

GPR40 (FFAR1) — Combined Gs and Gq signaling *in vitro* is associated with robust incretin secretagogue action *ex vivo* and *in vivo*



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ABSTRACT

Objectives: GPR40 (FFAR1), a clinically proven anti-diabetes target, is a Gq-coupled receptor for long chain fatty acids (LCFA) stimulating insulin secretion directly and mediating a major part of the dietary triglyceride-induced secretion of the incretins GLP-1 and GIP. In phase-II studies the GPR40 agonist TAK-875 decreased blood glucose but surprisingly without stimulating incretins.

Methods and results: Here we find that GPR40 can signal through not only Gq and IP3 but also Gs and cAMP when stimulated with certain agonists such as AM-1638 and AM-5262 in contrast to the endogenous LCFA ligands and agonists such as TAK-875 and AM-837, which only signal through Gq. In competition binding against [3H]AM-1638 and [3H]L358 the Gq + Gs and the Gq-only agonists either competed for or showed positive cooperativity by increasing the binding of the two different radio-ligands, in opposite ways. Nevertheless, both the Gq-only and the Gq + Gs agonists all docked surprisingly well into the binding site for TAK-875 in the X-ray structure of GPR40. In murine intestinal primary cell-cultures the endogenous LCFAs and the Gq-only agonists stimulated GLP-1 secretion with rather poor efficacy as compared with the high efficacy Gq + Gs GPR40 agonists and a prototype GPR119 agonist. Similarly, in fasting both male and female mice the Gq + Gs agonists showed significantly higher efficacy than the Gq-only agonists in respect of increasing plasma GLP-1 and plasma GIP in a GPR40-dependent manner.

Conclusions: It is concluded that stimulation of GPR40 by endogenous LCFAs or by Gq-only synthetic agonists result in a rather limited incretin response, whereas Gq + Gs GPR40 agonists stimulate incretin secretion robustly.

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Keywords G protein-coupled receptor; Glucagon like peptide 1 (GLP-1); Biased signaling; Ago-allosteric agonist; Long chain fatty acids (LCFAs)

1. INTRODUCTION

GLP-1 mimetics, such as exenatide and liraglutide have over the last years become widely used in the treatment of type 2 diabetes. The success of GLP-1 mimetics is based on the fact that they function not only as incretins, i.e. compounds which stimulate insulin secretion in a glucose dependent manner, but that they also suppress glucagon secretion, delay gastric emptying, decrease appetite and promote β -cell survival [1,2].

However GLP-1 itself is just one out of a handful of gut hormones with similar beneficial metabolic properties, which all are released in response to food ingestion from closely related enteroendocrine cells scattered along the small and large intestine [3,4]. The co-released gut hormones act in symphony and in several cases even synergistically [5]. Thus, it could be tempting to try to develop compounds, which

stimulate the secretion of the powerful mixture of endogenous gut hormones [6,7]; compounds, which may not even enter the body as such, but act locally in the gut [6].

Metabolites of dietary triglycerides, i.e. free fatty acids (FFA) and 2-monoacyl glycerol (2-MAG) are among the most efficacious gut hormone secretagogues [8,9] conceivably acting through the G protein-coupled receptors GPR40 [10–14] and GPR119 [9,15], respectively. Since these receptors are highly expressed on β -cells of the endocrine pancreas, they rapidly became high priority drug discovery targets for new anti-diabetes agents. A number of GPR119 agonists reached phase II [9,16], but, although the compounds stimulated GLP-1 and insulin secretion in preclinical models they apparently did not deliver the required clinical efficacy perhaps due to their ability to also stimulate the secretion of the counter regulatory hormone glucagon [9].

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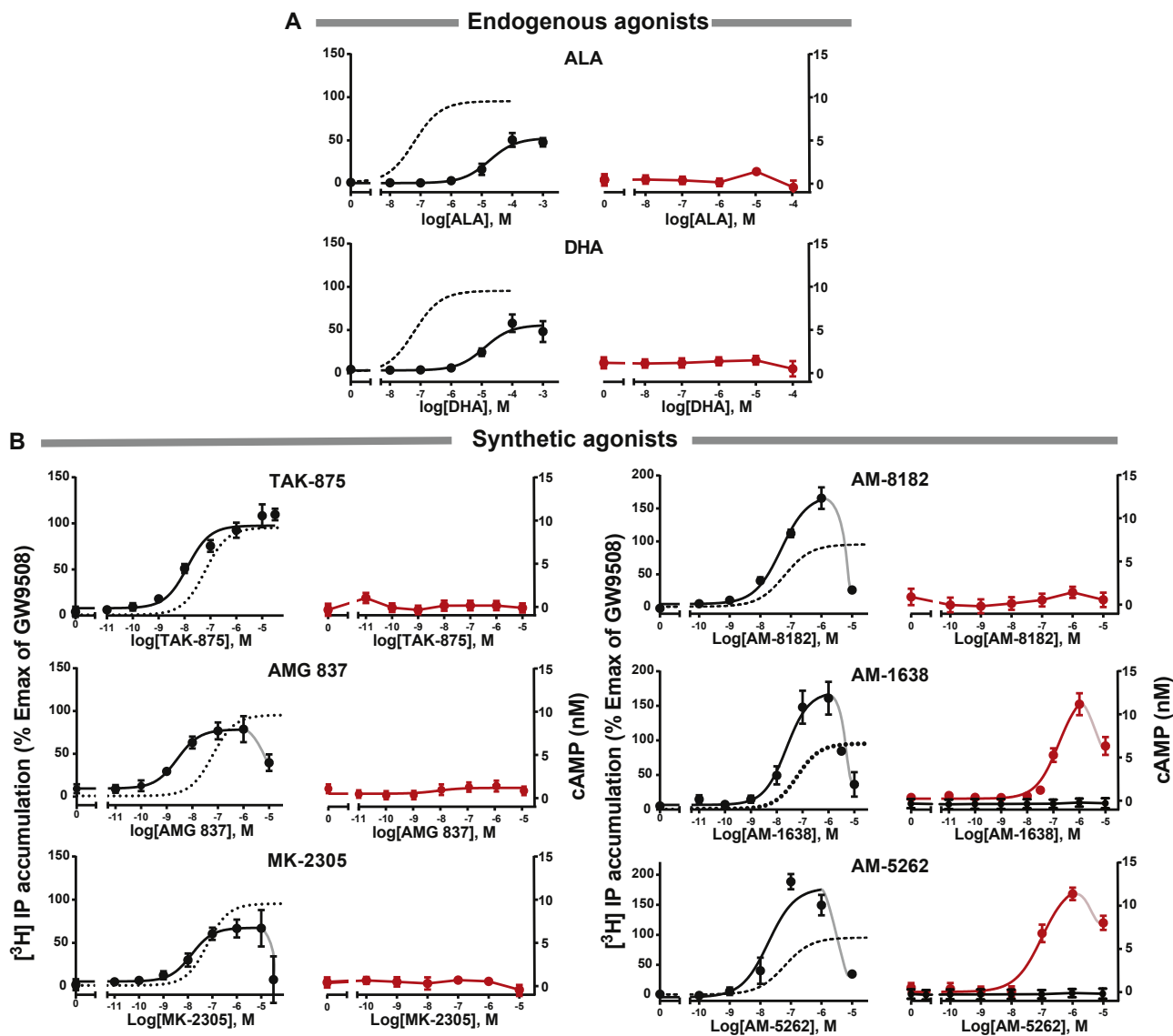


Figure 1: *In vitro* signaling properties of GPR40 agonists in transiently transfected COS7 cells. Effects on IP-turnover (black curves, left panels using GW9508 as positive control – stippled line) and cAMP accumulation (red curves, right panels) were determined for: (A) Endogenous lipid agonists α -linolenic acid (ALA) and docosahexaenoic acid (DHA); (B) synthetic agonists TAK-875, AMG 837, MK-2305, AM-8182, AM-1638 and AM-5262. Black curves in the cAMP accumulation panels for AM-1638 and AM-5262 represent empty vector control. Data are normalized to empty vector (0%) and E_{max} for GW9508 (100%) in IP-turnover. Concentrations of cAMP were interpolated based on cAMP standard curves for each experiment. Data represents mean \pm SEM and represents a minimum of three experiments.

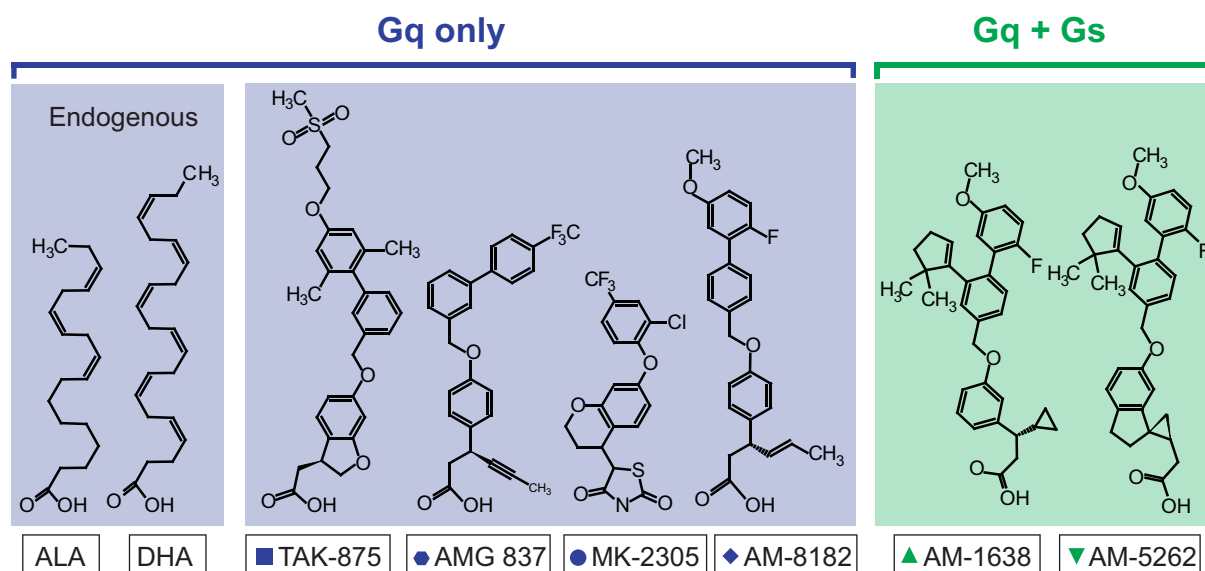
GPR40 was originally orphanized as a receptor for medium to long chain fatty acids being almost exclusively expressed in the β -cells and potentially responsible for the glucose-dependent stimulation of insulin secretion by long chain fatty acids (LCFA) [10–12,17]. However, the notion that GPR40 potentially was involved in lipotoxicity made it unclear whether you wanted agonists or antagonists and whether GPR40 would at all be a good drug target [17,18]. However, transgenic overexpression of GPR40 and administration of selective agonists later demonstrated that GPR40 activation augments insulin secretion and improves glucose tolerance and that GPR40 may even protect islets from the toxic effect of fatty acids [19–21]. Importantly, GPR40 was shown to be highly expressed and enriched also on enteroendocrine cells and to mediate GLP-1 and GIP secretion [22,23].

In respect of clinical development Takeda has pioneered the field with the GPR40 agonist TAK-875 or fasiglifam (Figure 2A), which in phase-II studies decreased HbA1c as efficiently as sulfonylurea without signs of

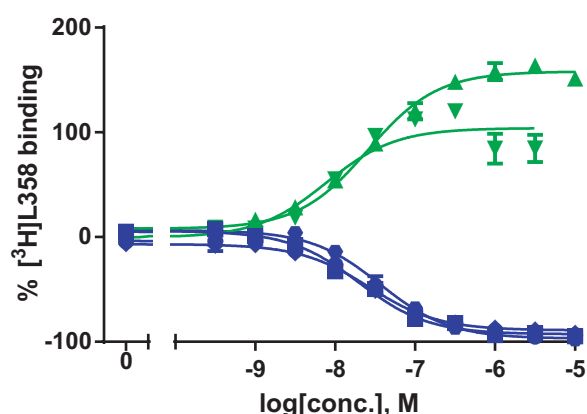
hypoglycemia [24]. This was obtained with surprisingly little effect on systemic insulin and no effect on plasma GLP-1 [25,26]. Recently the TAK-875 program was, however stopped in phase-III due to liver toxicity,⁵ which probably is not GPR40-mediated as the receptor is not expressed in the liver. A new series of GPR40 agonists were by the Amgen group shown to be positive ago-allosteric modulators [27–29], i.e. compounds which both act as agonists and as allosteric modulators increasing the potency and/or the efficacy of other agonists, for example the endogenous agonists [30]. Interestingly, this new class of ago-allosteric modulators improved glucose tolerance not only through direct stimulation of insulin secretion but also through stimulation of GLP-1 [28,29,31]. In the present study we compare a series of GPR40 agonists in respect of their signal transduction and binding properties and their ability to

⁵ Takeda press release, December 27, 2013; http://www.takeda.com/news/2013/20131227_6117.html.

A



B



C

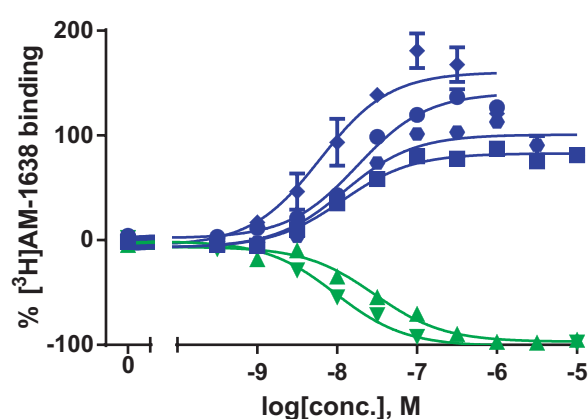


Figure 2: Chemical structures and binding properties of GPR40 agonists. (A) Chemical structures of endogenous agonists α -linolenic acid (ALA) and docosahexaenoic acid (DHA) as well as synthetic agonists TAK-875, AMG 837, MK-2305, AM-8182, AM-1638 and AM-5262. Agonists are grouped according to G-protein activation and competition binding is shown here with the Gq only agonist [³H]L358 (B) or the Gq+Gs agonist [³H]AM-1638 (C) to human GPR40. Data represent mean \pm SEM of two independent experiments performed in duplicates/triplicates.

stimulate GLP-1 and GIP release *ex vivo* and increase plasma concentrations of these incretins *in vivo*. Surprisingly, we find that GPR40 can couple efficiently not only through Gq but also through Gs to increase cAMP concentrations, which however is a property restricted to a sub-set of GPR40 agonists and is associated with the ability to stimulate incretin secretion in a robust manner as compared to the agonists, which like the endogenous lipid ligands only are able to signal through Gq. Unexpectedly both Gq-only and Gq + Gs agonists docked well into the binding site of the Gq-only agonist, TAK-875 in the recently published high resolution X-ray structure of GPR40 [32].

2. MATERIALS AND METHODS

2.1. Compounds

DHA, ALA and GW9508 were purchased from Sigma–Aldrich (Steinheim, Germany). Other GPR40 ligands were synthesized as published TAK-875 [33], AM-8182, AM-1638 and [³H]AM-1638 [27,34,35], AM-5262 [28], MK-2305 and [³H]L358 [36].

2.2. Molecular biology

Receptor cDNAs were cloned into the eukaryotic expression vector pCMV-Tag(2B) (Stratagene, CA, USA) (IP-turnover and cAMP accumulation) and pcDNA3.1+ (Life Technologies, CA, USA) (binding assay). All constructs were verified by DNA sequence analysis.

2.3. Cell culture and transfections

COS-7 cells were grown in Dulbecco's modified Eagle's medium 1885 (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. COS7 cells were transiently transfected (20 μ g/75 cm²) using the calcium phosphate precipitation method with chloroquine addition. CHO-k1 cells were stably transfected with human GPR40 using FuGENE6 transfection reagent (Roche Diagnostics) according to manufacturer's protocol. Stably expressing cells were cultured in Minimum Essential Medium Eagle (Sigma–Aldrich) supplemented with 10% FBS, 2 mM glutamine, 0.01 mg/ml gentamicin and

0.05 mg/ml zeocin and 1 mg/ml G418 for selection of GPR40 containing cells.

2.4. Binding assay

5 μg /well membrane together with 200 μg /well of PVT WGA SPA beads (GE #RPNP-0001) were suspended in a white 96 well plate with a final concentration of 50 μl /well in assay buffer (50 mM Tris, 10 mM MgCl_2 & 2 mM EDTA). The plate was shaken aggressively for 30 min before adding 20 μl /well of compound in 0.1% BSA assay buffer and 30 μl /well of radio-ligand in 0.5% assay buffer yielding a final concentration of 15,000 cpm/well. The plate is briefly mixed and incubated for 3–18 h at room temperature, before it is read on a MicroBeta plate counter (Perkin Elmer, MS, USA).

2.5. IP turnover assay

One day after transfection COS-7 cells were plated into poly-D-lysine-coated 96-well plates (20,000 cells/well) and incubated with 0.5 $\mu\text{Ci/ml}$ myo^3H inositol (Perkin Elmer) in 100 μl growth medium. The following day cells were washed twice with HBSS (Gibco, Life Technologies) and incubated for 30 min 37 °C in 100 μl buffer supplemented with 10 mM LiCl prior to ligand addition followed by 90 min incubation 37 °C. Cells were lysed with 50 μl 10 mM formic acid followed by incubation on ice for 30–60 min 20 μl of the extract were transferred to a white 96-well plate and 80 μl of 1:8 diluted YSi poly-D-lysine coated beads (Perkin Elmer) were added. After vigorously shaking the plate was centrifuged for 5 min at 1500 rpm, and γ -radiation was counted on a Packard Top Count NXT counter after an 8 h delay. Determinations were made in duplicates.

2.6. cAMP accumulation assay

Transient transfected COS-7 cells were plated into poly-D-lysine-coated white 96-well plates (20,000 cells/well). The following day, the cells were washed twice in HBS and incubated in HBS containing isobutyl-1-methylxanthine for 30 min at 37 °C. Varying concentrations of ligands were added and incubated in 30 min at 37 °C, followed by cAMP accumulation assay using the DiscoverX HitHunter™ cAMP XSKIT (Birmingham, UK) according to manufacturer's protocol. Determinations were made in duplicates. Chemiluminescence was read on an EnVision 2104 Multilabel Reader (Perkin Elmer).

2.7. Molecular modeling

The structure of the human GPR40 receptor (PDB code: 4PHU, solved to 2.3 Å resolution in complex with TAK-875) [32] was imported into the internal coordinate mechanics software package (ICM 3.7-3b, Molsoft L.L.C., San Diego, CA, USA). Crystallization water and co-crystal molecules were deleted and the structure was converted into an ICM object to optimize the position of hydrogen atoms. The compounds DHA, AMG-837, TAK-875, MK-2305, AM-8182, AM-5262, AM-1638 and TUG-761 were docked to the TAK-875 binding site (pocket #1) and two additional pockets (pocket #3 and Pocket #4 – see Figure 3) identified by the icmPocketFinder method (default threshold 4.6) using the step-by-step docking menu employed in ICM [37]. The docking procedure included: (i) *Setup receptor*; for each pocket the grid box was adjusted to cover all atoms within 15 Å from the center of the identified pockets. (ii) *Setup batch ligand*; (iii) *run docking batch*; thoroughness 10.0; assigning 3D ligand conformations, MMFF atom types and formal charges to ionizable groups. The ligand docking poses predicted for the three different pockets were ranked based on ICMs empiric ligand docking scoring function

weighted according to the following parameters: (i) internal energy of the ligand, (ii) entropy loss of the ligand between bound and unbound states, (iii) ligand-receptor hydrogen bond interactions, (iv) polar and non-polar solvation energy differences between bound and unbound states, (v) electrostatic energy, (vi) hydrophobic energy, and (vii) hydrogen bond donor or acceptor desolvation – and therefore unitless. Generally a score below –32 is regarded as a good docking score based on assessments of ligand docking scores of high resolution receptor and protein ligand complexes. Furthermore, the ligand conformational strain of all the best-scored docking poses was computed to confirm that the conformations of the bound ligands were accepted in low energy minimums. The final docking complexes were not refined further e.g. by including side chain flexibility to certain side chains.

2.8. Primary colonic crypt cultures

Colonic crypts were prepared from male C57BL/6 WT, *Gpr40* KO or *Gpr40/120* double KO littermate mice (obtained from Taconics, NY). The crypts were isolated by collagenase digestion as described by Reimann et al. [23] and seeded into 48-well plates coated with Matrigel (BD Biosciences). The following day, cells were incubated for 2 h with ligands (triplicates) at 37 °C in standard solution [23] containing 0.1% fatty acid-free BSA (Sigma–Aldrich) and 10 mM glucose. Synthetic ligands were dissolved in DMSO and ALA or DHA were complexed with free fatty acid BSA in a 6:1 molar ratio. Total GLP-1 was determined using Total GLP-1 (ver. 2) assay kit (Meso Scale Discovery, Gaithersburg, USA) (model number K150JVC-1) according to manufacturer's protocol.

2.9. In vivo

Adult (11–18 weeks) C57BL/6 *Gpr40* KO mice and WT littermate mice were group housed with up to 8 mice in each cage at 24 °C on a 12:12 h light–dark cycle. After an overnight fast a retro orbital blood sample was taken (app. 200 μl) and mice were orally gavaged with either vehicle solution containing 0.5% (w/v) carboxymethylcellulose sodium salt, medium viscosity, (Sigma–Aldrich) or 30 mg/kg of TAK-875, MK-2305, AM-1638 or AM-5262 dissolved in vehicle solution. PK experiments had shown the following C_{max} concentrations: TAK-875 – 2 μM ; MK-2305 – 22 μM ; AM-1638 – 11 μM ; and AM5262 – 19 μM . Each mouse was given 10 ml/kg of the test solution. 30 min after the oral gavage, the mice were decapitated and blood was collected in EDTA coated microvettes (Sarstedt AG & Co, Nümbrecht, Germany) containing 0.5 KIU aprotinin/ μl blood (Sigma Chemical, St. Louis, MO) and 1 μM DPP4 inhibitor (a kind gift from Novo Nordisk, Denmark). All blood samples were kept on ice at all times. The blood samples were centrifuged at 4 °C for 10 min at 9.6 g in a centrifuge (Heraeus Fresco 21 Microcentrifuge, Thermo Scientific, Denmark), plasma was collected and stored at –80 °C. Total GLP-1 was determined using Total GLP-1 (ver. 2) assay kit (Meso Scale Discovery, Gaithersburg, USA; model number K150JVC-1) according to manufacturer's protocol. GIP was measured using a Rat/Mouse Total GIP ELISA (Millipore, St. Charles, MO).

2.10. Calculations and statistical analyses

Data were visualized and tested for significance using the Prism 6.0 software (GraphPad Software, San Diego). E_{max} values, IC50 and EC50 values were determined by non-linear regression (variable slope). Differences in tGLP-1 release from primary colon cultures compared to vehicle were determined by non-parametric one-way

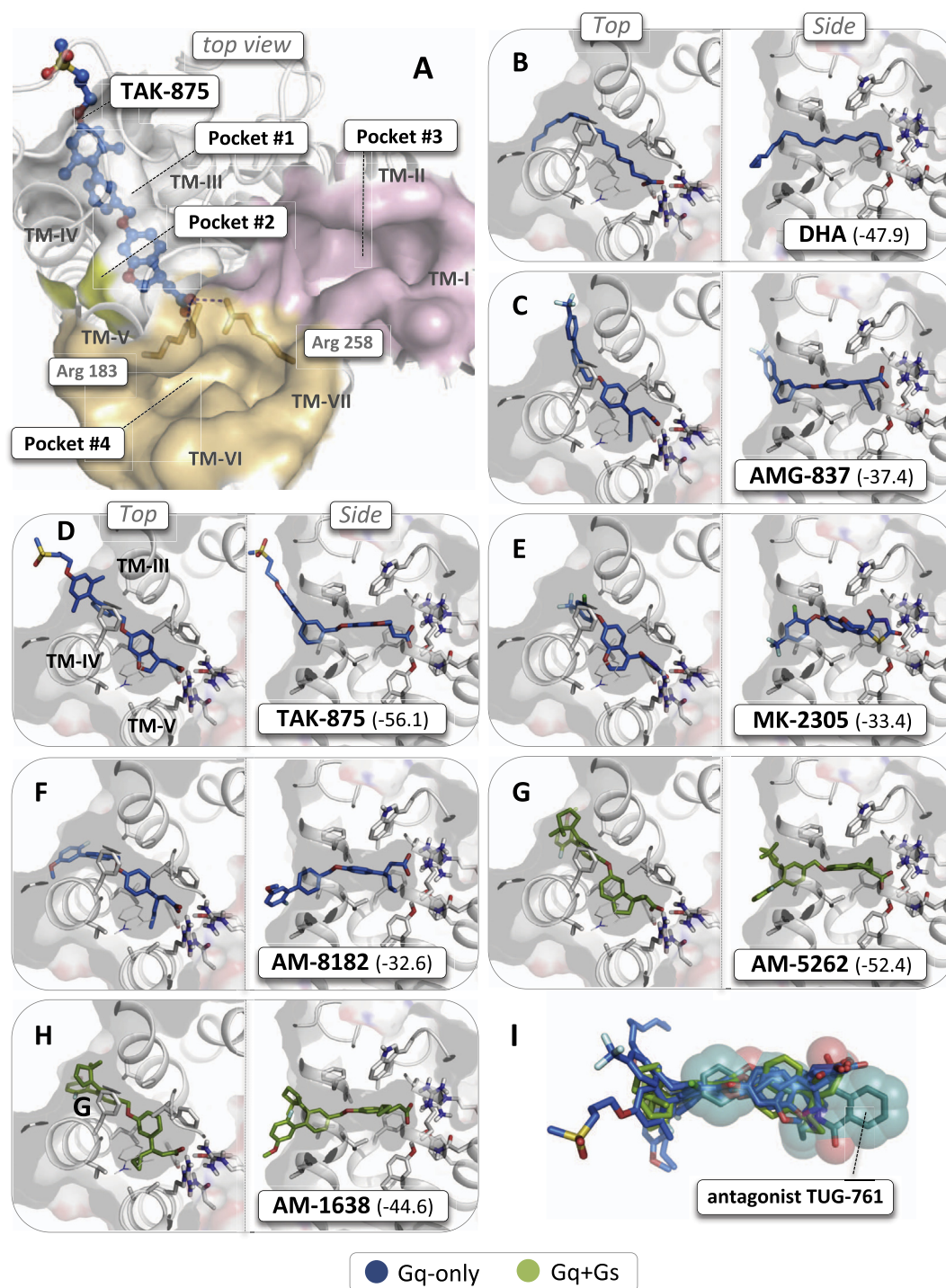


Figure 3: Molecular docking of GPR40 agonists in crystal structure of the human GPR40. (A) Overview of possible ligand binding pockets in the crystal structure of the human GPR40 receptor in complex with TAK-875 (PDB code: 4PHU, solved to 2.3 Å resolution) as viewed from the extracellular side [32]. The binding site for TAK-875 is covered by residues mainly from extracellular loop 3 (transparent) and the location of TM-I to TM-VII are indicated as well as the two Arg residues forming the anchor point for the carboxylic acid moiety of TAK-875. (B–H) Best scored docking poses of the Gq-only agonist shown in blue stick models, DHA, AMG-837, TAK-875, MK-2305, AM-8182 and the Gq-Gs agonists shown in green stick models, AM-1638 and AM5262 to the TAK-875 binding pocket (docking scores are specified in brackets – see Section 2). (I) Superimposition of the antagonist TUG-761 (transparent spheres and dark green stick model) onto the agonists in their docked binding conformations.

ANOVA (Kruskal–Wallis) with Dunn's posthoc test of multiple comparisons. Statistical significance between WT and *Gpr40* KO colonic crypt cultures was determined by a non-parametric unpaired two-tailed *t*-test (Mann–Whitney). *In vivo*, incretin release between

groups of mice receiving compound compared to vehicle was determined by a parametric unpaired *t*-test with Welch's correction. Statistical significance is denoted by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Table 1 — Summary of GPR40 ligand pharmacology and *ex vivo* response.

Ligand	Binding (<i>in vitro</i>)				IP3 (<i>in vitro</i>)		cAMP (<i>in vitro</i>)		tGLP-1 (<i>ex vivo</i>)		
	[3H]L358	LogIC50	[3H]AM-1638	LogIC50	LogEC50	E _{max}	LogEC50	E _{max}	WT	GPR40 (–/–)	
Endogenous	ALA	n.d.	n.d.		–4.8 ± 0.19	53 ± 4	>–3	1.39 ± 0.4	1.4 ± 0.06 ^a	1.0 ± 0.04 ^a	
	DHA	n.d.	n.d.		–4.9 ± 0.24	56 ± 6	>–3	1.50 ± 0.5	n.d.	n.d.	
Orthosteric	TAK-875	–7.7 ± 0.05	(pos)	–7.9 ± 0.07	(neg)	–7.9 ± 0.14	98 ± 4	>–5	0.13 ± 0.6	1.5 ± 0.08	1.1 ± 0.12
	MK-2305	–7.5 ± 0.05	(pos)	–7.7 ± 0.07	(neg)	–7.9 ± 0.24	68 ± 5	>–5	0.70 ± 0.4	2.8 ± 0.42	1.1 ± 0.06
	AM-8182	–7.6 ± 0.03	(pos)	–8.2 ± 0.17	(neg)	–7.3 ± 0.11	171 ± 9	>–5	1.48 ± 0.6	1.4 ± 0.19	1.4 ± 0.14
Ago-allosteric	AM-1638	–7.6 ± 0.05	(neg)	–7.5 ± 0.07	(pos)	–7.6 ± 0.20	170 ± 14	–6.8 ± 0.13	13.01 ± 1.1	3.0 ± 0.54	1.1 ± 0.07
	AM-5262	–8.1 ± 0.18	(neg)	–8.0 ± 0.05	(pos)	–7.7 ± 0.19	179 ± 14	–7.0 ± 0.13	12.63 ± 0.1	4.6 ± 0.77	1.2 ± 0.13

Data are representative of two or more experiments, and expressed as mean ± SEM. n.d.; not determined — binding was not carried out with the endogenous ligands, and *ex vivo* tGLP-1 release was not carried out with DHA. (pos) and (neg) indicates positive or negative competitive binding with radio labeled ligand. *Ex vivo* tGLP-1 release (fold change) as determined with 1 μM ligand, ^aexcept for ALA which was 30 μM.

3. RESULTS

3.1. *In vitro* signal transduction properties of GPR40 ligands

GPR40 is normally considered to be a Gq-coupled receptor. However, based on the knowledge that receptors often can signal through more than one pathway and the fact that Gs signaling very efficiently stimulates GLP-1 secretion, for example through activation by GPR119, we decided to study the effect of a series of six different synthetic GPR40 agonists and two endogenous lipid ligands in respect of stimulation of both IP accumulation and cAMP production in COS-7 cells transiently transfected with the human GPR40 receptor.

In the Gq mediated IP-turnover experiments the classical GPR40 agonist GW9508 [13] was used as a reference compound. The two endogenous ligands ALA and DHA stimulated IP-accumulation efficiently, but with somewhat lower E_{max} values than GW9508, i.e. 53 ± 4% and 56 ± 6% of the E_{max} of GW9508, and with EC50 values of 17 μM and 12 μM, respectively (Figure 1A).

The six synthetic GPR40 agonists stimulated IP-accumulation with rather similar potencies (Table 1) and with maximal efficacies ranging from 68 ± 5 (MK-2305) to 179 ± 14%, (AM-5262) of the E_{max} for GW9508 (Figure 1B).

In respect of Gs signaling, the two endogenous lipid ligands ALA and DHA were as expected not able to stimulate cAMP accumulation meaningfully through GPR40 (Figure 1A). Like the endogenous lipid ligands, four of the synthetic GPR40 agonists, TAK-875, AM-837, MK-2305, and AM-8182, showed no or very limited stimulation of cAMP production (Figure 1B).

However, surprisingly GPR40 could in fact signal through Gs, but only when stimulated with a subset of synthetic agonists, here represented by AM-1836 and AM-5262. Thus, in cells transfected with GPR40 as opposed to cells transfected with empty vector AM-1638 and AM-5256 stimulated cAMP accumulation robustly (Figure 1). The EC50 values for cAMP stimulation by AM-1638 and AM-5162 were 160 and 100 nM, respectively, which is 7 and 6 fold lower than the potencies for IP-accumulation (Table 1). In both the IP3 and cAMP assay, AM-1638 and AM-5262 displayed bell-shaped dose–response curves with a signal decline between 1 and 10 μM (Figure 1B). This phenomenon was also observed for several of the other GPR40 agonists in the IP3 assays (Figure 1B).

It is concluded that GPR40 can signal not only through Gq as observed with the endogenous lipid ligands but also in a ligand-biased fashion through Gs. In the following we differentiate between ‘Gq-only’ agonists, which here are TAK-875, AM-837, MK-2305 and AM-8182 and ‘Gq + Gs’ agonists, i.e. AM-1638 and AM-5262.

3.2. Binding properties of GPR40 agonists

Competition binding experiments were performed in membranes from stably transfected CHO-K1 cells using two different radio ligands: [3H] L358, which is a close analog of the Gq-only agonist MK2305, and the Gq + Gs agonist [3H]AM-1638.

As shown in Figure 2B, the Gq-only agonists TAK-875, AM-837, AM-8182 and MK-2305 all displaced [3H]L358 efficiently and with surprisingly similar affinities ranging from 20 to 36 nM. In contrast, the two Gq + Gs agonists AM-1638 and AM-5262 did not compete for [3H]L358 binding but instead displayed positive co-operativity by increasing [3H]L358 binding with apparent affinities of 26 and 8 nM, respectively (Figure 2B). However, when the Gq + Gs agonist [3H]AM-1638 was used as radio-ligand both AM-1638 and AM-5262 competed efficiently for binding with affinities of 29 and 10 nM, respectively (Figure 2C). In contrast, the Gq-only agonists TAK-875, AM-837, AM-8182 and MK-2305 did not displace but instead all increased the binding of the Gq + Gs agonist [3H]AM-1638 with apparent affinities ranging from 6 to 17 nM (Figure 2C).

These binding data indicate that the Gq + Gs agonists bind to GPR40 in a very different manner than the Gq-only agonists and that the use of the two distinct radio ligands, [3H]L358 and [3H]AM-1638, differentiate between these binding modes in a reciprocal manner.⁶

3.3. Molecular modeling of the binding mode of Gq-only versus Gq + Gs agonists in GPR40

The high resolution X-ray structure of GPR40 in complex with the Gq-only agonist TAK-875 was recently published in Ref. [32], which provides a unique basis for computational chemistry analysis of potential binding modes of other GPR40 ligands. As shown in Figure 3, TAK-875 binds in a pocket or channel extending out into the lipid bilayer between TM-III and TM-IV from the anchor point for the carboxylic acid group centered around the guanidinium groups of Arg183 (ArgV:05) and Arg258 (ArgVII:02). As viewed from the extracellular side, this binding pocket is totally covered by residues from TM-III, -IV and -V and extracellular loop 2. A small, empty ‘side-pocket’ is found between TM-IV and TM-V (originally called ‘pocket #2’) close to the carboxylic acid moiety of TAK-875. Two other relatively large, empty pockets are found on the opposite side of the carboxylic acid anchor

⁶ In the present report we do not use the terms orthosteric and allosteric binding modes and do not try to differentiate between the two GPR40 allosteric binding modes proposed by Lin and coworkers because we use [3H]L358 which gives a more simple competition binding picture where the different ligands basically fall into two rather well defined groups in contrast to the more complex picture obtained with [3H]AM-837 as used by the Amgen group [27].

point, i.e. an elongated pocket extending between TM-III and TM-VII over towards TM-I and TM-II (pocket#3) and a smaller pocket located between TM-V, -VI and -VII (pocket #4).

Using the ICM docking and scoring software all the GPR40 agonists employed in the signaling and binding assays described above docked well into the binding site for TAK-875 (pocket #1) using the unmodified GPR40 X-ray structure from which TAK-875 had been removed. In view of the clearly cooperative binding properties it was very surprising that both Gq-only and Gq + Gs agonists all docked into the same binding site (Figure 1B–H). As expected, TAK-875 itself was the top scorer (−56.1), whereas the docking score for the other agonists ranged from −52.4 (AM-5262) to −32.6 (AM-8182) which all are regarded as good scores. Surprisingly, the two Gq + Gs agonists, AM-1638 (−44.6) and AM5262 (−52.4) docked better into the TAK-875 pocket than the three Gq-only agonists, AM-8182 (−32.6), MK-2305 (−33.4) and AMG-837 (−37.4) (Figure 3B–H). Importantly, all the GPR40 agonists docked into the TAK-875 site with rather low intramolecular strain of the ligand i.e. accepted in low energy conformations. Interestingly, the different chemical moieties extending from the propionic acid group in several of the agonists nicely fitted into the small side-pocket (pocket #2) not used by TAK-875, for example the propyne, propene and cyclopropyl moiety of AMG-837, AM-8182 and AM-1638, respectively (Figure 3C,F and H). In contrast to the agonists, the potent dihydro-isoquinoline based GPR40 antagonist TUG-761 could not be docked into the TAK-875 binding pocket in any meaningful way, do to major structural differences compared to the agonists as illustrated in Figure 3I, where TUG-761 is overlaid on the structures of the collection of agonists as they docked into the TAK-875 site. Attempts to dock the agonists or the antagonist into the very polar pocket #3 or the shorter pocket #4 were also unsuccessful.

It is concluded that all the agonists in contrast to the antagonist TUG-761 dock very well into the TAK-875 binding site in the high resolution X-ray structure of GPR40 and that the Gq + Gs agonists surprisingly dock at least as good if not better than the Gq-only agonists in this site.

3.4. GPR40 agonist-mediated GLP-1 secretion *ex vivo*

In mouse colonic crypt cultures direct treatment with the endogenous LCFAs ALA or DHA led to 20- to 30-fold large increases in GLP-1 release above basal, which however were also observed in cultures from *Gpr40*-deficient mice (data not shown). However, treatment of the primary cell cultures with BSA-conjugated ALA resulted in small but GPR40-dependent GLP-1 release of 1.4 ± 0.06 fold above basal at 30 μ M (Figure 4A). Slightly larger increases in GLP-1 secretion of 2.0 ± 0.2 and 2.6 ± 0.3 fold above basal were observed with concentrations of 0.1 and 1 mM of BSA-conjugated ALA (Figure 4A). However, at these concentrations a large fraction of the responses was also observed in cells from *Gpr40*-deficient mice and even in cells from *Gpr40/Gpr120* double-deficient mice (Figure 4A), indicating that even when using BSA as a carrier ALA cause unspecific damage in this cell-based assay. Similar results were obtained with BSA-conjugated DHA (data not shown). Importantly, the small GPR40-dependent GLP-1 response to ALA was only a fraction of the 4.7 ± 0.3 fold increase in GLP-1 observed with the positive control, the GPR119 agonist AR231453.

In contrast, the two synthetic Gq-only GPR40 agonists, TAK-875 and MK-2305, both stimulated GLP-1 secretion from the colonic crypt cultures in a GPR40-dependent way, albeit still with efficacies less than the GPR119 agonist, AR231453, i.e. with responses of 1.5 ± 0.08 and 2.8 ± 0.4 fold above basal, respectively (Figure 4B). However, surprisingly the third Gq-only agonist, AM-8182, which in respect of stimulation of IP turnover *in vitro* acted as a highly

efficacious GPR40 agonist only gave a small GLP-1 response of 1.4 ± 0.2 fold above vehicle treated cells and a similar response, was observed in cells from *Gpr40*-deficient animals (Figure 4).

In contrast to the Gq-only the two Gq + Gs GPR40 agonists AM-1638 and AM-5262 both stimulated GLP-1 secretion from the mouse colonic crypt cultures with efficacies similar to and even higher than the GPR119 positive control agonist, i.e. 3.0 ± 0.5 fold and 4.6 ± 0.8 fold, respectively, which in the case of AM-5262 is the highest receptor mediated efficacy we have yet observed in this preparation. For both of the Gq + Gs agonists the GLP-1 stimulation was GPR40-dependent (Figure 4B).

3.5. Effect of GPR40 agonists on GLP-1 and GIP release *in vivo*

The two Gq-only GPR40 agonists, which were efficacious in the *ex vivo* colonic crypt cultures, TAK-875 and MK-2305 and the two Gq + Gs agonists, AM-1638 and AM-5262 were tested *in vivo* in lean mice after an overnight fast dosed orally with 30 mg/kg based on initial dose-range finding experiments. The four GPR40 agonists were all tested head-to-head and against vehicle with 6–8 mice in each group in two individual experiments one of which is shown in Figure 5A. In total, three to five experiments were done with each compound in various combinations and a summation of all the experiments is shown in Figure 5B.

Concerning the Gq-only agonists, TAK-875 increased plasma GLP-1 significantly in three experiments but only with a relatively small median increase from 5.1 to 10.8 pmol/L (Figure 5B left panel). MK-2305 increased plasma GLP-1 levels significantly in all five experiments with a median increase from 5 to 12 pmol/L (Figure 5B). The Gq + Gs GPR40 agonists both increased plasma GLP-1 significantly in all experiments and to a larger degree, i.e. both AM-1638 and AM-5262 increased plasma GLP-1 from 5 to 29 pmol/L (Figure 5B). In the head-to-head experiments the Gq + Gs agonists increased plasma GLP-1 significantly more than the Gq-only agonists (Figure 5A).

Concerning effects on GIP, TAK-875 only increased plasma GIP significantly in one of the three experiments, whereas MK-2305 increased plasma GIP in four out of the five experiments giving a median increase from 53 to 108 pmol/L (Figure 5B right panel). Also in the case of GIP, the two Gq + Gs agonists were more efficacious and increased plasma GIP to 383 (AM-1638) and 345 pmol/L (AM-5262) (Figure 5B). In the head-to-head experiments the increases in plasma GIP were significantly higher for the Gq + Gs agonists than for the Gq-only agonists (Figure 5A). The *in vivo* stimulatory effects of AM-5262 on both GLP-1 and GIP were GPR40-dependent, (Figure 5C). The *in vivo* stimulatory effects were similar in male and female mice (Figure 5B,C).

4. DISCUSSION

In the present study we find that GPR40, which generally is considered to be a Gq coupled receptor surprisingly can signal efficiently through Gs to increase intracellular cAMP; however not in response to the endogenous lipid ligands but in a ligand-dependent biased way, i.e. in response to some but not other synthetic agonists. Importantly, compounds which are capable of activating both the Gq and the Gs signaling pathway are particular efficient incretin secretagogues, i.e. these compound stimulate GLP-1 and GIP secretion robustly both *ex vivo* and *in vivo*.

4.1. Gq and Gs co-action on hormone secretion

Traditionally, both Gq and Gs signaling have been strongly implemented in peptide hormone secretion. However, the relative

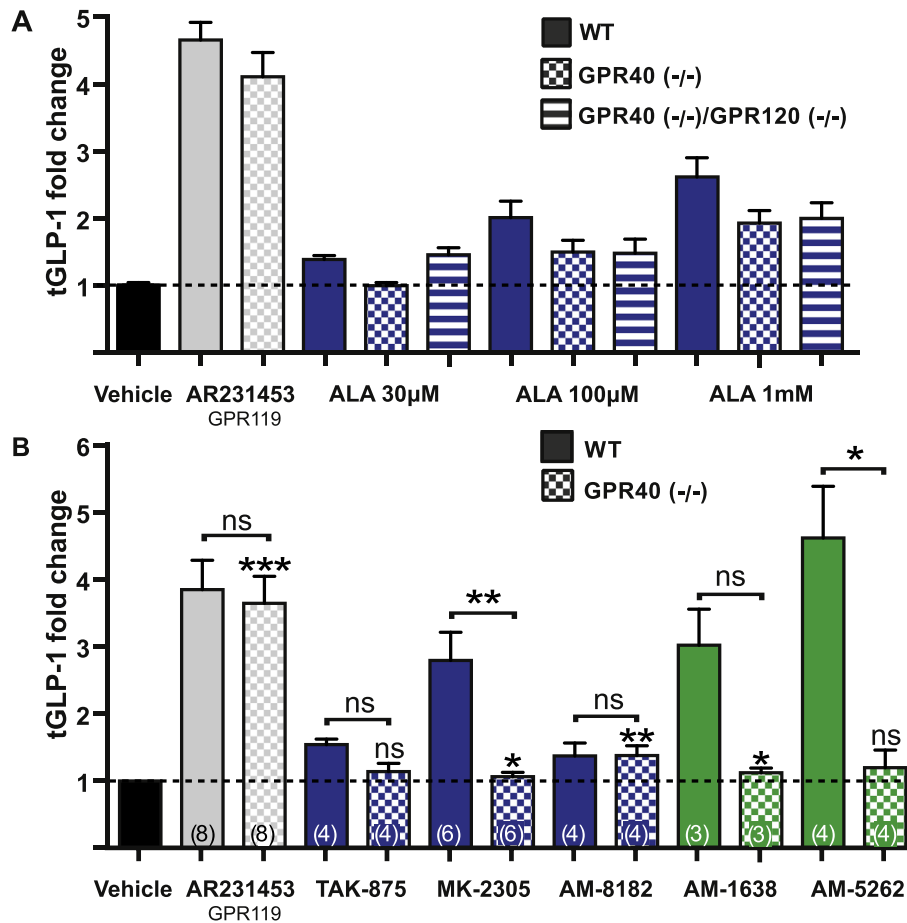


Figure 4: GPR40 agonist-dependent GLP-1 release *ex vivo* – in murine colonic crypt cultures. (A) Total GLP-1 (tGLP-1) release from primary cell cultures from WT (solid), GPR40(-/-) mice (grid) or GPR40/GPR120 double deficient mice (stripes) treated with 1 μ M GPR119 agonist AR231453 or varying concentrations of α -linolenic acid (ALA) as indicated in complex with BSA. One out of two experiments performed in triplicates is shown. (B) As panel A but treated with 1 μ M GPR40 synthetic agonists TAK-875, MK-2305, AM-8182, AM-1638 or AM-5262. Numbers in brackets indicate number of repeated experiments in triplicates. Data are normalized to the basal secretion of tGLP-1 from vehicle treated cells and are shown as mean \pm SEM. Statistical significance of WT compared to GPR40(-/-) (crotchets) or vehicle compared to GPR40(-/-) (above) is denoted by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) as determined by non-parametric unpaired two-tailed *t*-test (Mann–Whitney).

importance and precise mechanisms vary from system to system. In general, the main routes from Gq to hormone secretion goes through phospholipase C (PLC), IP3 and increase in intracellular Ca²⁺ released from ER stores which acts directly on the molecular exocytosis machinery for the hormone-containing secretory vesicles (Figure 6). On the other hand, the pathway from Gs classically goes through adenylyl cyclase, increase in cAMP and either PKA or Epac (exchange protein directly activated by cAMP) [38,39].

Potentiating interaction between the cAMP and Ca²⁺ signaling pathways in hormone secretion is probably best described in the β -cell where, for example the GLP-1 receptor-mediated increase in cAMP potentiates glucose-induced insulin secretion elicited by intracellular Ca²⁺, which in this case is initiated through the ATP-sensitive potassium channel and opening of voltage-dependent calcium channels in the cell membrane [40]. Thus, a combined increase in intracellular cAMP and Ca²⁺, which can be achieved in several different manners, is a highly efficient way of stimulating hormone secretion. In the case of incretin secretion this can be achieved physiologically through concomitant activation of different Gq- and Gs-coupled receptors and pharmacologically through activation of, for example GPR40 by Gq + Gs agonists as described in the present study.

4.2. GPR40 acts jointly with Gs-coupled receptors during physiological sensing of dietary triglycerides

When it originally was proposed that GPR40 mediates LCFA stimulation of incretin secretion, this was not based on LCFA challenges but only inferred from studies with triglycerides in wild type versus knock out animals [22,29,41]. Here we find that LCFAs like the Gq-only synthetic agonists stimulate GLP-1 secretion with low efficacy *ex vivo* and only signals through GPR40 via Gq and not Gs *in vitro* (Figure 2A). Interestingly, in order to get appropriate stimulation of GIP secretion by, for example linoleic acid, Parker and coworkers had to pre-treat the primary cell cultures with forskolin and IBMX, which increase cAMP concentrations [42]. Importantly, the selective orthosteric GPR40 agonist TAK-875 is also rather poor in stimulating incretin secretion and did not increase plasma GLP-1 at therapeutic doses in clinical trials [25]. Thus, it appears that stimulation of GPR40 by LCFAs or by orthosteric agonists is not an efficient stimulus for incretin release – on its own. However, under physiological circumstances dietary triglycerides are sensed by the enteroendocrine cells not only in the form of LCFAs but also by 2-MAG acting on the Gs coupled receptor GPR119 (Figure 6) [9]. Moreover, another important part of the physiological absorption and digestion of dietary triglycerides is their formation of

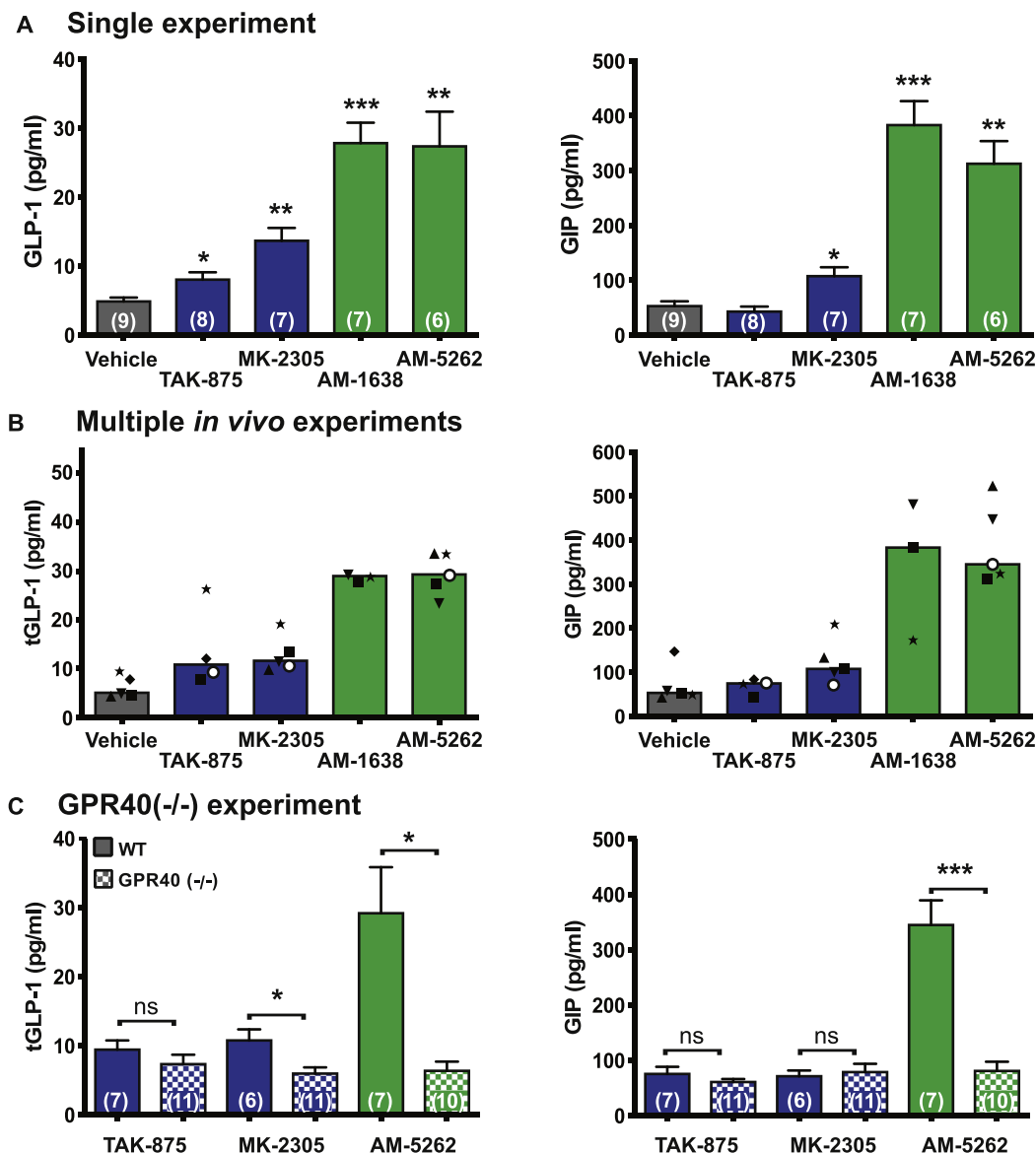


Figure 5: GLP-1 and GIP plasma responses to GPR40 agonists *in vivo* in mice. tGLP-1 and GIP plasma concentrations were measured 30 min after an oral administration of 30 mg/kg of the orthosteric agonists TAK-875, MK-2305 (in blue) and the ago-allosteric agonists, AM-1638 or AM-5262 (in green). (A) A representative head-to-head experiment in male B6 mice; (B) Summation of multiple experiment (X–Y) using the four ligands in various combinations, column indicate the median of incretin response in all experiments and symbols indicate response in each experiment (black squares – experiment in ‘A’); (C) Effect of the GPR40 agonists in female WT versus GPR40(–/–) littermate mice (this experiment is shown in open circle in ‘B’). Statistical significance compared to vehicle (A) or GPR40(–/–) (C) is denoted by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) as determined by unpaired *t*-test assuming Gaussian distribution with Welch’s correction.

micelles with bile acids, which are liberated in the distal small intestine to be sensed by the Gs-coupled TGR5 receptor (Figure 6). Thus, although the LCFAs by themselves only result in a rather inefficient Gq signaling through GPR40, dietary triglycerides are in fact highly efficient stimulators of incretins conceivably as a result of a joint action of LCFAs through the Gq-coupled GPR40 plus 2-MAG and bile acids acting through the Gs-coupled GPR119 and TGR5, respectively (Figure 6).

4.3. Pharmacological activation of both Gq and Gs by certain synthetic GPR40 agonists

As demonstrated in the present study, efficient pharmacological stimulation of both Gq and Gs can be obtained through GPR40 alone with agonists such as AM-1638 and AM-5262 and we propose that this

dual signaling property is the molecular pharmacological property associated with efficient stimulation of incretin release both *ex vivo* and *in vivo*. However, although these compounds are clearly able to stimulate cAMP *in vitro* in transfected ‘test tube cells’, we do not yet know whether such compounds actually exploit the Gs signaling pathway *in vivo* in the enteroendocrine GLP-1 and GIP cells or for that matter in the pancreatic β -cells. We just know that GPR40 agonists, who are able to increase cAMP also, are efficacious in stimulating incretin secretion. AM-1638 and AM-5262 have previously been referred to as ‘full agonists’, which is based on the fact that in certain *in vitro* Gq measuring systems such as Ca^{2+} , they display efficacies which are similar to ALA and DHA as opposed to, for example AMG-837 and TAK-875, which accordingly have been called ‘partial agonists’, i.e. in particular in

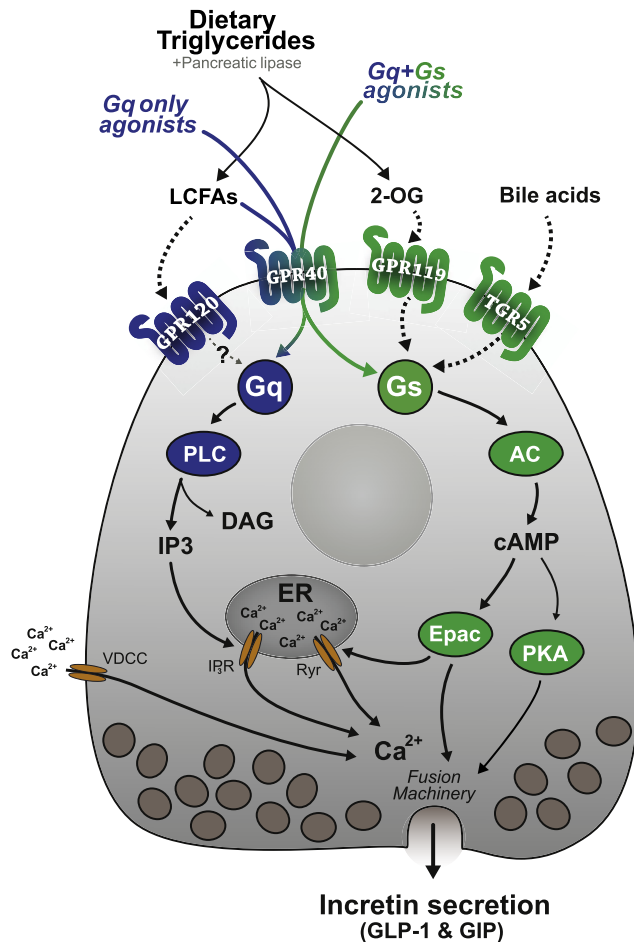


Figure 6: Schematic overview of signal transduction pathways connecting receptors sensing triglyceride metabolites with incretin secretion. In the physiological setting triglyceride ingestion will lead to concomitant LCFAs stimulation of the Gq-coupled GPR40 and potentially but probably not GPR120[14]; 2-monoacyl glyceride stimulation of Gs through GPR119 and bile acid stimulation of Gs through TGR5 resulting in strong incretin secretion. In the pharmacological setting orthosteric GPR40 agonists (blue) will like LCFAs only stimulate Gq pathways resulting in a rather limited incretin response on their own. In contrast, ago-allosteric agonists (blue and green) will stimulate both Gq and Gs pathways leading to a robust incretin response.

relation to stimulation of intracellular Ca^{2+} [26,27,31]. In the present study we also find that AM-1638 and AM-5262 have higher maximal efficacies in IP_3 signaling than, for example TAK-875 and AMG-837. However, in our hands all of these ligands display efficacies above the efficacy of the endogenous ligands ALA and DHA (Figure 1). Importantly, there is not a direct correlation between high efficacy in Gq signaling and high signaling through Gs and there is not a direct correlation between high efficacy in Gq signaling and robust incretin secreting effect, as exemplified by AM-8182, which is as efficient as AM-1638 and AM-5262 in respect of Gq signaling but very poorly if at all in signaling through Gs and does not release GLP-1. However, there are compounds such as MK-2305, which does not signal through Gs, but in certain experiments does increase plasma GLP-1 and GIP fairly well, albeit in total not as well as AM1638 and AM5262 (Figure 5).

4.4. Complex allosteric properties of GPR40 ligands in binding assays

The clear positive cooperative effects of the Gq-only and the Gq-Gs agonists on each other as determined in our radio ligand binding

assays confirm the complex allosteric properties of GPR40 ligands [26,27,31]. Swaminath and coworkers have pioneered the field of developing and characterizing ago-allosteric modulators for GPR40 where they recently showed that certain second generation ago-allosteric agonists can improve glucose tolerance not only through direct stimulation of insulin secretion but also through stimulation of incretins [27,29,31].⁷ They suggested that there may be even two allosteric sites or binding modes for the structurally closely related agonists besides the orthosteric binding site for the endogenous lipid ligands [27]. As observed in other systems the properties of the GPR40 ligands are probe-dependent and for example give very different allosteric pictures dependent upon which radio-ligand is used. Thus, in the present study we find that both AM-8182 and AM-837 compete with very similar high potency and full efficacy against [3H]-L358 indicating that all three ligands share a common binding mode (Figure 2). In contrast, Lin and coworkers found that AM-8182 displaced [3H]-AM837 with several orders of magnitude lower affinity and based on these binding data and on functional data, they suggest that AM-8182 binds to another site than AM-837 [27]. From a functional point-of-view positive cooperativity has been demonstrated between different types of synthetic agonists and between the synthetic agonists and the endogenous lipid ligands, such as DHA or ALA, even in cases where the ligands show negative cooperativity in binding experiments [27]. For example, although TAK-875 only stimulates insulin secretion with a rather low efficacy on its own, it has robust glucose-dependent stimulatory effects on insulin secretion in combination with the endogenous LCFA γ -linoleic acid [26].

4.5. Apparent simple, common binding modes for agonists in the GPR40 X-ray structure

In view of the fact that the different types of agonists show strong signs of allosteric binding properties, it was highly surprising that all agonists docked nicely with minimal internal strain and with high energy scores into a common binding site in the X-ray structure of GPR40 (Figure 3). Importantly, although several other potential binding pockets could be identified in the ‘main ligand-binding pocket’ of the receptor structure where ligands bind in other 7TM GPCRs [46–48] (Figure 3), it was not possible to dock any of the known agonists into these alternative pockets in any meaningful way.

It should be noted that in order to obtain the crystal structure a number of termo-stabilizing mutants were introduced in GPR40 one of which was an Ala-substitution of Phe88 (Phe11:10) in TM-III located just below the binding pocket for TAK-875. Most importantly, although the five termo-stabilizing mutants did not affect ligand binding, the receptor could not be activated by the agonists [32]. In other words, we are docking ligands into an X-ray structure of GPR40 which has been ‘frozen’ in a conformation where the allosteric mechanism between the agonist binding site and the G proteins, in this case Gq or Gs is no longer present and where a large Phe residue, which perhaps could interchange between different rotameric forms, has been exchanged with an Ala residue – close to the binding pocket. Thus, although the present GPR40 structure is a great starting point, it is only the first picture of a very complex allosteric receptor system, where agonists and allosteric modulators could stabilize active conformations at different or at more or less overlapping orthosteric and allosteric sites as previously proposed for ago-allosteric agonists [30,49]. GPR40

⁷ According to classical pharmacological concepts and definitions, an agonist could not be an allosteric modulator [43,44]. However, many synthetic agonists are in fact able to increase the potency and/or the efficacy of the endogenous agonist, i.e. they are ago-allosteric modulators [30], which also now is acknowledged by IUPHAR [45].

appears to be a particularly interesting system to study allosteric mechanisms in and with a wealth of pharmacological tools. With all the many X-ray structures available today, we are starting to get a picture of the structural basis for allosteric binding modes and for biased signaling in 'multi-state'-models allowing multiple ligand-specific conformations [50,51].

5. CONCLUSION

Under physiological conditions GPR40 is probably a strictly Gq-coupled receptor being activated by LCFAs which alone only provides a rather poor incretin response but conceivably act in symphony with the Gs-coupled receptors GPR119 sensing the other major triglyceride metabolite 2-MAG and TGR5 being stimulated by bile acids to collectively elicit a strong incretin response. A similar robust incretin response can be obtained pharmacologically through GPR40 alone with Gq + Gs agonists, which in contrast to LCFAs and synthetic Gq-only agonists can bias GPR40 to signal through both Gq and Gs at the same time. This biased signaling and strong incretin response by ago-allosteric agonists could make GPR40 an even more interesting target for the future treatment of diabetes.

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CONFLICT OF INTEREST

None declared.

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