The Effects of TAK-875, a Selective G Protein-Coupled Receptor 40/Free Fatty Acid 1 Agonist, on Insulin and Glucagon Secretion in Isolated Rat and Human Islets

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ABSTRACT

G protein-coupled receptor 40 (GPR40)/free fatty acid 1 (FFA1) is a G protein-coupled receptor involved in free fatty acid-induced insulin secretion. To analyze the effect of our novel GPR40/FFA1-selective agonist, [(3S)-6-[[2′,6′-dimethyl-4′-[3-(methylsulfonyl)propoxy]biphenyl-3-yl]methoxy]-2,3-dihydro-1-benzofuran-3-yl]acetic acid hemi-hydrate (TAK-875), on insulin and glucagon secretion, we performed hormone secretion assays and measured intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in both human and rat islets. Insulin and glucagon secretion were measured in static and dynamic conditions by using groups of isolated rat and human pancreatic islets. [Ca\(^{2+}\)]\(_i\), was recorded by using confocal microscopy. GPR40/FFA1 expression was measured by quantitative polymerase chain reaction. In both human and rat islets, TAK-875 enhanced glucose-induced insulin secretion in a glucose-dependent manner. The stimulatory effect of TAK-875 was similar to that produced by glucagon-like peptide-1 and correlated with the elevation of β-cell [Ca\(^{2+}\)]. TAK-875 was without effect on glucagon secretion at both 1 and 16 mM glucose in human islets. These data indicate that GPR40/FFA1 influences mainly insulin secretion in a glucose-dependent manner. The β-cell-specific action of TAK-875 in human islets may represent a therapeutically useful feature that allows plasma glucose control without compromising counter-regulation of glucagon secretion, thus minimizing the risk of hypoglycemia.

Introduction

Insulin and glucagon have opposite effects on the regulation of plasma glucose levels. Insulin, which is secreted from pancreatic β-cells in response to increased plasma glucose, decreases hepatic glucose production and increases glucose uptake in multiple tissues (Frayn, 2003). Impaired insulin secretion is a major cause of the onset and development of type 2 diabetes, and drugs that enhance insulin secretion, such as sulfonylureas and glinides, are commonly used for its treatment (Marchetti et al., 2008). On the other hand, glucagon secretion from pancreatic α-cells is suppressed in response to elevated blood glucose levels, whereas reduced blood glucose enhances glucagon release. Glucagon is the principal hormone stimulating hepatic glucose production. Glucagon secretion is inappropriately high at elevated plasma glucose levels in diabetic patients, which contributes significantly to the hyperglycemia that is a hallmark of the disease (Shah et al., 1999; Cryer, 2008). Thus, impaired regulation of both insulin and glucagon secretion plays a prominent role in the etiology of diabetes.

The mechanism of insulin secretion from β-cells is well understood: glucose metabolism increases the intracellular ATP/ADP ratio, which in turn closes ATP-sensitive potassium channels (K\(_{ATP}\) channels) and activates voltage-gated calcium channels (Ca\(^{2+}\) channels). The resultant stimulation of Ca\(^{2+}\) influx and associated elevation of [Ca\(^{2+}\)], triggers insulin exocytosis (MacDonald et al., 2005). The mechanisms regulating glucagon secretion from α-cells remain debated. It has been suggested that glucose can suppress glucagon secretion by enhanced calcium channel activity, which in turn increases intracellular Ca\(^{2+}\) concentration.
cretion through a direct effect on α-cell activity (Gromada et al., 2004), but there is also evidence that glucagon secretion is under paracrine and neuronal control (Miki et al., 2001; Gromada et al., 2007). Although the precise mechanism by which glucose regulates glucagon secretion is currently unclear, there is consensus that glucagon secretion from α-cells depends on an elevated [Ca$^{2+}$]i (Nadal et al., 1999). Spontaneous [Ca$^{2+}$]i oscillations are seen in α-cells exposed to low glucose concentrations (i.e., conditions associated with the stimulation of glucagon release). Glucose-induced suppression of glucagon secretion has been proposed to involve a reduction of [Ca$^{2+}$]i (Berts et al., 1995; Quesada et al., 1999; Barg et al., 2000; Göpel et al., 2000), but there are conflicting data (Le Marchand and Piston, 2010).

Free fatty acids (FFAs) act as a primary energy source in the body, but they also act as systemic and intracellular signaling modulators. Acute exposure to FFAs leads to the stimulation of insulin secretion (Olofsson et al., 2004b), whereas prolonged exposure leads to the inhibition of glucose-induced insulin secretion (Olofsson et al., 2007; Hoppa et al., 2009). FFAs also influence glucagon secretion from isolated mouse islets (Olofsson et al., 2004a). The acute effects involve stimulation of glucagon secretion at both high and low glucose concentrations, whereas long-term exposure is associated with stimulated release at low glucose levels and loss of glucose-induced suppression (Collins et al., 2008). These effects of FFAs on insulin and glucagon secretion require transport of the FFAs across the plasma membrane and metabolism by long-chain FA-

Intracellular Ca$^{2+}$ Measurements. Intact rat or human islets were loaded with 2 μM Fluo-4-acetoxymethyl ester (Invitrogen) in RPMI 1640 (Invitrogen) medium containing FFA-free BSA (0.01% for rat islets, and 0.2% for human islets), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 mM glucose for at least 2 h at room temperature before the imaging experiments commenced. Islets were immobilized with a wide-bore holding pipette in a continuously superfused and temperature-controlled (37°C) bath mounted on the stage of an Axioskop 2 FS-mot microscope (Carl Zeiss Inc., Thornwood, NY). KRH supplemented with 0.01% FFA-free BSA was used as a perfusion buffer. Images were collected by using an LSM 510 system (Carl Zeiss Inc.) at 1.48-s intervals. Excitation was with a 488-nm argon laser, and emitted fluorescence was collected through 500- to 550-nm band-pass filters for the Fluo-4 signal. Before experimental recordings, islets were perfused for 10 min with the perfusion buffer with 1 mM glucose and 0.1% DMSO. This was followed by perfusion with a buffer containing 16 mM glucose and 0.1% DMSO.
and finally by 16 mM glucose plus 3 μM TAK-875 (0.1% DMSO). The \(\left[\text{Ca}^{2+}\right]_i\) response was determined by baseline subtraction and plotted as change in fluorescence intensity (%Δ\(\text{F}\)/\(\text{F}\)), expressed as follows: %Δ\(\text{F}\)/\(\text{F}\) = \((\text{F}_{\text{max}} - \text{F}_{\text{min}})/\text{F}_{\text{max}} \times 100\). Single β- and α-cells within islets were identified by their reciprocal regulation by glucose as described previously (Berts et al., 1995; Quesada et al., 1999; Barg et al., 2000; Göpel et al., 2000). In brief, when glucose concentration is switched from low (1 mM) to high (16 mM), insulin-secreting β-cells show transient spikes or sustained increase of \(\left[\text{Ca}^{2+}\right]_i\). On the other hand, glucagon-secreting α-cells present \(\left[\text{Ca}^{2+}\right]_i\) oscillations in response to low glucose and tend to become silent at high glucose (MacDonald et al., 2007). To evaluate the effects of TAK-875, the area under the \(\left[\text{Ca}^{2+}\right]_i\) curve for each treatment period was calculated and reported as arbitrary units (AU).

Quantification of mRNA Expression of GPR40/FFA1 and Other Receptor Genes. Four batches of human islets were used in these experiments. Total RNA was extracted from 50 to 100 human islets and purified by using a QIA shredder and QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany). Genome DNA was digested by using RNase-free DNase (QIAGEN). First-strand cdNA was synthesized by random hexamer-primed reverse transcription using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). The mRNA levels were quantified by an ABI Prism 7900 TaqMan PCR system (Applied Biosystems) according to the manufacturer's instruction by using TaqMan Universal PCR Mastermix (Applied Biosystems). Copy numbers of the target genes were determined by the standard curve method using synthesized and polyacrylamide gel electrophoresis-purified oligo DNA fragments (Sigma) containing the PCR amplicon regions. The mRNA expression in each gene was normalized to that of GAPDH. The specific primers and probes were synthesized (Applied Biosystems) according to a published sequences (Tomita et al., 2006). Primer sequences are given in Supplemental Table 1.

Statistical Analysis. Results were expressed as mean ± S.E.M. Statistical analysis between two groups was assessed by Student's t test or the Aspin-Welch test as indicated depending on equal or unequal variances. A p value ≤0.05 was considered statistically significant. The dose-dependent effects of TAK-875 versus control on hormone secretion assays were evaluated by the one-tailed Williams' test, and a p value ≤0.025 was considered significant.

Results

Effects of TAK-875 on Insulin and Glucagon Secretion in Isolated Rat Islets. In rat islets, high glucose (16 mM) increased insulin secretion 3.3-fold compared with low glucose (1 mM). Addition of TAK-875 (0.1–1 μM) or GLP-1 (0.01 μM) potentiated the stimulatory effect of high glucose, but both compounds were ineffective at the low glucose concentration (Fig. 1A). As expected, increasing glucose from 1 to 16 mM resulted in reduced glucagon secretion. Consistent with what was observed in mouse islets (De Marinis et al., 2010), GLP-1 inhibited glucagon secretion at low glucose, and 0.1 μM TAK-875 was equally inhibitory. A 10-fold higher concentration of TAK-875 did not inhibit glucagon secretion in excess of the inhibition produced by 0.1 μM. Neither compound enhanced the inhibitory effect of high glucose (Fig. 1B).

Effects of TAK-875 on the First and Second Phases of Insulin Secretion at High Glucose in Perfused Rat Islets. To analyze the effect of TAK-875 on first- and second-phase insulin secretion, dynamic measurements of insulin secretion were obtained by perfusion of isolated rat islets. As shown in Fig. 2A, TAK-875 (3 μM) had no stimulatory effect on insulin secretion at 5 or 1 mM glucose and, if anything, inhibited secretion. However, a strong stimulation was observed at 16 mM glucose. The stimulatory effect was particularly pronounced for second-phase insulin secretion; the area under the curve (AUC) measured during the first phase (62–69 min) and second phase (70–82 min) increased 2.3- and 3.9-fold, respectively (Fig. 2B). The stimulatory effect of 16 mM glucose in both the absence and presence of TAK-875 was promptly reversed upon reduction of glucose to 5 mM.

Effects of TAK-875 on \(\left[\text{Ca}^{2+}\right]_i\) in Individual Cells within Intact Rat Islets. We next examined the effects of TAK-875 (3 μM) on \(\left[\text{Ca}^{2+}\right]_i\) responses in individual β- and α-cells within isolated intact rat islets (Fig. 3A). When glucose concentration was switched from low (1 mM) to high (16 mM), β-cells responded with either transient spikes or a sustained increase in \(\left[\text{Ca}^{2+}\right]_i\). The addition of TAK-875 at high glucose concentration resulted in a further elevation of \(\left[\text{Ca}^{2+}\right]_i\), in β-cells (Fig. 3B, Rβ1 and Rβ2). In α-cells, identified by the generation of \(\left[\text{Ca}^{2+}\right]_i\) oscillations at 1 mM glucose, increasing glucose to 16 mM reduced the amplitude of, but did not suppress, these \(\left[\text{Ca}^{2+}\right]_i\) oscillations. These effects of glucose were not changed by the inclusion of TAK-875 in the superfusion medium (Fig. 3B, Rα1 and Rα2). The effects of glucose and TAK-875 on β- and α-cell \(\left[\text{Ca}^{2+}\right]_i\) are summarized in Fig. 3, C and D.

Comparison of Gene Expression Levels of GPR40/FFA1, GLP-1R, and SUR1 in Human Islets. GPR40/FFA1 is highly expressed in rodent islets, but there is little information about the expression levels in human islets. There-
fore, we first quantified mRNA expression of GPR40/FFA1 in human islets from four nondiabetic organ donors. Donor characteristics are given in Supplemental Table 2. The expression of GPR40/FFA1 in human islets was comparable with that of the GLP-1 receptor (GLP-1R) (Fig. 4). Expression of GPR40/FFA1 was also compared with the K\textsubscript{ATP}-channel subunit SUR1. Again, GPR40/FFA1 expression was found to be at least as high as that of the reference gene. Thus, GPR40/FFA1 is expressed in human islets at levels comparable with or higher than those of two other genes that encode proteins with key roles in islet/β-cell function. This prompted us to investigate the effects of TAK-875 on insulin and glucagon secretion from human islets.

**Effects of TAK-875 on Insulin and Glucagon Secretion in Isolated Human Islets.** The effects of TAK-875 at 1 or 16 mM glucose in the absence and presence of TAK-875 resulted in a further elevation in many of the oscillations made it difficult to evaluate the effects of glucose and TAK-875, so we therefore refrained from quantitative analysis of these data from α-cells.

**Discussion**

Because activation of GPR40/FFA1 signaling evoked by medium- or long-chain FFAs enhances glucose-dependent insulin secretion (Itoh et al., 2003; Fujisawa et al., 2005), potent and specific GPR40/FFA1 agonists may be useful for the treatment of type 2 diabetes. Although published reports show that small-molecule agonists for GPR40/FFA1 stimulate glucose-dependent insulin secretion in both in vitro and in vivo rodent models (Briscoe et al., 2006; Tan et al., 2008; Bharate et al., 2009), there is as yet no evidence that GPR40/FFA1 agonists are effective in human islets. In addition, the effects of selective GPR40/FFA1 agonists on glucagon secretion have not been elucidated. Here, we have examined the effects of TAK-875, a novel GPR40/FFA1 agonist, on insulin and glucagon secretion and [Ca\textsuperscript{2+}]\textsubscript{i} in rat and human pancreatic islets. The significance of such comparative studies is highlighted by electrophysiological studies demonstrating major differences in the ion channel complements expressed in human and rodent islet cells (Braun et al., 2008, 2009; Ramracheya et al., 2010). It is unlikely that these differences will be restricted to ion channels and those already documented.

We found that GPR40/FFA1 is expressed at levels comparable with those found for GLP-1R and SUR1 in human islets. Similar results have been reported in rat islets (Itoh et al., 2003). GLP-1R and SUR1 play important roles in β-cell function, and they constitute the molecular targets of two groups of insulintropic drugs: GLP-1 analogs and sulfonylureas. We show that TAK-875 augments glucose-dependent insulin secretion in a way reminiscent of the action of GLP-1 in both rat and human islets. The dynamic measurements of insulin secretion reveal that although TAK-875 enhances
2 and Rβ2 and Rα1 and Rα2 are the representative [Ca2+]i response from single β- and α-cells, respectively, exposed to 1 mM glucose, 16 mM glucose, and 16 mM glucose plus 3 μM TAK-875 as indicated above the traces. C, summary of the [Ca2+]i response from 43 single β-cells within three independent islets and expressed as mean ± S.E.M. **, p ≤ 0.01 versus 1 or 16 mM glucose control (Aspin-Welch test). §§, p ≤ 0.01 versus 16 mM glucose control (Student’s t test).

Fig. 3. Effects of TAK-875 on the intracellular Ca2+ response of single β- and α-cells within intact rat islets. A, color image of rat islet located with the Ca2+-sensitive dye Fluo-4 at the beginning of Ca2+ recording. B, records of fluorescence intensity versus time from the cells indicated in A. Rβ1 and Rβ2 and Rα1 and Rα2 are the representative [Ca2+]i response from single β- and α-cells, respectively, exposed to 1 mM glucose, 16 mM glucose, and 16 mM glucose plus 3 μM TAK-875 as indicated above the traces. C, summary of the [Ca2+]i response from 43 single β-cells within three independent islets and expressed as mean ± S.E.M. D, summary of [Ca2+]i response from 23 single α-cells within three independent islets, expressed as mean ± S.E.M. **, p ≤ 0.01 versus 1 or 16 mM glucose control (Aspin-Welch test). §§, p ≤ 0.01 versus 16 mM glucose control (Student’s t test).

Fig. 4. Comparison of gene expression levels of GPR40/FFA1 (left), GLP-1R (center), and SUR1 (right) in human islets. The relative mRNA expression for an internal control (GAPDH) are expressed as mean and S.E.M. (n = 4).

both first- and second-phase insulin secretion in a strictly glucose-dependent fashion the stimulatory effect is particularly pronounced for second-phase release.

The insulin-releasing capacity of TAK-875 correlated with elevation of [Ca2+]i. It has been proposed that GPR40/FFA1, via inositol trisphosphate production, mobilizes Ca2+ from the endoplasmic reticulum (Fujiwara et al., 2005). Alternatively, TAK-875 may act by enhancing entry of extracellular Ca2+ via the voltage-gated Ca2+ channels. This could result from a direct effect on the voltage-gated Ca2+ channels or an indirect effect caused by closure of the KATP channels (Feng et al., 2006). The exact mechanism of the glucose-dependent insulinotropic action of TAK-875 remains to be elucidated and is beyond the scope of the current investigation, the primary objective of which was to compare the action of TAK-875 on hormone secretion from human and rat islets.

The human islet preps showed some heterogeneity in insulin secretion, and one batch (donor 7) was unresponsive to 1 μM TAK-875 (Supplemental Table 3), but it should be noted that the same islets responded to the agonist with an elevation of [Ca2+]i (Supplemental Fig. 1). Studies on islets from a greater number of donors are therefore needed to determine whether there is a true heterogeneity in responsiveness to TAK-875.

We also show that TAK-875 tends to inhibit rather than stimulate glucagon secretion induced by low glucose in rat islets, but it is without effect on glucagon secretion from human islets. The presence of GPR40/FFA1 in rodent α-cells is debated (Flodgren et al., 2007; Hirasa et al., 2008). However, GPR40/FFA1 expression in human islets seems dominant in insulin-positive cells (Itoh et al., 2003; Tomita et al., 2006). Thus, one explanation for the lack of glucagonostatic action in human islets might reflect the low GPR40/FFA1 expression in human α-cells. Another explanation is that TAK-875 might affect paracrine factors, such as somatostatin secretion, and the contribution might be different between species.

It was reported that linoleic acid, one of the endogenous agonists for GPR40/FFA1, stimulates glucagon secretion in rodent islets (Flodgren et al., 2007; Wang et al., 2011). By contrast, our results indicate that TAK-875 is without stimulatory effect on glucagon secretion in both rat and human islets and if anything tends to inhibit glucagon secretion in rat islets exposed to low glucose. TAK-875 is >400-fold more potent at activating human GPR40/FFA1 than oleic acid, which shows agonist activity almost similar to that of linoleic acid (Tsujihata et al., 2011). These results clearly suggest that, unlike physiological agonists, the activation of GPR40/FFA1 by our selective synthesized agonist has no potential to enhance glucagon secretion.

With regard to the interaction of TAK-875 with the endogenous ligands (FFAs), it is of interest that TAK-875 potently...
stimulates insulin secretion during oral glucose tolerance tests in diabetic rats fasted overnight, in which plasma FFA levels are elevated because of the stimulation of lipolysis (Tsujihata et al., 2011). These data suggest that TAK-875 may show positive cooperativity with endogenous ligands. The role of GPR40/FFA1 in FFA-regulated glucagon secretion merits further studies, and it deserves pointing out that FFAs may influence glucagon secretion by mechanisms that do not involve GPR40/FFA1 (Olofsson et al., 2004a).

As for the glucagonostatic action of TAK-875 at 1 mM glucose in rat islets, a lower dose (0.1 μM) is paradoxically more potent than a higher dose (1 μM). The underlying mechanisms are unclear but it is worth remembering that this "anomaly" is shared with other modulators of glucagon secretion. Thus, glucose, adrenaline, and forskolin inhibit glucagon secretion at a low concentration, whereas higher concentrations are less inhibitory (glucose; Vieira et al., 2007) or even stimulatory (adrenaline and forskolin; De Marinis et al., 2010).

Our data suggest that the effects of TAK-875 on glucagon secretion differ between rat and human islets. This underscores the importance of confirming in human islets observations made in rodent islets. The clinical relevance of the lack of effect of TAK-875 on glucagon secretion remains to be determined. Diabetes is associated with hypersecretion of glucagon at elevated plasma glucose levels. Pharmacological treatment of hyperglycemia and hypoinsulinemia is complicated by an impaired counter-regulation by glucagon during hypoglycemia. From this perspective, it may be argued that the selective action of TAK-875 on insulin secretion at high glucose may be clinically beneficial.

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Prof. D. R. Matthews (Oxford Centre for Diabetes, Endocrinology, and Metabolism) for valuable discussions and helpful suggestions.

Authorship Contributions

Participated in research design: Yashiro, Tsujihata, Takeuchi, Hazama, and Rorsman.

Conducted experiments: Yashiro, Tsujihata, Takeuchi, Hazama, and Rorsman.

Contributed new reagents or analytic tools: Johnson and Rorsman.

Performed data analysis: Yashiro and Tsujihata.

Wrote or contributed to the writing of the manuscript: Yashiro, Tsujihata, Takeuchi, and Rorsman.

References


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Title: The effects of TAK-875, a selective GPR40/FAA1 agonist, on insulin and glucagon secretion in isolated rat and human islets

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Journal: Journal of Pharmacology & Experimental Therapeutics

Supplemental Figure 1 Individual data of [Ca^{2+}]_i response to 3 µM TAK-875 in three non-diabetic donors.

The data are collected from 26 β cells from 4 islets, 57 β cells from 5 islets and 63 β cells from 6 islets from donor 7, 8 and 9, respectively. Data are expressed as mean ± S.E.M.
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Supplemental Table 1 Sequences of TaqMan primers and probes

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<th>Reverse primer</th>
<th>Probe</th>
<th>Accession number</th>
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<td>GPR40/FAA1</td>
<td>5'-GCCCGCTTCAGCCTC TCT-3'</td>
<td>5'-GAGGCAGCCCAGTA GCA-3'</td>
<td>FAM-5'-TCTGCCCTTGCCATCACAGCCT-3'-T AMRA</td>
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<td>GLP-1R</td>
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<tr>
<td>ABCC8</td>
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<td>5'-GAATGTCTTCCGACCTGG-3'</td>
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FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine
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Journal: Journal of Pharmacology & Experimental Therapeutics

**Supplemental Table 2 Clinical profiles of islet donors**

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<th>Sex(M/F)</th>
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<td>M</td>
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<td>2</td>
<td>55</td>
<td>M</td>
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<td></td>
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<td>M</td>
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<tr>
<td></td>
<td>4</td>
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<td>Hormone secretion assay (Table S3, Fig. 5)</td>
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<td>M</td>
</tr>
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<td>51</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>57</td>
<td>F</td>
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<tr>
<td>Intracellular Ca^{2+} measurements (Fig. 6)</td>
<td>7</td>
<td>51</td>
<td>M</td>
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<td></td>
<td>8</td>
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<td></td>
<td>9</td>
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Supplemental Table 3 Summary of the results of static incubation assay in human islets (from 4 organ donors)

<table>
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<th>No.</th>
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<th>16 mM Glucose</th>
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<tr>
<td></td>
<td>TAK-85 (1 μM)</td>
<td>GLP-1 (10 nM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.3 μM)</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.27±0.02</td>
<td>0.35±0.02a</td>
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<tr>
<td>2</td>
<td>1.68±0.23</td>
<td>1.48±0.18</td>
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<tr>
<td>3</td>
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<td>0.45±0.10</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>Glucagon</td>
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<td>2.44±0.24</td>
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<tr>
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<td>0.56±0.11</td>
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<tr>
<td>3</td>
<td>1.19±0.21</td>
<td>1.87±0.41</td>
</tr>
<tr>
<td>4</td>
<td>1.02±0.19</td>
<td>0.95±0.11</td>
</tr>
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</table>

Human islets had been preincubated for 1 h at 1 mM glucose. Following the preincubation, these islets were incubated with the assay buffer containing 1 or 16 mM glucose with or without test substances for 1 h. Insulin (upper panel) and glucagon (lower panel) secretion were shown as % of content/h. Data are expressed as mean ± S.E.M (n=2-4). a, p≤ 0.05; b, p≤ 0.01 vs. 1 mM glucose control (Aspin Welch test); c, p≤ 0.05, d, p≤ 0.01, vs. 1 mM glucose control (Student’s t-test); e, p≤ 0.025, vs. 16 mM glucose control (one-tailed Williams’ test); f, p≤ 0.05 vs. 16 mM glucose control (Aspin Welch test).