GPR119, a Major Enteroendocrine Sensor of Dietary Triglyceride Metabolites Coacting in Synergy With FFA1 (GPR40)

Jeppe H. Ekberg,* Maria Hauge,* Line V. Kristensen, Andreas N. Madsen, Maja S. Engelstoft, Anna-Sofie Husted, Rasmus Sichlau, Kristoffer L. Egerod, Pascal Timshel, Timothy J. Kowalski, Fiona M. Gribble, Frank Reiman, Harald S. Hansen, Andrew D. Howard, Birgitte Holst, and Thue W. Schwartz

NNF Center for Basic Metabolic Research (J.H.E., M.H., L.V.K., A.N.M., M.S.E., A.-S.H., R.S., K.L.E., B.H., T.W.S.), Section for Metabolic Receptology; Laboratory for Molecular Pharmacology (J.H.E., M.H., L.V.K., M.S.E., A.-S.H., R.S., K.L.E., B.H., T.W.S.), Department of Neuroscience and Pharmacology; and Danish Diabetes Academy (M.S.E.), 5000 Odense, Denmark; Novo Nordisk Foundation Center for Basic Metabolic Research (P.T.), Section for Metabolic Genetics, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2200 Copenhagen, Denmark; Merck Research Laboratories (T.J.K., A.D.H.), Kenilworth, New Jersey 07033; Cambridge Institute for Medical Research and MRC Metabolic Diseases Unit (F.M.G., F.R.), University of Cambridge, Cambridge, CB20QQ United Kingdom; Department of Drug Design and Pharmacology (H.S.H.), University of Copenhagen, DK-2100 Copenhagen, Denmark

Triglycerides (TGs) are among the most efficacious stimulators of incretin secretion; however, the relative importance of FFA1 (G Protein-coupled Receptor [GPR] 40), FFA4 (GPR120), and GPR119, which all recognize TG metabolites, ie, long-chain fatty acid and 2-monoacylglycerol, respectively, is still unclear. Here, we find all 3 receptors to be highly expressed and highly enriched in fluorescence-activated cell sorting-purified GLP-1 and GIP cells isolated from transgenic reporter mice. In vivo, the TG-induced increase in plasma GIP was significantly reduced in FFA1-deficient mice (to 34%, mean of 4 experiments each with 8–10 animals), in GPR119-deficient mice (to 24%) and in FFA1/FFA4 double deficient mice (to 15%) but not in FFA4-deficient mice. The TG-induced increase in plasma GLP-1 was only significantly reduced in the GPR119-deficient and the FFA1/FFA4 double deficient mice, but not in the FFA1, and FFA4-deficient mice. In mouse colonic crypt cultures the synthetic FFA1 agonists, TAK-875 stimulated GLP-1 secretion to a similar extent as the prototype GLP-1 secretagogue neuromedin C; this, however, only corresponded to approximately half the maximal efficiency of the GPR119 agonist AR231453, whereas the GPR120 agonist Metabolex-209 had no effect. Importantly, when the FFA1 agonist was administered on top of appropriately low doses of the GPR119 agonist, a clear synergistic, ie, more than additive, effect was observed. It is concluded that the 2-monoacylglycerol receptor GPR119 is at least as important as the long-chain fatty acid receptor FFA1 in mediating the TG-induced secretion of incretins and that the 2 receptors act in synergy, whereas FFA4 plays a minor if any role. (*Endocrinology* 157: 4561–4569, 2016)

Triglycerides (TGs) are known to be highly efficacious secretagogues for gut hormones such as cholecystokinin (CCK) and the incretins, GIP and GLP-1 (1, 2). It is, however, not the intact TGs, which are sensed by the enteroendocrine cells. TGs are digested by pancreatic lipase to generate long-chain fatty acids (LCFAs) and 2-mono-

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* J.H.E. and M.H. contributed equally to this work.

Abbreviations: CCK, cholecystokinin; FACS, fluorescence-activated cell sorting; GPCR, G protein-coupled receptor; KO, knock out; LCFA, long-chain fatty acid; 2-MAG, 2-monoacylglycerol; NMC, neuromedin C; 2-OG, 2-oleoylglycerol; qPCR, quantitative PCR; TG, triglyceride; WT, wild type.

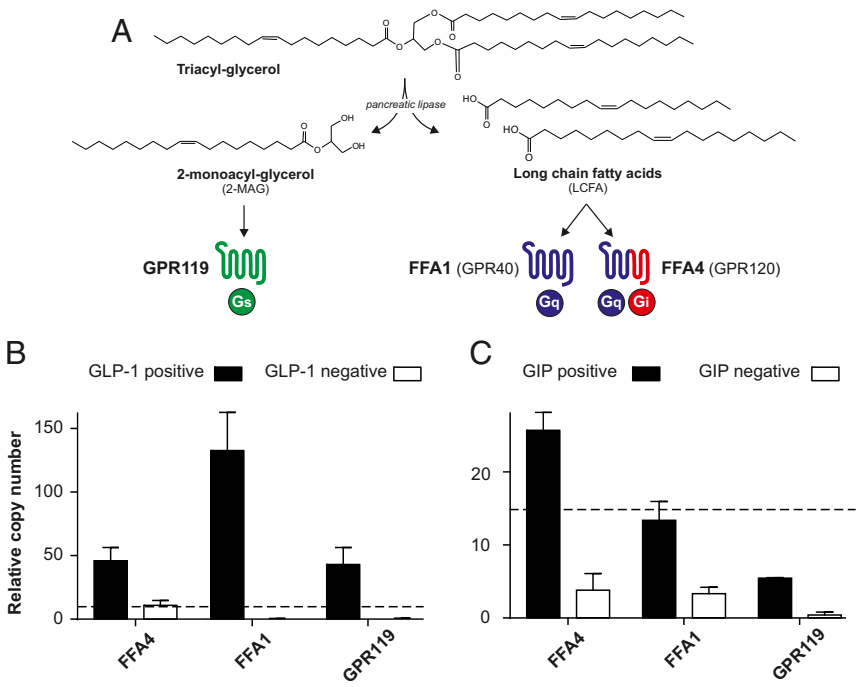

Figure 1. TG metabolite receptors and their expression in GIP and GLP-1 cells. A, Overview of the digestion of dietary TGs by pancreatic lipase generating LCFAs, which potentially could be acting through the 2 receptors GPR40 and GPR120, and 2-MAG, which could be acting through GPR119 to stimulate gut hormone secretion. B, Expression of the 3 TG metabolite receptors in FACS-purified murine GLP-1 cells, ie, Gcg-Venus-positive cells (closed columns) and in neighboring Gcg-Venus-negative cells (open columns) isolated from the Gcg-Venus reporter mice, n = 3. C, Expression of the 3 TG metabolite receptors in FACS-purified murine GIP cells, ie, Gip-Venus-positive cells and in neighboring Gip-Venus-negative cells isolated from the Gip-Venus reporter mice; n = 3. The dotted line indicates the median expression level for GPCRs expressed above noise level in the cells. The expression is given as relative copy number of transcripts as described in Materials and Methods (19).

acylglycerol (2-MAG), and it is generally assumed that it is the LCFAs which are stimulating the enteroendocrine cells to release hormones (3–5). Two G protein-coupled receptors (GPCRs), GPR40 (FFA1) and GPR120 (FFA4), are known to be activated by LCFAs (6, 7). Both receptors are coupled to Gαq and have been reported to be expressed in the gut and specifically in, for example, CCK, GIP, and GLP-1 cells (8–10). Both FFA1 and FFA4 have been implicated as important regulators of incretin hormone secretion (7, 11, 12), and it is accordingly assumed that the strong effects of TG on gut hormone secretion in general and incretin hormone secretion in particular is mediated through stimulation of FFA1 and FFA4 by LCFAs liberated from TGs by pancreatic lipase digestion (Figure 1A).

Recently, it was, however, shown that the other major TG metabolite, 2-MAGs and in particular 2-oleoylglycerol (2-OG), can act as an agonist for the Gαs-coupled receptor, GPR119, which is also expressed on enteroendocrine cells (10, 13, 14). Although the potency of 2-OG is only in the micromolar range, the local concentrations of 2-MAG can reach such high levels during ingestion of a lipid-rich meal (14). Importantly, although GPR119 agonists have not been particularly successful in the clinics as antidiabetes agents (15, 16), they are, nevertheless, efficacious incretin secretagogues (17, 18). Thus, the stimulatory effect of TGs on gut hormone release could potentially be mediated not only by the LCFAs but also by 2-MAG (Figure 1A).

The present study was designed to clarify the relative importance of the 3 receptors, which all recognize TG metabolites, FFA1, FFA4, and GPR119, for the strong stimulatory effect of TGs on the secretion of the 2 incretin hormones GIP and GLP-1. In vivo studies in animals deficient in either FFA1, FFA4, or both or deficient in the 2-MAG receptor GPR119 are here combined with ex vivo studies of hormone release in primary cell cultures using highly selective agonists for each of the 3 receptors either alone or in combinations. Surprisingly, we discovered that the 2-MAG receptor GPR119 is at least as important for the sensing of TGs as the LCFA receptor FFA1, whereas the other LCFA receptor FFA4 apparently plays a rather minor, if any, role in the direct regulation of hormone secretion.

Results

Receptors for TG metabolites are highly expressed on GLP-1 and GIP cells

The expressions of GPCRs were analyzed in fluorescence-activated cell sorting (FACS)-purified Gcg-Venus and Gip-Venus-positive cells isolated from small intestine of the corresponding, previously published, reporter mice (9, 10) using a dedicated quantitative PCR (qPCR) array (19, 20). As shown in Figure 1B, the LCFA receptors FFA1 and FFA4 as well as the 2-MAG receptor GPR119 are all highly expressed in the Gcg-Venus-positive cells, ie, GLP-1 cells. In fact, the transcript for FFA1 was among the most highly expressed GPCR in the GLP-1 cells. As shown in Figure 1B, all 3 receptors were highly enriched in the GLP-1-positive cells with GPR119 being enriched more than 1000-fold. FFA4 was equally highly expressed as GPR119 but only enriched 4-fold in the GLP-1 cells as

compared with the neighboring cells (Figure 1B). Also in the GIP-Venus-positive cells, all 3 receptors were relatively highly expressed, although the transcripts for FFA1 and GPR119 were not as enriched as observed in the GLP-1 cells (Figure 1C). In the GIP cells, FFA4 was apparently more highly expressed than FFA1 and GPR119.

In vivo dependency of the GLP-1 and GIP responses to TG on FFA1, FFA4, and GPR119

Oral TG is one of the most efficient stimuli for both GIP and GLP-1 secretion. Here, we used a standard oral olive oil challenge in mice deficient for FFA1, FFA4, and GPR119, respectively, as well as mice deficient in both FFA1 and FFA4 as compared with littermate wild type (WT) control mice. Plasma GIP and GLP-1 were measured 60 minutes after the TG challenge, ie, a time point determined to be optimal in previous time-course experiments (Supplemental Figure 1). Three experiments with 6–8 animals in each group were performed with littermates vs knockout (KO) animals and 1 experiment using wild-type C57BL/6 mice as controls. In Figure 2, A and C, are shown the GIP and GLP-1 responses from a representative experiment and in Figure 2, B and D, are shown normalized results from all 4 experiments. Statistical analysis of each experiment is shown in Supplemental Table 1, and statistical analysis for the multiple experiments is shown in Supplemental Table 2.

As expected, oral TG induced a robust response in both plasma GIP and GLP-1 in the WT animals. Although statistically significant reduction in the GIP response to TG was observed in 2 of the 4 experiments performed in the FFA4 KO animals compared with WT the overall effect of FFA4 deficiency was not nominally significant ($P = .063$) when all 4 experiments were analyzed together by a mixed effect model (see Materials and Methods). In contrast, a

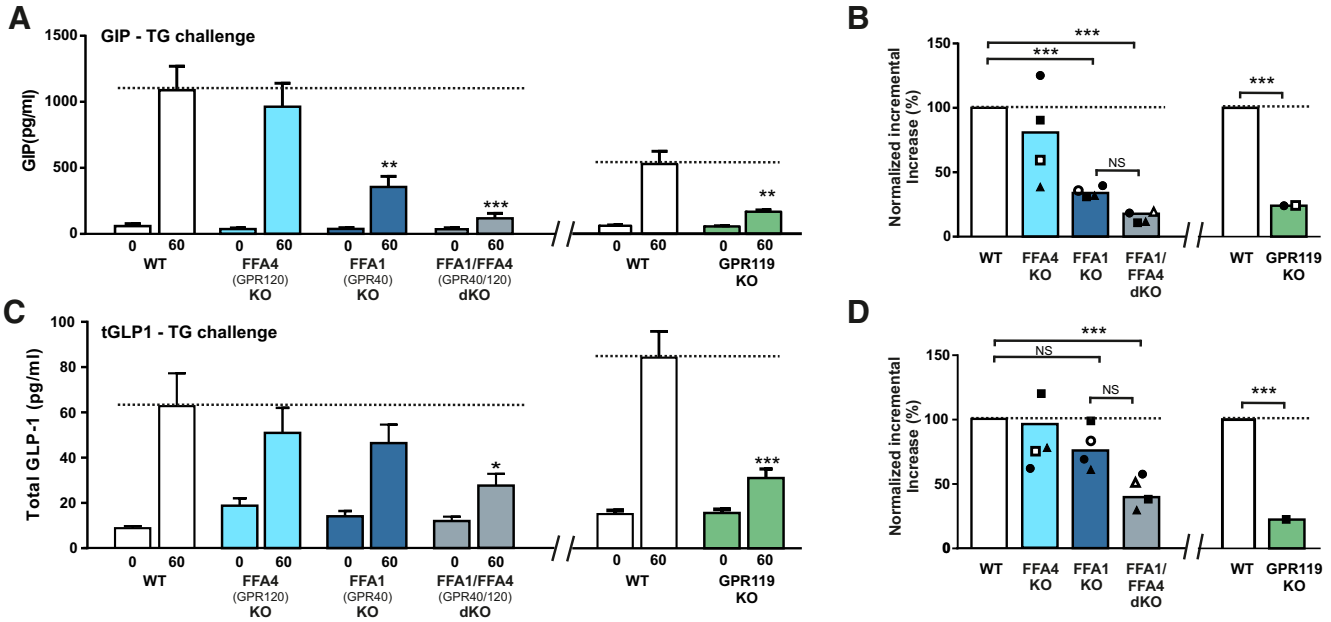

Figure 2. Plasma GIP and GLP-1 responses to oral TG challenge in mice, dependency on the metabolite receptors FFA1 (GPR40), FFA4 (GPR120), and GPR119. A, Plasma GIP levels before and 60 minutes after an oral gavage challenge with 10 mL/kg of olive oil in FFA4 (GPR120), FFA1 (GPR40), and double deficient mice vs littermate wild-type control mice (left panel) and in GPR119-deficient mice vs littermate control mice (right panel). A representative experiment out of 4 with n = 8–10 animals in each group is shown. The FFA1- and FFA4-deficient animals were taken from breeding to obtain the double deficient animals and accordingly the WT littermates are used as joint controls; *, $P < .05$; **, $P < .001$ according to one-way ANOVA Dunnett's test (see also Supplemental Table 1). B, Columns are the estimated differences according to Turkey's post hoc test for all 4 experiments similar to the one shown in A, left panel (indicated in filled square symbols), except that in 1 experiment, wild-type C57BL/6 mice were used as controls (open symbols). In the right panel is shown the mean GIP response from 2 experiments with GPR119-deficient mice and littermate controls; ***, $P < .001$ according to a linear mixed effect model (see Materials and Methods); NS, no significant difference (see also Supplemental Table 2). C, Plasma GLP-1 levels before and 60 minutes after an oral gavage challenge with 10 mL/kg of olive oil in FFA4 (GPR120), FFA1 (GPR40), and double deficient mice vs littermate wild-type control mice (left panel) and in GPR119-deficient mice vs littermate control mice (right panel). A representative out of 4 experiment each with n = 8–10 animals in each group is shown. The FFA1- and FFA4-deficient animals were taken from a breeding to obtain the double deficient animals and accordingly the littermate WT animals are used as joint controls; *, $P < .05$; **, $P < .001$ according to one-way ANOVA Dunnett's test (see also Supplemental Table 1). D, Columns are the estimated difference according to Turkey's post hoc test for 4 experiments similar to the one shown in A (indicated in filled square symbols), except that in 1 experiment, wild-type C57BL/6 mice were used as controls (open symbols). In the right panel is shown the mean GLP-1 responses from 2 experiments with GPR119-deficient mice vs littermate WT controls; ***, $P < .001$ according to a linear mixed effect model (see Materials and Methods; see also Supplemental Table 2).

highly significant reduction of the GIP response to TGs was observed both in the FFA1 KO animals ($P < .001$) and in the FFA1/FFA4 double KO animals as compared with WT animals ($P < .001$). Although the nominal reduction in the GIP response to TGs, ie, the estimated difference according to Turkey's post hoc test, was larger in the double KO animals as compared with the FFA1 KO animals, ie, −86% vs −66% (see Materials and Methods), this difference was not statistically significant ($P = .279$).

In the GPR119 KO mice, the GIP response to TG was reduced significantly ($P < .001$), ie, with 75.9% as compared with WT animals (green columns in Figure 2, A and B).

Concerning GLP-1, the TG response was not reduced significantly in the FFA4 and FFA1 single KO animals neither in any of the individual experiments nor when all 4 experiments were analyzed together by the linear mixed effect model ($P = .959$ and $P = .238$, respectively). In contrast, a highly significant reduction of the TG induced GLP-1 response was observed in the FFA1/FFA4 double KO animals of 59% ($P < .001$) and was significantly larger ($P < .001$) than the nonsignificant reduction of 6.3% estimated for the FFA4 KO animals but not significantly larger than the nonsignificant reduction of 24.7% estimated for the FFA1 animals.

As observed for GIP, a highly significant ($P < .001$) reduction of 78% of the GLP-1 response to TG was observed in the GPR119 KO animals as compared with littermate controls (Figure 2, C and D).

It is concluded that GPR119 plays a major role in the incretin response to TGs, which is clearly observed both for GIP and GLP-1, whereas the role for the 2 LCFA receptors is more unclear. Apparently, FFA1 was only important for GIP but not for GLP-1, whereas FFA4 alone was not important for the TG response of any of the 2 incretins; nevertheless, the effect of the FFA1/FFA4 double KO was significant for both incretins and of similar magnitude as the effect of the GPR119 KO.

Stimulation of GLP-1 secretion ex vivo by FFA1-, FFA4-, and GPR119-selective agonists

FFA1, FFA4, and GPR119 have all been proposed to stimulate GLP-1 secretion mainly based on experiments with not entirely selective ligands (7, 12, 17, 21). Today, highly selective, synthetic agonists are available for all 3 receptors (19, 22–24), which we here use to study effects on GLP-1 release from murine colonic crypt cultures. As shown in Figure 3A, the prototype GLP-1 secretagogue, neuromedin C (NMC), which is a bombesin 2 receptor agonist, increased GLP-1 secretion 1.5-fold. The selective FFA1 agonist, TAK-875 or fasiglifam (10^{-5}M), stimulated GLP-1 secretion to a similar extent (1.6-fold) as did NMC but in a FFA1-dependent manner, because no response was observed in cells isolated from FFA1-deficient mice (Figure 3A). No increase in GLP-1 secretion was observed in cells stimulated with the FFA4-selective agonist Metabolex-209 (10^{-5}M), ie, a compound which we previously have observed to efficiently inhibit ghrelin secretion in a FFA4-dependent manner (19). In contrast, the GPR119 prototype agonist AR231453 (10^{-6}M) stimulated GLP-1 release with high efficacy, ie, 2.5-fold and in a GPR119-dependent manner (Figure 3A). Dose-response experiments demonstrated that the EC_{50} for AR231453 in respect of stimulation of GLP-1 release from the murine colonic crypt cultures was 2.6nM (Figure 3B), previous measurements have shown an EC_{50} of 12nM from an in vitro HTRF cAMP assay (22).

Probing for synergy between the metabolite receptors ex vivo

Because FFA1 and FFA4 are generally believed to couple mainly through Gαq under physiological circumstances, although GPR119 is a strongly Gαs-coupled receptor, it is likely that these receptors could act in synergy in the enteroendocrine cells through activation of the 2 distinct signal transduction pathways. In order to be able to observe synergistic effects, it is necessary to use highly selective ligands and to use doses that, on their own, only provide a suitable small response. For the FFA1 agonist, we chose TAK-875 in doses of 10^{-6}M and 10^{-5}M, which gave appropriately small responses (Figure 3C, blue symbols), and for the GPR119 agonist AR231453, we used doses of 10^{-10}M and 10^{-9}M based on the dose-response experiments shown in Figure 1D (Figure 3C, green symbols).

When the GPR119 agonist AR231453 was administered in a dose of 10^{-10}M, which on its own gave a minimal GLP-1 response (green), together with the FFA1 agonist TAK-875 (blue) to mouse colonic crypt cultures, a synergistic, ie, more than additive, effect was observed in respect of stimulation of GLP-1 secretion (Figure 3C, orange being more than blue plus green). A more than additive effect was also observed with a 10^{-9}M dose of AR231453 resulting in GLP-1 release corresponding at least to the maximal response normally achieved with a dose of 10^{-6}M GPR119 agonist alone (Figure 3, B and C). Importantly, when all experiments using coadministration of the FFA1 and GPR119 agonists were evaluated together in a two-way ANOVA test, a significant ($P = .022$) (Supplemental Table 3) interaction term, and hence synergistic effect between the FFA1 and the GPR119 agonist, was determined.

In contrast, similar coadministration experiments using the by itself inactive FFA4 agonist and the GPR119

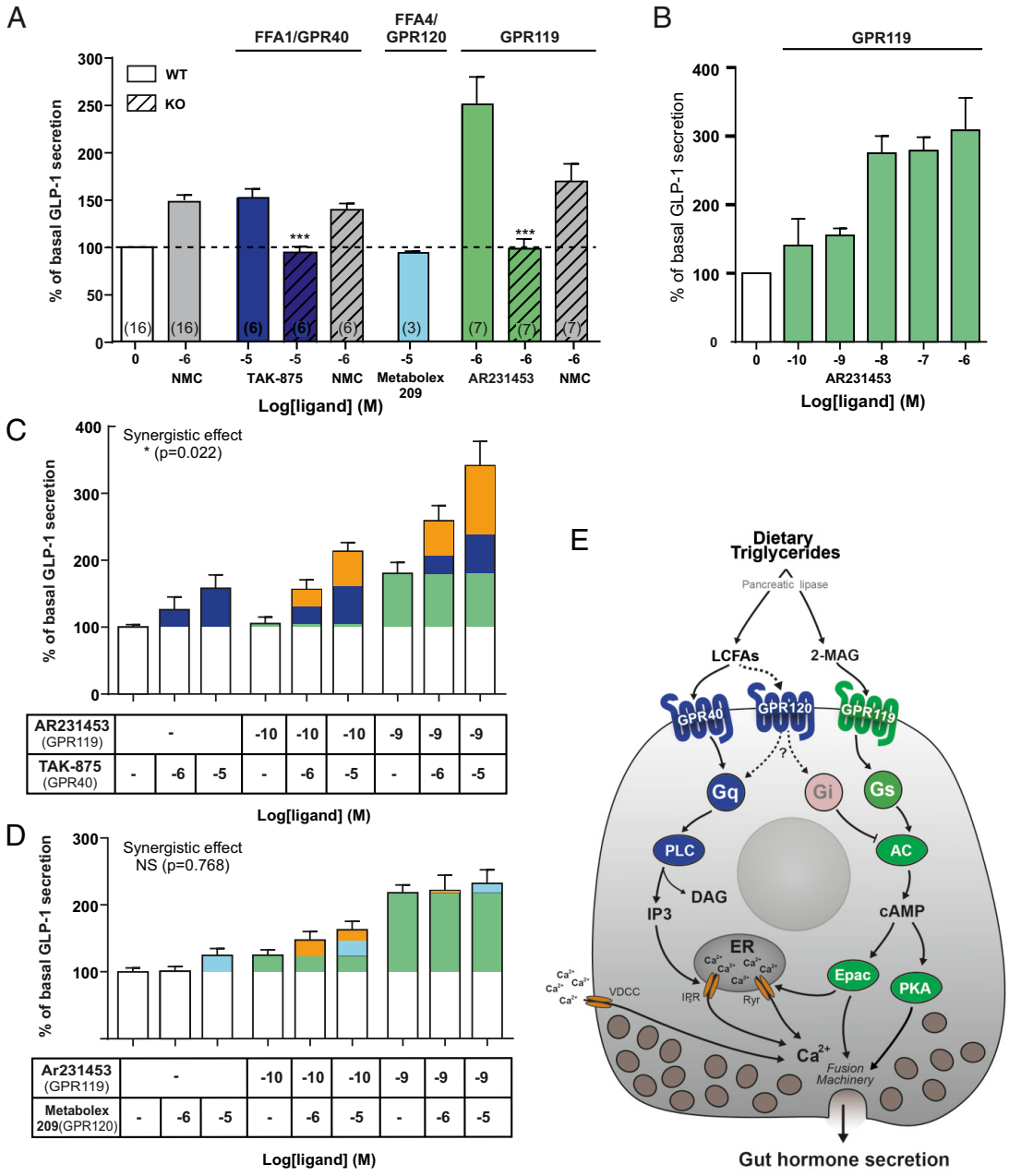

Figure 3. Secretion of GLP-1 in response to synthetic-selective FFA1, FFA4, and GPR119 agonists administered alone or coadministered to probe for potential synergistic effects in mouse colonic crypt cultures. A, Comparison of the effect on GLP-1 secretion in mouse colonic crypt cultures of maximal doses of the FFA1 agonist TAK-875 (dark blue), the GPR120 agonist Metabolex-209 (light blue), and the GPR119 agonist AR231453 (green) with the prototype GLP-1 secretagogue the bombesin 2 receptor agonist, NMC (gray). For FFA1 and GPR119, the responses in FFA1- and GPR119-deficient animals are indicated in hatched columns. Numbers of experiments are indicated in brackets in the columns. B, Dose-response experiment for the highly efficacious GPR119 agonist AR231453 performed to identify appropriately low doses to be used for coadministration studies (C and D). Each column consists of n = 3. C, GLP-1 secretion in response to the GPR40 agonist TAK-875 alone (10^{-5}M and 10^{-6}M, in dark blue) and in response to the GPR119 agonist AR231453 alone (10^{-9}M and 10^{-10}M, in green) or the GPR40 agonist together with the GPR119 agonist (orange corresponding to response above the sum of the "green" and the "blue" response). Data are shown with 4 replicates. D, GLP-1 secretion in response to the GPR120 agonist Metabolex-209 alone (10^{-5}M and 10^{-6}M, in light blue) and in response to the GPR119 agonist AR231453 alone (10^{-9}M and 10^{-10}M, in green) or the GPR120 agonist together with the GPR119 agonist (orange corresponding to response above the sum of the green and the "light blue" response); n = 4. E, Simplified schematic overview of the physiological ligand binding and signal transduction mechanisms for FFA1, FFA4, and GPR120 in a generic enteroendocrine cell. As indicated, LCFAs will induce Gαq signaling through FFA1 leading to PLC activation, IP3 accumulation, and increase in intracellular Ca++, whereas 2-MAG will induce Gαs signaling through GPR119 leading to adenylate cyclase activation, cAMP accumulation, and presumably activation of Epac and PKA, which will activate the fusion machinery of the secretory granules. As shown in C, coactivation of the FFA1/Gαq and the GPR119/Gαs signaling pathway act in synergy to provide a robust GLP-1 secretory response. GPR120 has little or no effect, conceivably due to the fact that the receptor mainly is coupled to Gαi in enteroendocrine cells (19). Data are normalized to the basal secretion of GLP-1 with data shown as mean ± SEM.

agonists did not show any significant synergistic effect ($P = .768$) (Supplemental Table 3).

It is concluded that clear synergy between FFA1 and GPR119 agonists is observed in respect of stimulation of GLP-1 secretion in the ex vivo setting but not between FFA4 and GPR119 agonists.

Discussion

In the present study, we find that GPR119 conceivably through sensing of 2-MAG is at least as important as the LCFA receptor FFA1 for the strong TG-mediated stimulation of incretin hormones. By use of highly selective pharmacological tools, we provide evidence that GPR119 and FFA1 act synergistically to stimulate GLP-1 secretion. The other LCFA receptor, FFA4, which also is highly expressed by the GLP-1 and GIP cells, appears to play only a minor, if any, role in the direct control of incretin secretion in response to TGs.

GPR119 is highly important for incretin secretion in response to TGs

GPR119 has been known for a while to stimulate GLP-1 secretion as demonstrated by the effect of selective synthetic GPR119 agonists that are among the most efficacious secretagogues for GLP-1 (17, 18, 25, 26). However, the physiological role of GPR119 has been rather unclear. Because the receptor originally was deorphanized as a sensor of n-acylethanolamines, such as oleoylethanolamide, a number of lipids have over the years been proposed to function as endogenous ligands for GPR119 (15). Although it is still rather unclear what the endogenous GPR119 ligand is in the endocrine pancreas, TG-derived 2-MAGs such as 2-OG appear to be the most likely physiological stimulator of GPR119 at least in enteroendocrine cells. 2-OG is rather efficacious but not particularly potent in respect of stimulating cAMP production in cells transfected with human GPR119 (13). Importantly, intrajejunal administration of 2-OG in amounts corresponding to what would be liberated from TGs during a meal stimulates both GLP-1 and GIP in normal human subjects (13). Moreover, ingestion of 1,3-dioctanoyl-2-oleoyl glycerol, ie, an artificial, synthetic TG, which upon digestion by pancreatic lipase generates 1 molecule of 2-OG plus 2 molecules of the "inactive" medium-chain fatty acid octanoic acid, stimulated GLP-1 to almost the same degree as olive oil in human subjects (14). Thus, 2-OG is a powerful stimulus of incretin secretion in vivo in man. In agreement with our results (Figure 2), Moss et al recently found that the GLP-1 release in response to oral lipids was dramatically reduced in mice lacking GPR119 selectively in proglucagon-expressing cells (27). These studies combined with the studies with 2-OG administration strongly indicate that sensing of 2-MAG by GPR119 is a key player in the stimulation of incretins by dietary TGs.

FFA1 sensing LCFAs acts in synergy with GPR119

For many years, it has been generally believed that it was the sensing of LCFA which was responsible for the strong stimulation of incretins by TGs (3–5). Based on similar arguments as presented above for GPR119 and 2-MAG, FFA1 was believed to be "the fat sensor" responsible for this presumed LCFA effect (28, 29). Thus, Edfalk et al demonstrated that FFA1, at that time known as GPR40, was expressed in GLP-1 cells and that insulin, GLP-1 and GIP responses to high-fat diet were reduced or eliminated in FFA1 KO mice (11) just like Xiong et al found that the GLP-1 response to corn oil was eliminated in FFA1 KO animals (12). Similarly, FFA1 has been shown to be expressed in CCK cells, and the CCK response to olive oil is reduced to 50% in FFA1 KO mice (8). However, although LCFAs apparently stimulate GLP-1 secretion from various cell lines and ex vivo preparations, we recently found that when care is taken to avoid unspecific effects of the LCFAs the actual FFA1 receptor-mediated effect on GLP-1 secretion from colonic crypt cultures was very small compared with the effect of, eg, a prototype GPR119 agonist (26). Most importantly, in the clinical trials, the FFA1 agonist TAK-875 had no effect on GLP-1 and GIP while efficiently lowering basal blood glucose and improving glucose tolerance in the diabetic patients (30). These ex vivo and in vivo studies indicate that LCFAs and in particularly FFA1 is not a major player in the stimulation of incretin secretion by TGs, ie, on its own.

In agreement with previous reports, we observe in the present study that the incretin response to TGs in the form of olive oil is reduced in FFA1 KO mice (11, 12). However, by use of synthetic-selective agonists, we find that FFA1 stimulation alone is not very efficacious in respect of stimulating GLP-1 secretion ex vivo but that FFA1 only acts efficiently in synergy with GPR119. That is, providing more than additive GLP-1 responses when the GPR40 agonist was administered on top of appropriately low doses of the GPR119 agonists (Figure 3). We believe these pharmacological experiments mimic the physiological situation where the Gαq-coupled FFA1 and the Gαs-coupled GPR119 receptors on the enteroendocrine cells are stimulated concomitantly by LCFAs and 2-MAG (Figure 3E).

Interestingly, although FFA1 in response to endogenous LCFAs and orthosteric FFA1 agonists such as TAK-875 only are able to signal through Gαq as originally described, we recently found that certain second generation ago-allosteric FFA1 agonists are able to make FFA1 signal

not only through Gαq but also through Gαs (26). Importantly, in contrast to the Gαq-only TAK-875, the Gαq+Gαs FFA1 agonists, as, for example, AM-5262, stimulated GLP-1 and GIP secretion robustly both ex vivo and in vivo (26). Thus, it is possible by use of Gαq+Gαs FFA1 agonists to obtain a similarly strong incretin response as obtained with TG presumably acting through its metabolites LCFAs and 2-MAG as Gαq-only FFA1 and as Gαs GPR119 agonists, respectively (Figure 3E).

FFA4 plays a minor if any role in the direct control of incretin secretion

Originally, it was reported that FFA4, at that time known as GPR120, was highly and exclusively expressed in enteroendocrine GLP-1 cells, and through circumstantial evidence, it was argued that FFA4 should be involved in the control of GLP-1 secretion (7). However, over the last years, it has become increasingly clear that this is not the case. Xiong et al observed that although the GLP-1 response to corn oil was eliminated by FFA1 KO, it was not affected by FFA4 KO (12). Paulsen et al observed FFA4 to be broadly expressed in enterocytes and also concluded that the receptor was not involved in the control of GLP-1 secretion (31). Here, we find that although FFA4 is highly expressed in both GLP-1 and GIP cells, it is only slightly enriched, meaning that it is almost as highly expressed in the neighboring cells. Most importantly, an otherwise very efficacious FFA4 agonist did not stimulate GLP-1 secretion ex vivo and the GIP and GLP-1 response to olive oil is only marginally if at all reduced in FFA4 KO animals. Thus, there is an increasing consensus that FFA4 in fact is not involved in the direct control of incretin secretion, although it is highly expressed in the enteroendocrine cells.

Nevertheless, there was a trend towards a reduced incretin response to TGs in the FFA4 KO animals (Figure 2, B and D), and recently, it was reported that the GIP response to lard was reduced in FFA4 KO animals (32). Thus, it is possible that FFA4 could, for example, affect the secretory capacity of the enteroendocrine cells without directly being involved in the secretory process as such.

In other endocrine cells of the gastrointestinal tract and pancreas, FFA4 appears to inhibit instead of stimulate hormone secretion. Thus, gastric ghrelin secretion is dose dependently and efficiently inhibited both ex vivo and in vivo by a selective, synthetic FFA4 agonist, and basal ghrelin levels are increased in FFA4 KO animals (19). Importantly, we found that the inhibitory effect of FFA4 agonists was blocked by pertussis toxin, indicating that GPR120 in these cells in fact couples through Gαi (19). FFA4 agonists also inhibit somatostatin secretion efficiently both in the stomach and in the pancreas (20, 33). Thus, it is unclear what the role of FFA4 is in the enteroendocrine cells of the intestine; however, future work should take into account that the receptor signals not only through Gαq but also through Gαi (19).

Materials and Methods

FACS and qPCR

The upper-half portion of the small intestine was collected from female GLP-1-Venus (10) and GIP-Venus (9) transgenic mice, washed, minced, and treated with collagenase to obtain a single cell suspension as previously described (10). The cells were sorted based on fluorescence at 530 nm and 580 nm directly into a lysis buffer (Ambion), and the mRNA was purified using RNAqueous-Micro micro scale RNA isolation kit (catalog 1931; Ambion). The mRNA was treated with deoxyribonuclease and converted to cDNA using Superscript III (Invitrogen). cDNA was prepared from 3 rounds of cell sorting. The GPCR analysis was performed using custom designed 384-well qPCR plates from Lonza containing primers for 379 GPCRs receptors and 3 receptor activity-modifying proteins together with primers for Rn18s and genomic DNA. Primer target regions have been published before (19). A gDNA sample was used as calibrator as described previously (19).

In vivo studies in mice

Fully backcrossed male C57BL/6J GPR40$^{-/-}$, C57BL/6J GPR120$^{-/-}$, C57BL/6J GPR119$^{-/-}$, and C57BL/6J GPR40$^{-/-}$/120$^{-/-}$ were obtained from Taconic. Heterozygous mice of GPR40$^{-/-}$, GPR120$^{-/-}$, and GPR40$^{-/-}$/120$^{-/-}$ strain were bred to give GPR40$^{-/-}$, GPR120$^{-/-}$, and GPR40$^{-/-}$/120$^{-/-}$ KO and littermate wild-type controls. GPR119 is X-linked, thus heterozygous female GPR119$^{-/-}$ mice were bred with C57BL/6J male mice to give KO and littermate controls. The mice were housed on a normal 12-hour light, 12-hour dark cycle and had free access to food and water. The animal studies were conducted in accordance with institutional guidelines and approved by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture, and Fisheries and the mice handled in a fully Association for Assessment and Accreditation of Laboratory Animal Care accredited facility.

TG challenge

Mice (9–29 wk) were fasted overnight (16–18 h) with ad libitum access to water. Olive oil (Sigma-Aldrich) was administered per os with a dose of 10 mL/kg. A retroorbital blood sample (100 μL) was taken before the dosage and was considered 0 minutes. Sixty minutes after the dosage, animals were decapitated, and blood was collected. All blood samples were collected in EDTA-coated tubes with 0.5-KIU aprotinin/μL blood (Sigma Chemical) and 1μM dipeptidyl peptidase-4 inhibitor. All blood samples were kept on ice at all times. Blood samples were spun at 9600*g* for 10 minutes at 4°C, and plasma was stored at −80°C. Total GLP-1 was determined using Total GLP-1 (version 2) assay kit (model number K150JVC-1; Meso Scale Discovery). GIP was measured using a Rat/Mouse Total GIP ELISA assay kit (Millipore).

GLP-1 secretion from primary colonic crypt cultures

NMC was purchased from Bachem, AR231453 was a generous gift from Rob Jones, Arena Pharmaceuticals, and TAK-875 (23) and Metabolex-209 (24) were synthesized as described. Colonic crypts were prepared from male C57BL/6, GPR119$^{-/-}$, or GPR40$^{-/-}$ mice by collagenase treatment of minced tissue as described previously (10). Cells were seeded into 24-well plates coated with Matrigel (BD Biosciences). The next day, cells were washed and incubated for 3 hours with ligands dissolved in dimethyl sulfoxide (quadruplicates) in standard solution (10) containing 0.1% fatty acid-free BSA (Sigma) and 10mM glucose. The liquid was aspirated from the wells, centrifuged, and the supernatant was kept at −80°C until further use. GLP-1 was measured according to the protocol "Total GLP-1 version 2" from Meso Scale Discovery (K150JVC-1).

Data analysis and calculations

Data were visualized and statistics calculated in GraphPad Prism software 6.0 (GraphPad Software).

Quantitative PCR

Statistical significant differences between receptor expression levels in Venus-positive vs Venus-negative cells were assessed using multiple unpaired *t* tests with corrections for multiple testing by the Holm-Sidak method.

In vivo studies

GIP and GLP-1 release from knockout mice and WT littermates was compared using one-way ANOVA Dunnett's multiple comparisons test for GPR40$^{-/-}$, GPR120$^{-/-}$, and GPR40$^{-/-}$/120$^{-/-}$. For the comparison of GIP and GLP-1 release from GPR119$^{-/-}$ and WT littermates, an unpaired *t* test was used. We used the software R (https://www.r-project.org/) for statistical analysis to test the effect of receptor knockout on incretin hormone secretion in response to TG. We used the lme() function to fit a linear mixed effect model with the different experimental trial as random effects. We performed Turkey's post hoc test to test for differences in receptor knockout effects using the glht() function from the multcomp package.

Ex vivo studies

Statistical significant differences between GLP-1 release from GPR119 and GPR40 KO and WT colonic crypt cultures were assessed using a nonparametric unpaired 2-tailed *t* test (Mann-Whitney). Same method was used when assessing significant increase in GLP-1 secretion with addition of 2 compounds to elicit synergistic effects, in cases where one of the used compound's concentration did not give a response by itself.

To test for synergistic effects in the ex vivo experiments, we used a two-way ANOVA test. For each experiment we fitted 2 linear models to the GLP-1 fold change, y. We modeled each compound dosage as a categorical variable. The reduced model contained only additive main effects of compounds, lm(y∼compund1+compund2); the full model included interaction terms of the compounds lm(y∼compund1+compund2+compund1:compund2). We used a likelihood ratio test (ANOVA(additive_model, full_model), test="Chisq") to assess the significance of the interaction term and hence the synergistic effect. Statistical significance is denoted by * ($P < .05$), ** ($P < .01$), and *** ($P < .001$).

Acknowledgments

Address all correspondence and requests for reprints to: Thue W. Schwartz, Laboratory for Molecular Pharmacology, The Panum Institute, Building 18.5, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark. E-mail: tws@sund.ku.dk.

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