Multivalent activation of GLP-1 and sulfonylurea receptors modulates β-cell second-messenger signaling and insulin secretion

Nathaniel J. Hart,1 Craig Weber,2 Klearchos K. Papas,1 Sean W. Limesand,3,5 Josef Vagner,5 and Ronald M. Lynch2,4,5

1Department of Surgery, University of Arizona, Tucson, Arizona; 2Department of Physiology, University of Arizona, Tucson, Arizona; 3School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson, Arizona; 4Department of Pharmacology, University of Arizona, Tucson, Arizona; and 5BIO5 Institute, University of Arizona, Tucson, Arizona

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Hart NJ, Weber C, Papas KK, Limesand SW, Vagner J, Lynch RM. Multivalent activation of GLP-1 and sulfonylurea receptors modulates β-cell second-messenger signaling and insulin secretion. Am J Physiol Cell Physiol 316: C48–C56, 2019. First published November 7, 2018; doi:10.1152/ajpcell.00209.2018.—Linking two pharmacophores that bind different cell surface receptors into a single molecule can enhance cell-targeting specificity to cells that express the complementary receptor pair. In this report, we developed and tested a synthetic multivalent ligand consisting of glucagon-like peptide-1 (GLP-1) linked to glibenclamide (Glb) (GLP-1/Glb) for signaling efficacy in β-cells. Expression of receptors for these ligands, as a combination, is relatively specific to the β-cell in the pancreas. The multivalent GLP-1/Glb increased both intracellular cAMP and Ca2+ responses in β-cells and islet β-cells, although Ca2+ responses were significantly depressed compared with the monomeric Glb. Moreover, GLP-1/Glb increased glucose-stimulated insulin secretion in a dose-dependent manner. However, unlike the combined monomers, GLP-1/Glb did not augment insulin secretion at nonstimulatory glucose concentrations in INS 832/13 β-cells or human islets of Langerhans. These data suggest that linking two binding elements, such as GLP-1 and Glb, into a single bivalent ligand can provide a unique functional agent targeted to β-cells.

β-cell; cell targeting; diabetes; GLP-1; GPCR signaling; incretin; multivalent; sulfonylurea

INTRODUCTION

Type 2 diabetes is a progressive disease that requires continual pharmacological treatment to modify insulin resistance and insulin release by β-cells (20, 34, 35, 37). Type 2 diabetes is treated with sulfonylureas, including glibenclamide (Glb), which enhance insulin secretion in a glucose-independent manner via their interaction with β-cell sulfonylurea-1 receptors (SUR1; 34). Additionally, agonists of the glucagon-like peptide-1 receptor (GLP-1R) have recently gained considerable traction as diabetic therapeutics because of their myriad antidiabetic properties (10), including glucose-dependent stimulation of insulin secretion from β-cells (26), inhibition of glucagon secretion from pancreatic α-cells (12, 32, 33), decreased gastric emptying (33), and central anorectic effects (12, 48). GLP-1R agonists improve glycemic control (8, 23, 39) and promote weight loss (7, 39, 51); however, GLP-1R agonists have been associated with side effects. The most common side effects are gastrointestinal in nature, including nausea, vomiting, and diarrhea. Concerns also have been raised related to long-term use of glucagon-like peptide-1 (GLP-1) agonists because of their potential induction of cell growth (1, 9). With off-target effects of GLP-1 analog treatment, diabetic therapeutics may benefit from improved cell type specificity.

Most natural peptide hormones and biological signaling agents bind to a receptor on the cell surface to activate downstream signaling events. Many of these interactions involve a single recognition element within the ligand that binds a complementary region on its receptor in a monovalent interaction. However, some agents exhibit higher specificity for their receptors by interacting with multiple sites on the receptor’s extracellular face, where one domain is required for activation and a second or multiple domains provide allosteric properties (21). GLP-1 exhibits multivalent binding to its receptor, wherein binding to a transmembrane domain is required for receptor activation while other sites on GLP-1 bind to the extracellular tail region of GLP-1R conferring allosteric regulation (24, 25).

Higher-order multivalent interactions also have been demonstrated by coupling multiple copies of a ligand into a single molecule. In seminal studies, Sharma et al. developed melanotropin (MSH) conjugates containing multiple copies of a potent melanotropin analog ([Nle4,D-Phe7]α-MSH; 43). These MSH analogs exhibited enhanced binding affinities, which were attributed to simultaneous interactions with multiple MSH receptors on the cell membrane (43). The effects of monovalent and multivalent homomeric MSH analogs on downstream signaling were also evaluated. The sensitivity for activation of cAMP formation was enhanced ~100-fold for trivalent MSH compounds compared with the monovalent control suggesting that simultaneous binding to multiple receptors not only enhanced binding affinity but also activated downstream signaling efficiently (3).

On the basis of theoretical modeling, the increased affinity of multivalent ligands (MVLs) is likely due to proximity of the second binding domain to the membrane once the initial ligand of the complex binds to its receptor. The enhanced proximity greatly increases the probability for binding to a second receptor and, once bound, reduces the likelihood of dissociation thereby yielding high-affinity interactions (4). Thus, cell type specificity could be achieved by linking different elemental

Address for reprint requests and other correspondence: R. M. Lynch, Dept. of Physiological Sciences, Univ. of Arizona, 1656 E. Mabel St., Tucson, AZ 85721 (e-mail: rlynch@email.arizona.edu).
binding domains [receptor recognition elements (22)] into heteromultivalent ligand (htMVL) complexes that allow them to simultaneously bind different receptor types (3, 19, 50, 53). Portoghese et al. evaluated a series of bivalent ligands composed of δ- and κ-opioid antagonists linked through a range of spacer lengths (36). Pharmacophores linked with a spacer of 21 atoms displayed the greatest enhancement in affinity (36). These studies concluded that the opioid receptors for the two unique ligands organized into functional heterodimers to obtain the enhanced binding (6, 36). We subsequently proposed that if the expression of a “receptor cohort” is unique to a cell type of interest, a htMVL could be developed with recognition elements for a cell type-specific receptor combination, which would enhance target specificity (19, 53). To test this possibility, a construct was synthesized with recognition elements for the melanocortin-1 receptor linked to a binding domain for the cholecystokinin B receptor. Again, a significant enhancement in binding affinity was observed, and this high-affinity binding was observed only to cells that expressed both complementary receptors (19, 52). Specific targeting of this MVL to dual receptor-expressing tumors was then demonstrated in vivo, indicating the targeting potential of these agents (52). However, the aforementioned studies were performed on engineered cell lines that overexpressed the complementary receptor pair (19, 52, 53). Thus, we sought to characterize MVL binding and signaling in cells that were not engineered to overexpress the receptor pair of interest. To target pancreatic β-cells, we created a GLP-1 analog where GLP-1(1–37) was linked to the α2-adrenergic receptor antagonist yohimbine. This GLP-1-yohambine htMVL was observed to bind at low concentration (1–5 nM) only to cells naturally expressing the complementary receptors and targeted islets of Langerhans in rodents (44). These findings demonstrated that htMVL can bind with high avidity and enhanced specificity to cells with endogenous expression of the complementary receptors.

Other unique dual ligands based on GLP-1 have been synthesized and tested for their efficacy in modulating β-cell function. Tschöp and colleagues synthesized estrogen coupled directly to a GLP-1 analog (11). In this case, GLP-1 was used to ferry the estrogen to any cell that expressed the GLP-1 receptor, but this ligand was unlikely to exhibit simultaneous binding to the complementary receptors; rather, the estrogen was expected to be released to the cytosol after cleavage from the construct within the lysosomal pathway. This approach demonstrates one of the potential uses of multivalent agents as cell-specific targeting/delivery agents. In other studies, Glb has been linked to glucose to improve the hydrophilicity of Glb and its usage as a targeting agent for imaging (41). This ligand exhibited high-affinity SUR binding with short-duration effects on blood glucose due to enhanced clearance (41). A truncated GLP-1 (residues 7–36) [GLP-1(7–36)] was synthesized on a resin with solid-phase chemistry, modified at Lys26 with a flexible oligoethylene glycol, and then conjugated to the carboxy-glibenclamide derivative. A thiol intermediate, GLP-1/Glb, was cleaved from the resin, purified by HPLC, and characterized by high-resolution mass spectrometry. Similarly, monomeric GLP-1(7–36) used in subsequent experiments was prepared by solid-phase chemistry on a resin cleaved from the resin, purified by HPLC, and characterized by mass spectrometry.

Cell culture. INS 832/13 (from C. Newgard, Durham, NC) and βTC3 (American Type Culture Collection) cell lines were selected because of their GLP-1R/SUR1 expression and insulin secretory response to elevated glucose and other secretagogues (15, 40). Passages 20–40 and 40–50 were used for INS 832/13 and βTC3 cells, respectively. Both cell lines were grown in RPMI 1640 medium with 1-glutamine (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 5.5 mM glucose (INS 832/13 media were supplemented with 10 mM HEPES) at 37°C in 5% CO2. Both cell lines were confirmed to be mycoplasma free upon receipt.

Cytosolic Ca2+ measurements. βTC3 cells were grown on 25-mm, no. 1 coverslips housed in six-well plates to ~70% confluency. For cytotoxic Ca2+2 concentration ([Ca2+]i) measurements, coverslips with βTC3 cells were washed with HBSS at 37°C for 10 min and then loaded with 2.5 μM of the acetoxymethyl ester (AM) form of fura-2 (Molecular Probes) with 0.0025% pluronic acid in HBSS-HEPES-buffered salt solution containing (in mM) 0.3 KH2PO4, 138 NaCl, 0.2 NaHCO3, 0.3 NaH2PO4, 20 HEPES, 1.3 CaCl2, 0.4 MgSO4, and 5.5 glucose (unless otherwise noted) at pH 7.4 for 20 min at 37°C. Cells were rinsed in HBSS for 20 min at 37°C to allow for hydrolysis of the fura-2 AM. The coverslip with the dye-loaded cells was then placed in a chamber held at 37°C while mounted on the stage of an inverted Olympus IX-70 microscope equipped with a ×40 1.4-numerical aperture ultrafluar objective and a 150-W Xe lamp as the excitation source. Fura-2 was alternately excited at 340 and 380 nm using a filter.
The emitted light was filtered at 510 nm (10-nm band pass) and captured with a charge-coupled device camera (CH-350; Photometrics). Control images were acquired before the addition of the selected ligand, GLP-1/Glb or Glb (glybenzycyclamide; Alfa Aesar, Ward Hill, MA). Following ligand addition, images were acquired at regular intervals from 30 s to 10 min. Following the 10-min experimental period, 10 μM ionomycin was added to the media, and images were acquired. On average, groups of 16–20 cells were analyzed for each coverslip, and the average response from these cells was considered a single experiment. A minimum of five independent coverslips were analyzed for each experimental condition. For quantification, regions of interest were drawn within the cytosol of the cell using ImageJ (National Institutes of Health, Bethesda, MD), and the average pixel intensity was measured at 340 and 380 nm throughout the sequential series of images. For data analysis, fluorescence values were converted to a ratio of excitation at 340 and 380 nm and, using an in vitro calibration of fura-2, subsequently converted to [Ca\(^{2+}\)]. Responses were calculated, and

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### Fig. 1. Combinatorial expression of glucagon-like peptide-1 receptor (GLP1R) and ATP-binding cassette subfamily C member 8 (ABCC8) was relatively specific to the pancreas and β-cells. RNA sequencing from the Human Protein Atlas (49; A), Genotype-Tissue Expression (GTEx; B), and Functional Annotation of Mammalian Genomes 5 (FANTOM5; C) data sets showed that the combinatorial expression of GLP1R and ABCC8 was relatively specific to pancreatic tissue. However, the GLP1R and ABCC8 expression combination was also present in the brain and heart. Single-cell RNA sequencing by Segerstolpe et al. (42) showed that combinatorial expression of GLP1R and ABCC8 was restricted to β-cells (D) within the pancreas (D–H). RPKM, reads per kilobase of transcript per million mapped reads; TPM, transcripts per million. The GTEx Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health and by the National Cancer Institute, National Human Genome Research Institute, National Heart, Lung, and Blood Institute, National Institute on Drug Abuse, National Institute of Mental Health, and National Institute of Neurological Disorders and Stroke. [Data used for tissue analysis (A–C) were accessed via the Human Protein Atlas (version 18; www.proteinatlas.org/ENSG00000112164-GLP1R/tissue and www.proteinatlas.org/ENSG00000006071-ABCC8/tissue), with permission under the Creative Commons Attribution-ShareAlike 3.0 International License.]
data are presented as the maximal change in $[Ca^{2+}]$, over baseline during the experimental course. EC$_{50}$ and $r^2$ values were obtained using non-linear regression analysis in GraphPad Prism (La Jolla, CA).

**cAMP assays.** βTC3 cells were seeded in a 96-well plate at a density of 70,000 cells per well in RPMI with 10% FBS and 1% penicillin-streptomycin and cultured for 72 h. Cells were rinsed in serum-free RPMI and then cAMP assay medium [serum-free RPMI with 1 mM isobutyl-1-methylxanthine (IBMX)] for 5 min at 37°C. Cells were incubated with ligands in fresh cAMP assay medium for 15 min at 37°C. After treatment, media were discarded, cells were lysed, and the amount of cAMP was determined using a cAMP Chemiluminescent Immunoassay Kit (Invitrogen, Carlsbad, CA) per the manufacturer’s instructions. EC$_{50}$ and $r^2$ values were obtained using non-linear regression analysis in GraphPad Prism. At least 10 wells per ligand concentration were analyzed, with up to 5 ligand concentrations evaluated per experiment.

**Insulin secretion assays.** Insulin secretion assays were performed using the INS 832/13 cell line, which has been shown to be an incretin-sensitive model with robust glucose-stimulated insulin secretion (GSIS; 17, 38). INS 832/13 cells were seeded into a 24-well tissue culture plate at a density of 600,000 cells per well and cultured for 96 h. After 72 h, media were replaced with RPMI media containing 1 mM glucose (1 G). Insulin secretion for each well was measured in 1 ml of oxygenated HBSS with 1 G for 40 min at 37°C with gentle agitation. After this initial period, one-half of the media was removed, and an equal volume of oxygenated HBSS containing the desired glucose concentration ± modulator was added. The incubation continued for an additional 40 min at 37°C; then media were collected, and insulin concentrations were measured with the High Range Rat Insulin ELISA Kit (ALPCO) per the manufacturer’s instructions. Samples were run in quadruplicate and averaged. Insulin secretion rates are presented as a percentage of the dynamic range (%dynamic range) of secretion measured within each experiment; that is, basal insulin secretion in the presence of 1 G (min) and the maximum cAMP-potentiated GSIS induced by 15.5 mM glucose + 10 mM forskolin (max) were measured for each experiment, and the %dynamic range for a given condition ($x$) was then calculated as

$$%\text{dynamic range} = \left( \frac{x - \text{min}}{\text{max} - \text{min}} \right) \times 100$$

This protocol provided a high degree of precision for normalizing between individual experiments wherein absolute rates might vary greatly; when evaluated as %dynamic range, these differences were reduced. Insulin secretion values were taken from at least eight replicate wells for each condition evaluated.

**Human islet culture and perfusion.** Human pancreatic islets (combined from 2 female non-diabetic donors, aged 52 and 61) were provided by the Integrated Islet Distribution Program (City of Hope, Duarte, CA). Islets were maintained in gas-permeable culture flasks (G-Rex100; Wilson Wolf) containing CMRL medium (cellgro, 99-603-CV) supplemented with 5% human serum albumin (Sigma-Aldrich), 10 U/ml heparin (Fisher Scientific, Hampton, NH), and 1% penicillin-streptomycin (Sigma-Aldrich) in humidified air with 5% CO$_2$ at 37°C. Media were changed every 3 days by removing ~50% of old media and adding an equal volume of fresh CMRL medium. Islets used for these experiments were in culture 11–16 days before measurement.

An automated perfusion system (model PERI-4.2; Biorep Technologies, Miami Lakes, FL) was used to collect 100-µl timed samples from the human pancreatic islets. A low-pulsatility peristaltic pump pushed Krebs-Ringer bicarbonate (KRB) through a sample chamber containing ~100 islet equivalents immobilized in Bio-Gel P-4 Gel at a rate of 100 µl/min. The KRB contained (in mM) 137 NaCl, 4.7 KCl, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 2.5 CaCl$_2$, 25 NaCO$_3$, and 20 HEPES and 0.25% BSA (pH 7.4). All islets were perfused with 2.8 mM glucose (2.8 G) KRB for 1 h before initiation of experimental measurements. The chambers containing the islets were maintained at 37°C. Islet response to 16.7 mM glucose (16.7 G) alone was measured in parallel with experimental perfusions. The perfusate was collected in an automatic fraction collector designed for a 96-well plate. Samples (100 µl) were collected every minute and were stored at -80°C. Insulin secreted into each sample was measured using a human ELISA kit (no. 10-1113; Mercodia, Uppsala, Sweden) according to the manufacturer’s instructions. If necessary, samples were diluted 1:10 in KRB to provide a concentration that fell within the range of the standard curve; otherwise, undiluted samples were used. Samples were run in duplicates. Insulin was normalized to the total DNA from the islets in each chamber. DNA was quantified using the Quant-it PicoGreen dsDNA kit (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Islets were diluted in 1.0 ml AT buffer (1 M ammonium hydroxide and 0.2% Triton X-100) and then sonicated for 15 s. Samples were run in quintuplicates. Experimental perfusion values are presented as a percentage of the maximum GSIS measured in parallel experiments. Area under the curve (AUC) values for 2.8 G + ligand(s) were calculated by subtracting the AUC of the initial 6 min of the perfusion where islets were exposed to 2.8 G alone from the AUC of 6 min of the 2.8 G + ligand(s) period (11–17 min). AUC values for 16.7 G + ligand(s) were calculated by subtracting the AUC of 12 min of the 16.7 G stimulation period of parallel experiments from the AUC of 12 min of the 16.7 G + ligand(s) period (23–35 min).

**Statistical analysis.** Data are presented as means ± SE. Statistical differences were determined using an unpaired two-sided Student’s t-test or a one-way ANOVA with a Tukey multiple-comparison correction in GraphPad Prism. A value of $P < 0.05$ was considered statistically significant for all statistical tests.

**RESULTS**

[Ca$^{2+}$], changes were diminished by the multivalent GLP-1/Glb ligand. Glb is known to elevate [Ca$^{2+}$], in β-cells via its inhibition of ATP-sensitive K$^+$ (K$_{ATP}$) channel activity and subsequent membrane depolarization. To measure the effect of GLP-1/Glb on [Ca$^{2+}$], we developed dose-response profiles of the maximal [Ca$^{2+}$], change elicited by GLP-1/Glb and monomeric Glb (Fig. 2). Both GLP-1/Glb and Glb elicited concentration-dependent increases in [Ca$^{2+}$]. However, the responses elicited by GLP-1/Glb were reduced over the entire concentration range compared with monomeric Glb, suggesting that the Glb moiety of the htMVL was not as efficacious as monomeric Glb (Fig. 2).

**cAMP signaling of GLP-1/Glb was equivalent to that of GLP-1.** GLP-1 is known to potentiate β-cell insulin secretion via elevation of cAMP, whereas Glb does not influence cellular cAMP levels, which we confirmed for the βTC3 cell line (Fig. 3). To investigate the signaling properties of the GLP-1 moiety of the GLP-1/Glb ligand, we compared cAMP production in βTC3 cells in response to several concentrations of GLP-1/Glb or monomeric GLP-1 (Fig. 4). Significant increases in cAMP production were observed at 1 nM for both the GLP-1/Glb ligand and GLP-1 monomer. However, no significant differences were observed between GLP-1/Glb ligand and monomeric GLP-1 over a wide concentration range (GLP-1/Glb, EC$_{50}$ = 7.22 nM and $r^2$ = 0.9924; GLP-1, EC$_{50}$ = 8.76 nM and $r^2$ = 0.9934), suggesting that the efficacy and potency of the GLP-1 moiety of the htMVL were preserved.

**GLP-1/Glb modulation of INS 832/13 cell insulin secretion.** After investigating the Ca$^{2+}$ and cAMP signaling properties of the GLP-1/Glb ligand we sought to determine the functional outputs of the htMVL by measuring insulin secretion from INS.
Fig. 2. β-Cell Ca^{2+} was diminished in the presence of heterobivalent glucagon-like peptide-1-glibenclamide (GLP-1/Glb). The maximum change in in βTC3 cytosolic Ca^{2+} concentration ([Ca^{2+}]_i) in response to GLP-1/Glb or Glb was measured using fura-2. GLP-1/Glb increased [Ca^{2+}]_i, in a dose-dependent manner, but GLP-1/Glb did not increase [Ca^{2+}]_i, as effectively as monomeric Glb at any concentration. Data represent means ± SE of separate experiments (GLP-1/Glb: n = 5–13 experiments, n = 98–216 total cells per concentration; monomeric Glb: n = 5–15 experiments, n = 100–219 total cells per concentration). An unpaired two-sided t-test was used to compare [Ca^{2+}]_i responses to the ligands at each concentration. *Significant differences (P < 0.05) between Glb and GLP-1/Glb.

In the presence of stimulatory concentrations of glucose (15.5 G), GLP-1/Glb increased GSIS in a dose-dependent manner (Fig. 6). At 5 nM, the enhancement of GSIS by GLP-1(7–36) was greater than that by GLP-1/Glb, but the levels of GSIS enhancement by 100 nM GLP-1 and GLP-1/Glb were

Fig. 3. Glibenclamide (Glb) did not stimulate cAMP production. Various concentrations of Glb did not change cAMP production in βTC3 cells.

832/13 cells and human islets. In INS 832/3 cells, the mean basal rate of insulin release at 1 G was 0.55 ± 0.03 ng insulin·mg cell protein\(^{-1}\)·min\(^{-1}\). At 15.5 mM glucose (15.5 G), the rate of insulin secretion was ~3–4-fold greater than that observed at basal glucose (1.7 ± 0.1 ng·mg protein\(^{-1}\)·min\(^{-1}\)). The maximal cAMP-potentiated secretory rate measured in the presence of 15.5 G and 10 μM forskolin was 8.47 ± 0.35 ng insulin·mg protein\(^{-1}\)·min\(^{-1}\). In the presence of nonstimulatory glucose concentrations (1 G), neither 5 nM GLP-1, 5 nM Glb, or 5 nM GLP-1/Glb significantly altered insulin secretion; however, a trend toward decreased nonstimulated insulin secretion in the presence of GLP-1/Glb was observed. The combination of 5 nM GLP-1 and Glb increased insulin secretion by ~3-fold relative to the other conditions (Fig. 5).

Fig. 4. Heterobivalent glucagon-like peptide-1-glibenclamide (GLP-1/Glb)-stimulated cAMP production was similar to that stimulated by monomeric GLP-1. There was no significant difference in cAMP production when βTC3 cells were exposed to GLP-1/Glb or monomeric GLP-1 across a range of concentrations. This suggests that the signaling induced by the GLP-1 moiety of the heterobivalent GLP-1/Glb ligand was preserved. Data represent means ± SE of wells per concentration; monomeric GLP-1: n = 2–5 experiments, n = 10–30 wells per concentration. An unpaired two-sided t-test was used to compare cAMP production response at each concentration, and no significant differences (P < 0.05) in βTC3 cAMP production were observed between GLP-1 and GLP-1/Glb.

Fig. 5. Basal insulin secretion was unaffected by heterobivalent glucagon-like peptide-1-glibenclamide (GLP-1/Glb). The heterobivalent GLP-1/Glb ligand, monomeric GLP-1, and monomeric Glb had no significant effect on insulin secretion in the presence of 1 mM glucose (1 G). In combination, GLP-1 and Glb significantly elevated insulin secretion relative to the other conditions. Data represent means ± SE of well replicates (n = 3–11 experiments, n = 8–26 replicates per condition). A one-way ANOVA with a Tukey multiple-comparison correction was used to compare insulin secretory responses. *,†,‡,§Statistically differences (P < 0.05) between 1 G, 5 nM GLP-1/Glb, 5 nM GLP-1, and 5 nM Glb, respectively.
and C phase insulin secretion (Fig. 8). Ligand(s) concentration with an apparent elevation of second-presence of 5 nM ligand(s) concentration compared with 1 nM concentrations. However, GSIS was enhanced ~1.5–2-fold in the presence of 5 nM ligand(s) concentration compared with 1 nM ligand(s) concentration with an apparent elevation of second-phase insulin secretion (Fig. 8B).

GLP-1/Glb modulation of human islet insulin secretion. We were particularly interested in investigating whether GLP-1/Glb could modulate insulin secretion by human islets given that the heterobivalent ligand 1) did not induce insulin secretion at nonstimulatory glucose concentrations and 2) enhanced GSIS, albeit at levels lower than those for the combination of monomeric GLP-1 and Glb (Figs. 5 and 6). To this end, we performed a focused study evaluating the relative effects of the GLP-1/Glb ligand and the combination of monomeric GLP-1 and Glb ligands on isolated human islet insulin secretion (Fig. 8). At 2.8 G, 1 nM GLP-1 and Glb significantly increased insulin secretion relative to GLP-1/Glb (Fig. 8, A and C). At 2.8 G, both the combination of 5 nM GLP-1 and Glb and GLP-1/Glb induced an increase in insulin secretion (Fig. 8, A and C). At 16.7 G, both the combination of GLP-1 and Glb and GLP-1/Glb increased insulin secretion at 1 and 5 nM concentrations. However, GSIS was enhanced ~1.5–2-fold in the presence of 5 nM ligand(s) concentration compared with 1 nM ligand(s) concentration with an apparent elevation of second-phase insulin secretion (Fig. 8B).

DISCUSSION AND CONCLUSIONS

Here we present a functional characterization of a β-cell-targeted hMVL composed of linked GLP-1(7–36) and Glb binding domains (14). Our findings demonstrate that the GLP-1/Glb hMVL differentially activated β-cell signal transduction and insulin secretion relative to monomeric GLP-1 and/or Glb.

An important consideration of GLP-1/Glb binding was that the individual binding moieties within the MVL had lower affinities for their respective receptors (GLP-1R and SUR1) compared with unconjugated monomers. We previously estimated that the binding constants for the individual recognition elements within the bivalent ligand were ~80 nM for GLP-1(7–36) and ~40 nM for Glb, and high-affinity binding of GLP-1/Glb (Kd ~5 nM) was observed only when both complementary receptors (i.e., GLP-1R and SUR1) were available (14). Thus, activity of GLP-1/Glb below ~10 nM was presumed to be mediated by bivalent interactions, whereas activity at higher concentrations may have included monovalent ligand-receptor interactions of individual binding moieties within the GLP-1/Glb (particularly Glb-SUR1) hMVL (14).

Previous data have shown that activation of GLP-1R signaling combined with elevated glucose causes a more pronounced inhibition of KATP channels [SUR1 and inward rectifier K+] channel 6.2 (Kir6.2) complex; 27]. Thus, we hypothesized that greater changes in [Ca2+]i would be observed in the presence of the bivalent GLP-1/Glb ligand and 5.5 mM glucose (a glucose concentration stimulatory to βTC3 cells) compared with monomeric Glb. However, live-cell Ca2+ imaging indicated that the magnitude of changes in [Ca2+]i was reduced at
all concentrations of GLP-1/Glb tested compared with monomeric Glb (Fig. 2). These data suggest that the Glb moiety of the htMVL was not as effective as monomeric Glb. However, GLP-1/Glb was an effective and potent activator of cAMP production with an EC50 similar to monomeric GLP-1 (Fig. 4) suggesting that GLP-1R second-messenger activation by the GLP-1/Glb was not impaired but inhibitory sensitization of the KATP complex mediated by actors downstream of cAMP was. Previous data have also shown that interaction of sulfonylureas with exchange protein directly activated by cAMP 2 isoform A (Epac2A)/Rap1 signaling is crucial for combinatorial incretin-sulfonylurea augmentation of insulin secretion (46, 47). However, the ability of sulfonylureas to directly activate Epac2A is controversial (16).

Given the complicated nature of incretin and sulfonylurea signaling, we predict that the observed reduction in [Ca2+]i, elicited by GLP-1/Glb was caused by multiple factors. First, the Glb moiety of GLP-1/Glb, when bound bivalently, did not inhibit the SUR1 as effectively as monomeric Glb because of its structural relationship with the polyethylene glycol linker and an engaged GLP-1-GLP-1R complex. At concentrations <100 nM, GLP-1/Glb was largely ineffective at changing [Ca2+]i. However, as GLP-1/Glb concentration was raised above 100 nM, larger changes in [Ca2+]i were observed (Fig. 2). Thus, at higher concentrations, we predict that the Glb moiety of the bivalent ligand bound to, and partially activated, additional SUR1 in a monovalent and less potent mode. Second, bivalent binding of GLP-1/Glb to GLP-1R and SUR1 did not appear to cause additional KATP channel inactivation or increased [Ca2+]i as observed by others (27, 46). Our data suggest that this interference did not occur through inhibition of cAMP (Fig. 4) but most likely through interference between PKA and SUR1 and/or disruption of Glb activation of the Epac2A/Rap1 axis.

Insulin secretion in the presence of GLP-1/Glb was also altered relative to monomeric GLP-1 and/or Glb. At nonstimulatory glucose concentrations, INS 832/13 cells in the presence of the monomeric combination of 5 nM GLP-1 and Glb significantly elevated insulin secretion, whereas neither 5 nM monomeric Glb nor 5 nM monomeric GLP-1 alone activated insulin secretion, indicating a sensitization of SUR1 inhibition and/or activation of Epac2A/Rap1 signaling, as previously reported (27, 46). Conversely, 5 nM bivalent ligand, at nonstimulatory glucose concentrations, had no significant effect on “basal” insulin secretion in INS 832/13 cells. In human islets, 1 nM bivalent GLP-1/Glb did not stimulate basal insulin secretion to the same degree as the monomeric combination of GLP-1 and Glb did, demonstrating that the bivalent ligand was not acting as a sum of its constituent monomers at low glucose concentrations, even though binding in bivalent mode was significant (14). On the other hand, 5 nM GLP-1/Glb did stimulate basal insulin secretion in human islets (to levels ~75% of that observed for GLP-1 and Glb in combination) suggesting that increasing concentrations of GLP-1/Glb may be able to restore some level of GLP-1R-mediated inhibitory sensitization of the KATP and/or Epac2A activation. Moreover, GLP-1/Glb and the monomeric combination of GLP-1 and Glb, at both 1 and 5 nM, enhanced GSIS to similar levels. Interestingly, at 5 nM ligand concentration, GLP-1 and Glb combined and GLP-1/Glb increased GSIS ~2-fold relative to that observed at 1 nM ligand concentration because of apparent prolonged elevation of second-phase insulin secretion not observed at 1 nM. Combined, these data suggest that the htMVL GLP-1/Glb interrupted inhibitory sensitization of KATP and/or
Epac2A signaling at nonstimulatory glucose concentrations but augmentation of insulin secretion at elevated glucose concentrations was preserved compared with its constituent monomers. Since the high-affinity binding of GLP-1/Glb was dependent on the availability of both receptors, our findings indicate that this bivalent ligand may, at concentrations below 20 nM, serve as an incretin targeted to β-cells. Importantly, GLP-1/Glb not only retained the capacity to promote GSIS but also had little effect on insulin secretion in the absence of stimulatory glucose at concentrations below 5 nM, contrary to its constituent monomers. Therefore, in addition to the potential for limiting off-target effects of GLP-1 agonists due to enhanced β-cell specificity, GLP-1/Glb may be useful in preventing hypoglycemic incursions during fasting periods while retaining considerable GLP-1 potency and potentiation of β-cell GSIS during nonfasted states. Further investigation of downstream signaling and optimization of heterobivalent ligands as a β-cell-specific therapeutic agent appear warranted. Moreover, the rules learned here guide further development for tuning both specificity and signaling properties of multivalent agents.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.J.H., C.W., K.K.P., S.W.L., J.V., and R.M.L. conceived and designed experiments; N.J.H., C.W., and S.W.L. analyzed data; N.J.H., C.W., and R.M.L. interpreted results of experiments; N.J.H. and C.W. performed experiments; N.J.H., C.W., and S.W.L. drafted manuscript; N.J.H., C.W., K.K.P., S.W.L., J.V., and R.M.L. edited and revised manuscript; N.J.H., C.W., K.K.P., S.W.L., J.V., and R.M.L. approved final version of manuscript.

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