Modulation of Glucagon-like Peptide-1 (GLP-1) Potency by Endocannabinoid-like Lipids Represents a Novel Mode of Regulating GLP-1 Receptor Signaling*

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Yu-Hong Cheng‡, Mei-Shang Ho§, Wei-Ting Huang§, Ying-Ting Chou‡, and Klim King‡,§

From the ‡Genomic Research Center and §Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan

Background: Glucagon-like peptide-1 receptors (GLP-1Rs) are expressed in many tissues that are accessed only by a basal circulating level of GLP-1.

Results: GLP-1 potency is enhanced by specific endocannabinoid-like lipids.

Conclusion: Enhancing GLP-1 potency is a novel mechanism regulating GLP-1R signaling.

Significance: Spatiotemporal regulation of GLP-1R becomes possible at basal physiological levels of GLP-1 because endocannabinoid-like lipids are known to be physiologically regulated.

Glucagon-like peptide-1 (GLP-1) analogs are approved for treatment of type 2 diabetes and are in clinical trials for disorders including neurodegenerative diseases. GLP-1 receptor (GLP-1R) is expressed in many peripheral and neuronal tissues and is activated by circulating GLP-1. Other than food intake, little is known about factors regulating GLP-1 secretion. Given a normally basal circulating level of GLP-1, knowledge of mechanisms regulating GLP-1R signaling, which has diverse functions in extraprancreatic tissues, remains elusive. In this study, we found that the potency of GLP-1, not exendin 4, is specifically enhanced by the endocannabinoid-like lipids oleoylethanolamide (OEA) and 2-oleoylglycerol but not by stearoylethanolamide (SEA) or palmitoylethanolamide. 9.2 μM OEA enhances the potency of GLP-1 in stimulating cAMP production by 10-fold but does not affect its receptor binding affinity. OEA and 2-oleoylglycerol, but not SEA, bind to GLP-1 in a dose-dependent and saturable manner. OEA but not SEA promoted GLP-1(7–36) amide to trypsin inactivation in a dose-dependent and saturable manner. Susceptibility of GLP-1(7–36) amide to trypsin inactivation is increased 40-fold upon binding to OEA but not to SEA. Our findings indicate that OEA binds to GLP-1(7–36) amide and enhances the potency that may result from a conformational change of the peptide. In conclusion, modulating potency of GLP-1 by physiologically regulated endocannabinoid-like lipids allows GLP-1R signaling to be regulated spatiotemporally at a constant basal GLP-1 level.

Glucagon-like peptide-1 receptor (GLP-1R)2 signaling is an established therapeutic target for type 2 diabetes. In addition to human pancreatic islet β cells, GLP-1R is expressed in a wide array of tissues, including lung, heart, kidney, blood vessels, neurons, and lymphocytes (1–4). Mice deficient in GLP-1R expression or with blunted GLP-1R function show impairment of physiologic features not limited to glucose homeostasis but also including learning and memory (4). Clinical trials targeting GLP-1 signaling to treat non-metabolic diseases include those for psoriasis, heart disease, and neurodegenerative diseases (5–7). Despite encouraging outcomes with GLP-1 analogs in reducing myocardial infarct size in acute coronary occlusion (7) and improving clinical symptoms in patients with Parkinson disease (5), the mechanisms of physiological regulation of GLP-1R signaling beyond energy homeostasis remain largely unknown.

GLP-1 is an incretin peptide hormone derived from post-translational processing of the precursor proglucagon in intestinal L cells (8). On food intake, the biologically active forms of GLP-1(7–36) amide and GLP-1(7–37) are secreted, thus increasing the basal plasma level by 3–4-fold, to maintain normoglycemia by enhancing glucose-dependent insulin secretion and suppressing glucagon function (8, 9). Circulating GLP-1 has a short plasma half-life of only a few min due to renal clearance after rapid enzymatic inactivation by a plasma enzyme, dipeptidyl peptidase 4 (10). Other cells outside of the gut shown to produce GLP-1 include pancreatic α cells and neurons in the localized area of the brain stem (4, 11–14), but our knowledge of the physiological regulation of GLP-1 secretion by these cells is limited.

In the brain, GLP-1 is synthesized primarily by a discrete group of neurons located in the nucleus of the solitary tract (12). These neurons send abundant projections to other regions of the brain, including the forebrain, hypothalamus, amygdala, stria terminalis, and thalamus, where GLP-1Rs are expressed; this neuronal circuit of GLP-1 signaling is considered relevant to satiety and energy homeostasis (2, 11). GLP-1R is also expressed in neurons in the hippocampus (1) and dopaminergic neurons in substantia nigra (3), where no known GLP-1-secretin-neuron innervation is found (3, 14). It has been suggested that the basal circulating GLP-1 level is the primary source of...
ligands accessible to GLP-1Rs in these brain regions and probably in the heart as well. Therefore, determining the mechanism by which the basal level of GLP-1 can activate receptors in these brain regions is germane.

The well-delineated functions of GLP-1 are mainly mediated by activation of GLP-1R (14). GLP-1R, as a member of the class B G protein-coupled receptor (GPCR) family, is the only known receptor with high specific affinity for GLP-1. GLP-1R activation leads to two major signaling pathways, namely Goq coupling and recruitment of β-arrestin to the agonist-occupied receptor; the former mainly leads to activation of adenyl cyclase, with subsequent generation of cAMP (15), and the latter leads to receptor endocytosis and activation of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling (4). In pancreatic β cells, the increased cAMP level is responsible for glucose-dependent insulin release (16) and contributes to maintaining glucose homeostasis. Thus, cAMP production is measured and used as a GLP-1R-mediated functional response in properly designed assays.

Analysis of food intake-induced increase in GLP-1 level and subsequent activation of GLP-1R has provided valuable insights into the role of GLP-1R signaling in energy homeostasis. However, the short half-life and low basal level of circulating GLP-1 (1–36) amide do not permit assessment of the physiological relevance of GLP-1R signaling other than energy homeostasis. During initial screening of GLP-1 potentiation activity from edible plants, we found that olive oil enhances GLP-1-dependent cAMP production in RINm5F cells. Here, we report that the specific endogenous endocannabinoid-like lipids oleoylethanolamide (OEA) and 2-oleoylglycerol (2-OG) can bind to GLP-1 peptide and are accompanied by an enhancement of cAMP production. Physiological levels of these lipids are subject to temporal and spatial regulation (17–19), and alone, they cannot activate GLP-1R signaling. Here, we show that the potency of GLP-1 is modulated by specific lipids, and this may represent a novel mode of spatiotemporal regulation of GLP-1R signaling.

**Experimental Procedures**

Reagents—A U2OS osteosarcoma cell line stably expressing a β-arrestin 2-GFP fusion protein was obtained from Norak Biosciences (Morrisville, NC) (now Molecular Devices, a part of MDS (Mississauga, Ontario, Canada)). The construct pcDNA-Rlu8, which expresses a variant of Renilla luciferase (Rlu8) (20), and a green fluorescent protein (GFP) cDNA were kind gifts of Drs. Sanjiv Gambhir (Stanford University, Stanford, CA) and Szu-Hao Kung (21), respectively. cDNA encoding human exchange protein activated by cAMP 1 (Epac1; Rap guanine nucleotide exchange factor 3) and cDNA of human GLP-1R were purchased from OriGene Technologies (Rockville, MD). All oligonucleotides were from Mission Biotech (Taipei, Taiwan). The Lipofectamine LTX transfection kit and TOPO TA cloning kit were from Invitrogen. Peptides of GLP-1 (1–36) amide, His-tagged GLP-1 (1–36), glucagon, and gastric inhibitory polypeptide (GIP) were from LifeTein (Hillsborough, NJ). Exendin 4 (Ex-4) and exendin 9 (Ex-9) were synthesized by Genomics BioSci & Technology (Taipei, Taiwan). The membrane-permeable cAMP 8-Br-2′-O-Me-cAMP-AM was from Axxora (Farmingdale, NY). Adenyl cyclase inhibitor MDL-12,330A hydrochloride, puromycin, G418, and 2-OG were from Sigma-Aldrich. OEA, oleic acid (OA), and all other fatty acids and lipids were from Cayman (Ann Arbor, MI). RPMI 1640 tissue culture medium, minimum essential medium (MEM), FBS, sodium pyruvate, l-glutamine, HEPES, penicillin/streptomycin, amphotericin B, gentamicin, phenol red-free MEM, and 0.05% trypsin-EDTA were from Life Technologies, Invitrogen Life Sciences. Copper-nitrilotriacetate resin was from Jena Bioscience (Jena, Germany).

**Construction of RG-cAMP Sensor**—We replaced the energy donor and acceptor of the previously described Epac1 biosensor MDL-12,330A hydrochloride, puromycin, G418, and 2-OG were from Sigma-Aldrich. OEA, oleic acid (OA), and all other fatty acids and lipids were from Cayman (Ann Arbor, MI). RPMI 1640 tissue culture medium, minimum essential medium (MEM), FBS, sodium pyruvate, l-glutamine, HEPES, penicillin/streptomycin, amphotericin B, gentamicin, phenol red-free MEM, and 0.05% trypsin-EDTA were from Life Technologies, Invitrogen Life Sciences. Copper-nitrilotriacetate resin was from Jena Bioscience (Jena, Germany).

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fusion protein Rluc8-Epac1 (r8-luc8-fyse2A)-GFP (RG-cAMP sensor) (Fig. 1C).

**Cell Culture and Transfection of Cell Lines**—The RINm5F cell line is derived from a rat islet cell tumor (25, 26) and expresses receptors for GLP-1, GIP, and glucagon (27, 28). RINm5F cells were seeded at a density of $3 \times 10^5$ cells/well in 24-well plates and cultured at 37 °C and 5% CO$_2$ in complete RPMI 1640 medium supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, 14 mM glucose, 1:1000 penicillin/streptomycin, 2 mM glutamine, and 35 mM sodium bicarbonate, pH 7.4, for 16 h. Before transfection, the medium was removed and replaced with 500 µl of fresh complete RPMI 1640. Transfection was carried out using the Lipofectamine LTX transfection kit according to the manufacturer’s protocol. Briefly, 0.5 µg of plasmid RG-cAMP sensor in 0.1 ml of Opti-MEM containing 1 µl of Plus reagent and 3 µl of LTX reagent was used to transfected the cells. Transfection complexes were added dropwise to the cells. After a 12-h incubation, the medium containing the lipoplexes was removed and replaced with 500 µl of complete RPMI 1640. The cultures were incubated for another 48 h, the population of green fluorescent cells was counted, and transfection efficiency was estimated. Cells from each well were transferred to a 15-cm culture dish with 20 ml of complete RPMI 1640 containing 400 µg/ml G418 to select the stably transfected cells.

**BRET and cAMP Response Assay**—RINm5F cells stably expressing the RG-cAMP sensor were seeded at $3 \times 10^5$ cells/well in 96-well white plates in 0.15 ml of RPMI 1640 containing 400 µg/ml G418. On the next day, cells were washed twice with 0.1 ml of phenol red-free MEM containing 5 mM HEPES and incubated in the same medium for 1 h. The medium was replaced with 90 µl of the same medium containing 1 mg/ml BSA and 5 µM Deep Blue C. The whole plate was immediately loaded onto a SpectraMax paradigm detection platform equipped with a dual-color luminescence detection cartridge and SoftMax Pro 6.2.2 (Molecular Devices, Sunnyvale, CA) to obtain the background BRET signal based on the sequential integration of the luminescence detected at 370–450 and 500–530 nm over 60–150 s. Each well was then stimulated by adding 10 µl of 10× solutions of peptide and lipids and 1 mg/ml BSA in phenol red-free MEM containing 5 mM HEPES, and BRET signals were obtained immediately under identical settings. The BRET ratio is the ratio of light emitted between 90 and 300 s at 500–530 nm to that emitted at 370–450 nm. The cAMP response was expressed as a percentage of cAMP production and was calculated as $100 \times (\text{BRET ratio from 0.01 nM GLP-1 (7–36) amide} - \text{BRET ratio from the indicated concentration of peptide with or without lipids}) / (\text{BRET ratio from 0.01 nM GLP-1 (7–36) amide} - \text{BRET ratio from 250 nM GLP-1 (7–36) amide})$. The dose-response curve, maximal response, and concentration of peptide needed to yield half-maximal response ($EC_{50}$) were obtained by nonlinear regression to fit the data to the agonist versus response equation using Prism software version 5.0 (GraphPad, San Diego, CA). Unless otherwise specified, all cAMP response data are mean ± S.E. of three independent experiments performed in triplicate.

**GLP-1 and OEA Binding Assay**—The putative GLP-1 peptide and lipid interaction were examined with a binding assay between tritium-labeled OEA ([³H]OEA) and His-tagged GLP-1 (7–36) peptide (Fig. 6A). [³H]OEA bound to His-tagged GLP-1 (7–36) peptide was separated from free [³H]OEA by incubating the binding reaction with copper-nitritotriacetate resin (Cu²⁺-NTA) and subsequent centrifugation. [³H]OEA in the supernatant is the amount of free [³H]OEA. Specific bound [³H]OEA was obtained by subtracting the supernatant [³H]OEA of the reaction from supernatant [³H]OEA of the reaction without GLP-1. For binding of [³H]OEA to an increasing amount of His-tagged GLP-1 (7–36), 0.2 µM [³H]OEA and increasing concentration of His-tagged GLP-1 (7–36) peptide were mixed in 50 µl of Dulbecco’s PBS containing 0.02 mg/ml bovine serum albumin (BSA) at room temperature for 90 min. Copper-nitritotriacetate resin (Cu²⁺-NTA) (3 µl in 30 µl of Dulbecco’s PBS) was added to capture all of the His-tagged GLP-1 (up to 60 µM in an 80-µl reaction), further incubated with rotation at room temperature for 30 min. The mixture was centrifuged at 4 °C, 20,600 × g for 10 min to precipitate the His-tagged GLP-1 (7–36) trapped in resin, and 20 µl of supernatant containing the unbound free [³H]OEA was mixed with 120 µl of Microscint 40 (PerkinElmer Life Sciences) for quantification of tritium by using single-photon counting (60 s/well read) on a TopCount scintillation counter (PerkinElmer Life Sciences). Bound [³H]OEA = supernatant [³H]OEA of binding reaction in the absence of His-tagged GLP-1 (7–36) − supernatant [³H]OEA of binding reaction with the indicated amount of His-tagged GLP-1 (7–36). For the binding of 0.1 µM His-tagged GLP-1 (7–36) to increasing concentration of [³H]OEA, the indicated concentrations of [³H]OEA were incubated in the presence (total binding) or absence (nonspecific binding) of 0.1 µM His-tagged GLP-1 (7–36), and the free and bound [³H]OEA were separated as described above. Bound [³H]OEA = supernatant [³H]OEA of nonspecific binding − supernatant [³H]OEA of total binding. For the competition assay to evaluate the binding of SEA and 2-OG to His-tagged GLP-1 (7–36), increasing concentrations of 2-OG or SEA were incubated with 0.2 µM [³H]OEA in the presence (total binding) or absence (nonspecific binding) of 0.2 µM His-tagged GLP-1 (7–36). The bound [³H]OEA and free [³H]OEA were separated as described above, and specific binding was calculated; bound [³H]OEA = supernatant [³H]OEA of nonspecific binding reaction − supernatant [³H]OEA in the total binding reaction.

**Preparation of GLP-1 Receptor Membrane**—The pcDNA3 GLP-1R-V2R chimeric construct contains the first 440 amino acids of the GLP-1R (Met¹–Thr⁴⁴⁰) fused to the last 29 amino acids of the vasopressin V2 receptor (Ala³⁴³–Ser³⁷¹) (29) and separated by two alanine residues as linker. The GLP-1R-V2R chimeric construct was inserted into the EcoRI site of pcDNA3 (pcDNA3-GLP-1R-V2R) such that expression of the chimeric protein was under the control of the CMV promoter. pcDNA3-GLP-1R-V2R was used to transfect U2OS osteosarcoma cells stably expressing β-arrestin-2-GFP to obtain cell lines stably co-expressing GLP-1R-V2R and β-arrestin-2-GFP. U2OS cells stably expressing GLP-1R-V2R were grown to 90% confluence (about 10⁷ cells/15-cm dish). The media was removed and washed twice with 30 ml of PBS, followed by adding 2.2 ml of ice-cold homogenization buffer (20 mM HEPES, 1 mM EDTA, 0.7% protease inhibitor mixture (Sigma-Aldrich,
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P8340) per dish. The cells were scraped immediately, and the scraped cells were centrifuged at 3000 \times g and 4 °C for 30 min. The pellet was resuspended with 5 ml of homogenization buffer and then homogenized with the maximal speed of a Polytron 3000 system for 10 s on ice with a 30-s interval rest three times. The homogenized cells were centrifuged for 10 min at 4 °C and 1000 \times g. The supernatant was transferred to a fresh transparent centrifuge tube and centrifuged for 60 min at 4 °C and 55,000 \times g. The pellets were resuspended with resuspension buffer (20 mM HEPES, 1 mM MgCl\(_2\), 0.7% protease inhibitor) by passing through the 22-gauge needle one time, a 25-gauge needle three times, and then a 26-gauge needle one time. The protein concentration of the membrane preparation was determined according to the instructions for the Qubit fluorometer (Life Technologies). 4–5 μg of this membrane will yield greater than 5-fold signal/background with \(^{125}\)I-labeled GLP-1 at 5 nm in a 0.22-ml assay volume.

Receptor Binding Assay—Assays were conducted in 0.22 ml of 50 mM HEPES, pH 7.4, 5 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 1 ml/ml BSA containing 3.9 μg of GLP-1 receptor membranes, varying concentrations of \(^{125}\)I-GLP-1(7–36) radioactive ligand and in the absence (total binding) or presence (nonspecific binding) of a 500-fold excess of unlabeled exendin 4. The reactions were incubated for 90 min. Prior to filtration, an FC 96-well harvest plate (Millipore, MAFC N0B 10) was coated with 0.5% polyethyleneimine for 30 min and then washed with 50 mM HEPES, pH 7.4. 0.1 ml of the binding reaction was transferred to the filter plate and washed 15 times (0.1 ml/well/wash) with ice-cold 25 mM HEPES, pH 7.4, and 50 mM NaCl. The plate was dried, followed by the addition of 30 μl of Microscint 20 (PerkinElmer Life Sciences) per well, and the activity was determined by using single-photon counting (60 s/well read) on a TopCount scintillation counter (PerkinElmer Life Sciences). Specific bindings were obtained by subtracting nonspecific binding from total binding. The dissociation constant (\(K_d\)) for GLP-1(7–36) was obtained using Prism software version 5.0 (GraphPad). Data shown are the means ± S.E. of three independent experiments performed in duplicates.

In Vitro Assay of Trypsin Activity in the Presence of OEA—To determine whether OEA affects trypsin activity, we used the Trypsin Activity Colorimetric Test Kit (BioVision, Milpitas, CA). Briefly, 2 μl of substrate was added to a well in a 96-well plate containing 48 μl of 0.000125% trypsin in the presence or absence of 92 μM OEA. Reactions were conducted at room temperature, and the extent of cleavage was monitored by real-time reading of optical density at 405 nm.

Limited Trypsin Digestion of GLP-1(7–36) Amide—Trypsin digestion was carried out at 37 °C for 30 min in 0.1 ml of phenol red-free MEM containing 5 mM HEPES, pH 7.0, 2 μM GLP-1(7–36) amide, \(2 \times 10^{-3}\) to \(2.5 \times 10^{-5}\) μg trypsin (prepared by diluting 0.05% trypsin-EDTA), and 92 to 1 μM OEA. The reaction was terminated by incubation at 94 °C for 30 min and then at 4 °C for 10 min, followed by the addition of PMSF to 1 mM.

Determination of Concentrations of GLP-1(7–36) Amide and Glucagon and GIP Peptides—Molar concentrations were determined using an equation whereby peptide concentration (\(M\)) = (\(A_{280} \times \text{fold dilution}\))/(1200 + 5560), where 1200 and 5560 are the molar extinction coefficients for tyrosine and tryptophan, respectively.

Results

Construction of RG-cAMP Sensor—To reduce BRET background and obtain better separation of the donor and acceptor energy emission peaks, we replaced the energy donor and acceptor in the early version Epac1 cAMP biosensor (22, 23) with RLuc8 (20) and GFP2, respectively, for use in enhanced BRET2 (24) (Fig. 1, A–C). For a detailed description of the expression construct for the RG-cAMP sensor, see “Experimental Procedures.”

Validation of the RG-cAMP Sensor in RINm5F Cells—The use of the RG-cAMP sensor (Fig. 2A) as an intracellular cAMP biosensor in the RINm5F cell line, which is derived from a rat islet cell tumor, in the BRET assay was validated using the membrane-permeable cAMP analog 8-Br-2’-O-Me-cAMP-AM, the adenylyl cyclase activator forskolin, and the adenylyl cyclase inhibitor MDL-12330A. As shown in Fig. 2B, increasing the level of cAMP analog resulted in reduction of the BRET ratio in a concentration-dependent and saturable manner. The BRET ratio started to decrease from 1.0 as the concentration of 8-Br-2’-O-Me-cAMP-AM increased to 0.2 μM and stopped dropping at 0.55 when the concentration of 8-Br-2’-O-Me-cAMP-AM reached 50 μM. The concentration of 8-Br-2’-O-Me-cAMP-AM at which half-maximal reduction of BRET ratio is elicited (EC\(_{50}\)) was determined to be 2.6 μM. This analysis shows that 0.45 units of BRET ratio reduction corresponds to a concentration change of 8-Br-2’-O-Me-cAMP-AM from 0.2 to 50 μM. This observation led us further to test whether this cAMP biosensor would respond to forskolin, which activates adenylyl cyclase and leads to cAMP production. As shown in Fig. 2C, forskolin treatment resulted in reduction of the BRET ratio in a dose-dependent and saturable manner; the BRET ratio started to decrease at a concentration of 1.8 μM forskolin, and reduction became saturated as the forskolin concentration reached 5000 nM. The forskolin-elicted BRET response was eliminated in the presence of 250 μM MDL-12,330A, a potent adenylyl cyclase inhibitor. The change in basal cAMP level to that maximally stimulated by forskolin corresponds to a BRET ratio reduction of 0.3 units. The EC\(_{50}\) for forskolin was determined to be 70 nM. This observation demonstrated that activation of adenylyl cyclase leads to increased intracellular cAMP. MDL-12,330A, which in turn inhibits the enzyme, abolished the activating effect of forskolin. Both processes can be sensed by the RG-cAMP sensor in the cells. RINm5F cells endogenously express G\(_{\alpha}\)-coupled receptors for GLP-1, GIP, and glucagon (27, 28). cAMP production in RINm5F cells expressing the RG-cAMP sensor showed dose-dependent and saturable responses to GLP-1(7–36) amide, GIP, and glucagon (Fig. 3, A–C). These responses were curtailed by the corresponding antagonists, Ex-9, GIP(8–42) (30), and [des-His1,Glu9]glucagon amide (31), respectively, indicating that the cAMP responses were mediated by specific activation of the cognate receptors (Fig. 3, A–C). The EC\(_{50}\) was 1.3, 6, and 43 nM for GLP-1(7–36) amide, GIP, and glucagon, respectively. These results validated the
applicability of the RG-cAMP sensor in detecting intracellular cAMP level in response to incretin and glucagon stimulation in RINm5F cells.

**OEA and 2-OG Potentiate cAMP Production in Response to GLP-1(7–36) Amide**—In a preliminary test of the ability of the 18-carbon fatty acids and their lipid derivatives to enhance the GLP-1R cAMP response using a BRET assay, OA (18:1) and linoleic acid (18:2) at 10^6 and 10^7 M H9262 M, respectively, consistently but mildly left-shifted the dose-response curve of GLP-1(7–36) amide (Fig. 4, A and B), whereas stearic acid, α-linolenic acid, and γ-linolenic acid did not show such an effect (Fig. 4C). Other lipids tested that did not affect GLP-1 potency in receptor signaling included stearoylethanolamide (SEA), palmitoylethanolamide, and n-oleoyldopamine (Fig. 4, D and E). We then examined endogenous lipid derivatives containing OA as their acyl-lipid backbone. OEA and 2-OG, at a concentration of 9 to 8 μM, markedly left-shifted the dose-response curve for GLP-1(7–36) amide (Fig. 5, A and B). Responses were enhanced when the concentration of GLP-1 reached 20 pm, and the EC_{50} of GLP-1(7–36) amide was reduced from 1.29 nM to 0.12 and 0.11 nM by 9.2 μM OEA and 8.4 μM 2-OG, respectively (Table 2). The enhancement was dependent on the GLP-1(7–36) amide, because the effect diminished when the concentration of GLP-1(7–36) was reduced to 2 pm. To examine whether this enhancement is specific for GLP-1(7–36) amide, we further examined whether OEA and 2-OG could enhance the potency of exendin 4 (Ex-4), an analog of mammalian GLP-1. Ex-4 dose-dependently stimulated cAMP production and reached saturation at a concentration of 10 nM, with an EC_{50} of 0.5 ± 0.13 nM (Fig. 5, C and D); however, the potency of Ex-4 was unaltered by the addition of OEA or 2-OG to a concentration up to 27 μM (Fig. 5, C and D). Therefore, OEA and 2-OG do not enhance the Ex-4-stimulated cAMP response in RINm5F cells. RINm5F is a rat insulinoma cell line, which should express many receptors involved in OEA- or 2-OG-mediated enhancement of cAMP production by GLP-1. In order to investigate whether receptors other than GLP-1 receptor participate in OEA- or 2-OG-mediated enhancement of cAMP production by GLP-1, we examined whether this enhancement and cAMP responses still occur when GLP-1 receptor signaling was blocked by its antagonist exendin 9. As shown in Fig. 5E, 9 μM OEA or 8.4 μM 2-OG remarkably enhanced the cAMP

**FIGURE 1. Generation of construct to express fusion protein RG-cAMP sensor.** (A) overlap extension PCR to generate Rluc8-Epac1148 - 430pcDNA* encoding Epac1 amino acids 148 – 430. 8, generation of Rluc8-Epac1148 – 881pcDNA* encoding Epac1 amino acids 148 – 881. C, generation of construct encoding the three-protein fusion of Rluc8-Epac1148 – 881(T781A,F782A)-GFP2 (RG-cAMP sensor) containing Epac1 sequence encoding amino acids 148 – 881, where Thr_{781} and Phe_{782} in the Epac1 have been changed to alanine.
responses to 200 pm GLP-1(7–36) amide in RINm5F cells (Student’s t test; p < 0.001). This enhancement and cAMP responses were barely detectable when GLP-1 receptor signaling was blocked by the presence of 500 nM exendin 9. This analysis clearly illustrated that most of OEA- or 2-OG-mediated enhancement of cAMP response by GLP-1 is through GLP-1 receptors. This analysis revealed the specificity of these lipids in enhancing GLP-1(7–36) amide potency and not that of structurally related peptides or receptors other than GLP-1R.

GLP-1 Specifically Binds to OEA and 2-OG—Because both OEA and 2-OG enhance the potency of GLP-1(7–36) but not that of exendin 4, one possible explanation is that these lipids may interact specifically with GLP-1(7–36) amide. We further looked into the binding between His-tagged GLP-1(7–36) (Fig. 6A) and [3H]OEA. His-tagged GLP-1(7–36) displays comparable response to OEA to enhance cAMP response as GLP-1(7–36) amide does. In a binding reaction, the peptide-bound [3H]OEA together with the free peptide were captured by Cu²⁺-NTA resin and separated from free [3H]OEA after centrifugation. 3 μl of Cu²⁺-NTA resin in an 80-μl binding reaction volume has been validated to be sufficient to capture all of the His-tagged GLP-1(7–36). Fig. 6B shows that the binding of 0.2 μM [3H]OEA was increased with increasing concentration of His-tagged GLP-1(7–36), indicating that His-tagged GLP-1(7–36) binds to [3H]OEA in a dose-dependent and saturable way; the estimated concentration of GLP-1 to achieve half-maximal binding of OEA is 0.16 ± 0.05 μM. Fig. 6C showed an increased binding of [3H]OEA to 0.1 μM His-tagged GLP-1(7–36) with increasing concentration of [3H]OEA, also indicating that [3H]OEA binds to His-tagged GLP-1(7–36) in a dose-dependent way, and the concentration of [3H]OEA to achieve half-maximal binding is estimated to be 0.28 ± 0.07 μM. Because the potency of GLP-1 to stimulate cAMP production is enhanced by OEA and 2-OG but not by SEA, we also analyzed binding of 2-OG and SEA to His-tagged GLP-1(7–36) in a competition assay. As shown in Fig. 6D, the His-tagged GLP-1(7–36)-bound [3H]OEA decreased as the concentration of 2-OG increased from 0.125 to 2.0 μM, and the Kᵢ is estimated to be 0.1 ± 0.03 μM. SEA, on the other hand, did not affect the binding of [3H]OEA to His-tagged GLP-1(7–36). These observations indicate that OEA and 2-OG specifically bind to His-tagged GLP-1(7–36).

Effect of OEA on Saturation Binding of GLP-1 to GLP-1 Receptor—Because OEA binds to and enhances the potency of GLP-1(7–36), we further investigated whether this effect may result from an enhanced binding affinity of GLP-1(7–36) to GLP-1 receptor. We performed a saturation binding assay to examine the effect of OEA on GLP-1 receptor binding affinity. As shown in Fig. 7A, binding of [125I]GLP-1 to GLP-1 receptor membrane is dose-dependent and saturable; the dissociation constant Kᵦ and the maximal binding (Bₘₐₓ) were measured to be 1.5 ± 0.25 nM and 51 ± 2.9 pmol/mg of membrane, respectively. The saturation binding curve was barely affected by 9.2 μM OEA, which maximally enhances the potency of cAMP production by GLP-1 (Fig. 7B). The Kᵦ and Bₘₐₓ measured in the presence of 9.2 μM OEA were 1.4 ± 0.5 nM and 51 ± 6.5

FIGURE 2. Detection of intracellular cAMP by RG-cAMP sensor in RINm5F cells. A, schematic diagram of RG-cAMP sensor stably expressed in RINm5F cells and comprising Epac1 (amino acids 148–881 with point mutations T781A, F782A) and GFP2, respectively. B, reduction in BRET ratio in response to titration of the membrane-permeable cAMP analog 8-Br-2‘-O-Me-cAMP-AM in RINm5F cells stably expressing the RG-cAMP sensor. C, BRET responses to increasing concentration of adenylyl cyclase inhibitor MDL-12330A. Red squares show responses to increasing concentration of adenylyl cyclase inhibitor MDL-12330A.
Specific binding of [125I]GLP-1(7–36) to GLP-1 receptor in the presence and absence of 9.2 μM OEA are almost superimposed (Fig. 7C). This analysis revealed that OEA has no discernible effects on the affinity or capacity of GLP-1 to bind GLP-1R. It is concluded that 9.2 μM OEA, although it maximally enhances the
potency of GLP-1(7–36) to stimulate cAMP response, does not affect the physical affinity between GLP-1 and its cognate receptor.

Effect of OEA on Trypsin Inactivation of GLP-1(7–36) Amide—OEA specifically binds to GLP-1(7–36) amide and enhances GLP-1 to stimulate cAMP production while not affecting the receptor binding affinity, suggesting a potential lipid-induced structural change of GLP-1 peptide. We probed the potential conformation of GLP-1(7–36) amide based on its susceptibility to trypsin digestion. The two trypsin cleavage products of GLP-1(7–36) amide are an inactive GLP-1(7–26) and a partially active GLP-1(7–34) (Fig. 8A) (32). We carried out limited trypsin digestion to determine whether the putative lipid-peptide interaction may alter the susceptibility of GLP-1(7–36) amide to trypsin and result in a change in activity measured by cAMP responses. Residual activity in stimulating cAMP production after trypsin treatment was used to examine susceptibility of the peptide to trypsin digestion. SEA and OEA were tested as representative non-signal-enhancing and signal-enhancing lipids, respectively. To obtain a workable concentra-

TABLE 2
Effect of endocannabinoid-like lipids on the EC50 (nM) of GLP-1 peptides to elicit cAMP response
Assay of the effects of the indicated concentrations of OEA and 2-OG on cAMP response to the titration of GLP-1(7–36) amide was carried out in RINm5F cells expressing RG-cAMP sensor. The cAMP response is determined by the reduction in BRET ratio, which is the ratio of light emitted at 500–530 nm to that emitted at 370–450 nm. The concentration needed to give half-maximal response (EC50) was derived using nonlinear regression to fit the data to the agonist versus response equation from the Prism software version 5.0 (GraphPad, Inc., San Diego, CA). Data are means ± S.E. of triplicate determinations from three independent experiments.

| Lipids | 0 | 9.2 | 28.6 | 100 |
|---|---|---|---|---|
| OEA | 1.29 ± 0.13 | 0.12 ± 0.02 | 0.44 ± 0.12 | 1.03 ± 0.35 |
| 2-OG | 1.29 ± 0.13 | 0.11 ± 0.04 | 0.26 ± 0.09 | 0.94 ± 0.39 |

FIGURE 6. Binding of GLP-1 to OEA, 2-OG, and SEA. A, sequence of His-tagged GLP-1(7–36). Six consecutive histidine residues are tagged to the C terminus of the GLP-1(7–36) peptide. B, binding of 0.2 μM [3H]OEA to increasing concentrations of His-tagged GLP-1(7–36). C, binding of 0.1 μM His-tagged GLP-1(7–36) to increasing concentration of [3H]OEA. D, competition of 2-OG and SEA for the binding of 0.2 μM OEA to 0.2 μM His-tagged GLP-1(7–36). Binding reactions, separation of His-tagged GLP-1(7–36)-bound [3H]OEA and free [3H]OEA, and quantitation of specific bound [3H]OEA are described under “Experimental Procedures.” All data are means ± S.E. (error bars) of triplicate determinations from three independent experiments.

FIGURE 7. Effect of OEA on saturation binding for GLP-1R. Shown are total (black circles) and nonspecific binding (blue squares) of [125I]GLP-1(7–36) to GLP-1R-V2R membrane in the absence (A) or presence (B) of 9.2 μM OEA. C, specific binding of [125I]GLP-1(7–36) to GLP-1R-V2R membrane in the absence (black circles) or presence (red squares) of 9.2 μM OEA. Binding reactions (220 μl) were carried out in the absence (Total Binding) or presence (Non-specific Binding) of a 500-fold excess of unlabeled exendin 4. Separation of bound and free [125I]GLP-1(7–36) and calculation of specific binding are described under “Experimental Procedures.” Specific binding was determined by subtracting nonspecific binding from total binding. All data are means ± S.E. (error bars) of three independent experiments performed in duplicate. Data were fitted globally to a one-site saturation isotherm.
tion of trypsin for the limited digestion of 2 μM GLP-1(7–36) amide, we titrated trypsin concentration from 2.5 × 10⁻⁵ to 0.002% versus GLP-1(7–36) amide activity to stimulate cAMP production in RINm5F cells. As shown in Fig. 8C, the residual activity of GLP-1(7–36) to stimulate cAMP production was gradually reduced as trypsin concentration increased from 2.5 × 10⁻⁵ to 0.002%. The activity was decreased by a factor of 10 when GLP-1(7–36) was digested with 0.002% of trypsin. This analysis demonstrated that trypsin inactivates GLP-1(7–36) amide in a dose-dependent manner. When the trypsin cleavage reactions were carried out in the presence of 92 μM OEA (Fig. 8D), GLP-1 activity was dramatically reduced by a factor of more than 300 (Fig. 8D). This analysis revealed that OEA remarkably facilitated GLP-1(7–36) to trypsin digestion. This is not due to an activation of trypsin by OEA, because OEA does not affect the innate trypsin activity (Fig. 8B). To analyze whether the effect of OEA on trypsin inactivation of GLP-1 was dependent on the concentration of OEA, digestion of GLP-1(7–36) amide with 0.00067% trypsin was carried out with increasing concentrations of OEA. Fig. 8E shows that as the
concentration of OEA increased from 1 to 92 μM, the residual activity of GLP-1(7–36) amide after trypsin digestion was reduced by a factor of more than 40. The effect of OEA became saturated as it reached a concentration of 9 μM. These analyses revealed that OEA dose-dependently and saturably promotes GLP-1(7–36) peptide to trypsin cleavage. In contrast, SEA at concentrations ranging from 1 to 92 μM did not affect trypsin digestion of GLP-1(7–36) amide (Fig. 8F). Our findings clearly revealed that trypsin digestion of GLP-1(7–36) amide was facilitated by OEA. This is not due to the activation of trypsin by OEA, because OEA did not affect the innate enzymatic activity of trypsin (Fig. 8B). Taken together, these data show that the susceptibility of GLP-1 peptide to trypsin cleavage is specifically increased by OEA, most likely due to a conformational change in GLP-1(7–36) amide.

Discussion

The real-time intracellular cAMP assay used in our study is an enhanced BRET2 (24) assay that has better separation of the energy emitted by the donor and acceptor (22, 33) and yields data comparable with that obtained by other previously reported methods (22, 33). This enhanced assay has the advantage of being able to monitor intracellular cAMP production (not cAMP accumulation) in the absence of phosphodiesterase inhibition (e.g. by 3-isobutyl-1-methylxanthine) as well as being a higher throughput assay, based on the use of 96- or 384-well plates.

OEA and 2-OG are both endogenous fatty acids reported to be ligands for GPCR (GPR119) but, when present alone in our assay, did not elicit cAMP responses even with concentrations as high as 10 times (33 μM for OEA and 28 μM for 2-OG) the reported EC50 (2.9 μM) for these lipids to activate GPR119 (34). These data agree with the observation that the RINm5F cell line is devoid of GPR119 receptor signaling (35, 36), and the assay is thus free from the interference of GPR119 signaling.

In the present work, we present evidence that specific endocannabinoid-like lipids (OEA and 2-OG) increase the potency of GLP-1 to stimulate cAMP production, whereas SEA showed little effect. Lipid modification of glucagon has been reported to alter receptor selectivity between GLP-1R and glucagon receptor (GCGR). It is also possible that binding of OEA or 2-OG to GLP-1 peptide may activate receptors other than GLP-1R in RINm5F cells and results in enhanced cAMP production. The present studies show that the enhancement effect of OEA or 2-OG and cAMP response was barely detected when GLP-1Rs in RINm5F cells were blocked by 500 nM of GLP-1R antagonist (Fig. 5E). This observation indicated that lipid enhancement and cAMP response is most likely mediated by GLP-1R. The result also shows that other receptors in RINm5F cells will not respond to the enhancement of potency by OEA or 2-OG. Several mechanisms may account for this phenomenon. At least three possible mechanisms depend on GLP-1R signaling. Binding of lipids to GLP-1R could enhance its coupling to Goαs. Alternatively, the GLP-1 peptide, which may also interact with lipids, could enhance its potency to stimulate the coupling of GLP-1R to Goαs. Alternatively, both of the mechanisms mentioned above could account for the observation. Several lines of evidence imply that endocannabinoid-like lipids interact with GLP-1(7–36) peptide. First, we are not able to observe detectable specific binding of [3H]OEA to GLP-1 receptor. Second, both OEA and 2-OG selectively enhance the potency of the GLP-1(7–36) amide, but not that of Ex-4, indicating interaction with selective peptide. Third, a signal-enhancing lipid, OEA, but not the non-signal-enhancing lipid, SEA, facilitates trypsin inactivation of the peptide, also suggesting a lipid-peptide interaction. Finally, GLP-1(7–36) binds to OEA and 2-OG but not to SEA, revealing a specific binding of OEA or 2-OG to GLP-1(7–36) peptide. Thus the available experimental data are in line with a simple model that specific endocannabinoid-like lipids can bind to GLP-1 and form a complex with enhanced potency to stimulate cAMP response. Surprisingly, interaction of OEA with GLP-1 does not affect the binding affinity to GLP-1 receptor, and the lipid-peptide interactions are mostly dedicated to enhancing the potency to stimulate the coupling of GLP-1 receptor to cAMP production. It has been demonstrated that the binding energy of single point mutant GLP-1 to GLP-1R may not be realized to its signaling potency (37). OEA but not SEA facilitates susceptibility of GLP-1 to trypsin cleavage, which is consistent with the specific binding of OEA and not SEA to GLP-1. Putting these findings together, it is most likely that binding of OEA to GLP-1 may induce a conformational change of the peptide and is accompanied with an enhancement in potency. This proposed model is evocative of a scenario in which peptide ligands for class B GPCRs can be induced to undergo a conformational change in the presence of lipids (38) or upon interaction with their cognate receptors (39).

Apart from the well known effects of postprandial GLP-1 secretion on pancreatic β cells for glycemic control, our knowledge of how GLP-1Rs broadly expressed in tissues serving different functions can be differentially regulated is far from complete (for a review, see Ref. 4). By changing the potency, our results present a novel way to regulate GLP-1 receptor signaling without altering the extracellular level of GLP-1 peptide. GLP-1Rs in the brain stem are considered activated by the centrally derived GLP-1, which is transmitted through neural fibers in most of brain areas (11, 12, 40). However, neurons expressing GLP-1Rs in notable areas, such as caudal hippocampus and the dopaminergic neurons in the substantia nigra, have no apparent innervations from GLP-1-producing neurons (1, 3, 14). Both areas are anatomic sites of deficient functions in disease that are ameliorable by GLP-1 analog therapy, which improves memory/learning in an Alzheimer mouse model and Parkinson symptoms in humans (5, 6, 41). It has been suggested that GLP-1Rs in these areas might be activated by the circulating GLP-1 in cerebrospinal fluid and blood (40), which is normally at a level that is probably below the threshold concentration for GLP-1R activation (42). Given that a 2–5-fold postprandial surge in plasma GLP-1 level can activate pancreatic β cells to release insulin, induction of a 10-fold enhancement in potency by lipids may be sufficient to allow the basal level of GLP-1 to activate its receptors. The lipids OEA and 2-OG are described as endocannabinoid-like because they share structural homology with the endocannabinoids but do not activate the cannabinoid receptors. Our knowledge of the biology of these endocannabinoid-
like lipids is sketchy because they have attracted research interest only in recent years, because each is known to activate other receptors, including GPR119 and peroxisome proliferator-activated receptor (for a review, see Ref. 43). In general, endocannabinoid-like lipids are present in variable amounts in different tissues (44). Their levels are also subject to change following chronic feeding of a diet rich in select types of oil (e.g., olive oil predominantly yields OEA and 2-OG (43)). Because the levels of OEA and 2-OG are much higher (19, 45, 46), these endocannabinoid-like lipids would seem similarly well situated as signal modulators in the brain. OEA is generated in tissues in a stimulus-dependent manner and is quickly removed by enzymatic hydrolysis, indicating a function in signaling (for a review, see Ref. 17). The concentration of 2-OG is significantly elevated in the blood of hibernating animals compared with that of active animals (18). The level of 2-OG is in the range of 2–3 nmol/g of whole brain tissues but in specific regions of the brain can go up to 100 nmol/g of tissue and is also subject to physiologic regulation (19). Distinct from the mode of all lipid mediators that activate receptors in their own right, these endocannabinoid-like lipids regulate GLP-1R signaling by enhancing the potency of GLP-1. In conclusion, local tissue levels of these lipids are varied and regulated physiologically; thus, they can modulate the potency of GLP-1 in a tissue- and physiology-dependent manner. This will allow temporal and spatial regulation of GLP-1R signaling without changing the basal level of GLP-1.

Finally, deterioration in GLP-1 signaling response in type 2 diabetes rather than reduced GLP-1 secretion is associated with the pathogenesis of the disease (47). Such patients have impaired incretin effect, despite normal GLP-1 secretion (48) and normal GLP-1 secretion in response to oral glucose or meal tests (47). Clinically, the administration of GLP-1 analog has been successful in ameliorating reduced GLP-1 signaling in type 2 diabetes; likewise, similar therapeutic effects may be achieved with positive modulators for GLP-1 peptide, which may restore the function of GLP-1 signaling that is more in line with physiological demands. Because cAMP is sufficient for glucose-dependent insulin release (49), one could investigate whether compounds capable of enhancing the cAMP response to GLP-1(7–36) amide stimulation in mouse islets may represent a new class of therapeutic agents. Or nutrient derivatives display similar activities can be used to treat or prevent type 2 diabetes and other GLP-1 signaling-related diseases.

In summary, our present study shows that the potency of a GLP-1 ligand is modulated by specific endogenous lipids, which in turn are subjected to temporal and spatial regulation. This novel mode of regulation allows GLP-1R signaling in different tissues to be differentially activated without changing the basal level of GLP-1. This mode of regulation is distinct from the traditional model, which requires that the extracellular levels of ligand be the primary factors contributing to regulation of GPCR signaling. Our results show that the potency of the ligand itself can be a target for modulation. This finding could further lead to exploration of a distinct class of endogenous peptide ligands that represent novel targets for drug discovery.

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