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# Biased signaling of lipids and allosteric actions of synthetic molecules for GPR119

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## ABSTRACT

GPR119 is a  $G\alpha s$ -coupled lipid-sensor in the gut, where it mediates release of incretin hormones from the enteroendocrine cells and in pancreatic  $\alpha$ -cells, where it releases insulin. Naturally occurring lipids such as monoacylglycerols (MAGs) and N-acylethanolamines (NAEs), like oleylethanolamide (OEA), activate GPR119, and multiple synthetic ligands have been described. Here, we extend the GPR119 signaling profile to  $G\alpha q$  and  $G\alpha i$  in addition to  $\beta$ -arrestin recruitment and the downstream transcription factors CRE (cAMP response element), SRE (serum response element) and NFAT (nuclear factor of activated T cells). The endogenous OEA and the synthetic AR231453 were full agonists in all pathways except for NFAT, where no ligand-modulation was observed. The potency of AR231453 varied <16-fold ( $EC_{50}$  from 6 to 95 nM) across the different signaling pathways, whereas that of OEA varied >175-fold (from 85 nM to 15  $\mu$ M) indicating a biased signaling for OEA. The degree of constitutive activity was 1–10%, 10–30% and 30–70% of OEA-induced  $E_{max}$  in  $G\alpha i$ ,  $G\alpha q$  and  $G\alpha s$ -driven pathways, respectively. This coincided with the lowest and highest OEA potency observed in  $G\alpha i$  and  $G\alpha s$ -driven pathways, respectively. Incubation for 2 h with the 2-MAG-lipase inhibitor JZL84 doubled the constitutive activity, indicating that endogenous lipids contribute to the apparent constitutive activity. Finally, besides being an agonist, AR231453 acted as a positive allosteric modulator of OEA and increased its potency by 54-fold at 100 nM AR231453. Our studies uncovering broad and biased signaling, masked constitutive activity by endogenous MAGs, and ago-allosteric properties of synthetic ligands may explain why many GPR119 drug-discovery programs have failed so far.

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## 1. Introduction

The number of people suffering from type 2 diabetes mellitus (T2DM) has escalated worldwide as a consequence of unhealthy lifestyle and obesity [1]. T2DM is characterized by insulin-resistance, decreased insulin secretion from the pancreatic  $\beta$ -cells and loss of intestinal glucagon-like-peptide (GLP)-1 release in

response to diet; deficits that are even more pronounced in obese individuals [2]. The G protein-coupled receptor (GPR) 119 acts as a lipid-sensor in the gut, where it induces the release of incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) from the intestinal L and K-cells, respectively, in response to dietary lipids [3–6]. Besides its expression in intestinal enteroendocrine cells (EEC), GPR119 is also expressed in pancreatic  $\beta$ -cells,

**Abbreviations:** CREB, cAMP response element-binding protein; EEC, enteroendocrine cell; FAAH, fatty acid amide hydrolase; FBS, Fetal Bovine Serum; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like-peptide 1; GPCR, G protein-coupled receptor; LPC, lysophosphatidylcholine; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase; MEM, minimal essential medium; NAE, N-acylethanolamine; OEA, oleylethanolamide; OLDA, N-oleoyldopamine; SRE, serum response element; 7TM, seven transmembrane receptor; 2-OG, 2-oleoyl glycerol; NFAT, nuclear factor activated T-cells; PAM, positive allosteric modulator.

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where it leads to insulin secretion in a glucose-dependent manner [7–9]. GPR119 in pancreas may possibly be stimulated by 2-monoacylglycerol (MAG) generated by lipoprotein lipase [10]. GPR119 couples to  $G_{\alpha s}$  with high constitutive activity and in response to lipid-based agonists like lysophosphatidylcholines (LPCs), oleic-acid containing N-acyl-amino compounds like N-oleoyldopamine (OLDA) and N-acylethanolamines (NAEs) [3,8,9,11–14]. Of these, the oleic-acid containing OEA is the most potent lipid-based GPR119 agonist [3]. It stimulates cAMP accumulation in GPR119 transfected cells with high potency ( $EC_{50}$  of 0.2–2.9  $\mu$ M) [3,12] and releases GLP-1 *in vitro* from GLUTag-cells, an EEC cell-line with endogenous GPR119 expression, and *in vivo* after intraluminal injection in mice [15]. Monoacylglycerols (MAGs) such as 2-oleoyl glycerol (2-OG), which is generated in high amounts during digestion of the diet, also activate GPR119 as shown in recent *in vivo* studies in humans, where ingested 2-OG stimulated GLP-1 release [3,4]. Similar experiments in wild type and GPR119-deficient mice proved that the GLP-1 secretion induced by 2-OG (and the more stable ether analog of 2-OG) indeed is mediated via GPR119 activation [5].

The massive interest in GPR119 as a pharmaceutical target for the treatment of T2DM and obesity has resulted in numerous synthetic GPR119 ligands, for recent reviews see [14,16,17]. In 2007, the prototypical GPR119 agonist, AR231453, was published as a GPR119-specific glucose-dependent insulin secretagogue with similar efficacy as GLP-1 [8], and shortly thereafter was shown to stimulate incretin hormone release in mice [13]. This inspired many companies to create new GPR119 agonists, though only few have reached clinical trials [16,18,19]. GPR119 antagonists and inverse agonists have also been described, like AR436352, used in the present study.

The signaling through  $G_{\alpha s}$  is well established for GPR119 [3,8,9,11–14,20–23] as measured by cAMP accumulation and/or downstream activation of the transcription factor CREB (cAMP response element binding protein) in response to both lipids (e.g. OEA, OLDA, LPC, 2-OG) and multiple synthetic GPR119 agonists [3,8,9,11,12,20,24,25]. In addition, calcium release has been described for ligand-mediated GPR119 activation [24,26], whereas G protein-independent signaling via  $\beta$ -arrestin recruitment has only been sparsely described in the patent literature [27].

Here, we present an in depth analysis of the signaling profile of GPR119 with and without ligand addition. We compare the activity of the endogenous lipid OEA and the synthetic non-lipid-based agonist AR231453 through G protein-dependent and -independent pathways to search for signaling bias. Given their structural differences, and only partly overlapping binding sites [28], these two ligands were furthermore combined in order to study allosteric modulation properties. The synthetic GPR119 inverse agonist AR436352 [28] was included to study ligand-modulation of the constitutive activity at the transcriptional activity level (CRE, SRE and NFAT). As the activity of lipid-receptors may be under influence of endogenous lipids revealing apparent constitutive activity, as shown for GPR40 and EBI2 (also known as GPR183) [29–32] – we tested whether this was the case for GPR119 by addition of two inhibitors of the enzymes that degrade the endogenous GPR119 agonists MAGs and NAEs. Our studies expand the basic pharmacological knowledge of GPR119, and introduce signaling bias, allosteric actions and apparent constitutive activity as important traits for this receptor.

## 2. Materials and methods

### 2.1. Materials

OEA (Cayman Chemicals, Ann Arbor, USA), AR231453 (C21H24FN7O5S), AR436352 (C20H18FN5O4S) (Arena Pharmaceutical Inc., San Diego, USA) and Glucose-dependent insulinotropic

polypeptide (GIP) (Human, H-5645, Bachman, Germany) were all dissolved in 1% DMSO (Sigma–Aldrich, MO, USA). Fatty acid amide hydrolase (FAAH) inhibitor URB597 and monoacylglycerol lipase (MAGL) inhibitor JZL184 (Cayman Chemicals, Ann Arbor, USA) [33,34] were dissolved in 0.5% DMSO. GPR119 and the human GIP receptor were cloned into the pcDNA3 vector. Gqs5 that turns  $G_{\alpha s}$ -coupling into a  $G_{\alpha q}$  signal was used as a positive control in IP3 turnover assay [35]. Gqi5 and Gqi4myr that both turn  $G_{\alpha i}$  coupling into a  $G_{\alpha q}$  signal were used to test for  $G_{\alpha i}$  coupling of GPR119. The three chimeric G proteins Gqs5, Gqi5 and Gqi4myr were kindly provided by 7TM pharma A/S (Hørsholm, Denmark).

### 2.2. Cell culture and transfections

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) 1885 (Gibco, Life Technologies, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, CA, USA), 26 mg/ml L-glutamine and 180 IU/ml penicillin + 45ug/L streptomycin (Pen Strep, P/S) (Life Technologies, CA, USA) at 37 °C and 10% CO<sub>2</sub>. HEK293 cells were cultured in DMEM with 10% FBS and Pen-Step as for the COS-7 cells, at 37 °C and 10% CO<sub>2</sub>. HEK293 medium for transcription factor reporter assay was DMEM, (Gibco, Life Technologies, USA) with 0.5% FBS for SRE and 10% FBS for both CREB and NFAT. U2OS cells, stably expressing  $\beta$ -arrestin2 coupled to the large  $\beta$ -galactosidase fragment and GPR119, were cultured in DMEM alpha + GlutaMAX™-I (Gibco, Aukland, New Zealand), 10% FBS, 26 mg/ml L-glutamine, 180 IU/ml penicillin + 45ug/L streptomycin (PenStrep) at 37 °C and 10% CO<sub>2</sub>. COS-7 cells were transiently transfected with GPR119 or pcDNA3 by the calcium phosphate transfection method for 5 h at 37 °C, 10% CO<sub>2</sub> [36] for the cAMP assay and the inositol tris-phosphate (IP3) turnover assay. For the luciferase reporter assays and the calcium assay, HEK293 cells were transfected for 5 h using Lipofectamine2000 and serum free optiMEM (both from Life Technology (TermoFischer), MA USA). For the luciferase reporter assays, cells were co-transfected with GPR119 DNA or pcDNA3 and *cis* reporter SRE/NFAT luciferase or *trans* reporter CREB luciferase.

### 2.3. cAMP accumulation assay

COS-7 cells were transfected with 40  $\mu$ g GPR119 DNA or pcDNA3 per 175 cm<sup>2</sup> flask. One day after transfection, the cells were seeded out (35,000 cell/well) in a 96 well white plate (Perkin Elmer, Massachusetts, USA). The cells were washed twice and incubated for 30 min at 37 °C with HBS + 1 mM IBMX (Sigma Aldrich, USA). Increasing concentrations of agonists were added to the cells and incubated for 30 min. Upon co-administration with the two agonists, a 5 min pre-incubation period was introduced for the agonist given in a fixed concentration before addition of increasing concentration of the other agonist. The enzyme inhibitors, URB597 (fatty acid amino hydrolase (FAAH) inhibitor) and JZL84 (monoacylglycerol lipase (MAGL) inhibitor) were added for 2 or 24 h at 37 °C. The Hithunter® cAMP XS+ procedure (DiscoverX, Fremont, USA) was carried out following the manufacturers' description.

### 2.4. Calcium mobilization experiment

HEK293 cells were transfected with 5 ng GPR119 DNA or pcDNA3 per well in black 96 well plate (35,000/well) (Corning Incorporated, Berlin, Germany). One day after transfection the loading buffer, containing calcium sensitive fluorophore, Fluo-4, (FLIPR calcium assay kit) (Invitrogen CA, USA), was added to the cell plate and incubated for 1 h at 37 °C. Agonists were prepared in a reagent plate. 25  $\mu$ l were transferred to the cell plate, while

measuring calcium mobilization at the NOVOstar (Isogen Life Science, PW De Meern, Netherlands).

### 2.5. $\beta$ -Arrestin recruitment experiment

$\beta$ -Arrestin recruitment was measured using a Pathhunter  $\beta$ -arrestin assay (DiscoverX, Fremont, USA). U2OS were cultured in minimal essential medium (MEM)  $\alpha$  supplemented with GlutaMAX (1x) (Gibco, Aukland, New Zealand) and adjusted with 10% (v/v) FBS, 1% (v/v) P/S and 0.5% (v/v) hygromycin B. Cells were seeded out (35,000/well) in 96 well white plates (Perkin Elmer, Massachusetts, USA). Agonist stimulation was performed for 90 min at 37 °C followed by incubation with the Detection Reagent Solution<sup>®</sup> for 1 h. The chemiluminescence was measured at the EnVision 2104 multi lab reader (Perkin Elmer, Massachusetts, USA).

### 2.6. IP3 turnover experiment

COS-7 cells were transfected with 40  $\mu$ g GPR119 or pcDNA3 with or without 30  $\mu$ g of the chimeric proteins Gqs5, Gqi5 or Gqi4myr per 175m2 flask. One day after transfection, cells were incubated with Myo-[2 <sup>3</sup>H(N)]-inositol (Perkin Elmer, Massachusetts, USA) for 24 h and afterward seeded out in a white bottom, 96 well plate (35,000/well) (Perkin Elmer, Massachusetts, USA). Agonists were added in HBSS + 10 mM LiCl to the cells for 90 min at 37 °C. The cells were lysed with 10 mM formic acid, and incubated with SPA-YSI beads (1 mg/well) (Perkin Elmer, Massachusetts, USA) at room temperature (RT) for 8–9 h. IP3 response was measured on the Top counter (Perkin Elmer, Massachusetts, USA).

### 2.7. Transcription factor reporter experiment

HEK293 cells were seeded out in 96 well plates (35,000/well) (Sigma–Aldrich, MO, USA) 24 h prior to transfection. The cells were transfected with increasing amount of GPR119 DNA or pcDNA3 (5, 10, 15 ng/well) together with *cis* reporter SRE/NFAT luciferase (50 ng/well) or *trans* reporter CREB luciferase (6 ng/well CREB + 25 ng/well luciferase). The cells were incubated with and without 10  $\mu$ M pertussis toxin (PTx, Sigma–Aldrich<sup>®</sup>). 24 h after transfection the cells were washed in phosphate buffered saline (PBS, Dulbecco) (Sigma–Aldrich, MO, USA), luminescence was measured at the luminescence counter (Topcounter, Packard) (Perkin Elmer, MA, USA) after 10 min incubation with 50  $\mu$ l PBS + 50  $\mu$ l Luciferase lite substrate (Perkin Elmer, MA, USA).

### 2.8. Statistics

All data are presented as mean  $\pm$  SEM. Statistical analysis (unpaired *t*-test) was performed in Fig. 6 using Graph pad Prism 6. Statistically significant difference was observed as *p* < 0.05 (\*), *p* < 0.01 (\*\*), *p* < 0.005 (\*\*\*) or *p* < 0.001 (\*\*\*\*).

## 3. Results

### 3.1. Equal efficacies of OEA and AR231453 in G protein-dependent, as well as G protein-independent pathways

We initially tested whether lipid-based and synthetic agonists for GPR119 activate the same pathways. OEA was chosen due to its previously described high potency [3–12] and tested in parallel with the synthetic molecule AR231453 for the G protein-mediated cAMP production and calcium release, as well as the G protein-independent  $\beta$ -arrestin recruitment (Fig. 1). Consistent with previously published data [3–9,20], OEA and AR231453 initiated cAMP

accumulation in a dose-dependent manner with 33-fold higher potency for AR231453 than that of OEA (Table 1) and with comparable efficacies (Fig. 1A). A considerable basal constitutive activity of  $\sim$ 30% of the maximal stimulation by OEA ( $E_{\max}$ ) was observed (Fig. 1A), consistent with the literature [8,13]. We next measured the increase in intracellular calcium for both agonists and the results were similar to those obtained from the cAMP accumulation assay. This too was in agreement with previous results of an OEA-induced GPR119 response [37]. AR231453 had a superior potency compared to OEA with a 25-fold higher  $EC_{50}$  (Table 1) and with comparable efficacies and a considerable constitutive activity of  $\sim$ 30% of the  $E_{\max}$  for OEA was observed (Fig. 1B). The patent literature describes  $\beta$ -arrestin recruitment for cyanopyrazole-based agonists through GPR119 [27], but nothing is known about other synthetic molecules as well as lipid signaling through this pathway. By employing stably transfected U2OS cells expressing GPR119, we tested both ligands through this pathway. A robust activation with similar efficacies was observed (Fig. 1C), and again AR231453 had higher potency (166-fold) than OEA (Table 1). In this assay the constitutive activity reached  $\sim$ 60% of the  $E_{\max}$  for OEA (Fig. 1C).

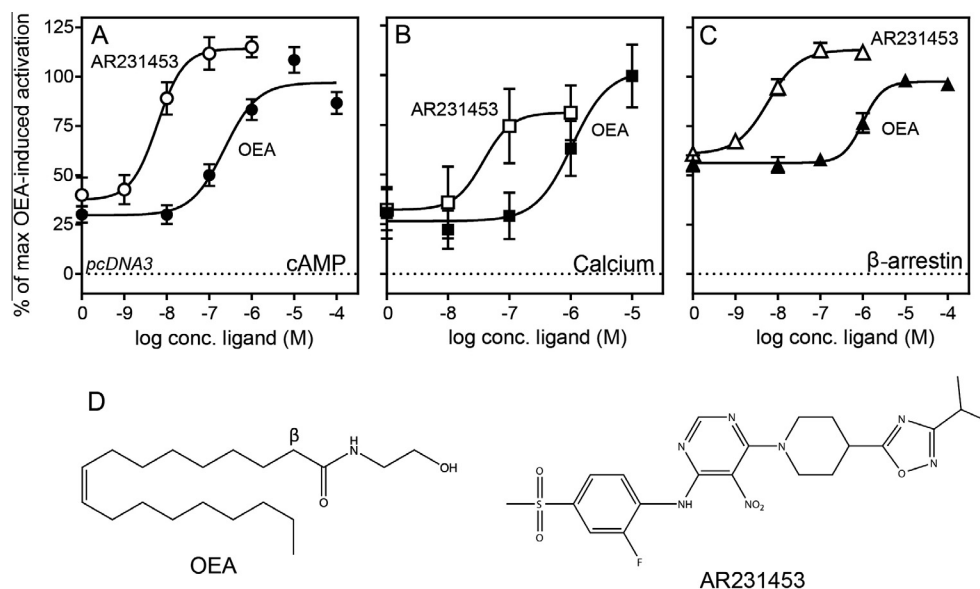
### 3.2. Expansion of the G protein-dependent signaling of GPR119

Besides  $G_{\alpha s}$ , a recent paper presented  $G_{\alpha i}$  as possible signaling pathway for GPR119 [38], and also  $G_{\alpha q}$  has been tested previously [8]. We therefore looked more detailed into the G protein-signaling profile of GPR119 in transiently transfected COS-7 cells. To measure  $G_{\alpha q}$  activity, we investigated the IP3 turnover and as a positive control, we co-transfected with the chimeric Gqs5 G protein, a  $G_{\alpha q}$  subunit which contains the last 5 amino acids from  $G_{\alpha s}$  (recognition motif for the  $G_{\alpha s}$  coupled receptors), and turns the activity of a  $G_{\alpha s}$ -coupled receptor into a  $G_{\alpha q}$  read-out [35]. Indeed, both OEA and AR231453 increased IP3 turnover in a dose-dependent manner with potencies similar to those observed in the signaling pathways presented in Fig. 1, but with much lower efficacies (7–10%) compared to the positive control (white symbols, Fig. 2A and B, respectively and Table 1). The GIP receptor was included as another control. Consistent with its  $G_{\alpha s}$  coupling [39], co-transfection with Gqs5 resulted in a dose-dependent response of GIP with similar potencies as described previously [40], whereas no signaling was observed in the absence of Gqs5 (Fig. 2A and B). Due to the strong  $G_{\alpha s}$  coupling of GPR119 (Fig. 1), we decided to use an indirect measurement of  $G_{\alpha i}$  activity by co-transfection with Gqi5, a  $G_{\alpha q}$  subunit, which contains the last 5 amino acids from  $G_{\alpha i}$  (recognition motif for the  $G_{\alpha i}$  coupled receptors), and turns the activity of a  $G_{\alpha i}$ -coupled receptor into a  $G_{\alpha q}$  read-out [35]. Here, both OEA and AR231453, but not GIP elicited a dose-dependent activation, indicating that GPR119 also couples to  $G_{\alpha i}$  (Fig. 2C, Table 1). The efficacy was however much lower, reaching levels of 27–40% of the positive controls co-transfected with Gqs5. Similar results were found with the variant of Gqi5 denoted Gqi4myr (Table 1), and we thus had obtained signaling data for GPR119 in  $G_{\alpha s}$ ,  $G_{\alpha q}$  and  $G_{\alpha i}$  in a highly comparable setup, which is not found in earlier literature on GPR119.

### 3.3. Differential signaling pattern of GPR119 through an array of transcription factors

Inspired by the broad G protein-signaling profile of GPR119, we tested the receptor activity through a number of transcription factors: CRE, NFAT and SRE in transiently transfected HEK293 cells. These are controlled by different G proteins:  $G_{\alpha s}$  and  $G_{\alpha q}$  (CRE),  $G_{\alpha q}$  and  $G_{\alpha i}$  (NFAT) and  $G_{\alpha i}$  and  $G_{\alpha 12/13}$  (SRE) thereby representing a suitable battery for further signaling characterization of GPR119. HEK293 cells were transfected with increasing amounts of GPR119, and the basal activity was measured together with





**Fig. 1.** GPR119 activation by OEA and AR231453 via G protein-dependent and -independent pathways. cAMP formation (A), increased intracellular calcium (B) and β-arrestin recruitment (C) induced by increasing doses of AR231453 (white) or OEA (black symbols) in COS-7 (cAMP and Ca<sup>2+</sup>), or in U2OS A<sub>2</sub> (β-arrestin) cells transfected with GPR119 or empty vector pcDNA3 as negative control. Data are normalized to maximum OEA-induced GPR119 activity (100%) and pcDNA3.1 in the absence of ligand (0%). Data are shown as mean ± SEM (n = 3–4). EC<sub>50</sub> values and numbers of experiments are provided in Table 1. Chemical structures of OEA and AR231453 (D).

**Table 1**

Potencies of OEA and AR231453 in all implemented signaling pathways. From left column: experimental condition, signaling pathway, EC<sub>50</sub>, log EC<sub>50</sub> ± SEM) and number of experiments of OEA and AR231453. Upper third: GPR119 expressed alone. Middle third: GPR119 co-expressed with chimeric G proteins. Lower third: ligands administered together with dose-response curves of OEA together with low (10 nM) and high (100 nM) concentrations of AR231453 (middle column) and dose-response or AR231453 together with low (10 nM) and high (100 nM) OEA (right column).

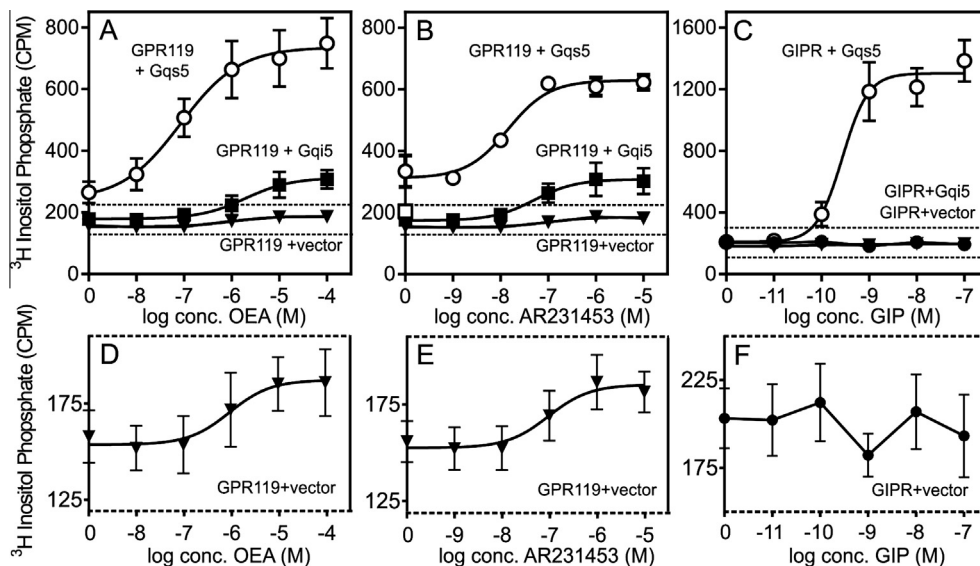
Experimental condition	Signaling pathway	OEA			AR231453		
		EC <sub>50</sub> (μM)	Log EC <sub>50</sub> ± SEM	N	EC <sub>50</sub> (nM)	Log EC <sub>50</sub> ± SEM	N
GPR119 expressed alone	cAMP	0.2	−6.6 ± 0.2	4	6	−8.2 ± 0.2	4
	CRE	0.3	−6.6 ± 0.9	5	57	−7.3 ± 0.4	5
	Calcium	1	−6.0 ± 0.3	3	40	−7.4 ± 0.8	3
	IP <sub>3</sub>	0.9	−6.0 ± 0.9	5	95	−7.0 ± 0.7	5
	SRE	15	−4.8 ± 0.2	5	43	−7.4 ± 0.2	5
	β-arrestin	1	−6.0 ± 0.1	4	6	−8.2 ± 0.1	4
GPR119 co-expressed with chimeric G protein	IP <sub>3</sub> (+Gqs5)	0.085	−7.0 ± 0.5	5	14	−7.8 ± 0.2	3
	IP <sub>3</sub> (+Gqi5)	2	−5.7 ± 0.2	5	52	−7.3 ± 0.5	3
	IP <sub>3</sub> (+Gqi4myr)	1.8	−5.7 ± 0.2	5	26	−7.5 ± 0.2	3
Ligands co-administered (10 nM co-ligand, low) (100 nM co-ligand, high)	cAMP (one ligand)	0.2	−6.7 ± 0.4	5	20	−7.7 ± 0.5	5
	cAMP (two ligands, low)	0.023	−7.6 ± 0.6	5	3.5	−8.5 ± 0.7	5
	cAMP (two ligands, high)	0.0037	−8.4 ± 0.8	5	37	−7.4 ± 0.5	5

the activity in the presence of an agonist (0.1 μM AR231453) or an inverse agonist (0.1 μM AR436352) of GPR119 (Fig. 3). Confirming the Gαs activity, increasing doses of GPR119 resulted in an increase in CRE-activation. At 10 ng GPR119, the AR231453-induced activity was ~20% higher than the basal activity, whereas the presence of AR436352 reduced the basal activity by ~16% (Fig. 3A). Intriguingly, no constitutive SRE activity was observed for increasing concentrations of GPR119, whereas AR231453 induced a substantial increase (Fig. 3B). As expected from the lack of the basal activity through this signaling pathway, AR436352 had no impact on the signaling through SRE (Fig. 3B). Like the CRE-activity, NFAT was activated constitutively (agonist-independent) in a gene-dose dependent manner (Fig. 3C). However in contrast to the CRE-activity, NFAT activity could not be modulated further by AR231453 or AR436352 (Fig. 3C). Knowing that NFAT is mainly regulated by Gαq and Gαi and due to the fact that the activation of this pathway is mainly dependent on the GPR119 constitutive activity (Fig. 3C), NFAT activation was included to confirm the Gαi coupling observed using the Gαqi5 chimeric

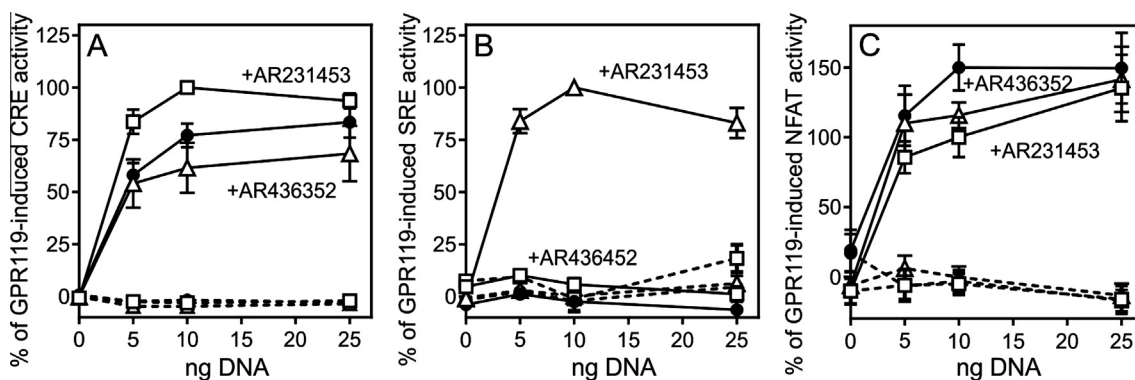
signaling (Fig. 2). To this end, we investigated the GPR119-induced NFAT activation in the presence of the Gαi inhibitor pertussis toxin (PTx). The PTx effect was investigated in comparison to the two control receptors ORF74-human herpes virus (HHV) 8 and the ghrelin receptor. ORF74-HHV8 is constitutively active through Gαi and Gαq [41], whereas the ghrelin receptor facilitates an efficacious Gαq signal [42]. PTx significantly inhibited the GPR119-induced NFAT activation (in the absence of added ligand) to the same extent as for ORF74-HHV8, whereas there was no effect on the ghrelin receptor. The activity level of both GPR119 and ORF74-HHV8 was reduced to around 75% of the maximum receptor activity when PTx was used, which confirms the observed Gαi signaling of GPR119 (Fig. 2).

### 3.4. Signaling pathway-dependent potencies of OEA, but not of AR231453

The substantial variation in basal- and agonist-induced activity among the three transcription factors (Fig. 3) urged further phar-



**Fig. 2.** Broad G protein-mediated signaling by GPR119. IP<sub>3</sub> formation induced by increasing doses of OEA (A), AR231453 (B) or GIP (C) in COS-7 cells transfected with either GPR119 (A and B) or the GIP receptor (C) alone (black triangles) or co-transfected with Gqi5 (black squares) or Gqs5 (white circles). COS-7 cells transfected with GPR119 are again pictured in Fig. 2D–F as a zoomed enlargement of the area between the dotted lines in Fig. 2A–C for a more detailed perspective. Data are shown as mean  $\pm$  SEM ( $n = 3–5$ ). EC<sub>50</sub> values and numbers of experiments are provided in Table 1.

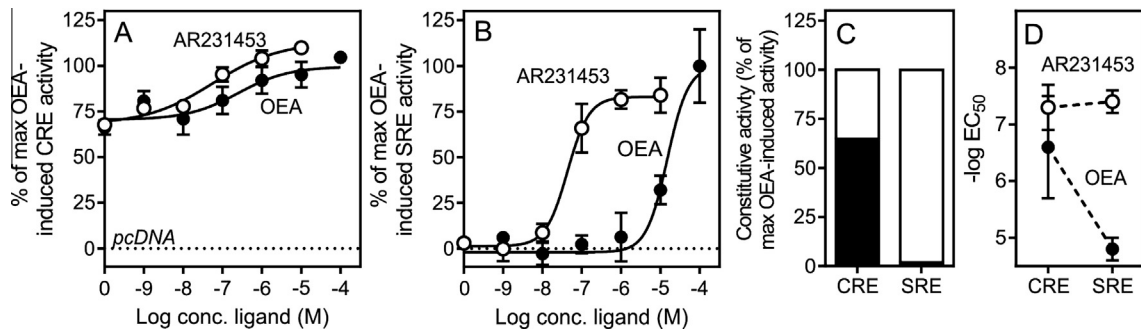


**Fig. 3.** Constitutive and ligand-modulated transcriptional activity by GPR119. Transcription factor activity via CRE (A), SRE (B) and NFAT (C) in response to increasing doses of GPR119 DNA (full lines) or pcDNA3 (dashed lines) in HEK293 cells. The cells were incubated for 5 h without (black symbols) or with (white symbols) AR231453 (square) or AR436352 (triangle) at a concentration of 0.1  $\mu$ M. Data are normalized to 10 ng GPR119 in the presence of AR231453 (100%). Data are shown as mean  $\pm$  SEM,  $n = 4–5$ .

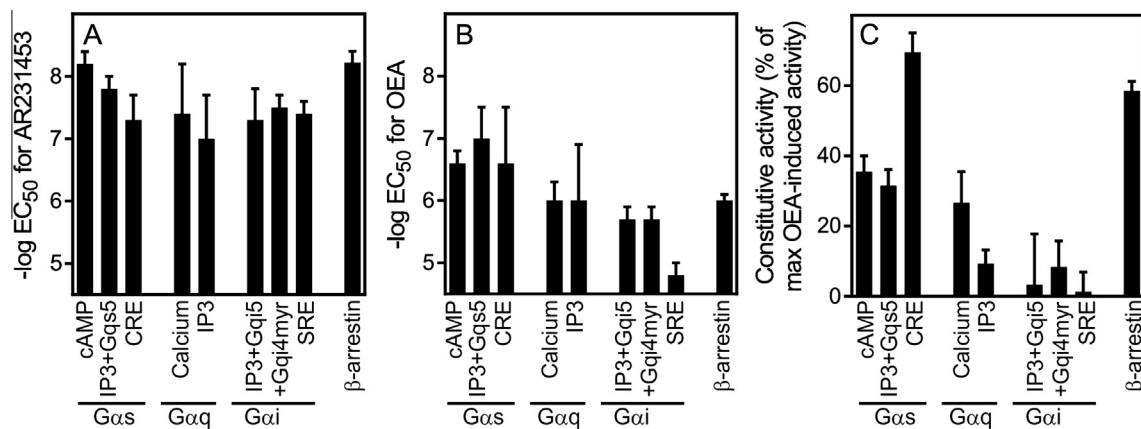
macrodynamics studies. We therefore tested both the lipid-based OEA and the synthetic agonist AR231453 in a dose-dependent manner for the two transcription factors (CRE and SRE) that were responsive to AR231453 (Fig. 4). Consistent with the largely unaltered, and high potency of AR231453 through cAMP accumulation, calcium release and  $\beta$ -arrestin recruitment (Fig. 1, Table 1), AR231453 stimulated CRE and SRE with similar high potency (Fig. 4A and B, Table 1). However, this was not the case for OEA, as its potency through SRE activation was remarkably lower ( $\sim 50$ -fold) compared to the CRE-activation (Fig. 4B, Table 1). Notwithstanding, the efficacy of OEA was similar to that of AR231453 (Fig. 4B). Thus OEA acts as a full agonist through SRE-activation, despite a very low potency. Consistent with the gene-dose experiments (Fig. 3), the basal activity leading to CRE-activation was high, and reached 65% of the maximum activity ( $E_{\max}$ ) induced by OEA, whereas no basal activity was observed through SRE ( $<2\%$  of the  $E_{\max}$  for OEA (Fig. 4C)). Thus, the degree of basal activity was congruent with the variation in OEA potencies, as high potency was observed in the system with high basal activities (CRE), whereas the absence of basal activity concurred with a very low potency of OEA (Fig. 4D). The CRE and NFAT activity was measured in media containing 10% serum, while 0.5%

serum was used for the SRE activity. To test if this low serum concentration was causing the low basal activity observed in SRE activation, we repeated the SRE activity in the presence of 10% serum. Under these settings, the level of basal activity was comparable to that in the presence of 0.5% serum, indicating no significant difference in the basal receptor activity in the presence of high serum content (10%) and low serum content (0.5%).

Summarizing all data for the potencies of the two agonists and the degree of constitutive activity obtained in the implemented pathways, the fluctuations in EC<sub>50</sub> values for AR231453 were small (Fig. 5A), but varied a lot for OEA (Fig. 5B). Here, the highest potencies were observed in  $G_{\alpha s}$ -mediated pathways (cAMP increase, IP<sub>3</sub> measured by co-transfection with Gqs5 and the CRE transcription factor), followed by assays mediated by  $G_{\alpha q}$  (IP<sub>3</sub> turnover and calcium release) and lowest for  $G_{\alpha i}$ -mediated pathways (IP<sub>3</sub> measured by co-transfection with Gqi5 and the SRE transcription factor). Importantly, the percentage of constitutive activity relative to the efficacy of OEA followed the same pattern, as high basal activity was observed in pathways with high potency of OEA, whereas it dropped in pathways with low potency (Fig. 5C). This indicates that the observed high basal activity of GPR119 could be caused by, or influenced by lipids present in the cells or cell media – a



**Fig. 4.** Potencies of OEA and AR231453 in the CRE and SRE-transcription factors. Transcription factor CRE (A) and SRE (B) activation by increasing doses of OEA (closed circles) or AR231453 (open circles) in HEK293 cells transfected with GPR119 or pcDNA3. Data are normalized to  $E_{\max}$  for OEA (100%) and the activity of pcDNA (0%). Data are shown as mean  $\pm$  SEM,  $n = 8$ . Graphical illustrations of the degree of constitutive activity (black columns) relative to the  $E_{\max}$  for OEA (100%) (C) and the potencies of AR231453 and OEA (D).



**Fig. 5.** Overview of potencies and degree of constitutive activity for all pathways tested. Potencies of AR231453 (A) and OEA (B) in all included signaling pathways. From left the Gas coupled pathways (CRE, IP3 measured by co-transfection with the chimeric Gqs5 and cAMP measurements), the Gαq coupled pathways (calcium release and IP3 measurements), the Gαi coupled pathways (IP3 measured by co-transfection with the chimeric Gqi5 and Gqi4myr and the SRE transcriptional activity) and β-arrestin recruitment. (C) Degree of constitutive activity relative to  $E_{\max}$  of OEA in the same signaling pathways. Data are shown as mean  $\pm$  SEM,  $n = 3-5$ .

phenomenon that is unmasked by the low potency of lipids through SRE-activation – in turn pointing at OEA as a biased ligand.

### 3.5. Endogenous lipids may contribute to the apparent high basal activity of GPR119

To further assess the contribution of endogenous lipids in the observed high basal activity, we included two inhibitors (JZL184 and URB597) for the enzymes responsible for the degradation of 2-MAGs and NAEs (i.e. MAGL and FAAH, respectively). Both inhibitors were added 2 or 24 h before measuring the basal cAMP activity in HEK293 cells transfected with either GPR119 or empty vector control. None of the inhibitors had any impact on cAMP formation in cells without GPR119 (Fig. 6, white bars), whereas the basal activity of GPR119 (Fig. 6, black bars) was significantly ( $p < 0.01$ ) increased by pre-incubation with 0.1  $\mu$ M JZL184 for 2 h (Fig. 6A) and for 24 h (Fig. 6B). In contrast, 1  $\mu$ M URB597 did not affect the basal activity of GPR119 at any of these settings (Fig. 6C and D). This indicates that endogenous 2-MAGs, but not NAEs, are generated during a process of lipid metabolism in COS-7 cells, and may contribute to the apparent constitutive activity of GPR119.

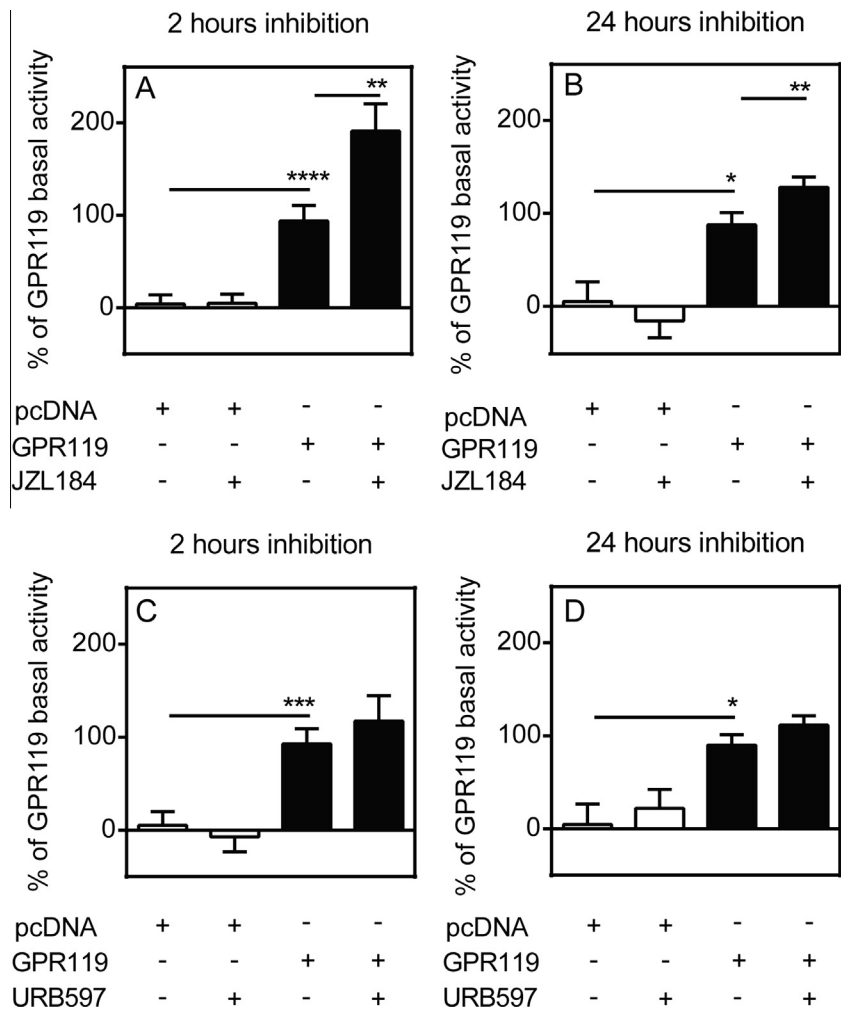
### 3.6. Agonist (-ago) allosteric modulation of OEA-induced GPR119 activation

As a consequence of the uncovering of endogenous lipids as putative players in the observed high basal activity of GPR119

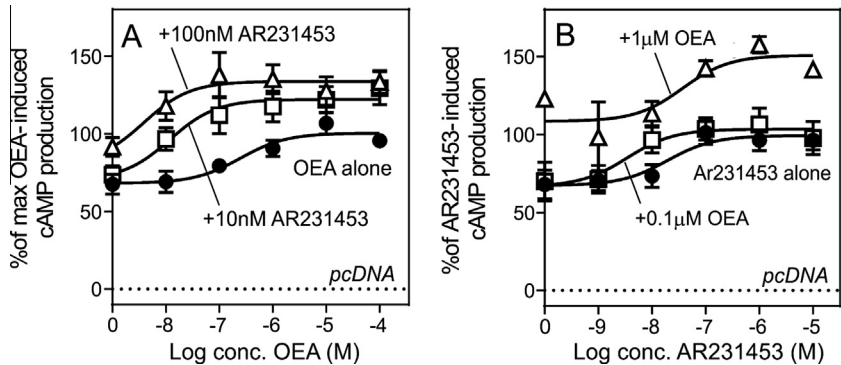
(Fig. 6), we decided to test whether the synthetic agonist AR231453 acted as allosteric modulator of the lipid-induced activity. A series of dose-response curves for OEA-induced cAMP formation was performed in the absence and presence of low (10 nM) or high (100 nM) concentration of AR231453. Both concentrations of AR231453 increased the potency positively with 8-fold increase in the presence of 10 nM AR231453, and more than 50-fold for 100 nM AR231453 ( $p < 0.05$ ) (Fig. 7A, Table 1). This was not the case in the opposite situation (Fig. 7B, Table 1). Thus, AR231453 seems to act as a positive allosteric modulator of OEA, concomitant with being an agonist itself.

## 4. Discussion

In the present study, we extend the signaling profile of GPR119 to include not only G $\alpha$ s, but also other G proteins, transcription factors as well as β-arrestin recruitment. The endogenous OEA and the synthetic AR231453 were full agonists through most pathways, and whereas the potency of AR231453 was rather constant, that of OEA varied in a pathway-dependent manner, that paralleled the basal receptor activity. This establishes OEA as a biased ligand. Together with the observation that endogenous MAGs influence the basal activity of GPR119, this indicates that the basal activity of GPR119 is – in part – mediated by endogenous lipids. Finally, AR231453 was shown to act as a positive allosteric modulator of OEA activity.



**Fig. 6.** Impact on the constitutive activity of GPR119 by inhibition of MAG and NAE degradation. cAMP formation in COS-7 cells transfected with GPR119 (black columns) or pcDNA3 (white columns) in the presence and absence of the monoacylglycerol (MAG) lipase inhibitor JZL184, 0.1  $\mu$ M (A and B) or the FAAH inhibitor URB597, 1  $\mu$ M (C and D), for 2 h (A and C) or 24 h (B and D). Data are normalized to the basal activity of GPR119 in the absence of inhibitors (100%) and zero (0%) and shown as mean  $\pm$  SEM,  $n = 3$ . The unpaired  $t$ -test was used to test for statistical significance ( $p < 0.05^*$ ,  $< 0.01^{**}$ ,  $< 0.005^{***}$  and  $< 0.0001^{****}$ ).



**Fig. 7.** Allosteric modulation of GPR119. cAMP formation in transiently transfected COS-7 cells was stimulated by increasing concentrations of OEA (A) or AR231453 (B) with or without co-administration of fixed concentrations of OEA (1  $\mu$ M and 0.1  $\mu$ M) (B) or AR231453 (10 nM/0.1  $\mu$ M) (A). Data are normalized to  $E_{max}$  of OEA (100%) and pcDNA-transfected cells (0%). Data are shown as mean  $\pm$  SEM,  $n = 5$ .

4.1. Signaling properties of GPR119 through multiple G proteins

The signaling through  $G_{\alpha s}$  is well established for GPR119 [3,8,9,12,13,21–23], and we confirm this both by direct cAMP measurements and by CRE activity.  $G_{\alpha s}$  signaling has been shown to be a driving pathway for the insulinotropic effect of many GPCRs like

GPR119 and TGR5 expressed in pancreatic  $\beta$ -cells and for GLP-1 release from EECs in the gut [8,13]. However, growing evidence suggests that  $G_{\alpha s}$  and  $G_{\alpha q}$  may act in synergy on hormone secretion for instance via a cAMP-mediated potentiation of glucose-induced insulin secretion triggered by intracellular  $Ca^{2+}$  through the ATP-sensitive potassium channel and opening of



voltage-dependent calcium channels located in the cell membrane [43], as recently described for GPR40 [44].  $G_{\alpha q}$  activity has not been observed before for GPR119. In the present study, we observed a minute  $G_{\alpha q}$  coupling measured by IP<sub>3</sub> turnover, yet the robust agonist-induced calcium release and basal NFAT activity supports this activity. It should be noted however, that calcium release as well as NFAT activation can be mediated not only by  $G_{\alpha q}$ , but also by for instance  $G_{\alpha s}$  and  $G_{\alpha i}$  (for calcium release) and  $G_{\alpha i}$  (for NFAT activity) [41–46]. It is therefore likely that the strong calcium release reported here and previously [24,26] is mediated not only by  $G_{\alpha q}$  activity, but also by the other pathways. It is possible that the  $G_{\alpha q}$  activity may contribute to, and maybe even synergize, the insulinotropic action of GPR119 as in GPR40 [44]; however like in GPR40, where  $G_{\alpha s}$  is the main insulinotropic pathway [44], the  $G_{\alpha s}$  activity of GPR119 is far superior to that of  $G_{\alpha q}$ . The  $G_{\alpha i}$  activity observed here, and previously [38], by IP<sub>3</sub> release during co-transfection setups with Gq15 and confirmed with the inhibition of the receptor-induced NFAT activation in the presence of the  $G_{\alpha i}$  inhibitor PTx is somehow paradoxical to the strong  $G_{\alpha s}$  coupling. However, dual coupling to these two G proteins have been observed before in for instance the adrenergic A<sub>2a</sub> receptor with the most potent activation through  $G_{\alpha i}$  and a weaker signaling through  $G_{\alpha s}$  [47,48]. In this receptor, distinct regions of selective coupling to these two G proteins were identified in ICL2 and ICL3 [49,50]. The M2 and M4 muscarinic receptors display similar dual coupling with the same preponderance toward  $G_{\alpha i}$  coupling [51], whereas the GnRH receptors in addition to  $G_{\alpha q}$ , couples to  $G_{\alpha s}$  and  $G_{\alpha i}$ , where the  $G_{\alpha s}$  coupling occurs with higher potency than the  $G_{\alpha i}$  activity [52] (like for GPR119, Fig. 5). It can be envisioned that the dual regulation of the activity of adenylate cyclase may fine-tune the downstream signaling in GPR119 and thereby provide a more tightly regulated hormone release, as shown in the GnRH receptor, where the dual action is necessary for the pulsatile hormone release [52]. In support of this as a relevant mechanism for GPR119, it was shown earlier that epinephrine administration to pancreatic  $\alpha$ -cells resulted in both  $G_{\alpha i}$  and  $G_{\alpha s}$  activation by the A<sub>2a</sub> receptor [53].

#### 4.2. The role of $\beta$ -arrestin recruitment for GPR119 action

Classically,  $\beta$ -arrestin recruitment is an initial step in receptor desensitization and subsequent internalization [54,55], yet it may also be involved in the initiation of G protein-independent signaling pathways, like MAP-kinases [56].  $\beta$ -Arrestin recruitment by lipids acting via GPR119 has never been described before. We observed potent activation via this pathway for both ligands and thereby show for the first time, that also endogenous lipids activate recruitment of  $\beta$ -arrestin via GPR119. However it remains to be determined if this signaling of GPR119 is involved in its biological effects similar to the reported involvement of GLP-1 receptor-mediated  $\beta$ -arrestin recruitment for insulin release from pancreatic  $\beta$ -cells [57]. In support of the relevance of  $\beta$ -arrestin recruitment for GPR119, a recent publication described desensitization of GPR119 by different synthetic agonists [58]. Furthermore, the strong  $\beta$ -arrestin coupling by GPR119 may be involved in the high degree of constitutive internalization, rapid recycling and preferential intracellular location of GPR119 [59], a distribution also known for other constitutively active GPCRs, like the viral US28 chemokine receptor [60,61].

#### 4.3. Ligand-bias of OEA and apparent constitutive activity of GPR119

Signaling bias describes a situation where different ligands elicit different signals through a given receptor (ligand bias), when the same ligand acts on different receptors with different signaling outcomes (receptor bias) or when a given ligand-receptor pair

results in different signaling outcome in an expression-dependent manner (tissue/cell bias) [62]. In contrast to the potency of AR231453, that only varied very little, consistent with previous data [8–13], the potency of OEA was pathway-dependent and thereby acted as a biased ligand with the same efficacy as AR231453 in all tested pathways. The highest potency of OEA was observed in  $G_{\alpha s}$ -dependent pathways, where previously reported potencies were confirmed [3,12,28] followed by the  $G_{\alpha q}$ -mediated pathways, and with lowest potency via  $G_{\alpha i}$  (Fig. 5, Table 1). The lowest potency was observed in the SRE pathway concomitant with a complete lack of constitutive activity, whereas the  $G_{\alpha s}$ -mediated pathways displayed the highest degree of constitutive activity concomitant with the highest OEA potencies. Thus, the observed constitutive activity via  $G_{\alpha s}$  correlates with the biased action of this endogenous lipid. This was further emphasized by the increase in activity upon pre-incubation with JZL184 – an inhibitor of the enzyme MAGL that degrades 2-MAGs, demonstrating that endogenous MAGs are indeed produced in a common (fibroblast-like) cell line, and implying that MAGs potentially contribute to the observed basal activity of GPR119 in any cells that employ lipoprotein lipase to metabolize lipids, if they negatively regulate MAGL activity. Interestingly, we could not determine that NAEs play the same role, as the NAE inhibitor URB597 did not affect GPR119 signaling, which could both indicate that NAEs are not being produced – or degraded – endogenously in our setup.

Constitutive activity is commonly observed among GPCRs as for example the ghrelin receptor [42], and many virus-encoded receptors [63,64,46,65]. However, apparent constitutive activity may be a result of – or at least influenced by – endogenous agonist(s) present in the media or in the cells that may lead to receptor activity, thereby masking the “true” pharmacology of a given receptor – a situation that our results indicate for GPR119. This was for instance the case for GPR40 (FFAR1); another lipid receptor with similar expression pattern as GPR119 and similar insulinotropic action mediated via  $G_{\alpha s}$  and  $G_{\alpha q}$  activation by FFA [44,66]. In GPR40, FFA in the added serum, resulted in an apparent high constitutive activity, that was eliminated in fatty acid-depleted serum [29]. Another example is the lipid receptor GPR183 (also known as Epstein Barr virus-induced receptor 2, EB12). Prior to its deorphanization as a receptor for oxysterols [67,68], high constitutive  $G_{\alpha i}$  activity was observed in several cell lines [30,69]. However, this activity was, at least in part, mediated by oxysterols in the medium/cells as an inhibition of the CYP7B1 enzyme, involved in oxysterol formation, by clotrimazole, reduced EB12 activity [30,68,69].

#### 4.4. Intrinsic and allosteric actions of GPR119 agonists

In the present study we observed that, besides its intrinsic activity, AR231453 acted as a positive allosteric modulator of OEA. A recent study by Zhang et al. also investigated the putative synergy between these two ligands, not by a direct measurement of cAMP accumulation as it is the case here, but by measuring downstream signaling mediated by the induced cAMP, i.e. the transcription factor CRE [70]. In contrast to our data, they did not observe any allosteric action of AR231453. This however could be due to insufficient amounts of AR231453 in their studies (0.008–1 nM) as compared to the concentrations used in our study (10 and 100 nM); differences that may have an even higher impact given the chosen experimental settings, as AR231453 displays a 10-fold lower potency in the CRE transcription factor assay than in the cAMP accumulation assay (Table 1). The structural basis for this allosteric action of AR231453 is rather challenging, as a recent study described largely overlapping binding pockets for AR231453 and OEA, yet with minor differences in that AR231453

seemed to dock deeper into the main binding pocket with direct interaction with residues as deep as position III:09 and III:12 (3.36 and 3.39, respectively) [28]. Furthermore, OEA is a highly flexible ligand that may allow several binding poses, some of which could permit the action of AR231453 at the same time, or it could be envisioned that AR231453 constrains the receptor in an OEA-preferential conformation. Interestingly, a large degree of overlap was identified for residues involved in binding of OEA and AR231453 and residues involved in the constitutive activity [28], again pointing toward a possible role of endogenous lipids as contributors to the apparent high constitutive activity of GPR119.

#### 4.5. Perspectives

Owing to the expression pattern and physiological role of GPR119, this receptor is an attractive metabolic target – an insulinotropic “lipid sensor” in the gut with 2-OG as the most relevant food-derived ligand [5,14] recently proven to act *in vivo* in humans [3]. Exogenous OEA has also been proven effective *in vivo* in rodent studies [12,15], but it is unclear whether endogenous OEA participate in activation of GPR119. The observed positive allosteric action of AR231453 is important, as allosteric modulation drugs encompass obvious advantages as compared to orthosteric counterparts by potentially modulating and fine tune the action of the endogenous ligands, possibly without the same risk of overdosing effects and with an action that depends on the endogenous ligands. However, allosteric action by a ligand may also possess intrinsic activity, as in the case of AR231453. We also show that endogenous lipids may contribute to the apparent constitutive activity of GPR119. An intriguing question is therefore whether AR231453 indeed possesses intrinsic activity, or whether its observed agonism is a result of an allosteric action of the effect of endogenous lipids. Further studies are needed to clarify this. From a clinical point of view, synthetic GPR119 agonists have not yielded much success yet and in fact several studies have been disappointing [16,18,19]. Thus, we are still waiting for a successful outcome of clinical trials for GPR119 agonists. The lack of success – despite numerous potent GPR119 agonists – could be due to inadequate characterization of basic pharmacological properties of these compounds, hereunder allosteric actions and well-defined pathway targeting, maybe in the sense of biased drugs targeting one or several of the pathways described in the current study.

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#### Author contributions

HAH, SF, HP, MMR, TWS and HSH designed the experiments. HAH, HP, SF, OL, MH made the experiments. RJ provided the ARENA compounds. HAH, SF, HP, OL, MH and MMR analyzed data. HAH, HSH, SF and MMR wrote the manuscript. All authors have approved the manuscript.

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