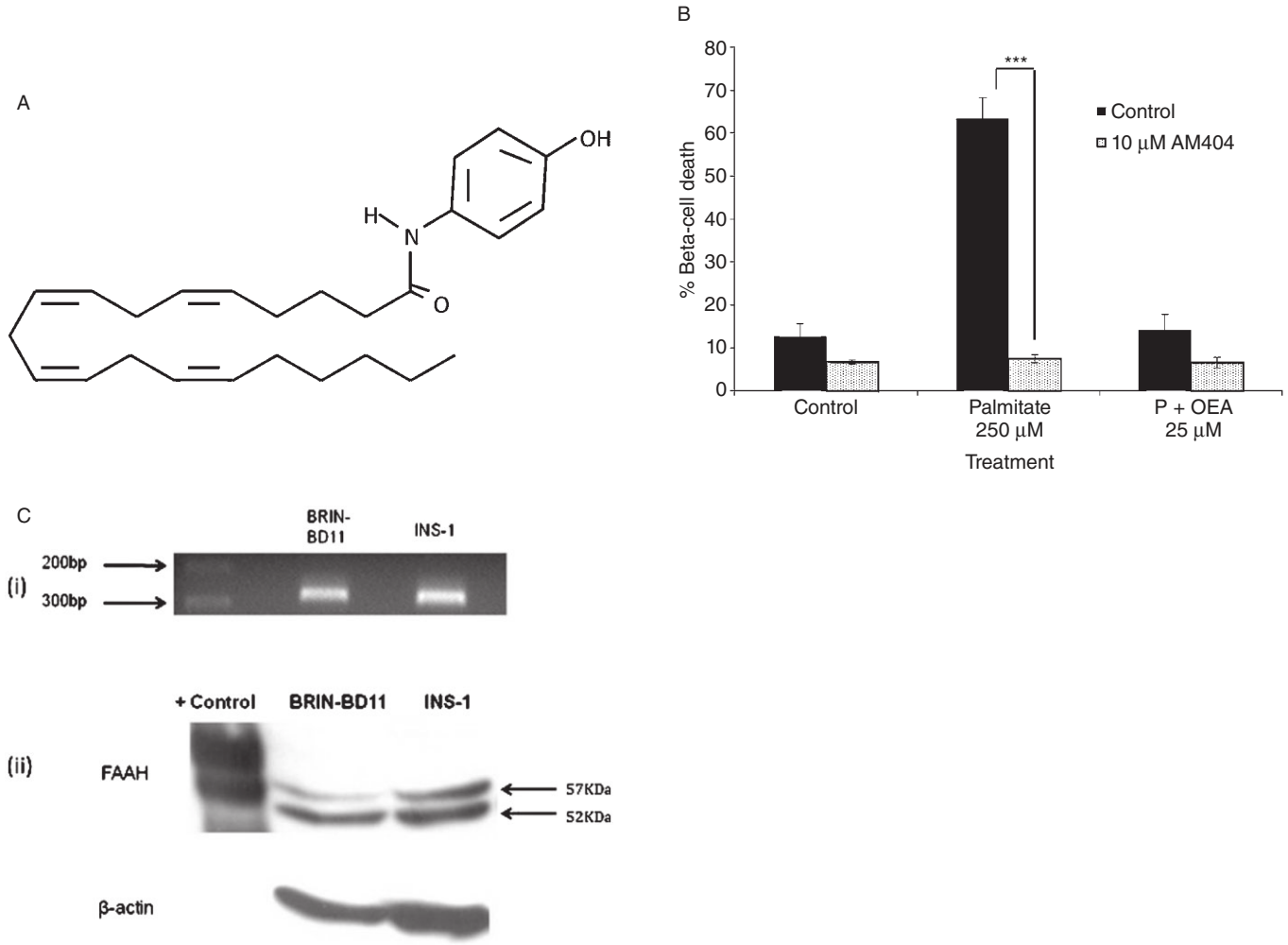

Figure 6

(A) Structure of the putative anandamide transport inhibitor AM404. (B) Effects of AM404 on BRIN-BD11 cell viability. BRIN-BD11 cells were treated with vehicle (control), 250 μM palmitate or 250 μM palmitate and 25 μM OEA in the absence or presence of 10 μM AM404 for 18 h. The cells were then harvested and viability assessed. ***$P < 0.001$ compared with palmitate alone. (C) – Expression of fatty acid amide hydrolase in cultured β-cells. (i) – mRNA was extracted from either BRIN-BD11 or INS-1 cells, reverse transcribed and amplified by PCR with primers designed to FAAH. (ii) – protein was extracted from BRIN-BD11 or INS-1 cells and 80 μg was loaded before fractionation by electrophoresis prior to Western blotting with an anti-FAAH antibody. The molecular weights of relevant bands were determined by reference to markers run in parallel.

thetic ligands of GPR119 or by OEA. In contrast however, cAMP levels were elevated during exposure to the direct activator of adenylate cyclase, forskolin.

These results lead to two important conclusions. Firstly, they reveal that the cytoprotective actions of OEA are unlikely to be mediated by the activation of GPR119. Secondly, they raise doubts about the functionality of GPR119 in commonly used rat β-cell lines. Our examination of the literature suggests that the majority of studies whereby GPR119 agonists have been shown to increase cAMP levels and insulin secretion have been were performed with a hamster β-cell line, HIT-T15 (Chu *et al.*, 2007; 2008; Overton *et al.*, 2008) or in MIN6 cells (Ning *et al.*, 2008). Thus, it seems possible that these cells may be more sensitive to GPR119 agonists than rat β-cells. In making this point, we also note that clear evidence exists for an insulinotropic effect of GPR119 agonists in rats although that this could reflect an indirect actions of the ligands (mediated via incretins) (Chu *et al.*, 2007; 2008). It is also possible that primary rat β-cells are more responsive to GPR119 agonists than clonal β-cell lines although, as noted elsewhere (Dhayal and Morgan, 2010), there is little direct evidence in the literature to suggest that GPR119 mediates a rise in cAMP in primary rat islet cells.

Having established that the cytoprotection achieved with OEA in rat β-cells is unlikely to be mediated via GPR119, it was important to evaluate the potential involvement of other proteins shown to bind OEA. These include anion channels such as TRPV1 as well as the nuclear receptor, PPARα and the putative cannabinoid receptor homologue, GPR55 (Ryberg *et al.*, 2007; Ho *et al.*, 2008; Swaminath, 2008; Borrelli and Izzo, 2009; Cluny *et al.*, 2009; Martinez de Ubago *et al.*, 2009; Ropero *et al.*, 2009; Eder and Ringseis, 2010). However, selec-

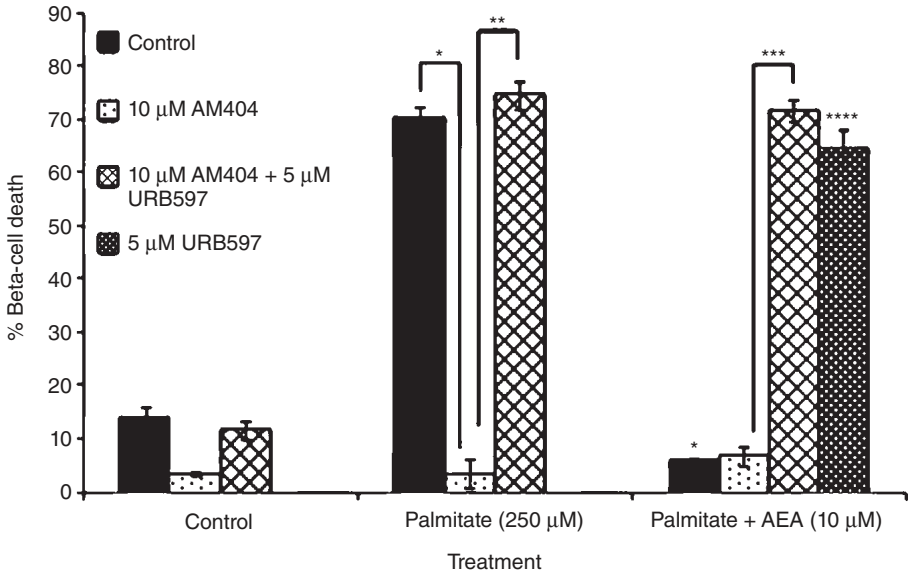

Figure 7

Influence of URB597 and AM404 on the actions of the polyunsaturated fatty acid derivative, anandamide (AEA) in BRIN-BD11 cells. BRIN-BD11 cells were treated with combinations of vehicle (control), 250 μM palmitate, 10 μM AEA, 10 μM AM404 and/or 5 μM URB597 as shown, for 18 h. After this period the cell viability was assessed. *$P < 0.001$ compared with palmitate alone; **$P < 0.001$ compared with palmitate + AM404; ***$P < 0.001$ compared with palmitate + AEA + AM404; ****$P < 0.001$ compared with palmitate + AEA alone.

tive ligands of each of these molecules failed to reproduce the cytoprotection achieved with OEA. Furthermore, a TRPV1 antagonist did not attenuate the response to OEA. Thus, we conclude that the ability of OEA to maintain β-cell viability in the presence of cytotoxic stimuli, does not involve any of the putative receptors known to bind OEA.

In an attempt to identify the target for OEA's cytoprotective actions, experiments were undertaken with AM404, a compound that acts as an inhibitor of the uptake of certain fatty acid ethanolamides into cells (Hillard and Jarrahian, 2000; 2003; Fowler and Jacobsson, 2002; Hogestatt *et al.*, 2005). This agent did not influence the cytoprotective action of OEA, a result which, taken at face value, might imply that OEA acts extracellularly or, alternatively, that it enters cells by a mechanism that is insensitive to AM404. In fact, careful analysis of the actions of AM404 revealed a third possibility. It was noted that in control studies AM404 prevented the loss of viability seen when cells were exposed to palmitate. Thus, rather than attenuating the cytoprotection achieved with OEA, AM404 produced a direct cytopretective response (in the absence of OEA).

Because AM404 contains a phenylamide substituent attached to arachidonic acid, it might be susceptible to hydrolysis by the enzyme fatty acid amide hydrolase, which has a broad specificity for fatty acid amides (Hillard and Jarrahian, 2003; Hogestatt *et al.*, 2005). Enzymatic cleavage of AM404 could result in the release of free arachidonic acid, which is cytoprotective in β-cells (Dhayal and Morgan, 2011). In support of this hypothesis, exposure of cells to inhibitors of FAAH resulted in complete loss of the cytoprotection achieved with AM404. These results imply that AM404 serves as a substrate for FAAH in β-cells and that the resultant free arachidonic acid released during hydrolysis of AM404 is responsible for mediating the improvement in cell viability. In accordance with this proposition, it was confirmed that FAAH is present in BRIN-BD11 and INS-1 cells at both the mRNA (by RT-PCR) and protein levels (by Western blotting).

The validity of this hypothesis was tested in a second set of studies employing the cannabinoid ligand, anandamide. This molecule is the ethanolamide derivative of arachidonic acid and, as in the case of AM404, its hydrolysis by FAAH will yield free arachidonic acid (Patel *et al.*, 2005). Anandamide prevented the loss of viability seen when BRIN-BD11 cells were exposed to palmitate and this response was abrogated by inhibition of FAAH. Hence, it is likely that cytoprotection was mediated by free arachidonic acid, released during anandamide hydrolysis.

In view of these results, we then examined the possibility that OEA might also require hydrolysis to mediate cytoprotection because previous studies have established very firmly that free oleate minimises the loss of β-cell viability seen when cells are exposed to palmitate (Welters *et al.*, 2004; Morgan *et al.*, 2008). Investigation of the effects of FAAH inhibitors confirmed this possibility. Exposure of cells to URB597, URB532 or the structurally unrelated inhibitor, JNJ-1661010, caused complete loss of the cytoprotection seen upon incubation of cells with palmitate plus OEA in the absence of inhibitors.

In order to verify these conclusions still further, the experiment was repeated again, but this time with a saturated fatty acid derivative, PEA. In this case, rather than causing cytoprotection (as seen with OEA), culture of the cells with PEA resulted in loss of viability, as would be expected upon the release of free palmitate. This response was prevented by inhibition of FAAH. Hence, it can be deduced that hydrolysis of PEA by FAAH leads to the generation of free palmitate,

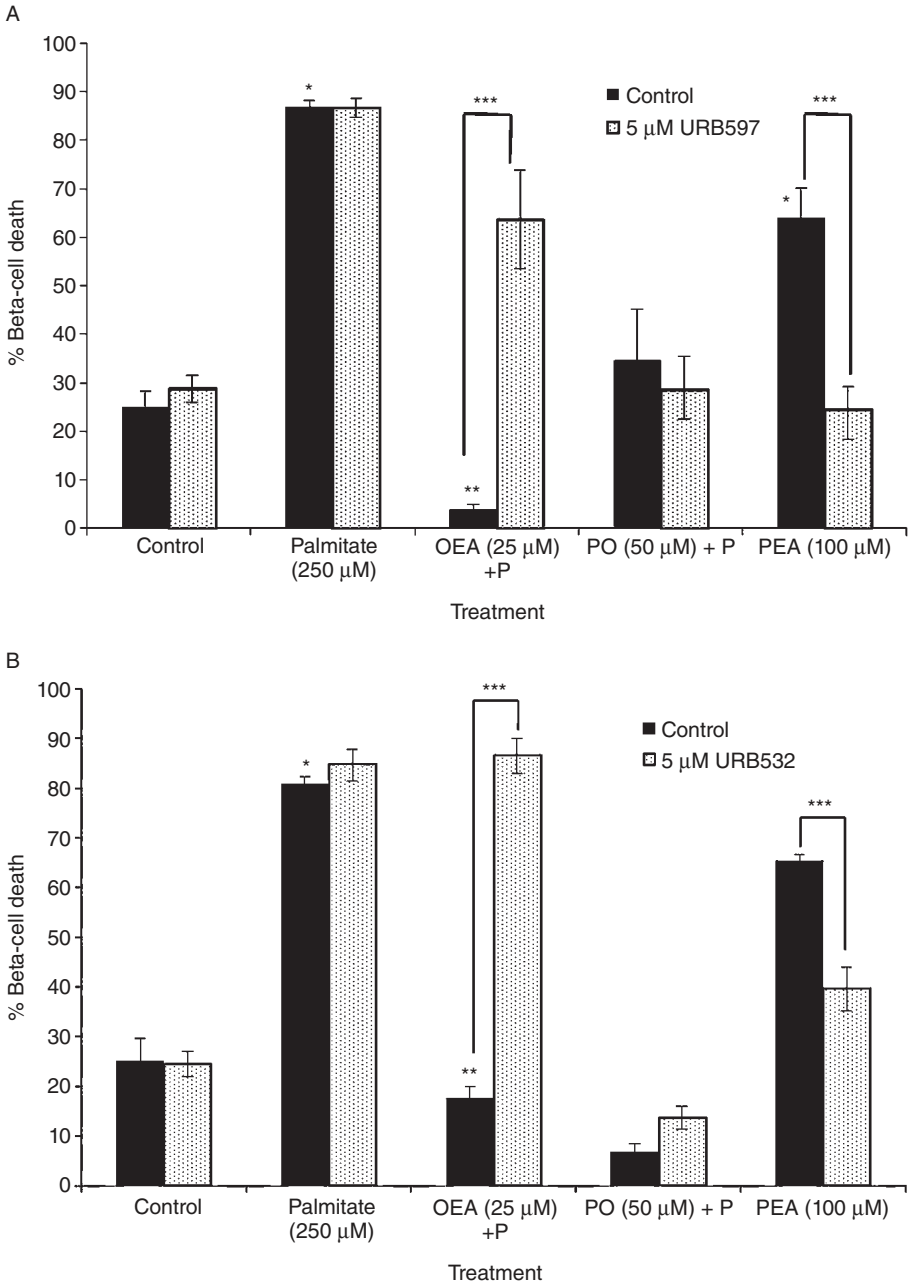

Figure 8

Effects of synthetic FAAH inhibitors on OEA mediated cytoprotection in BRIN-BD11 cells exposed to palmitate. BRIN-BD11 cells were treated with combinations of vehicle (control), 250 μM palmitate (P), 25 μM OEA, 50 μM palmitoleate (PO), 50 μM oleate (O), 100 μM palmitoylethanolamide (PEA) as shown, in the absence or presence of 5 μM URB597 (A), 5 μM URB532 (B) or 5 μM JNJ-1661010 (C) for 18 h. Cell viability was then assessed. *$P < 0.001$ compared with control; **$P < 0.001$ compared with palmitate alone; ***$P < 0.001$ compared with OEA or PEA with no FAAH inhibitor.

which is lipotoxic. Prevention of PEA hydrolysis attenuates this response.

Overall, the present data suggest that pancreatic β-cells produce endogenous FAAH and that this enzyme regulates their responses to fatty acid ethanolamides and to structurally related molecules. In particular, the results imply that fatty acid ethanolamides are readily hydrolysed within β-cells to yield their equivalent free fatty acids, which are then available to mediate biological responses within the cells. These considerations also lead to a further important conclusion; namely, that the generation of unsaturated free fatty acids (including oleate and arachidonate) at an intracellular site is associated with cytoprotection. This conclusion arises because FAAH is confined within the cell and is not thought to be present at the surface (Fegley *et al.*, 2005; Labar and Michaux, 2007). This, in turn, implies that the cytoprotective

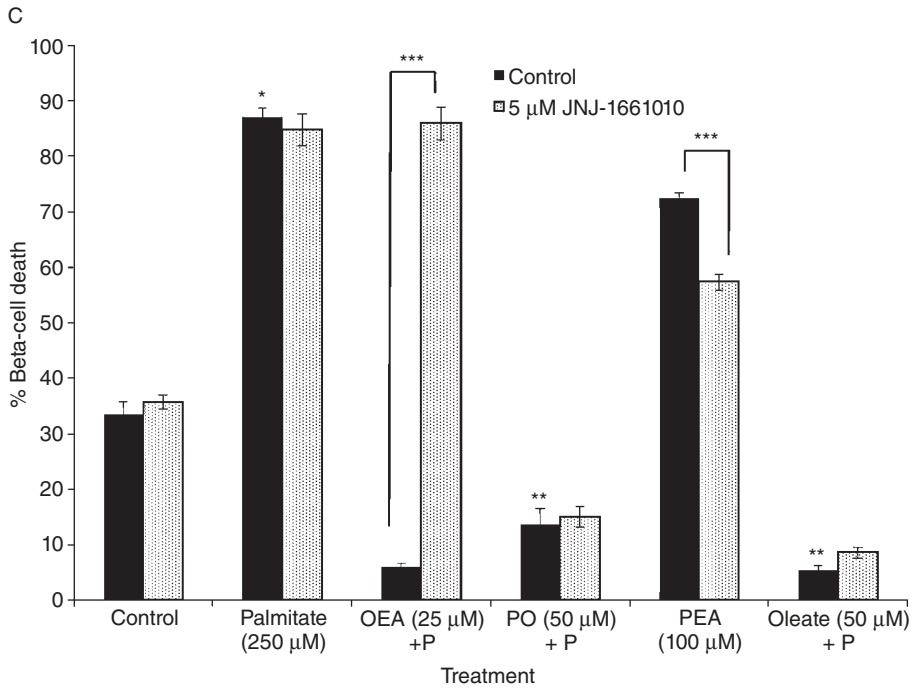

Figure 8

Continued.

response is unlikely to require the activation of a cell surface receptor but, rather, that an intracellular target is involved.

The results also suggest that fatty acyl-ethanolamides (including OEA) may penetrate into cells more effectively than their cognate free fatty acids (perhaps by virtue of their reduced polarity). In so doing, they then act as vehicles to deliver and release free fatty acids in close proximity to their primary site(s) of action, under the influence of FAAH. This may then account for the seemingly anomalous observation that, when added exogenously, OEA appears to be more potent than free oleate as a cytoprotective agent, even though oleate is the active species.

Finally, it should be emphasised again that the current data do not exclude the possibility that GPR119 might be capable of propagating a cytoprotective signal in β-cells when it is activated appropriately. This will require further study in other model systems. The results do, however, reveal that, in BRIN-BD11 and INS-1 cells, the ability of OEA to promote cell viability during exposure of cells to palmitate, does not require the intervention of GPR119.

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Conflict of interest

Employees of AstraZeneca plc may be stockholders in the company.

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