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β -Arrestin Recruitment and Biased Agonism at Free Fatty Acid Receptor 1*

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Background: FFAR1/GPR40 is a potential target to enhance insulin secretion in type 2 diabetes, yet knowledge of the pharmacobiology of GPR40 remains incomplete.

Results: GPR40 functions via both G protein-mediated and β -arrestin-mediated mechanisms; endogenous and synthetic ligands differentially engage these pathways to promote insulin secretion.

Conclusion: GPR40 is subject to functionally relevant biased agonism.

Significance: Biased agonism at GPR40 could be exploited for therapeutic purposes.

FFAR1/GPR40 is a seven-transmembrane domain receptor (7TMR) expressed in pancreatic β cells and activated by FFAs. Pharmacological activation of GPR40 is a strategy under consideration to increase insulin secretion in type 2 diabetes. GPR40 is known to signal predominantly via the heterotrimeric G proteins $G_{q/11}$. However, 7TMRs can also activate functionally distinct G protein-independent signaling via β -arrestins. Further, G protein- and β -arrestin-based signaling can be differentially modulated by different ligands, thus eliciting ligand-specific responses (“biased agonism”). Whether GPR40 engages β -arrestin-dependent mechanisms and is subject to biased agonism is unknown. Using bioluminescence resonance energy transfer-based biosensors for real-time monitoring of cell signaling in living cells, we detected a ligand-induced GPR40- β -arrestin interaction, with the synthetic GPR40 agonist TAK-875 being more effective than palmitate or oleate in recruiting β -arrestins 1 and 2. Conversely, TAK-875 acted as a partial agonist of $G_{q/11}$ -dependent GPR40 signaling relative to both FFAs. Pharmacological blockade of G_q activity decreased FFA-induced insulin secretion. In contrast, knockdown or genetic ablation of β -arrestin 2 in an insulin-secreting cell line and mouse pancreatic islets, respectively, uniquely attenuated the insulinotropic activity of TAK-875, thus providing functional validation of the biosensor data. Collectively, these data reveal that in addition to coupling to $G_{q/11}$, GPR40 is functionally linked to a β -arrestin 2-mediated insulinotropic signaling axis. These observations expose previously unrecognized complexity for GPR40 signal

transduction and may guide the development of biased agonists showing improved clinical profile in type 2 diabetes.

The free fatty acid receptor 1/G protein-coupled receptor 40 (FFAR1/GPR40) is a cell surface, seven-transmembrane domain receptor (7TMR)⁴ activated by medium-to-long chain FFAs (1, 2). GPR40 is predominately expressed in insulin-secreting pancreatic β -cells and mediates part of the acute stimulatory effects of various FFAs on insulin secretion. However, GPR40 does not mediate the long-term lipotoxic effects of FFAs on β -cell function (3, 4). Given that FFAs stimulate insulin secretion only when glucose levels are elevated, GPR40 could be targeted to enhance insulin secretion in type 2 diabetes (T2D) without the risk of iatrogenic hypoglycemia. Accordingly, GPR40 is generating substantial interest as a therapeutic target to enhance insulin secretion in T2D (5–7). However, despite significant investment in the development of selective GPR40 agonists, our understanding of the pharmacobiology of GPR40 remains incomplete (8).

To date, the predominant view holds that GPR40 signals primarily via the heterotrimeric G protein $G_{q/11}$ and that its biological effects are mediated by downstream second messenger molecules, namely diacylglycerol and inositol 1,4,5-trisphosphate as well as Ca^{2+} mobilization (9, 10). Additionally, coupling of GPR40 to the G_s /PKA/cAMP pathway has also been reported (11, 12). It is becoming increasingly apparent, however, that 7TMRs are more than mere bimodal molecular switches that signal via linear G protein-dependent transduction pathways. Indeed, 7TMRs can also engage functionally distinct, G protein-independent signaling mechanisms via the multifunctional adapter proteins β -arrestins 1 and 2. Once believed to solely mediate receptor desensitization and internalization, β -arrestins are now recognized as key signaling hubs

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⁴ The abbreviations used are: 7TMR, seven-transmembrane domain receptor; T2D, type 2 diabetes; BRET, bioluminescence resonance energy transfer; OA, oleate; PA, palmitate; KRB, Krebs-Ringer buffer.

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acting downstream of 7TMRs. According to the concept of “biased agonism,” binding of structurally/chemically different ligands to a given 7TMR can stabilize unique receptor conformations, each differentially modulating β -arrestin- and G protein-dependent signaling (13–15). As a result, different ligands may impart distinct signaling and biological attributes to a given receptor. By selectively engaging only part of a receptor’s potential intracellular partners and signaling cascades, biased ligands may provide refined pharmacological specificity and therapeutic efficacy with fewer side effects.

Recently, Qian *et al.* (16) demonstrated that β -arrestin 2 is implicated in linoleate-induced internalization of GPR40. Whether β -arrestin 1 and/or 2 partake in GPR40-dependent signaling and whether this receptor is subject to biased agonism, however, is unknown. This question is of particular relevance to the pharmacotherapy of T2D as several synthetic agonists of GPR40 are under clinical development. Among them, TAK-875 demonstrated encouraging therapeutic potential in Phase I and II clinical trials (17, 18) but was subsequently discontinued during Phase III trials due to hepatotoxicity (19). Given the important implications of biased agonism at this emerging drug target, we aimed to ascertain (i) whether GPR40 engages β -arrestin-dependent signaling and (ii) whether $G_{q/11}$ - and β -arrestin-mediated signaling can be differentially modulated by distinct ligands. Our results provide the first account of a functionally relevant, ligand-biased interaction between β -arrestin 2 and GPR40 that is involved in regulating the insulinotropic activity of GPR40.

Experimental Procedures

Reagents, Solutions, and Plasmids—DMEM was from Multi-Cell Technologies, whereas FBS and RPMI 1640 were from Invitrogen (Burlington, Ontario, Canada). TAK-875 was purchased from Selleckchem (Houston, TX); palmitate and oleate were obtained from Sigma. Murine GPR40 cDNA was purchased from OriGene (pCMV6-mGPR40; SKU MC212962). The GPR40-GFP10 fusion construct was created by deleting the GPR40 stop codon in pCMV6-mGPR40 with subsequent downstream insertion of linker-GFP10 cDNA (all performed by GenScript).

Cell Culture—HEK-293T cells were grown in DMEM supplemented with 10% FBS. INS832/13 cells were cultured in RPMI 1640 medium supplemented with 11 mM glucose, 10% FBS, 10 mM HEPES (pH 7.4), 1 mM sodium pyruvate, and 50 μ M β -mercaptoethanol. Cells were maintained at 37 °C with 5% CO₂.

Obelin Ca²⁺ Flux Assay—HEK-293T cells were plated at 0.5×10^6 cells/well in 6-well tissue culture plates. The following day, cells were co-transfected with 1 μ g of cDNA encoding the Ca²⁺ biosensor obelin and 100 ng of either pcDNA3.1 or GPR40 cDNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After overnight (18 h) incubation, transfected HEK-293T cells were passed and plated at $\sim 1.0 \times 10^5$ cells/well in poly-L-ornithine-coated 96-well white-walled microtiter plates. The following day, HEK-293T cells were washed twice with 200 μ l of Hanks’ balanced salt solution (Invitrogen) supplemented with 1.8 mM CaCl₂, 0.8 mM MgSO₄, and 0.2% glucose (pH 7.4) and then incubated for 2–3 h with 1 μ M coelenterazine CP (Biotium) in the dark. Agonist-induced

intracellular Ca²⁺ flux is reflected by the magnitude of the bioluminescent signal emitted by the obelin biosensor, which was recorded before and every 0.3 s after agonist injection in the SpectraMax L Microplate reader (Molecular Devices) for a total of 62.5 s. The indicated concentrations of palmitate, oleate, and TAK-875 were precomplexed to a fixed concentration (20 μ M final) of fatty acid-free BSA at 37 °C for 1 h. Control wells were stimulated with vehicle (20 μ M fatty acid-free BSA and 0.1% v/v dimethyl sulfoxide (TAK-875 solvent) or 0.17% v/v ethanol (palmitate solvent)). The area under the resulting curves was calculated using Prism GraphPad 6.0 as a measure of total Ca²⁺ flux; area under the resulting curves values were used to construct dose curves. Data were expressed as a percentage *versus* the maximal response, which was consistently obtained with 80 μ M palmitate.

G_q Activation Biosensor Assay—HEK-293T cells were plated at 0.5×10^6 cells/well in 6-well tissue culture plates. The following day, the three subunits of the multimolecular G_q activation biosensor (20 ng each of RLucII-G α_q and G β_1 and 100 ng of GFP10-tagged G γ_1) and 100 ng of pcDNA3.1 or GPR40 cDNA were transfected using 25-kDa linear PEI (Polysciences, Warrington, PA) (20) at a 3:1 μ l of PEI/ μ g of DNA ratio. The total amount of DNA transfected in each well was consistently adjusted to 2 μ g with salmon sperm DNA (Invitrogen). At 48 h after transfection, HEK-293T cells were washed once with Tyrode’s buffer (140 mM NaCl, 1 mM CaCl₂, 2.7 mM KCl, 0.49 mM MgCl₂, 0.37 mM NaH₂PO₄, 5.6 mM glucose, 12 mM NaHCO₃, and 25 mM HEPES, pH 7.5), detached, and plated in poly-L-ornithine-coated 96-well white-walled plates ($\sim 0.2 \times 10^6$ cells/well). Cells were allowed to adhere for 3 h at 37 °C, after which time vehicle or BSA-complexed GPR40 agonists (as for obelin Ca²⁺ flux assay above) were added to cells for 5 min at 37 °C. Coelenterazine 400a (Biotium) was then added to a final concentration of 5 μ M in Tyrode’s buffer for 5 min. Readings were subsequently collected with a Mithras LB 940 multidetector plate reader (Berthold Technologies), allowing the sequential integration of the signals detected at 410 ± 40 nm (RLucII light emission) and 515 ± 15 nm (GFP10 light emission). The bioluminescence resonance energy transfer (BRET) signal was calculated as the ratio of the GFP10 light emission to RLucII light emission. Values are expressed as net BRET by first subtracting vehicle-induced BRET from ligand-induced BRET (*i.e.* Δ BRET) and then subtracting the Δ BRET values calculated for cells transfected with pcDNA3.1 from the Δ BRET values obtained in cells transfected with GPR40.

β -Arrestin Recruitment Biosensor Assays—HEK-293T cells were seeded as for obelin and G_q activity biosensor experiments. Cells were co-transfected (using 25-kDa linear PEI) with 12.5 ng of either β -arrestin 1-RLucII or β -arrestin 2-RLucII and 500 ng of pcDNA3.1 or GPR40-GFP10 cDNA. The total amount of DNA transfected in each well was always adjusted to 2 μ g. After 48 h, cells were washed twice with Tyrode’s buffer, detached, and plated in poly-L-ornithine-coated 96-well microplates ($\sim 0.2 \times 10^6$ cells/well). Cells were allowed to adhere for 3 h at 37 °C. Total fluorescence was subsequently recorded with a FlexStation (Molecular Devices; excitation filter at 400 nm; emission filter at 510 nm) to ensure equal GPR40-GFP10 expression across different wells and plates. For experiments in

which G_q activity was inhibited, cells were preincubated with 100 nM UBO-QIC. Vehicle or BSA-complexed GPR40 agonists were then added to all wells, and cells were incubated at 37 °C in the dark for 10 min. Thereafter, coelenterazine 400a (Biotium) was added to each well at a final concentration of 2.5 μ M. Cells were incubated at 37 °C in the dark for an additional 5 min, after which time ligand-induced BRET was recorded with the Mithras LB940 multimode microplate reader (Berthold Technologies) equipped with the BRET400-GFP2/10-filter set (acceptor at 515 \pm 20-nm filter and donor at 400 \pm 70-nm filter). Values are expressed as net BRET by first subtracting vehicle-induced BRET from ligand-induced BRET (*i.e.* Δ BRET) and then subtracting the Δ BRET values calculated for cells transfected with pcDNA3.1 and either RLucII-linked β -arrestin 1 or RLucII-linked β -arrestin 2 from the Δ BRET values obtained in cells co-transfected with GPR40-GFP10 and the β -arrestin biosensors.

siRNA-mediated Knockdown of β -Arrestin 2 and Analysis by Western Blot—INS832/13 ($\sim 6 \times 10^6$) cells were electroporated with 350 pmol of either non-targeting (negative control) or anti- β -arrestin 2 siRNAs via nucleofection (Amaxa[®] Nucleofector[®]) and subsequently seeded in 24-well culture plates at $\sim 0.4 \times 10^6$ cells/well (for insulin secretion) and in 12-well plates at 1.2×10^6 cells/well (for protein extraction). Medium was replaced the day following nucleofection. Protein extraction and immunoblotting were performed as described (21) using an anti- β -arrestin 2 monoclonal antibody (Cell Signaling, clone C16D9; diluted 1:1000 in 5% nonfat milk/Tris-buffered saline with Tween). Membranes were subsequently stripped using Re-Blot Plus stripping solution (Millipore) and reprobed for α -tubulin (Abcam).

Static Insulin Secretions in INS832/13—Electroporated cells were seeded as described above. For experiments with UBO-QIC, cells were seeded in 24-well culture plates at $\sim 0.25 \times 10^6$ cells/well. At ~ 48 h after seeding, electroporated cells and those used in UBO-QIC experiments were starved for 2 h in RPMI 1640 medium supplemented with 1% FBS and 1 mM glucose, followed by a wash and 1-h incubation in KRB supplemented with 1 mM glucose and 0.1% BSA. For UBO-QIC experiments, 100 nM of the inhibitor was added 30 min into the 1-h KRB incubation. Following the aforementioned incubations, cells were treated with the reagents indicated in the text in KRB and insulin secretion was assayed following a 1-h static incubation. Insulin content was extracted with acid-alcohol. Secreted and intracellular insulin levels were measured using a rat insulin RIA kit (Millipore, St. Charles, MO). Each experimental condition was performed in triplicate.

Animals, Islet Isolations, and Static Insulin Secretions—Pancreatic islets were isolated from whole-body β -arrestin 2^{-/-} mice or WT littermates as described in Ref. 22. The original heterozygous β -arrestin 2^{+/-} mice were from R. J. Lefkowitz (Duke University Medical Center, Durham, NC; described in Ref. 23). All animal studies complied with the authorization of the Ministry of Agriculture, France (D34-172-13). Islets were hand-picked after collagenase digestion of the pancreas and maintained overnight in RPMI 1640 supplemented with 7.5% FBS and 10 mM glucose. The following day, insulin secretion was assessed in 1-h static incubations using batches of 10 islets

in bicarbonate KRB supplemented with 2.8 or 16.7 mM glucose with or without TAK-875 or palmitate. Both TAK-875 and palmitate were complexed for 1 h at 37 °C with fatty acid-free BSA to a final molar ratio of 1:5 as described previously (9). Control conditions contained an equal amount of BSA and vehicle (50% (v/v) ethanol). Secreted and intracellular insulin levels were measured using a rat insulin RIA kit (Millipore).

Data Analysis—Results were analyzed using GraphPad Prism 6.0 (GraphPad Software) and are presented as mean \pm S.E. Dose-response curves were fitted using the log(agonist) *versus* response function (four parameters). All ligand concentrations shown (including EC₅₀ values) correspond to those used for complexing to BSA and not free (uncomplexed) fractions. Statistical significance between was determined via unpaired Student's *t* test or analysis of variance (with Tukey's post hoc adjustment for multiple comparisons) as appropriate. *p* < 0.05 was considered significant.

Results

TAK-875 and FFAs Promote β -Arrestin Recruitment to GPR40 with Different Efficacies—Recent work by Qian *et al.* (16) demonstrated that β -arrestin 2 is recruited to GPR40 in response to the FFA linoleate. However, it remains unknown whether different GPR40 ligands exhibit qualitative differences in the β -arrestin isoform they recruit and whether such ligands are equi-efficacious in recruiting β -arrestins to the receptor. Thus, as a first step in establishing the existence of possible biased agonism at GPR40, we compared the potencies and efficacies of three distinct GPR40 agonists for β -arrestin 1 and 2 recruitment: the saturated and monounsaturated FFAs palmitate (PA; (C16:0)) and oleate (OA; (C18:1)), respectively, as well as the synthetic GPR40 agonist TAK-875. PA and OA were selected as the two most abundant circulating FFAs in humans (24). The choice of TAK-875 was justified by its high selectivity for GPR40 *versus* other fatty acid receptors (5), its lack of GPR40-independent insulinotropic action (25), and its overall structural dissimilarity to endogenous GPR40 ligands. Ligand-induced engagement of each β -arrestin by GFP10-tagged GPR40 was determined using a BRET-based assay that enables real-time monitoring of protein-protein interactions in living cells. These experiments were performed in HEK-293T cells, which lack endogenous GPR40 expression (26).

PA and OA (conjugated to a fixed concentration of 20 μ M BSA) dose-dependently promoted the recruitment of β -arrestins 1 and 2 to GPR40 (Fig. 1, *A* and *B*) with EC₅₀ values in the low μ M range (β -arrestin 1: 43.7 μ M for PA; 51.7 μ M for OA; β -arrestin 2: 42.4 μ M for PA; 58.4 μ M for OA). The synthetic GPR40 ligand TAK-875 (conjugated to a fixed concentration of 20 μ M BSA) was much more potent (EC₅₀: 64.1 nM for β -arrestin 1; 54.7 nM for β -arrestin 2) and efficacious in promoting β -arrestin recruitment to GPR40. At its maximally effective concentration of 100 μ M, the efficacy of PA for β -arrestin 1 and 2 recruitment was 38 \pm 4 and 52 \pm 7%, respectively, of that of 10 μ M TAK-875. Similarly, the maximal effect of OA (150 μ M) on β -arrestin 1 and 2 was 51 \pm 4 and 55 \pm 7%, respectively, of that of 10 μ M TAK-875. These findings establish that relative to the synthetic GPR40 agonist TAK-875, the two endogenous GPR40

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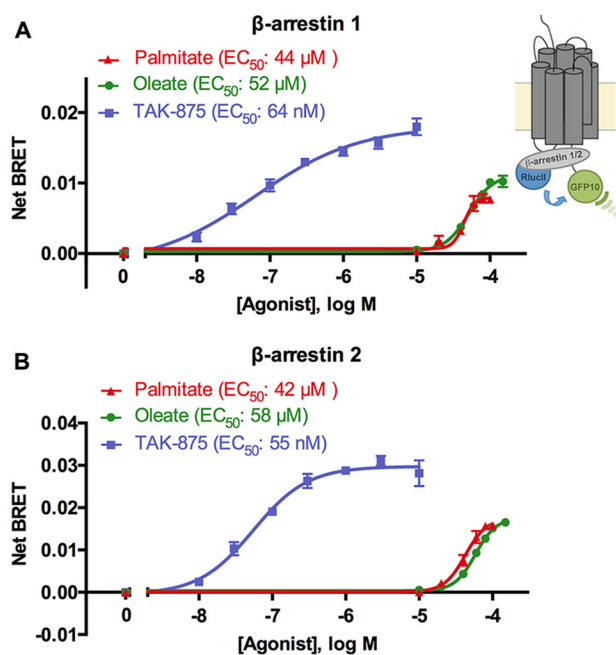


FIGURE 1. The synthetic GPR40 agonist TAK-875 is more efficacious than the endogenous agonists palmitate or oleate in promoting β -arrestin recruitment to GPR40. A and B, HEK-293T cells transiently overexpressing GPR40-GFP10 and the β -arrestin 1 (A) or β -arrestin 2 (B) recruitment biosensor were treated with vehicle or increasing concentrations of BSA-complexed palmitate (10–100 μ M), oleate (10–150 μ M), or TAK-875 (0.01–10 μ M). Agonist-induced recruitment of each β -arrestin was determined by measuring BRET at 15 min after agonist stimulation. Data shown are mean \pm S.E. of 4 and 7 independent experiments for β -arrestin 1 and β -arrestin 2, respectively.

agonists PA and OA behave as partial agonists for the recruitment of β -arrestins.

TAK-875 and FFAs Exhibit Distinct Signaling Signatures and Biased Agonism at GPR40—Having established that TAK-875 is more efficacious than either PA or OA at promoting β -arrestin 1 and 2 coupling to GPR40, we next determined whether these ligands exhibit similar relative efficacies for activation of heterotrimeric G protein ($G_{q/11}$)-dependent signaling. We used a BRET-based biosensor to directly monitor agonist-induced activation of G_q . The G_q activation biosensor consists of an RLucII-tagged G_{α_q} subunit, an untagged G_{β_1} subunit, and a GFP10-tagged G_{γ_1} subunit and monitors the physical separation of G_{α_q} from $G_{\beta/\gamma}$ subunits upon agonist stimulation and receptor activation. Consequently, G protein activation results in a decreased BRET signal whose magnitude is tightly correlated to ligand efficacy (27, 28). G_q activation was assayed in response to increasing concentrations of BSA-conjugated PA, OA, or TAK-875. As shown in Fig. 2A, TAK-875 weakly activated G_q with an apparent EC_{50} around 60 nM. G_q activation by PA or OA displayed no sign of saturation up to the highest testable concentrations (100 μ M for PA and 150 μ M for OA), due to insolubility (Fig. 2A). The maximal response produced by TAK-875 was $70 \pm 4\%$ of that observed with 100 μ M PA and $58 \pm 10\%$ of that observed for 150 μ M OA, thus demonstrating that relative to FFAs, TAK-875 is a partial agonist of GPR40-coupled $G_{q/11}$ activity. Analysis of downstream Ca^{2+} levels using the Ca^{2+} biosensor obelin corroborated the G_q activation data by showing that TAK-875 was 57 ± 7 and $66 \pm 9\%$ as effective as 80 μ M PA and 100 μ M OA, respectively, in increas-

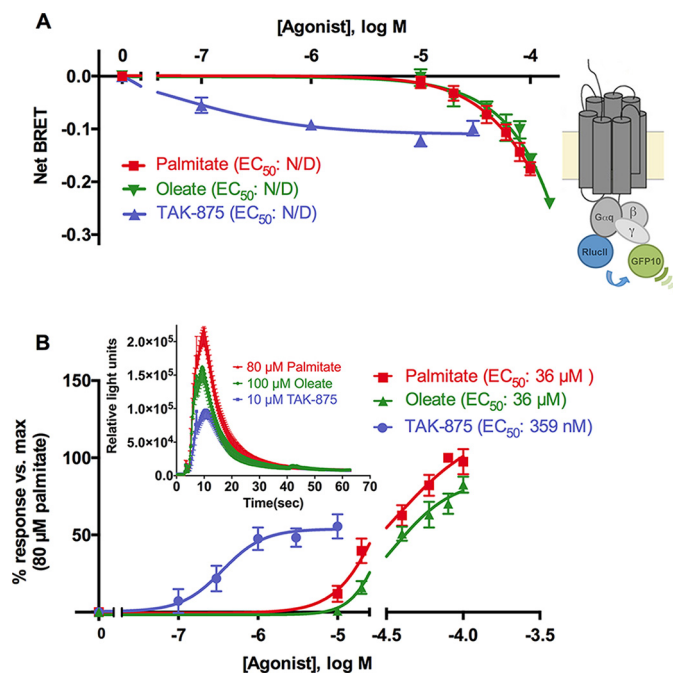


FIGURE 2. Relative to palmitate or oleate, TAK-875 is a partial agonist of heterotrimeric G protein ($G_{q/11}$)-dependent signaling downstream of GPR40. A and B, G_q pathway activation was determined in HEK-293T cells expressing pcDNA3.1 or GPR40 with the G_q activation biosensor (A) or the intracellular $[Ca^{2+}]_i$ biosensor obelin (B). A, transiently transfected cells were stimulated with vehicle or increasing concentrations of BSA-conjugated palmitate (10–100 μ M), oleate (10–150 μ M), or TAK-875 (0.1–30 μ M) for 5 min, after which time BRET between G_{α_q} -RLucII and GFP10- $G_{\beta\gamma}$ was measured. Activation of G_q , which results in the physical dissociation of G_{α_q} and $G_{\beta\gamma}$ subunits, is represented by a decreased BRET signal, the magnitude of which is directly correlated to G_q activation. Data are mean \pm S.E. of 5–9 independent determinations. N/D: non-determinable due to lack of saturation. B, cytosolic $[Ca^{2+}]_i$ was assessed following stimulation of transfected cells with 10–100 μ M palmitate, 10–100 μ M oleate, or 0.1–10 μ M TAK-875. Inset: intracellular Ca^{2+} flux kinetics immediately following stimulation of transfected HEK-293T cells with maximally effective concentrations of BSA-conjugated palmitate (80 μ M), oleate (100 μ M), or TAK-875 (10 μ M). Data are expressed as a percentage of Ca^{2+} flux observed with 80 μ M palmitate (max) and represent the mean \pm S.E. of 8–10 independent experiments. Kinetics and dose-response data are derived from same experiments.

ing intracellular Ca^{2+} concentrations (Fig. 2B). Examination of $[Ca^{2+}]_i$ mobilization kinetics revealed that at maximally effective concentrations, all three ligands quickly (within 2 s after stimulation) triggered intracellular Ca^{2+} flux and elicited a maximal response at ~ 10 s after stimulation (Fig. 2B, inset).

These data establish that relative to the FFAs PA and OA, TAK-875 is a partial agonist of the G_q/Ca^{2+} signaling axis downstream of GPR40 and behaves as a β -arrestin-biased ligand. Conversely, the endogenous GPR40 ligands PA and OA act as $G_{q/11}$ -biased GPR40 ligands by favoring $G_{q/11}$ activation over β -arrestin recruitment.

G_q Activity Is Implicated in TAK-875- and FFA-induced β -Arrestin Recruitment to GPR40—It is now recognized that β -arrestin recruitment to 7TMRs can occur independently of G protein coupling (29). Interplay between these two processes, however, has also been described (30). To study the potential impact of GPR40-mediated G_q activation on β -arrestin recruitment, we assessed β -arrestin coupling to GPR40 in cells treated with a pharmacological inhibitor of G_q (UBO-QIC, 100 nM). Inhibition of G_q was confirmed using the G_q activation biosensor and was complete in all experiments (Fig. 3A). Inhibition of

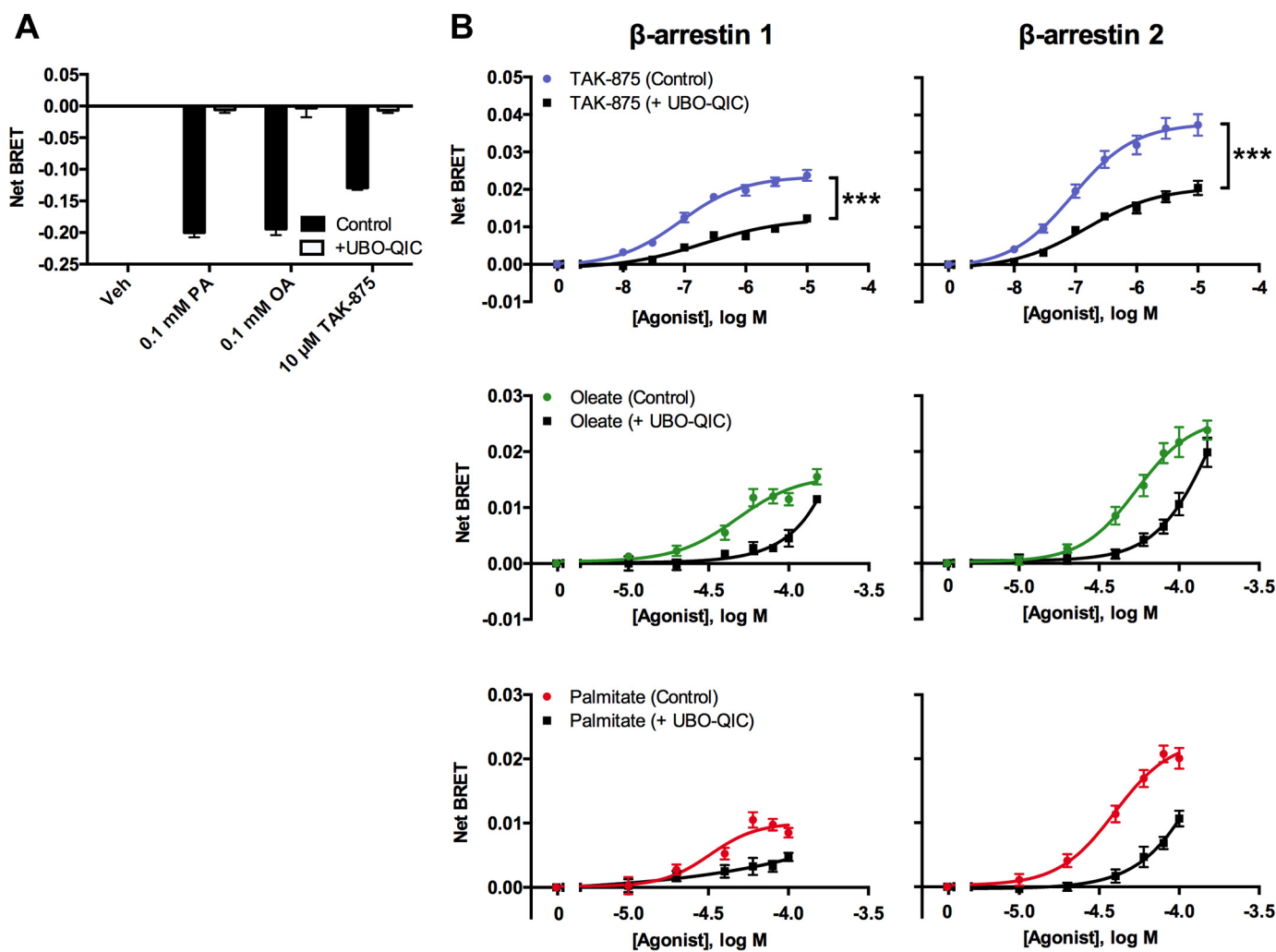


FIGURE 3. Pharmacological inhibition of G_q activity influences β -arrestin recruitment to GPR40 in a ligand-specific manner. A and B, HEK-293T cells transiently overexpressing GPR40-GFP10 and either the G_q activation biosensor (A) or the β -arrestin 1 or β -arrestin 2 (B) recruitment biosensors were pre-treated (30 min) with vehicle (Veh) (control) or with the G_q inhibitor UBO-QIC (100 nM). Cells transfected with β -arrestin recruitment biosensors were subsequently stimulated with increasing concentrations of BSA-complexed palmitate (10–100 μ M), oleate (10–150 μ M), or BSA-complexed TAK-875 (0.01–10 μ M). Agonist-induced recruitment of each β -arrestin was determined by measuring BRET at 15 min after agonist stimulation (B and C). Data shown are mean \pm S.E. of 4–7 independent determinations. For each experiment, validation of G_q inactivation efficiency by UBO-QIC was performed in parallel using cells transfected with the G_q activation biosensor (A). ***, $p < 0.001$ by unpaired Student's t test.

G_q reduced the potency of FFAs to recruit β -arrestins 1 and 2 to GPR40 (as evidenced by a rightward shift of the dose-response curves). However, whereas the potency of TAK-875-induced β -arrestin recruitment to GPR40 was not affected by G_q inhibition, a clear reduction in maximal BRET was observed (48 and 45% reduction for β -arrestin 1 and β -arrestin 2, respectively) (Fig. 3B). These data imply that β -arrestin recruitment to GPR40 occurs via G_q -dependent and G_q -independent mechanisms. The different impact of G_q inhibition on TAK-875- and FFA-induced β -arrestin recruitment further highlights differences in how these two ligand classes engage β -arrestins.

TAK-875 and the FFA Palmitate Require Different GPR40 Downstream Effectors for Their Maximal Insulinotropic Activity in INS832/13—Given the role of GPR40 in insulin secretion, we assessed the implication of β -arrestin 2 in mediating the insulinotropic action of TAK-875 and PA in insulin-secreting INS832/13 cells, which express GPR40. First, we measured insulin secretion in response to increasing concentrations of

BSA-complexed TAK-875 in the presence of 11 mM glucose (Fig. 4A). At 10 μ M, TAK-875 potentiated glucose-stimulated insulin secretion by 2.1-fold ($p < 0.05$ versus 11 mM glucose; $n = 3$). Next, insulin secretion assays were conducted with TAK-875 and PA in β -arrestin 2-depleted INS832/13 cells. A $\sim 50\%$ knockdown of β -arrestin 2 protein levels (Fig. 4B) significantly reduced TAK-875-induced insulin secretion ($52 \pm 5\%$ reduction versus non-targeting negative control siRNA siNC1; $p < 0.01$, $n = 3-6$). Conversely, insulin secretion in response to PA was not significantly reduced by β -arrestin 2 knockdown ($30 \pm 17\%$ reduction versus siNC1; NS; Fig. 4C). In light of our previous observation linking GPR40-mediated G_q activation to β -arrestin recruitment, we investigated the impact of G_q inhibition on TAK-875- and PA-induced insulin secretion. Treatment of INS832/13 with the G_q inhibitor UBO-QIC (100 nM) did not significantly alter the insulinotropic potential of TAK-875, whereas the effects of PA were significantly reduced by $53 \pm 11\%$ ($n = 4$, $p < 0.05$; Fig. 4D). Collectively, these data indicate that the relative contributions of G_q and β -arrestin 2

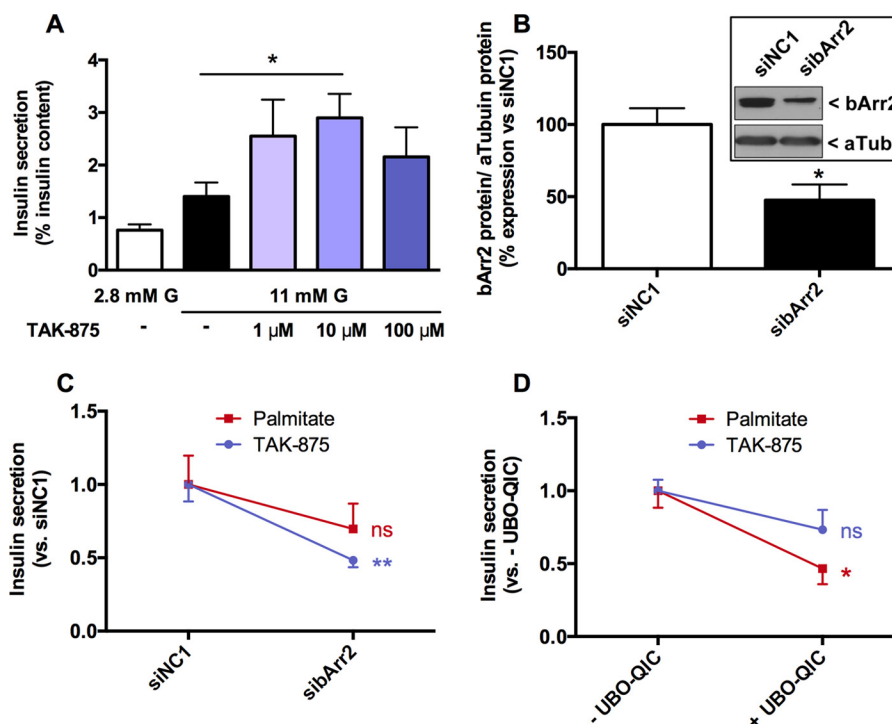


FIGURE 4. The insulinotropic activity of TAK-875 and palmitate requires different GPR40 downstream effectors. *A*, determination of maximally effective insulinotropic dose of TAK-875. Insulin-secreting INS832/13 cells were treated with increasing concentrations of BSA-complexed TAK-875 (1, 10, or 100 μ M) in the presence of 11 mM glucose. Data are mean \pm S.E. of 3 independent experiments. *, $p < 0.05$ versus 11 mM glucose by one-way analysis of variance. *B*, validation of β -arrestin 2 knockdown efficiency via Western blot. INS832/13 cells were electroporated with either a non-targeting negative control siRNA (siNC1) or an anti- β -arrestin 2 siRNA (sibArr2). At 48 h after electroporation, immunoblotting was performed with an anti- β -arrestin 2 antibody (bArr2) or α -tubulin (aTub) antibody (loading control). β -Arrestin 2 knockdown efficiency is shown as percentage versus siNC1 electroporated cells; all data were normalized by α -tubulin. The mean \pm S.E. of 4 independent experiments is shown. *C*, INS832/13 cells electroporated with siNC1 or sibArr2 were stimulated with BSA-complexed TAK-875 (10 μ M) or palmitate (500 μ M) at 11 mM glucose. Secreted insulin was measured after 1 h via RIA. Data were normalized by total insulin content and represented as -fold versus siNC1-electroporated cells. The mean \pm S.E. of 3–7 independent determinations is displayed. *D*, insulin secretion was assessed in 1-h static incubations of INS832/13 with 11 mM glucose in the absence (–) or presence (+) of the G_q inhibitor UBO-QIC (100 nM). Data were normalized by total insulin content and represented as -fold versus non UBO-QIC-treated cells. Shown is the mean \pm S.E. of 4–5 independent determinations. ns, non-significant by unpaired Student's *t* test; *, $p < 0.05$ by unpaired Student's *t* test; **, $p < 0.01$ by unpaired Student's *t* test.

activity favor G_q for the insulinotropic response to PA but β -arrestin 2 for the response to TAK-875.

TAK-875-, but Not FFA-induced Insulin Secretion Is Reduced in β -Arrestin 2^{-/-} Mouse Islets of Langerhans—To validate the insulinotropic function of the GPR40- β -arrestin 2 axis in a physiologically relevant experimental system, insulin secretion was assessed *ex vivo* in 1-h static incubations of islets of Langerhans isolated from male β -arrestin 2^{-/-} mice and their WT littermates. One hundred μ M BSA-complexed TAK-875 significantly potentiated glucose-stimulated insulin secretion in WT islets ($p < 0.01$ versus 16.7 mM glucose; $n = 4$ –5; Fig. 5A), and this concentration was chosen for subsequent experiments. Treatment of WT and β -arrestin 2^{-/-} islets with 16.7 mM glucose resulted in a similar induction of insulin secretion (Fig. 5B), as reported previously (22). PA or OA similarly potentiated glucose-stimulated insulin secretion from WT and β -arrestin 2^{-/-} islets. In contrast, potentiation of glucose-stimulated insulin secretion by TAK-875 was significantly dampened in β -arrestin 2^{-/-} islets (31 \pm 6% reduction versus WT, $p < 0.001$; $n = 5$ –8). These results corroborate those obtained following siRNA-mediated knockdown of β -arrestin 2 in INS832/13 and provide key functional validation of the biosensor data. They show, in a physiologically relevant system, that TAK-875 promotes insulin secretion at least in part via a β -arrestin 2-dependent mechanism, whereas FFA insulinotropic activity is largely

β -arrestin-independent, confirming the bias between these ligands.

Discussion

The free fatty acid receptor GPR40 has emerged as a promising therapeutic target to enhance insulin secretion in T2D. Knowledge of GPR40-mediated signal transduction remains limited to classical notions of 7TMR biology, with the prevailing view holding that GPR40 primarily acts through G protein-dependent mechanisms (9, 10, 12). Unknown was this receptor's ability to function via “non-canonical” and mechanistically distinct β -arrestin-dependent (G protein-independent) mechanisms. We show here, using BRET-based molecular biosensors, that in addition to coupling to the $G_{q/11}$ /Ca²⁺ pathway, GPR40 interacts with β -arrestins 1 and 2 and is functionally linked to a β -arrestin 2-mediated signaling axis implicated in insulin secretion. Further, our findings identify ligand-specific signaling signatures downstream of GPR40, with the synthetic agonist TAK-875 acting as a β -arrestin-biased GPR40 agonist and the endogenous GPR40 agonists PA and OA being $G_{q/11}$ -biased ligands. With these findings, we have established the existence of biased agonism at GPR40 (Fig. 6).

Biased agonism reportedly emanates from the ability of structurally/chemically distinct ligands of a given 7TMR to sta-

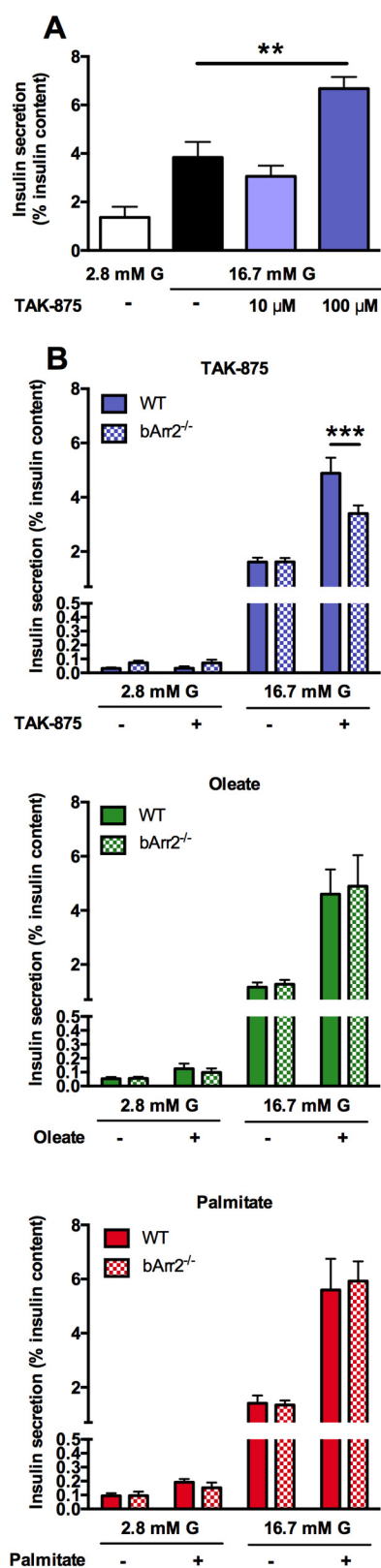


FIGURE 5. β -Arrestin 2 is implicated in the insulinotropic action of TAK-875 but not FFAs in pancreatic islets. A, determination of maximally effective insulinotropic dose of TAK-875. Pancreatic islets isolated from WT C57Bl/6 mice were stimulated with 10 or 100 μ M BSA-complexed TAK-875 in the presence of 16.7 mM glucose. Data are mean \pm S.E. of 4–5 independent determinations. **, $p < 0.01$ versus 16.7 mM glucose by one-way analysis of variance. B, pancreatic islets isolated from WT or β -arrestin 2^{-/-} male C57Bl/6 mice were incubated with KRB buffer containing 2.8 or 16.7 mM glucose in the

presence of BSA-complexed palmitate (500 μ M), oleate (500 μ M), or TAK-875 (100 μ M). Insulin secretion was assessed in 1-h static incubations. The mean \pm S.E. of at least 5 independent experiments is shown. ***, $p < 0.001$ by two-way analysis of variance.

bilize different receptor conformations (14, 31) that may influence the receptor's affinity for various downstream signaling effectors. It is therefore conceivable that TAK-875-bound GPR40 adopts a conformation that displays higher affinity for the β -arrestin versus $G_{q/11}$ pathway, whereas the conformation(s) stabilized by PA and OA preferentially activate(s) $G_{q/11}$. Various studies assessing the interaction of GPR40 with different ligands (both endogenous and synthetic) have revealed the existence of multiple ligand-binding sites (32). Of particular interest, mutation of residues previously shown to be important for the binding of the GPR40 synthetic agonist GW9508 (33) influenced TAK-875-induced Ca^{2+} influx without significantly impacting the activity of the FFA γ -linolenic acid (25). Mutation of two of the three residues shown to be indispensable for GPR40 activity (*i.e.* Arg-183 and Arg-258) rendered TAK-875 completely inactive in Ca^{2+} flux assays, whereas only reducing the potency of γ -linolenic acid in this regard. Further, Hauge *et al.* (12) described agonists promoting dual coupling of GPR40 to $G_{q/11}$ and G_s pathways and used radioligand binding studies to show that dual $G_{q/11}/G_s$ agonists bind GPR40 at a site distinct from that used by agonists stimulating $G_{q/11}$ signaling only. Finally, the recent crystallization of GPR40 co-complexed with TAK-875 (Protein Data Bank accession number 4PHU) revealed the existence of three putative ligand-binding sites and showed that TAK-875 exhibits a binding mode that is likely distinct from that for FFAs (34). Altogether, these results denote a substantial degree of ligand binding diversity and binding pocket plasticity at GPR40 that may translate into ligand-specific, functionally distinct receptor states. Still, direct proof that FFAs and TAK-875 exhibit unique binding modes and stabilize functionally different receptor conformations remains to be provided.

The demonstration of biased agonism at GPR40 was greatly enabled by our ability to directly assess GPR40-linked $G_{q/11}$ activation. Indeed, our study is the first to directly measure $G_{q/11}$ activation following ligand stimulation of native GPR40 in living cells. In a previous work, Yabuki *et al.* (25) used Ca^{2+} flux assays to define TAK-875 as a partial agonist of GPR40-coupled G_q activity. In our study, measures of G_q activation and Ca^{2+} flux were used in a complementary manner to directly demonstrate that relative to PA and OA, TAK-875 is a partial agonist of the G_q signaling axis (Fig. 2). Together, these independent but interrelated measures were instrumental in demonstrating the existence of biased agonism at GPR40.

To demonstrate that the biased agonism observed with biosensors in a heterologous expression system was functionally and physiologically relevant, we assessed insulin secretion in two experimental models: 1) the insulin-secreting cell line INS832/13 in which G_q activity was inhibited or following siRNA-mediated knockdown of β -arrestin 2, and 2) WT and β -arrestin 2^{-/-} islets of Langerhans. Based on the biosensor-derived data, we predicted that the blockade of G_q activity would primarily affect FFA-induced insulin release, whereas

Biased Agonism at FFAR1/GPR40

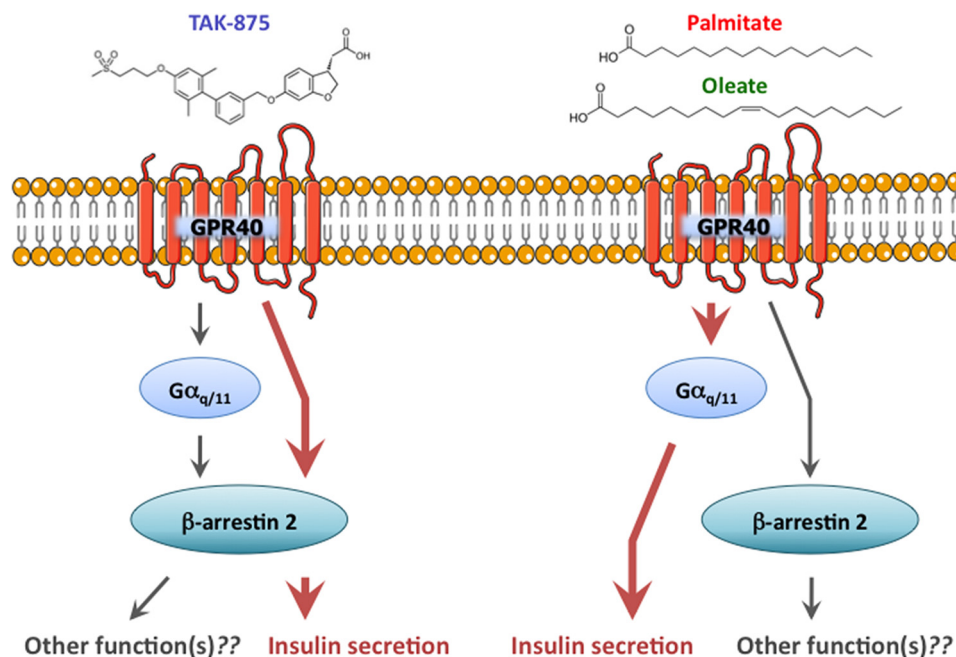


FIGURE 6. Hypothetical model for biased agonism at GPR40. Activation of GPR40 promotes insulin secretion via G protein ($G_{q/11}$)-dependent and β -arrestin-dependent mechanisms, with the endogenous GPR40 agonists PA and OA and the synthetic agonist TAK-875 displaying converse relative efficacies for $G_{q/11}$ and β -arrestin pathway activation (as depicted by *arrow thickness*). *Left side*, TAK-875 acts as a partial agonist of $G_{q/11}$ activation. β -Arrestin recruitment to GPR40 occurs via $G_{q/11}$ -dependent and -independent mechanisms. *Right side*, the $G_{q/11}$ pathway serves as the main GPR40-downstream signaling pathway responsible for the insulinotropic activity of PA and OA. PA and OA also engage β -arrestins, but with lower efficacy than is observed with TAK-875. Activation of β -arrestins by PA and OA is not implicated in mediating their insulin secretagogue effect.

the loss of β -arrestin 2 would impact the secretagogue activity of TAK-875 to a greater degree than that of FFAs. Indeed, this prediction proved to be correct, as shown in Figs. 4 and 5. These data are in agreement with previous data in mouse islets demonstrating that GPR40-dependent G_q signaling accounts for $\sim 50\%$ of FFA-induced potentiation of insulin secretion (35). Importantly, these data also establish a novel role for β -arrestin 2 in GPR40-mediated insulin secretion. Of note, TAK-875-induced insulin secretion was only partially affected by loss of β -arrestin 2, thus implying that β -arrestin 2-independent mechanisms are also operative in this regard. Such mechanisms may involve β -arrestin 1, which is also recruited to GPR40 and has been shown to interact with and mediate insulinotropic signaling downstream of two 7TMRs (*i.e.* the glucagon-like peptide-1 receptor and M_3 -muscarinic receptor) (36–38). β -Arrestin recruitment to GPR40 was also detected in response to PA and OA; however, potentiation of insulin secretion by these FFAs was insensitive to the ablation of β -arrestin 2 expression. These data may indicate that a minimal recruitment “threshold,” which is unattained by PA- or OA-stimulated GPR40, must be reached for the production of a functional outcome. Alternatively, β -arrestins recruited in response to TAK-875 and FFAs may serve distinct functions. Indeed, recent immunofluorescence microscopy data from Qian *et al.* (16) demonstrated that the FFA linoleate results in β -arrestin 2-specific internalization of GPR40.

Our data have also unveiled cross-talk between the G_q -dependent and β -arrestin-mediated signaling pathways downstream of GPR40. Interestingly, pharmacological inhibition of G_q activity differentially affected TAK-875- and FFA-induced β -arrestin recruitment to GPR40 (Fig. 3). Inactivation of G_q

reduced the potency of FFAs to engage β -arrestins, whereas (in a manner comparable with carbachol-stimulated muscarinic acetylcholine receptor M_1 (30)) it reduced the maximal BRET in response to TAK-875 without influencing potency. This combination of BRET outcomes is consistent with an alteration in the overall β -arrestin conformational landscape recruited to GPR40. β -Arrestin recruitment to 7TMRs occurs via both G protein-dependent and G protein-independent mechanisms. In the former, activation of the G protein results in the dissociation of $G\alpha$ and $G\beta/\gamma$ subunits. Consequently, $G\beta/\gamma$ recruits G protein-coupled receptor kinase 2 (GRK2) to the receptor, which in turn phosphorylates cytosolic residues on the 7TMR to promote β -arrestin recruitment (39). Additionally, β -arrestin recruitment can also be activated without G protein coupling (40). This may explain why in our study, inactivation of G_q (which affects β -arrestin recruitment to GPR40) does not impact insulin secretion in response to a ligand whose insulinotropic effect is partly β -arrestin 2-dependent.

The discovery of β -arrestin-induced insulinotropic signaling and biased agonism at GPR40 may have important pharmacotherapeutic implications. Despite the sudden termination of the clinical development of TAK-875 during Phase III trials, GPR40 remains a pharmacological target of interest for the treatment of T2D. Our data demonstrate that GPR40 signaling is neither linear nor entirely $G_{q/11}$ -dependent, which, together with the failure of TAK-875, highlights the importance of characterizing the full complexity of the signaling mechanisms of this 7TMR. The existence of G protein-dependent and β -arrestin-mediated signaling pathways downstream of GPR40 opens the possibility of developing pathway-selective agonists that, through the fine differential regulation of both pathways,

will afford maximal long-term therapeutic benefit and safety. β -Arrestins have been shown to promote β -cell cytoprotection and survival via promoting ERK1/2-dependent phosphorylation of the anti-apoptotic factor Bad (36). Further, β -arrestins negatively regulate the production of pro-inflammatory cytokines by interfering with NF- κ B signaling, a pathway implicated in β -cell dysfunction in T2D (41). Finally, β -arrestins have been implicated in compensatory β -cell proliferation and insulin signaling (22). It is therefore tempting to speculate that contrary to unbiased GPR40 agonists, which would simply act as insulin secretagogues, β -arrestin-biased GPR40 agonists may also afford additional cytoprotective, anti-inflammatory, and mitogenic actions, although this needs to be directly tested.

In conclusion, our data expose unrecognized diversity and texture in GPR40 signal transduction and function. We have shown that GPR40-mediated insulinotropic signaling can occur via a $G_{q/11}$ -dependent pathway as well as a novel β -arrestin 2-dependent axis. Further, these insulinotropic signaling axes are differentially engaged by different GPR40 ligands, with the endogenous ligands PA and OA preferentially activating $G_{q/11}$ signaling and the synthetic ligand TAK-875 promoting β -arrestin 2-mediated signal transduction. Such biased agonism at GPR40 may instigate the development of new pathway-selective GPR40 agonists showing improved clinical efficacy and safety in T2D.

Author Contributions—A. D. M., J. G., M. B., and V. P. conceived and coordinated the study and wrote the manuscript. A. D. M., K. V., E. C., C. T., and J. G. performed and analyzed the experiments shown in Figures 1–4 and 5A. G. B. performed and analyzed the experiments shown in Figure 5B. All authors reviewed the results and approved the final version of the manuscript.

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