26 Yang J, Chen JM, Liu WY, Song CY, Lin BC. Through V2, not V1 receptor relating to endogenous opiate peptides, arginine vasopressin in periaqueductal gray regulates antinociception in the rat. Regul Pept. 2006;137:156–161.

27 Tribollet E, Barberis C, Jard S, Dubois-Dauphin M, Dreifuss JJ. Localization and pharmacological characterization of high affinity binding sites for vasopressin and oxytocin in the rat brain by light microscopic autoradiography. Brain Res. 1988; 442:105–118.

28 Vaccari C, Lolait SJ, Ostrowski NL. Comparative distribution of vasopressin V1B and oxytocin receptor messenger ribonucleic acids in brain. Endocrinology. 1998;139:5015–5033.

29 Hernando F, Schoots O, Lolait SJ, Burbach JPH. Immunohistochemical localization of the vasopressin V1b receptor in the rat brain and pituitary gland: Anatomical support for its involvement in the central effects of vasopressin. Endocrinology. 2001;142:1659–1668.

30 Young WS, Li J, Wersinger SR, Palkovits M. The vasopressin 1b receptor is prominent in the hippocampal area CA2 where it is unaffected by restraint stress or adrenalectomy. Neuroscience. 2006;143:1031–1039.

31 Nakamura K, Fujiwara Y, Mizutani R, Sanbe A, Miyauchi N, Hiroyama M, et al. Effects of vasopressin V1b receptor deficiency on adrenocorticotropin release from anterior pituitary cells in response to oxytocin stimulation. Endocrinology. 2008;149:4883–4891.

32 Barberis C, Morin D, Durroux T, Mouillac B, Guillon G, Seyer R, et al. Molecular pharmacology of AVP and OT receptors and therapeutic potential. Drug News Perspect. 1999;12:279–292.

33 Luttinger D, Hernandez DE, Nemeroff CB, Prange AJ Jr. Peptides and nociception. Int Rev Neurobiol. 1984;25:185–241.

34 Recht LD, Abrams GM. Neuropeptides and their role in nociception and analgesia. Neurol Clin. 1986;4:833–852.

35 Raggenbass M. Overview of cellular electrophysiological actions of vasopressin. Eur J Pharmacol. 2008;583:243–254.

36 Berson BS, Berntson GG, Zipf W, Torello MW, Kirk WT. Vasopressin-induced antinociception: an investigation into its physiological and hormonal basis. Endocrinology. 1983;113: 337–343.

37 Berntson GG, Berson BS. Antinociceptive effects of intraventricular or systemic administration of vasopressin in the rat. Life Sci. 1980;26:455–459.

38 Kordower JH, Bodnar RJ. Vasopressin analgesia: Specificity of action and non-opioid effects. Peptides. 1984;5:747–756.

39 Kordower JH, Sikorszky V, Bodnar RJ. Central antinociceptive effects of lysine-vasopressin and an analogue. Peptides. 1982;3: 613–617.

40 Madrazo I, Franco-Bourland RE, Leon-Meza VM, Mena I. Intraventricular somatostatin-14, arginine vasopressin, and oxytocin: Analgesic effect in a patient with intractable cancer pain. Appl Neurophysiol. 1987;50:427–431.

41 Bodnar RJ, Wallace MM, Kordower JH, Nilaver G, Cort J, Zimmerman EA. Modulation of nociceptive thresholds by vasopressin in the Brattleboro and normal rat. Ann N Y Acad Sci. 1982;394:735–739.

42 Bodnar RJ, Zimmerman EA, Nilaver G, Mansour A, Thomas LW, Kelly DD, et al. Dissociation of cold-water swim and morphine analgesia in Brattleboro rats with diabetes insipidus. Life Sci. 1980;26:1581–1590.

43 Bodnar RJ. Neuropharmacological and neuroendocrine substrates of stress-induced analgesia. Ann N Y Acad Sci. 1986;467:345–360.

44 Ahn DK, Kim KH, Ju JS, Kwon S, Park JS. Microinjection of arginine vasopressin into the central nucleus of amygdala suppressed nociceptive jaw opening reflex in freely moving rats. Brain Res Bull. 2001;55:117–121.

45 Yang J, Yang Y, Xu HT, Chen JM, Liu WY, Lin BC. Arginine vasopressin induces periaqueductal gray release of enkephalin and endorphin relating to pain modulation in the rat. Regul Pept. 2007;142:29–36.

46 Watkins LR, Suberg SN, Thurston CL, Culhane ES. Role of spinal cord neuropeptides in pain sensitivity and analgesia: thyrotropin releasing hormone and vasopressin. Brain Res. 1986;362:308–317.

47 Thurston CL, Culhane ES, Suberg SN, Carstens E, Watkins LR. Antinociception vs motor effects of intrathecal vasopressin as measured by four pain tests. Brain Res. 1988;463:1–11.

48 Millan MJ, Schmauss C, Millan MH, Herz A. Vasopressin and oxytocin in the rat spinal cord: analysis of their role in the control of nociception. Brain Res. 1984;309:384–388.

49 Zubrzycka M, Janecka A. Effects of centrally administered vasopressin on orofacial pain perception in rats. Brain Res. 2005;1051:112–116.

50 Yang J, Yang Y, Xu Ht, Chen Jm, Liu Wy, Lin Bc. Arginine vasopressin enhances periaqueductal gray synthesis and secretion of enkephalin and endorphin in the rat. Brain Res Bull. 2006;71:193–199.

51 Martin R, Voigt KH. Enkephalins co-exist with oxytocin and vasopressin in nerve terminals of rat neurohypophysis. Nature. 1981;289:502–504.

52 Watson SJ, Akil H, Fischli W, Goldstein A, Zimmerman E, Nilaver G, et al. Dynorphin and vasopressin: common localization in magnocellular neurons. Science. 1982;216:85–87.

53 Liotta AS, Suda T, Krieger DT. beta-Lipotropin is the major opioid-like peptide of human pituitary and rat pars distalis: lack of significant beta-endorphin. Proc Natl Acad Sci U S A. 1978;75:2950–2954.

54 Rubinstein M, Mogil JS, Japon M, Chan EC, Allen RG, Low MJ. Absence of opioid stress-induced analgesia in mice lacking beta-endorphin by site-directed mutagenesis. Proc Natl Acad Sci U S A. 1996;93:3995–4000.

55 Misfeldt DS, Goldstein A. Hypophysectomy relieves pain not via endorphins. N Engl J Med. 1977;297:1236–1237.

56 Kovacs GL, Telegdy G. Hypothalamo-neurohypophyseal neuropeptides and experimental drug addiction. Brain Res Bull. 1988;20:893–895.

57 Terrillon S, Barberis C, Bouvier M. Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with beta-arrestin and their trafficking patterns. Proc Natl Acad Sci U S A. 2004;101:1548–1553.

58 Terrillon S, Durroux T, Mouillac B, Breit A, Ayoub MA, Taulan M, et al. Oxytocin and vasopressin V1a and V2 receptors form constitutive homo- and heterodimers during biosynthesis. Mol Endocrinol. 2003;17:677–691.

59 Honda K, Takano Y. New topics in vasopressin receptors and approach to novel drugs: Involvement of vasopressin V1a and V1b recepotrs in nociceptive responses and morphine-induced effects. J Pharmacol Sci. 2009;109:38–43.

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Flow Cytometry-Based Binding Assay for GPR40 (FFAR1; Free Fatty Acid Receptor 1)

Takafumi Hara, Akira Hirasawa, Qi Sun, Taka-aki Koshimizu, Chisato Itsubo, Keiko Sadakane, Takeo Awaji, and Gozoh Tsujimoto

Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan (T.H., A.H., Q.S., T.K., C.I., K.S., G.T.); and Department of Pharmacology, Saitama Medical University, Saitama, Japan (T.A.)

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ABSTRACT

GPR40 is a G protein-coupled receptor (GPCR) whose endogenous ligands have recently been identified as medium- and long-chain free fatty acids (FFAs), and it is thought to play an important role in insulin release. Despite recent research efforts, much still remains unclear in our understanding of its pharmacology, mainly because the receptor-ligand interaction has not been analyzed directly. To study the pharmacology of GPR40 in a more direct fashion, we developed a flow cytometry-based binding assay. FLAG-tagged GPR40 protein was expressed in Sf9 cells, solubilized, immobilized on immunomagnetic beads, and labeled with the fluorescent probe C1-BODIPY-C12. Flow cytometry analysis showed that C1-BODIPY-C12 specifically labels a single class of binding site in a saturable and reversible manner with an apparent dissociation constant of ~3 μM. The FFAs that activate GPR40 competed with C1-BODIPY-C12 binding; thus, medium- to long-chain FFAs could compete, whereas short-chain FFAs and methyl linoleate had no inhibitory effect. Furthermore, ligands that are known to activate GPR40 competed for binding in a concentration-dependent manner. All the ligands that inhibited the binding promoted phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 in human embryonic kidney (HEK) 293 cells that expressed GPR40 and $[Ca^{2+}]_i$ responses in mouse insulinoma (MIN6) cells that natively express GPR40; however, pioglitazone, a thiazolidinedione that failed to compete for the binding, did not activate ERK or $[Ca^{2+}]_i$ response. This study showed that a flow cytometry-based binding assay can successfully identify direct interactions between GPR40 and its ligands. This approach would be of value in studying the pharmacology of GPCRs.

Free fatty acids (FFAs) have been demonstrated as ligands for orphan GPCRs (GPR40, GPR41, GPR43, GPR84, and GPR120) and have been proposed to play important roles in a various physiological responses (Briscoe et al., 2003; Brown et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003; Hirasawa et al., 2005; Wang et al., 2006). They are activated by FFAs of different chain lengths with varying degrees of specificity, such that GPR40 (known as free fatty acid receptor 1; FFAR1), GPR84, and GPR120 prefer medium- to long-chain fatty acids (Itoh et al., 2003; Hirasawa et al., 2005; Wang et al., 2006), whereas GPR41 and GPR43, which are now called FFAR3 and FFAR2, respectively, prefer chain lengths of C2 to C5 (Le Poul et al., 2003; Nilsson et al., 2003). GPR40 is coupled to G_q, which results in the activation of phospholipase C (Hardy et al., 2005) and subsequent increases in the intracellular calcium concentration ($[Ca^{2+}]_i$) (Itoh et al., 2003). In addition, GPR40 has been reported to promote the phosphorylation of ERK-1/2 (Yonezawa et al., 2008). GPR40 mRNA is expressed primarily in the pancreas, brain, and monocytes (Briscoe et al., 2003; Itoh et al., 2003). A number of in vitro

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ABBREVIATIONS: FFA, free fatty acid; GPCR, G protein-coupled receptor; FFAR, free fatty acid receptor; ERK, extracellular regulated kinase; BODIPY, 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3*a*,4*a*-diaza-s-indacene; C1-BODIPY-C12, 4,4-difluoro-5-methyl-4-bora-3*a*,4*a*-diaza-s-indacene-3-dodecanoic acid; C4-BODIPY-C9, 5-butyl-4,4-difluoro-4-bora-3*a*,4*a*-diaza-s-indacene-3-nonanoic acid; C8-BODIPY-C5, 4,4-difluoro-5-octyl-4-bora-3*a*,4*a*-diaza-s-indacene-3-pentanoic acid; BODIPY-C5, 4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-s-indacene-3-pentanoic acid; GW9508, 3-[4-({[3-(phenyloxy)phenyl]methyl}amino)phenyl]propanoic acid; MEDICA16, β,β'-tetramethyl hexadecanedioic acid; hGPR40, human GPR40; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Dox, doxycycline; PBS, phosphate-buffered saline; LA, linolenic acid; MIN6, mouse insulinoma cell line.

and in vivo studies have now demonstrated that FFAs promote glucose-stimulated insulin secretion in pancreatic β-cells via GPR40 (Briscoe et al., 2003; Itoh et al., 2003; Poitout, 2003; Steneberg et al., 2005; Feng et al., 2006); therefore, research into GPR40 has the potential to lead to the development of antidiabetes drugs.

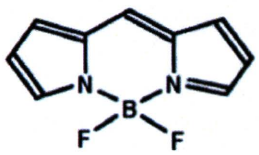
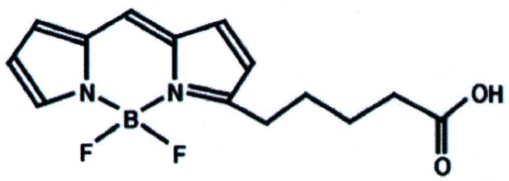
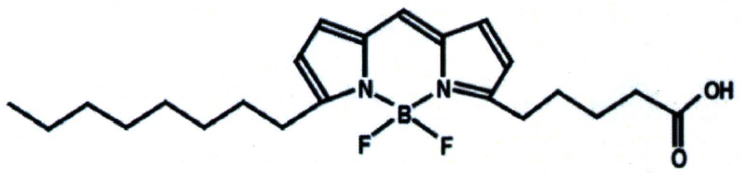
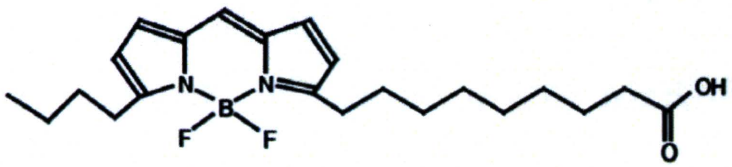
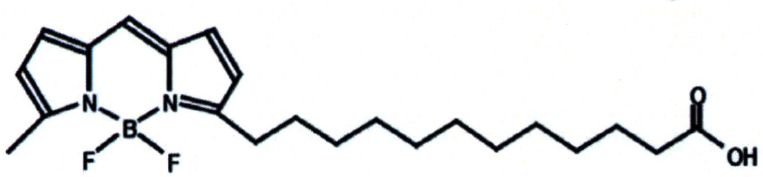
Despite intensive research efforts, the pharmacology of GPR40 is not yet fully understood because most of the data that have been collected were obtained by monitoring the GPR40-mediated second messengers, and direct analysis of the receptor-ligand relationship has not yet been performed. The development of a direct binding assay for GPR40 (and other FFARs) has been hindered mainly by the lack of specific labeled probes. Here, we show that some fluorescent-labeled FFAs can specifically label GPR40. Furthermore, we show that a flow cytometry-based binding assay that uses one of these ligands, C1-BODIPY-C12, as a specific probe can be used to monitor successfully the interaction of GPR40 with its ligands.

Materials and Methods

Materials. Fluorescent-labeled FFA analogs were purchased from Invitrogen (Carlsbad, CA) (Table 1). They included: 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3*a*,4*a*-diaza-s-indacene (BODIPY), 4,4-difluoro-5-methyl-4-bora-3*a*,4*a*-diaza-s-indacene-3-dodecanoic acid (C1-BODIPY-C12), 5-butyl-4,4-difluoro-4-bora-3*a*,4*a*-diaza-s-indacene-3-nonanoic acid (C4-BODIPY-C9), 4,4-difluoro-5-octyl-4-bora-3*a*,4*a*-diaza-s-indacene-3-pentanoic acid (C8-BODIPY-C5), and 4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-s-indacene-3-pentanoic acid (BODIPY-C5). Troglitazone was a gift from Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Pioglitazone and rosiglitazone were purchased from Alexis Biochemicals (San Diego, CA). Ciglitazone and MEDICA16 were purchased from Sigma (St. Louis, MO). GW9508 was synthesized according to the previously reported procedure (Garrido et al., 2006) and purchased from Namiki Shoji Co., Ltd. (Tokyo, Japan). The FLAG-human GPR40 (hGPR40)/pcDNA5/FRT/TO plasmid was prepared as described previously (Hirasawa et al., 2008). In brief, hGPR40 cDNA was obtained by polymerase chain reaction using genomic DNA as a template and ligated into the multicloning site of the mammalian expression vector pcDNA5/FRT/TO (Invitrogen) together with an N-terminal FLAG-tag. All other materials were from standard sources and of the highest purity commercially available.

Cell Culture. The Flp-In T-REx 293 cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) that had been supplemented with 10% fetal bovine serum (FBS), 10 μg/ml blasticidin S (Funakoshi, Tokyo, Japan) and 100 μg/ml Zeocin (Invitrogen). In this study, we used FBS, which we had confirmed to have no inducible effect for T-REx system. MIN6 cells were cultured in DMEM containing 25 mM glucose supplemented with 10% fetal calf serum, 60 μM β-mercaptoethanol, and 0.5% streptomycin and penicillin sulfate. All mammalian cells were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air. The *Spodoptera frugiperda* ovarian (Sf9) cells were cultured in Grace's medium (Invitrogen) that had been supplemented with 10% FBS and grown in monolayer culture at 27°C in 200-ml flasks.

TABLE 1
The chemical structures of BODIPY and BODIPY-FFA derivatives

Compound Name	Structure
BODIPY	
BODIPY-C5	
C8-BODIPY-C5	
C4-BODIPY-C9	
C1-BODIPY-C12	

T-REx Expression System. We used Flp-In T-REx 293 cells to develop stable cell lines (T-Rex hGPR40) in which the expression of GPR40 could be induced with doxycycline (Dox). Cells were transfected with FLAG-hGPR40/pcDNA5/FRT/TO using Lipofectamine reagent (Invitrogen), and selected with DMEM that had been supplemented with 10% FBS, 10 μg/ml blasticidin S, and 100 μg/ml hygromycin B (Sigma). GPR40 protein expression was induced with 10 μg/ml Dox for 24 h.

ERK Assay. Activation of ERK-1/2 in T-REx hGPR40 cells was assayed by Western blotting as described previously (Hirasawa et al., 2008). In brief, cells were serum-starved for 2 h and treated with each compound that was being tested at a concentration of 100 μM. After 10 min of incubation with each compound, total cell extracts were prepared and subjected to Western blotting using anti-phospho- and anti-total-kinase antibodies.

Baculovirus Expression System. To construct the pBAC-FLAG-hGPR40 plasmid, the FLAG-hGPR40 cDNA fragment was prepared from the FLAG-hGPR40/pcDNA5/FRT/TO plasmid and inserted into the pBAC transfer plasmid (Takara Bio, Shiga, Japan). To generate recombinant baculovirus, Sf9 cells were cotransfected with the pBAC-FLAG-hGPR40 plasmid and BacVector-1000 Triple Cut Virus DNA (Novagen, Madison, WI). The recombinant baculovirus was then purified by plaque assay. For expression, the Sf9 cells were grown to a density of 60 to 70% and then infected with recombinant baculovirus at a multiplicity of infection of 10 for 72 h.

Preparation of the GPR40-Bead Complex. GPR40 was complexed to magnetic beads according to the following procedure. Cells that had been infected with recombinant baculovirus were collected and solubilized with 1% digitonin/PBS that contained 1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The lysates were centrifuged in a microcentrifuge for 5 min at 15,000 rpm to sediment the insoluble material. The soluble fraction was removed and incubated with 4 μg of anti-FLAG M2 antibody (Sigma) for 1 h on a rotator at 4°C. After 1 h of incubation with 15 μl of Magnabind protein G beads (Pierce, Rockford, IL), the GPR40-bead complex was collected using an external magnetic field and transferred into 170 μl of 1% digitonin/PBS that contained 1% protease inhibitor cocktail. For some Western blotting experiments, the GPR40-bead complex that had been collected was heated with SDS-polyacrylamide gel electrophoresis sample buffer for 5 min at 70°C, the beads were removed using an external magnetic field, and the supernatant was analyzed.

Flow Cytometry Analysis. The fluorescence emissions were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA). For the binding analysis, samples of the GPR40-bead complex that had been resuspended in 1% digitonin/PBS that contained 1% protease inhibitor cocktail were used. Thirty thousand events were analyzed per sample, using forward scatter versus side scatter dot-plot gating to resolve the primary population of bead particles. The data were collected from FL1 (BODIPY fluorescence) in log mode. The data were normalized by using fluorescence calibration beads (BD Biosciences, San Jose, CA).

Flow Cytometry-Based Binding Assay. A 10-μl aliquot of the bead complex (~8.8×10^4 counts/μl) was incubated for 5 min at room temperature in a 3.5-ml U-bottomed plastic tube with test compounds that had been diluted with 0.1% digitonin/PBS containing 1% protease inhibitor cocktail. Each BODIPY-labeled FFA was diluted in 0.1% digitonin/PBS containing 1% protease inhibitor cocktail and added to the bead-complex. After the samples had been allowed to equilibrate for 5 min, their fluorescence was measured using the FACSCalibur.

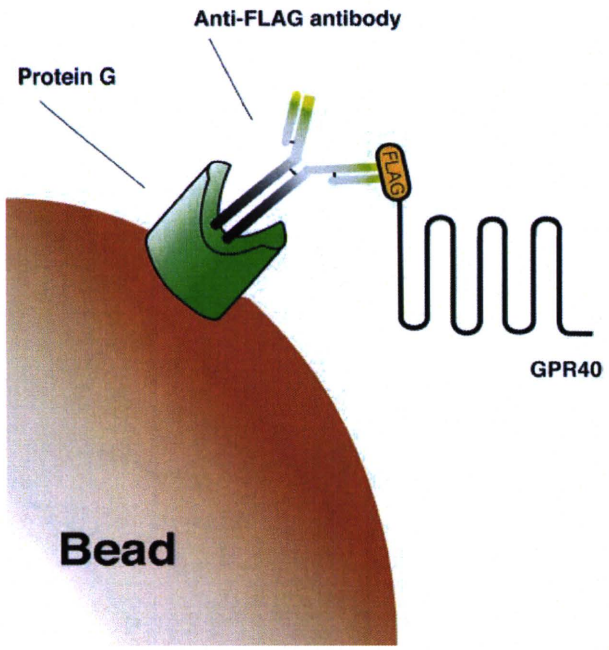


Fig. 1. Schematic diagram of the GPR40-bead complex. The solubilized FLAG-tagged GPR40 fusion protein is depicted as a snakeview GPR40 with an oval FLAG-tag at its *N terminus*. Solubilized GPR40 protein was immobilized on protein G magnetic beads via an anti-FLAG antibody.

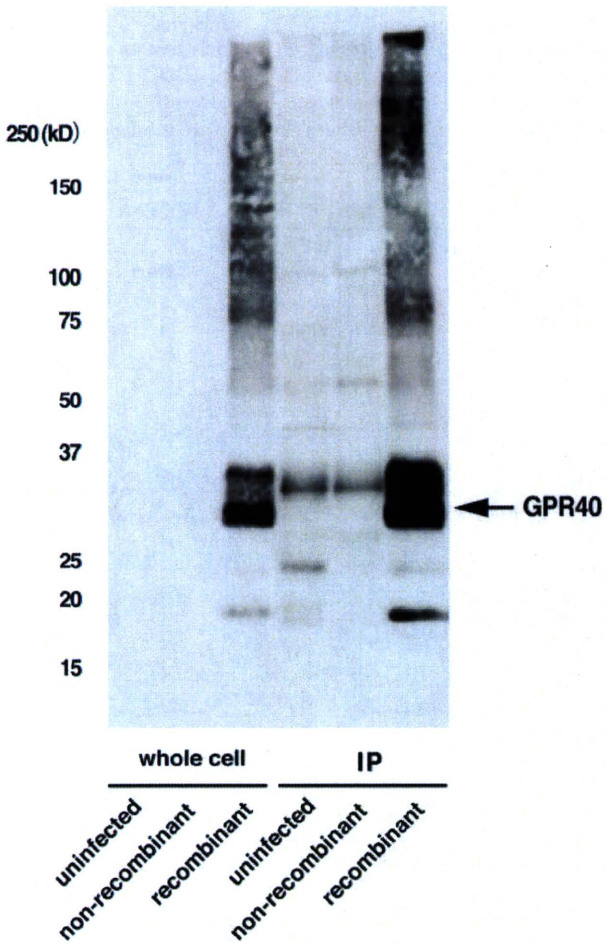


Fig. 2. Immunoblot analysis of FLAG-tagged GPR40 protein with an anti-FLAG antibody. Left, whole-cell lysates were prepared from uninfected Sf9 cells, and cells that had been infected with either recombinant or nonrecombinant baculovirus. Right (IP, immunoprecipitation), the supernatant was collected after immunoprecipitation of cell lysates under the condition described under *Materials and Methods*. Both whole-cell lysates and supernatant were analyzed by immunoblotting using an anti-FLAG antibody. Representative results from one of the three independent experiments are shown. Two additional experiments gave similar results.

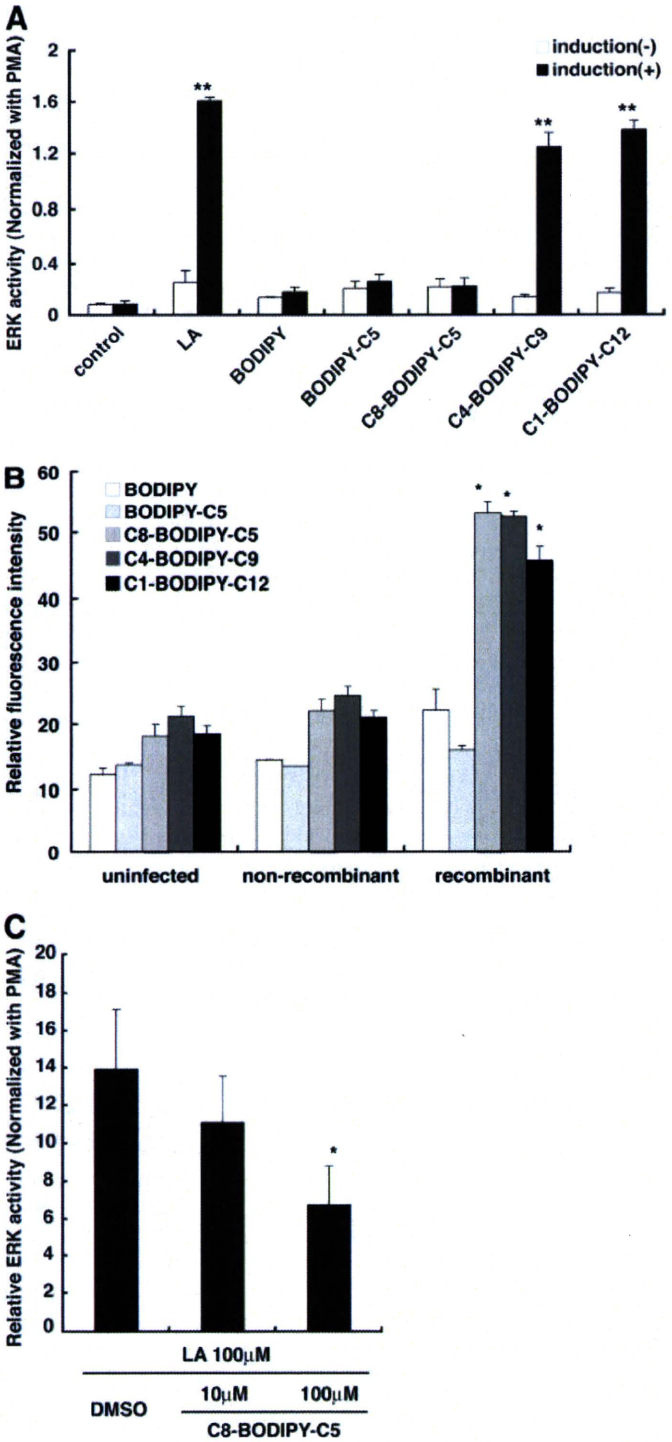


Fig. 3. Effects of BODIPY-FFAs on GPR40. A, effects of BODIPY-FFAs on ERK-1/2 activation in T-REx hGPR40 cells. T-REx hGPR40 cells that had been incubated with Dox [■, induction (+)] or without Dox [□, induction (−)] for 24 h were stimulated with each compound at a concentration of 100 μM. Cell lysates were analyzed by immunoblotting using anti-phospho- and anti-total-kinase antibodies. The amount of phosphorylated ERK-1/2 was normalized to the amount of total ERK-1/2. Then the data were expressed as -fold difference relative to the amount of ERK-1/2 phosphorylation that was obtained in the presence of phorbol 12-myristate 13-acetate (PMA). Results are means ± S.E. of three independent experiments. Significant differences (*, $p < 0.05$; **, $p < 0.01$) between treatment with the control (DMSO only) and with the compound. B, interaction of BODIPY-FFAs with solubilized GPR40 protein that had been immobilized on the beads. BODIPY-FFAs (3 μM) were added to the GPR40-bead complex. After 5 min of incubation at room temperature, fluorescence was measured by flow cytometry. Results are means ± S.E. of three independent experiments. Significant difference (*, $p < 0.05$) between samples from uninfected cells or cells that had been infected with the nonrecombinant and cells that had been infected with the recombinant baculovirus. C, inhibition effects of C8-BODIPY-C5 on LA-induced ERK-1/2 activation in T-REx hGPR40 cells. T-REx hGPR40 cells that had been incubated with Dox for 24 h were stimulated with LA at a concentration of 100 μM. After stimulation with LA, cells were loaded for 5 min with C8-BODIPY-C5 at the concentrations of 10 and 100 μM. Results are means ± S.E. of three independent experiments. Significant differences (*, $p < 0.05$) between treatment with the control (LA only) and with the C8-BODIPY-C5.

The reversibility of binding was monitored in following procedure. In brief, GPR40-bead complex was preincubated for 5 min with C1-BODIPY-C12 until equilibrium was obtained. After the addition of 100 μM troglitazone at the 0-s time point, the fluorescence intensity was monitored. Data were best fitted using a two-phase exponential decay function.

$[Ca^{2+}]_i$ Measurement. $[Ca^{2+}]_i$ were recorded by conventional Ca^{2+} imaging method using an image processor (Argus 50; Hamamatsu Photonics, Hamamatsu, Japan). For $[Ca^{2+}]_i$ measurement, MIN6 cells were loaded with fura-2 acetoxymethyl ester (fura-2 AM; Dojindo, Tokyo, Japan) by incubation in 2 μM fura-2 acetoxymethyl ester for 30 min at 37°C. $[Ca^{2+}]_i$ measurement was performed at 30°C in Tyrode's solution. Fluorescence of fura-2 was measured by applying UV light at 340 and 380 nm alternatively and by leading emission light through a 505-nm dichroic mirror (DCLP; Omega Optical, Brattleboro, VT). Fluorescence was detected by an SPD-CCD camera (MC681APD-R0B0; Texas Instruments, Dallas, TX). Ca^{2+} images were acquired at intervals of 20 s and processed to calculate F_{340}/F_{380} later using NIH Image (http://rsbweb.nih.gov/nih-image/).

Data Analysis. Curve fitting and parameter estimations were performed by using the Igor Pro 3.1.4 software (WaveMetrics, Lake Oswego, OR). The level of significance for the difference between sets of data was assessed using an unpaired Student's t test. Data were expressed as means ± S.E. $p < 0.05$ was considered statistically significant.

Results

Analysis of the GPR40-Bead Complex. The first step in the development of the binding assay was to immobilize solubilized FLAG-tagged GPR40 protein on magnetic protein G beads via an anti-FLAG antibody, as shown in Fig. 1. To determine whether the GPR40 protein was enriched and immobilized on the beads, Western blotting was performed. The first three lanes of Fig. 2, left, show the Western blotting of whole-cell lysates. The anti-FLAG antibody detected a protein of approximately 30 kDa and a smear of proteins from 75 to 250 kDa in the lysate from cells that had been infected with the recombinant baculovirus, which expressed FLAG-tagged GPR40. These proteins were not detected in the lysates from cells that were uninfected or that had been infected with nonrecombinant baculovirus.

The Western blotting performed on the supernatant collected after removing beads from the GPR40-bead complex was shown in Fig. 2, right. For the samples that were derived from either uninfected cells or cells that had been infected with nonrecombinant baculovirus, Western blotting with the anti-FLAG antibody showed that there were several bands in common between these two samples, which presumably correspond to proteins that are bound nonspecifically by the antibody. In contrast, the anti-FLAG antibody produced very strong signals at approximately 30 kDa and in a smear from

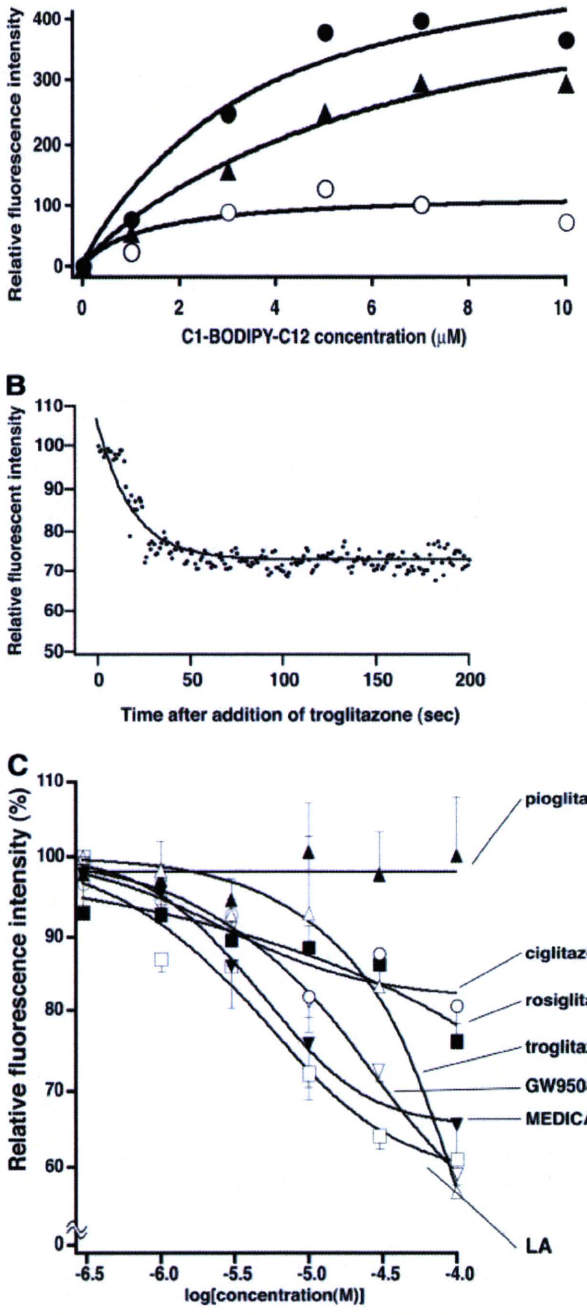


Fig. 4. Flow cytometry-based binding of GPR40 with C1-BODIPY-C12. A, saturation of C1-BODIPY-C12 binding sites. Increasing concentrations of C1-BODIPY-C12 were incubated with the GPR40-bead complex for 5 min at room temperature: total binding (●), nonspecific binding in the presence of 100 μM troglitazone (▲), specific binding (○). Results are given as means ± S.E. of three independent experiments. B, reversibility of specific C1-BODIPY-C12 binding to GPR40-bead complex. GPR40-bead complex were preincubated with C1-BODIPY-C12 until equilibrium was obtained. Dissociation reaction was initiated by adding 100 μM (final concentration) troglitazone at the indicated time point (0 s). The data shown, which are expressed as means of 110 to 124 beads, are the results from a representative experiment. Data were best fitted using a two-phase exponential decay function. Representative results from one of the three independent experiments are shown. Two additional experiments gave similar results. C, competition curves of C1-BODIPY-C12 with ligands. Concentration-dependent inhibition of C1-BODIPY-C12 (3 μM) binding to the GPR40-bead complex by LA and chemical compounds as monitored by flow cytometry: pioglitazone (▲), ciglitazone (○), rosiglitazone (■), troglitazone (△), GW9508 (▽), MEDICA16 (▼), and LA (□). Results are given as means ± S.E. of three independent experiments.

75 to 250 kDa in the sample from the cells that had been infected with the recombinant baculovirus. The results confirmed that GPR40 protein that had been prepared from cells that had been infected with recombinant baculovirus was enriched by using immunomagnetic beads.

Fluorescent Ligands for GPR40. To develop a binding assay with the GPR40-bead complex, we examined a variety of radiolabeled ligands, which included [^{3}H]rosiglitazone, but it was difficult to detect specific binding (data not shown). In an attempt to find a fluorescence-labeled probe that allowed the detection of GPR40, we then examined the effects of BODIPY-FFAs on ERK-1/2 activation in T-Rex hGPR40 cells, in which GPR40 expression can be induced by Dox. As shown in Fig. 3A, C4-BODIPY-C9 and C1-BODIPY-C12, but not BODIPY, BODIPY-C5, or C8-BODIPY-C5, resulted in potent ERK-1/2 activation after Dox induction. These two compounds activated ERK-1/2 to a degree similar to that of linolenic acid (LA), which is the natural ligand for GPR40. Furthermore, we examined whether these BODIPY compounds could specifically label GPR40 protein that had been immobilized on the immunomagnetic beads. We performed flow cytometry analysis with beads that had been prepared with lysates from either uninfected Sf9 cells, cells that had been infected with nonrecombinant baculovirus, or cells that had been infected with recombinant baculovirus. As shown in Fig. 3B, C8-BODIPY-C5, C4-BODIPY-C9, and C1-BODIPY-C12, but not BODIPY or BODIPY-C5, showed strong fluorescence intensity with beads to which GPR40 protein was bound but not with beads that had been incubated with lysate from either the uninfected cells or cells that had been infected with the nonrecombinant baculovirus. Similar fluorescence intensity was observed with C8-BODIPY-C5, C4-BODIPY-C9, and C1-BODIPY-C12, but only C4-BODIPY-C9 and C1-BODIPY-C12, not C8-BODIPY-C5, activated ERK-1/2. Hence, we further examined whether C8-BODIPY-C5 acts as an antagonist for GPR40. As shown in Fig. 3C, C8-BODIPY-C5 inhibited LA-induced ERK-1/2 phosphorylation in a concentration-dependent manner, indicating that C8-BODIPY-C5 may act as an antagonist for GPR40. These results indicated that C8-BODIPY-C5, C4-BODIPY-C9, and C1-BODIPY-C12 could specifically label solubilized GPR40 protein that had been immobilized on immunomagnetic beads; however, C8-BODIPY-C5 has an antagonistic effect, whereas C4-BODIPY-C9 and C1-BODIPY-C12 have agonistic effects, respectively.

Flow Cytometry-Based Binding Assay. Using these BODIPY compounds, we performed saturation experiments. The relative fluorescence intensity of the BODIPY compound that was bound to the GPR40-bead complex was determined at five different concentrations ranging from 1 to 10 μM. Nonspecific binding was determined in the presence of 100 μM troglitazone, which is known to activate GPR40 (Stoddart et al., 2007). Because the three BODIPY compounds showed similar specific binding (in a typical study, specific binding was approximately 20% of total binding at 7 μM BODIPY compound), we used C1-BODIPY-C12 as a specific probe in the following experiments. As shown in Fig. 4A, C1-BODIPY-C12 binding was saturable, with an apparent equilibrium dissociation constant (K_d) of 2.7 ± 0.7 μM (n = 3). In addition, the reversibility of specific C1-BODIPY-C12 binding was assessed. As shown in Fig. 4B, addition of 100 μM troglitazone at equilibrium produced a rapid dissociation

(apparent half-time of dissociation was ~15 s) of the bound C1-BODIPY-C12, demonstrating the reversibility of C1-BODIPY-C12 binding. After approximately 60 s, almost 25% of the C1-BODIPY-C12 was dissociated.

The ability of FFAs to compete for C1-BODIPY-C12 binding sites was examined. FFAs competed for the C1-BODIPY-C12 binding sites in a concentration-dependent manner, and apparent inhibitory binding activities were detected in saturated FFAs of C12 and C16 length and in C18 length unsaturated FFAs. However, methyl-linoleate did not show any inhibitory activity, which suggests that the carboxyl group is indispensable for this interaction. Some eicosanoids also showed inhibitory activity at levels that were comparable with those of long-chain FFAs. The apparent K_i values that were obtained are summarized in Table 2.

We then examined whether the binding to GPR40 of the chemical compounds MEDICA16, GW9508, rosiglitazone, and troglitazone, which were previously shown to activate GPR40 (Kotarsky et al., 2003; Stoddart et al., 2007; Sum et al., 2007), could be monitored by this flow cytometry-based binding assay. As shown in Fig. 4C, these compounds, together with an additional thiazolidinedione, ciglitazone, inhibited the C1-BODIPY-C12 binding in a concentration-dependent manner; however, another thiazolidinedione that was examined, pioglitazone, had no effect on the C1-BODIPY-C12 binding. We further examined whether the binding profile of these compounds correlated with GPR40-mediated ERK activation in T-REx hGPR40 cells as well as with the $[Ca^{2+}]_i$ response in MIN6 cells, which endogenously express GPR40. MEDICA16, GW9508, and all the thiazolidinediones that had been shown to inhibit the binding of C1-BODIPY-C12 were found to activate ERK-1/2 in cells expressing GPR40 (Fig. 5), and they also increased $[Ca^{2+}]_i$ in MIN6 cells (Fig. 6A and B). On the other hand, pioglitazone, which had been shown to have no direct effect on C1-BODIPY-C12 binding, did not activate the GPR40-mediated ERK-1/2 (Fig. 5) or $[Ca^{2+}]_i$ response (Fig. 6, A and B). Thus, the binding profile of the chemical compounds obtained by C1-BODIPY-C12 binding assay seemed to correlate well with their biological effect via GPR40.

Discussion

This study demonstrated that specific C1-BODIPY-C12 binding sites that were identified by a flow cytometry-based assay had the characteristics that were expected for binding sites on the FFAR GPR40. The binding of C1-BODIPY-C12 was of apparently high affinity and was saturable. FFAs competed for the C1-BODIPY-C12 binding sites in a concentration-dependent manner. In addition, as summarized in Table 2, there was good agreement between the apparent K_i values (obtained using the flow cytometry-based binding assay) and apparent EC_{50} values (obtained by $[Ca^{2+}]_i$ monitoring) that were estimated for the FFAs. Thus, the binding properties of the C1-BODIPY-C12 binding sites closely resembled those of GPR40 as delineated by pharmacological procedures ($[Ca^{2+}]_i$) in CHO cells that stably express human GPR40 (Itoh et al., 2003), although both parameters were apparent. Furthermore, the binding profile of not only the FFAs but also the synthetic chemical compounds correlated well with their ability to stimulate ERK-1/2 activity; thus, all the synthetic chemical compounds that inhibited the binding of C1-BODIPY-C12 were shown to activate ERK-1/2 in cells that expressed GPR40, whereas the thiazolidinedione that had no direct effect on the C1-BODIPY-C12 binding (pioglitazone) did not activate the GPR40-mediated ERK response. Moreover, these ligand binding profiles were also found to correlate well with their effect on $[Ca^{2+}]_i$ response in MIN6 cells.

This study showed that the assay we developed can directly monitor the interaction between the FFAR GPR40 and its ligands. Besides GPR40, we have recently succeeded in monitoring the interaction between the FFAR GPR120 and its ligands by this technique (T. Hara, A. Mirasawa, Q. Sun, T. Koshimizu, T. Awaji, and G. Tsujimoto, manuscript in preparation). In addition to these FFARs, we had confirmed that this assay was applicable to other GPCRs (such as adrenergic receptors) in a preliminary series of experiments (data not shown). Hence, this technique seems to be applied for GPCRs in general.

TABLE 2

Comparison of FFA potencies between the competition binding assay, and the $[Ca^{2+}]_i$ assay for GPR40

Apparent IC_{50} values were converted to apparent K_i values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Results are means ± S.E. of three independent experiments. EC_{50} values were adapted from Itoh et al. (2003).

Compound	K_i	EC_{50}
	μM	
Caprylic acid (C8)	Inactive	>300
Lauric acid (C12)	5.8 ± 0.9	5.7 ± 1.4
Palmitic acid (C16)	2.4 ± 1.6	6.8 ± 0.5
Elaidic acid (C18:1)	2.9 ± 1.1	4.7 ± 0.4
Oleic acid (C18:1)	1.3 ± 1.0	2.0 ± 0.3
α-Linolenic acid (C18:3)	2.6 ± 0.4	2.0 ± 0.3
γ-Linolenic acid (C18:3)	2.8 ± 0.5	4.6 ± 1.6
Methyl linoleate	Inactive	Inactive
Eicosapentaenoic acid (C20:5)	3.1 ± 1.0	2.3 ± 0.4
Docosahexaenoic acid (C22:6)	3.3 ± 1.0	1.1 ± 0.3

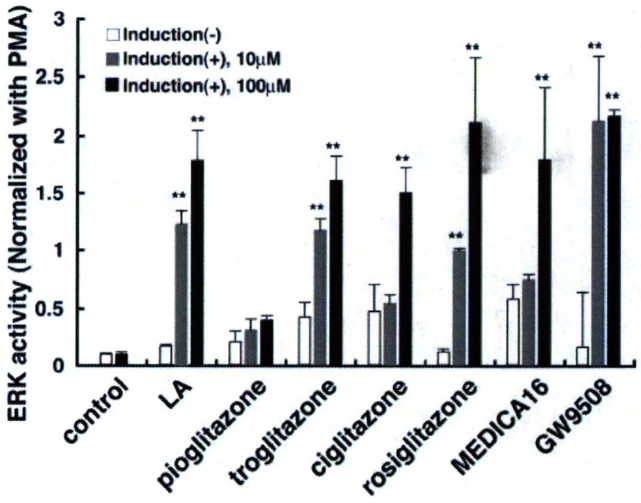


Fig. 5. Effects of ligands on ERK-1/2 activity in T-REx hGPR40 cells. T-REx hGPR40 cells that had been incubated with Dox [■, induction (+)] or without Dox [□, induction (−)] were stimulated with each compound at the concentrations of 10 and 100 μM. Cell lysates were analyzed by immunoblotting using anti-phospho- and anti-total-kinase antibodies. The amount of phosphorylated ERK-1/2 was normalized to the amount of total ERK-1/2. Then the data were expressed as a -fold difference relative to the amount of ERK-1/2 phosphorylation that was obtained in the presence of PMA. Results are means ± S.E. of three independent experiments. Significant differences (**, $p < 0.01$) between treatment with the control (DMSO only) and with the compound.

This flow cytometry-based assay has advantages and disadvantages compared with other established methods. Similar to radioligand binding assay, this technique also requires specific probe to label the receptor, a fluorescent one in this case. Becasue this technique assesses the receptor-ligand interaction by monitoring the fluorescent emission, and the fluorescent measurement is less rigorous in quantifying the probe-labeled receptor compared with other methods (such as radioligand binding assay), the pharmacological parameters on receptor-ligand interactions obtained by this technique should be considered as "apparent" ones. Despite these problems, this technique has several advantages. As described in this study, the radioligand binding assay with [^{3}H]rosiglitazone on the putative membrane preparation of the cloned GPR40 could not detect the receptor-ligand interactions, mainly because of its high nonspecific binding. The reasons for the successful monitoring by the flow cytometry-based binding assay would include that this technique uses the immunopurified receptor protein, which may minimize the nonspecific binding of probe. In addition, this assay measures fluorescent intensity continuously and does not need to separate the free and bound ligands. With these advantages and disadvantages, the availability of the direct flow cytometry-based binding assay will make it possible to perform further pharmacological characterization of GPCRs, especially those difficult to be monitored by other methods. In addition, the assay should prove useful for high-throughput screening of ligands for such GPCRs.

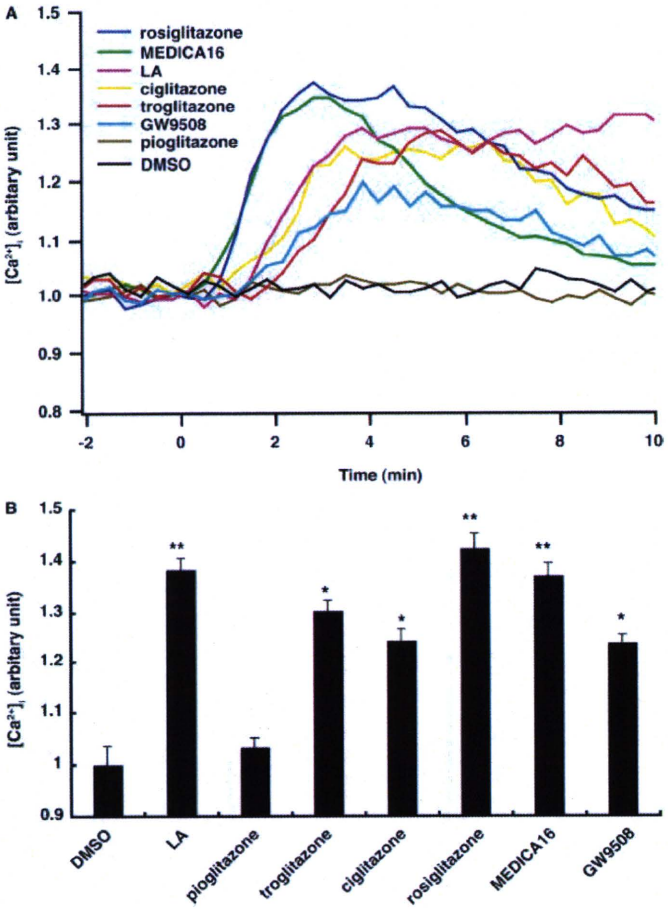


Fig. 6. Effects of chemical compounds on $[Ca^{2+}]_i$ in MIN6 cells. MIN6 cells were stimulated with each chemical compounds (100 μM). A, the representative results shown, which are expressed as means of five to six cells, are obtained from one of three independent experiments. Two additional experiments gave similar results. The time point when indicated compounds were administered was considered 0 s. B, the maximum response of $[Ca^{2+}]_i$ induced by indicated compounds between 0 and 10 min was summarized. Results are means ± S.E. of three independent experiments. The data were normalized to the maximum response observed from DMSO. Significant differences (*, $p < 0.05$; **, $p < 0.01$) between treatment with the control (DMSO only) and that with the indicated compound.

References

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR Jr, Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, and Muir AI (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *J Biol Chem* **278:**11303–11311.

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, et al. (2003) The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J Biol Chem* **278:**11312–11319.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Feng DD, Luo Z, Roh SG, Hernandez M, Tawadros N, Keating DJ, and Chen C (2006) Reduction in voltage-gated K+ currents in primary cultured rat pancreatic beta-cells by linoleic acids. *Endocrinology* **147:**674–682.

Garrido DM, Corbett DF, Dwornik KA, Goetz AS, Littleton TR, McKeown SC, Mills WY, Smalley TL Jr, Briscoe CP, and Peat AJ (2006) Synthesis and activity of small molecule GPR40 agonists. *Bioorg Med Chem Lett* **16:**1840–1845.

Hardy S, St-Onge GG, Joly E, Langelier Y, and Prentki M (2005) Oleate promotes the proliferation of breast cancer cells via the G protein-coupled receptor GPR40. *J Biol Chem* **280:**13285–13291.

Hirasawa A, Itsubo C, Sadakane K, Hara T, Shinagawa S, Koga H, Nose H, Koshimizu TA, and Tsujimoto G (2008) Production and characterization of a monoclonal antibody against GPR40 (FFAR1; free fatty acid receptor 1). *Biochem Biophys Res Commun* **365:**22–28.

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, and Tsujimoto G (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med* **11:**90–94.

Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, et al. (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* **422:**173–176.

Kotarsky K, Nilsson NE, Flodgren E, Owman C, and Olde B (2003) A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. *Biochem Biophys Res Commun* **301:**406–410.

Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, et al. (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J Biol Chem* **278:**25481–25489.

Nilsson NE, Kotarsky K, Owman C, and Olde B (2003) Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. *Biochem Biophys Res Commun* **303:**1047–1052.

Poitout V (2003) The ins and outs of fatty acids on the pancreatic beta cell. *Trends Endocrinol Metab* **14:**201–203.

Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, and Edlund H (2005) The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. *Cell Metab* **1:**245–258.

Stoddart LA, Brown AJ, and Milligan G (2007) Uncovering the pharmacology of the G protein-coupled receptor GPR40: high apparent constitutive activity in guanosine 5′-O-(3-[35S]thio)triphosphate binding studies reflects binding of an endogenous agonist. *Mol Pharmacol* **71:**994–1005.

Sum CS, Tikhonova IG, Neumann S, Engel S, Raaka BM, Costanzi S, and Gershengorn MC (2007) Identification of residues important for agonist recognition and activation in GPR40. *J Biol Chem* **282:**29248–29255.

Wang J, Wu X, Simonavicius N, Tian H, and Ling L (2006) Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. *J Biol Chem* **281:**34457–34464.

Yonezawa T, Haga S, Kobayashi Y, Katoh K, and Obara Y (2008) Unsaturated fatty acids promote proliferation via ERK1/2 and Akt pathway in bovine mammary epithelial cells. *Biochem Biophys Res Commun* **367:**729–735.

Address correspondence to: Dr. Akira Hirasawa, Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University, 46–29 Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan. E-mail: akira_h@pharm.kyoto-u.ac.jp

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ORIGINAL ARTICLE

Cloning and characterization of the rat free fatty acid receptor GPR120: in vivo effect of the natural ligand on GLP-1 secretion and proliferation of pancreatic β cells

Toshiki Tanaka · Takeaki Yano · Tetsuya Adachi · Taka-aki Koshimizu · Akira Hirasawa · Gozoh Tsujimoto

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Abstract We have recently found that GPR120, which is abundantly expressed in intestine, functions as a receptor for unsaturated long-chain free fatty acids (FFAs) and that GPR120 stimulation promotes the secretion of glucagons-like peptide-1 (GLP-1) in the mouse (Hirasawa et al., Nat Med 11:90–94, 2005). In this study, we cloned and characterized rat GPR120 (rGPR120), and then we examined the in vivo effects of acute and long-term administration of the natural ligand α-linolenic acid (α-LA). The cloned rat GPR120 complimentary DNA had a seven transmembrane structure, and a homology comparison of human, mouse, and rat GPR120 revealed that the rat GPR120 (rGPR120) shares 85 and 98% sequence identity with the human and mouse GPR120 proteins, respectively. The tissue distribution and ligand properties of rGPR120 were similar to those of mouse GPR120. In addition, α-LA provoked a transient increase in $[Ca^{2+}]_i$ levels in HEK293 cells expressing rGPR120. Furthermore, administration of α-LA to the rat increased plasma GLP-1 levels, and long-term administration of α-LA led to proliferation of pancreatic β cells, probably because of the enhanced GLP-1 secretion. These results show that rat GPR120 is a G-protein-coupled receptor whose ligand is a free fatty acid, and it may play an important role in the FFA-associated physiological responses.

Keywords Rat · GPR120 · Free fatty acid · GLP-1 · Ca^{2+} signaling · Pancreatic islet

Abbreviations

FFA	free fatty acid
FFAR	free fatty acid receptor
GPCR	G-protein-coupled receptor
GLP-1	glucagon-like peptide-1
α-LA	α-linolenic acid
OA	octanoic acid
rGPR120	rat GPR120

Introduction

Free fatty acids (FFAs) are now known to act as endogenous signaling molecules that play a variety of roles in physiological regulation. They carry out their functions via G-protein-coupled receptors (GPCRs) such as the GPR40 family (Briscoe et al. 2003; Brown et al. 2003; Itoh et al. 2003; Le Poul et al. 2003; Xiong et al. 2004). GPR40 prefers to interact with fatty acids with a medium- to long-chain length (Briscoe et al. 2003; Itoh et al. 2003); it has also been called the free fatty acid receptor 1 (FFAR1). GPR41 and GPR43 prefer chain lengths of between two to five carbonyl groups (Brown et al. 2003); they are now referred to as FFAR3 and FFAR2, respectively. GPR40 couples to Gq (Latour et al. 2007; Stoddart et al. 2007), which results in activation of phospholipase C

Toshiki Tanaka, Takeaki Yano, and Tetsuya Adachi equally contributed to this study.

T. Tanaka · T. Yano · T. Adachi · T.-a. Koshimizu · A. Hirasawa · G. Tsujimoto (✉)
Department of Genomic Drug Discovery Science,
Graduate School of Pharmaceutical Sciences, Kyoto University,
Kyoto 606-8501, Japan
e-mail: gtsuji@pharm.kyoto-u.ac.jp

A. Hirasawa · G. Tsujimoto
Innovation Plaza Kyoto, Japan Science and Technology Agency,
Kyoto, Japan

(Fujiwara et al. 2005; Shapiro et al. 2005) and subsequent increases in intracellular calcium concentration ($[Ca^{2+}]_i$; Briscoe et al. 2003; Itoh et al. 2003). GPR40 has also been reported to inhibit the activity of potassium channels via a cAMP-dependent pathway (Feng et al. 2006).

We recently showed that an orphan GPCR, GPR120, which is abundantly expressed in the intestine, also functions as a receptor for unsaturated long-chain FFAs such as α-linolenic acid (α-LA) and docosahexaenoic acid (Hirasawa et al. 2005). Furthermore, we demonstrated that the stimulation of GPR120 by FFAs promotes secretion of glucagon-like peptide-1 (GLP-1) and cholecystokinin (Hirasawa et al. 2005; Tanaka et al. 2007) and activates the extracellular signal-regulated kinase cascade (Hirasawa et al. 2005). In addition, FFAs were found to inhibit serum-deprivation-induced apoptosis through GPR120 in the murine enteroendocrine cell line STC-1 (Katsuma et al. 2005).

GLP-1 is a potent intestinal insulinotropic hormone (incretin; Schmidt et al. 1985) that can be secreted from specialized enteroendocrine cells (L-cells) to digest fat (Herrmann et al. 1995); our study mentioned above showed that this response is mediated via GPR120 (Hirasawa et al. 2005). Furthermore, we showed that administration of α-LA, the natural ligand for GPR120, to the colon induces GLP-1 secretion (Adachi et al. 2006; Hirasawa et al. 2005). GLP-1 acutely lowers plasma glucose levels in rodents (Wang and Brubaker 2002) and even in type 1 and type 2 diabetic patients (Dupre et al. 1995; Nauck et al. 1993). Previous in vivo studies showed that GLP-1 and exendin-4, a long-acting and potent GLP-1 receptor agonist, may improve glucose tolerance in animal models of diabetes (Xu et al. 1999). Moreover, GLP-1 receptor agonists enhance the proliferation, differentiation, and regeneration of pancreatic β-cells (Brubaker and Drucker 2004; de la Tour et al. 2001).

Previous studies of GPR120 were performed mostly in the mouse. For pharmacological studies, however, rat is frequently used to evaluate drugs as well as disease models. However, the rat and mouse are not always the same with regard to nutrition and/or metabolism, and receptor pharmacology in particular. Free fatty acid metabolism can be markedly different depending on the species (Kushiro et al. 2004; Hirai et al. 1983), and GPR120 is the receptor whose ligand is free fatty acid. In addition, many previous studies have shown that amino acid difference (even a single amino acid) can cause dramatic pharmacological variation between species homologues of the same GPCR (Ghanekar et al. 1997; Shibata et al. 1995; Hirasawa et al. 1994; Oksenberg et al. 1992). Furthermore, ligand–receptor relationship of a given GPCR cannot be extrapolated across species without independent validation even in the context of very high interspecies sequence identity. In this study, hence, we aimed to clone and characterize rat GPR120 (rGPR120), and we further examined the physiological role of GPR120 in the rat.

Materials and methods

Materials

Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Free fatty acids and the other chemicals were from Sigma (St. Louis, MO, USA).

Cloning and in vitro characterization of rGPR120

RNA preparation

Male Wistar rats (8 weeks old) were purchased from SLC Japan (Hamamatsu, Japan). The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Cerebellum, cerebral, cortex, pons/medulla oblongata mesocephalon, striatum, thalamus, hypothalamus, thymus, heart, spleen, pancreas, lung, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, kidney, and adrenal gland were surgically removed and frozen in liquid nitrogen. Total RNA was extracted using Isogen reagent (Nippon Gene, Tokyo, Japan).

Reverse transcription–polymerase chain reaction

Reverse transcription reaction was performed using the Superscript-II enzyme (Invitrogen, Carlsbad, CA, USA). Quantitative detection of specific messenger RNA (mRNA) transcripts was carried out by real-time polymerase chain reaction (PCR) using the DNA Engine Opticon2 System and the DyNAmo HS SYBR Green qPCR kit (Bio-Rad, Tokyo, Japan; Hirasawa et al. 2005) with primers for rGPR120 (forward primer: ccaaccgcataggagaaatc, reverse primer: gaaggaaaccatgagcagga).

Cloning of rat GPR40 and GPR120

The coding regions of rat GPR40 (rGPR40) and GPR120 (rGPR120) were obtained by PCR amplification using genome and colon complimentary DNA (cDNA) as templates, respectively. PCR was performed using primers for rGPR40 (forward primer: aagaggaacagtgacgagagg, reverse primer: ctccagaggtggctgctact) and for rGPR120 (forward primer: gcagatgaacgctctcacag, reverse primer: tacctggcaccagcagttag). Amplified fragments were cloned into pGEM-T easy by TA cloning kit (Promega Japan, Tokyo, Japan) and sequenced. The rGPR40 and rGPR120 cDNAs were subcloned into the pcDNA5/FRT/TO vector

(Invitrogen) to give the plasmids rGPR40/pcDNA5/FRT/TO and rGPR120/pcDNA5/FRT/TO, respectively.

Expression of rGPR40 and rGPR120

We used Flp-In™ T-REx™-293 cells (Invitrogen) to develop stable cell lines for rGPR40 and rGPR120. Flp-In™ T-REx™-293 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS), 10 μg/ml blasticidin S (Funakoshi, Tokyo, Japan), and 100 μg/ml zeocin (Invitrogen). They were transfected using Lipofectamine™ Reagent (Invitrogen) with rGPR40/pcDNA5/FRT/TO or rGPR120/pcDNA5/FRT/TO. They were first selected and then maintained in DMEM supplemented with 10% FBS, 10 μg/ml blasticidin S, and 100 μg/ml hygromycin B (Invitrogen), thus, establishing the stable cell lines rGPR120/T-REX-293 and rGPR40/T-REX-293.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ assays were carried out as described previously (Hirasawa et al. 2005). rGPR120/T-REX-293 and rGPR40/T-REX-293 were seeded into 96-well black plates without FBS. The $[Ca^{2+}]_i$ was measured using a fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA, USA) and a FLIPR Calcium 3 Assay kit (Molecular Devices). FFAs were tested at a final concentration between 30 nM and 100 μM.

In vivo study

Acute administration of α-LA

Male Wistar rats (8 weeks old) were maintained in a temperature-controlled room (23°C) on a 12-h light/dark cycle. This study was approved by the Kyoto University Animal Care and Use Committee. The animals were fed a standard rodent chow diet (MF; Oriental Yeast, Osaka, Japan), and had free access to food and water. The animals fasted for at least 18 h before the experiments. α-LA was used as a test fatty acid, and polyethylene glycol (PEG) 400 (Sigma) was used as vehicle solution for administration to animals (Adachi et al. 2006; Hirasawa et al. 2005). Oral fatty acid administration (3 μmol/100 μl, α-LA or vehicle, n=12 each) was performed 5 min before administration of glucose, and then rats were orally administered of glucose. Rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight), and blood samples were collected from the portal vein 15 min after fatty acid administration and centrifuged. Plasma obtained from blood samples was used to measure GLP-1 levels. The plasma level of GLP-1 was measured using blood samples and GLP-1 enzyme-linked immunosorbent assay (ELISA) kit (Wako Pure Chemical, Osaka, Japan).

Long-term administration of α-LA

Male SD rats (4 weeks old) were purchased from Kiwa experimental animal (Wakayama, Japan). The animals were maintained in a temperature-controlled room (23°C) on a 12-h light/dark cycle. This study was approved by the Kyoto University Animal Care and Use Committee. The animals were fed a high-fat rodent chow diet (Quick Fat; Crea Japan, Osaka, Japan), and they had free access to food and tap water. Octanoic acid (OA) and α-LA were used as test fatty acids, and PEG400 was used as a vehicle solution for administration to the animals. Oral administration of fatty acids (3 μmol/100 μl, α-LA, OA or vehicle, n= 8 each) was performed everyday for 4 weeks. After 4 weeks, the animals were fasted for at least 18 h before scarification, and they were then anaesthetized with sodium pentobarbital (60 mg/kg body weight). Blood samples were obtained from heart and centrifuged. The plasma obtained from these blood samples was used for the measurement of GLP-1 and insulin level. GLP-1 and insulin were measured from blood samples using GLP-1 ELISA kit or Revis Insulin Kit (Shibayagi, Maebashi, Japan), respectively, as described above.

After collection of blood, the pancreas was removed and fixed in 10% formalin solution (Nakalai Tesque, Kyoto, Japan). A piece of the pancreas was embedded in OCT compound (Sakura Finetechnical Tokyo, Japan). Frozen continuous sections (20 μm) were cut, and immunostaining of each section was performed using an anti-insulin antibody and an anti-ki67 antibody. In a series of random fields from regions 5 to 10 of the insulin-positive area (pancreatic islet), ki67-positive cells were counted, and data were represented as the number of ki67-positive cells in a pancreatic islet.

Statistical analysis

Results were expressed as mean±SEM. Statistical significance was evaluated using analysis of variance, and statistical significance was defined as $p<0.05$.

Results

Cloning of rGPR120

An approximately 1-kb DNA fragment was PCR-amplified from rat colon cDNA using two primers designed from the putative rGPR120 sequence. The DNA sequence is unique and shares greater than 85% sequence identity to the human

and mouse GPR120 sequences, suggesting that it is indeed a rGPR120 cDNA fragment. Our cDNA sequence of rGPR120 has been submitted to the DNA Data Bank of Japan (Accession no.: AB207868). The complete coding region of the rGPR120 cDNA encodes a putative protein of 361 amino acids. Homology comparison among human, mouse, and rat GPR120 revealed that the rat GPR120 shares 85 and 98% amino acid sequence identity with the human and mouse GPR120, respectively (Fig. 1).

Tissue distribution of rGPR120

Analysis of the tissue distribution of the rGPR120 mRNA real-time polymerase chain reaction with reverse transcription (RT-PCR) showed abundant expression of rGpr120 mRNA in the rat intestinal tract (Fig. 2).

$[Ca^{2+}]_i$ response

Effects of FFAs on the elevation of $[Ca^{2+}]_i$ levels were examined in rGPR120/T-REX-293 and rGPR40/T-REX-293 cells preloaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4 and then measured using the FLIPR. T-REX-293 cells, rGPR120/T-REX-293, and rGPR40/T-REX-293 cells without doxycycline induction did not elicit a $[Ca^{2+}]_i$ response by α-LA. In contrast, when the rGPR120/T-REX-293 or rGPR40/T-REX-293 cells were induced by doxycycline, 100 μM of α-LA caused a rapid increase in $[Ca^{2+}]_i$. To compare the potency of fatty acids in the elevation of $[Ca^{2+}]_i$, the total fatty acid concentration—$[Ca^{2+}]_i$ response relationships for OA, lauric acid, linoleic acid, and α-LA were determined for rGPR120 and rGPR40 (Fig. 3). α-LA was the most potent for both rGPR120 and rGPR40, with a pEC50 of 5.06±0.03 (n=3) and 4.83±0.02 (n=3), respectively. As shown in Fig. 3, only linoleic acid and α-LA provoked transient increases in $[Ca^{2+}]_i$ in rGPR120/T-REX-293 cells. By contrast, lauric acid could provoke $[Ca^{2+}]_i$ in rGPR40/T-REX-293 cells.

Acute administration of α-LA

The plasma GLP-1 level after fatty acid administration was measured. Add-on administration of α-LA significantly enhanced the plasma GLP-1 level over that measured after vehicle treatment (Fig. 4a).

Long-term α-LA administration

Plasma GLP-1 and insulin levels on administration of fatty acid for 4 weeks while feeding with a high-fat diet were measured. The plasma GLP-1 level was not significantly different between OA-administered rats and those receiving vehicle, but α-LA treatment increased the plasma GLP-1 level significantly more than OA or vehicle treatment (Fig. 4b). Moreover, the plasma insulin level was higher for α-LA treatment than for OA and vehicle treatment (Fig. 4c).

The in vivo effect of long-term stimulation of GPR120 on pancreatic β cells was examined by performing immunohistochemical analysis. Figure 5a shows immunostaining using anti-insulin and anti-ki67 antibody of the rat pancreas, to which, vehicle, OA, or α-LA was administered for 4 weeks. The number of ki67-positive cells in the insulin-positive area (pancreatic islet) revealed that the number of ki67-positive cells was increased after α-LA

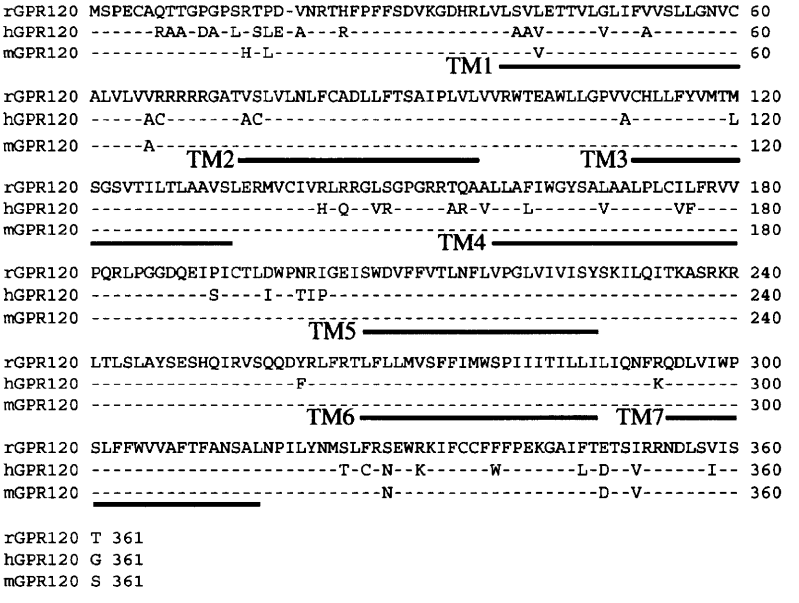

Fig. 1 Homology in the deduced amino acid sequences of rGPR120, hGPR120, and mGPR120. Putative transmembrane regions are *underlined*, and amino acid residues that are identical with those of the other proteins sequence are indicated by a *dash*

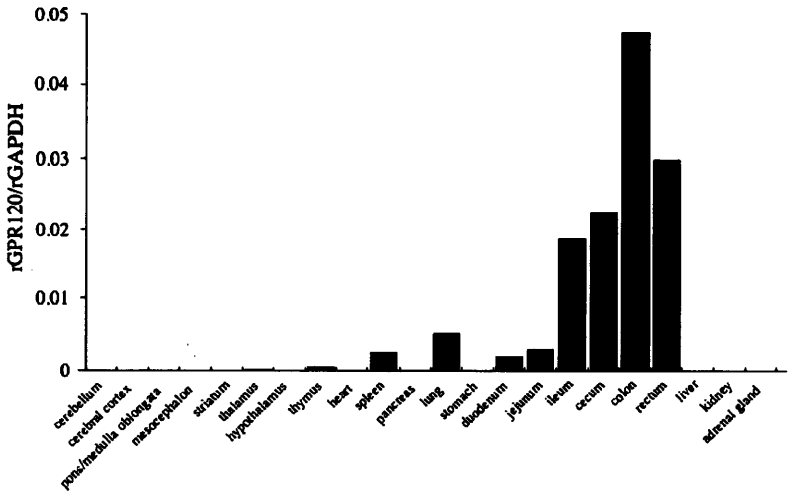

Fig. 2 Distribution of mRNA encoding rGPR120 in rat tissues. cDNAs from the reverse transcription of mRNAs from multiple tissues of three Male Wistar rats were analyzed by DNA Engine Opticon2 System and the DyNAmo HS SYBR Green qPCR kit. Data represent the ratios of rGPR120 and rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) cDNAs. Data shown are representative experiments

administration, compared with vehicle or OA administration, in the pancreatic islet (Fig. 5b).

Discussion

In this study, we, for the first time, cloned and characterized GPR120 in rat (rGPR120). The open reading frame of rGPR120 mRNA encodes a 361-amino acid protein that has high homology to the human and mouse GPR120. In addition, the pharmacology and ligand properties of the rGPR120 expressed in vitro exhibit strong similarity to those of the human and mouse GPR120 (Hirasawa et al. 2005). We examined the effect of FFAs on rGPR120; α-LA raised $[Ca^{2+}]_i$ but not OA in HEK293 cells expressing rGPR120. Additionally, the pharmacological characteristics of rGPR120 and rGPR40 differ at the lauric acid, suggesting that there exists a pharmacological difference between rGPR40 and rGPR120. This kind of pharmacological difference has been noticed in the mouse and human counterparts; thus, mouse and human GPR40 can be activated by medium- and long-chain free fatty acids with chain length above 10, including lauric acid (Briscoe et al. 2003; Itoh et al. 2003), while mouse GPR120 cannot be activated by free fatty acids with chain length less than 14 (Hirasawa et al. 2005).

Next, we examined in vivo function of rGPR120, especially focusing on GLP-1 secretion. It is already known that FFAs induce GLP-1 secretion in rat fetal intestine (Rocca and Brubaker 1995) and that stimulation of GPR120 by FFAs promotes the secretion of GLP-1 in the mouse (Hirasawa et al. 2005). We, hence, examined the effect of acute and long-term administration of α-LA on GLP-1 secretion. Acute administration of α-LA increased plasma GLP-1 levels. Interestingly, long-term administration of α-LA led to enhanced insulin secretion and

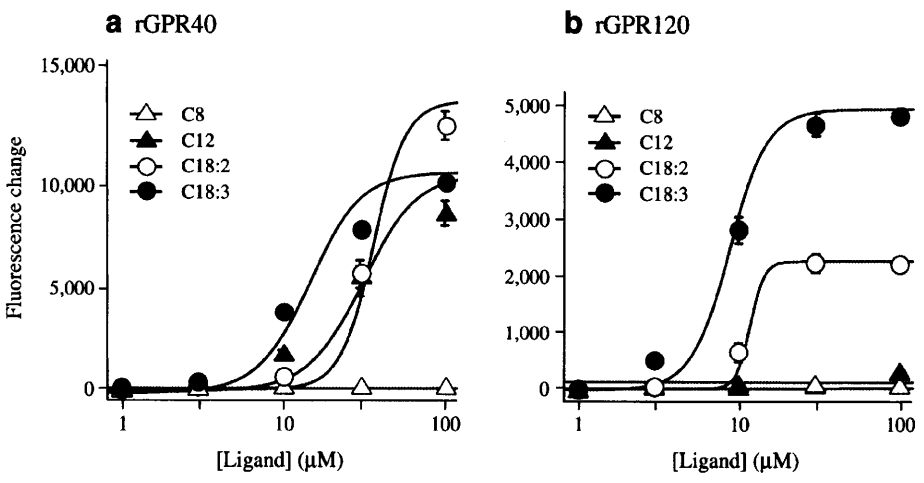

Fig. 3 Free-fatty-acid-induced concentration–$[Ca^{2+}]_i$ response relationships in HEK293 cells expressing rGPR40 (**a**) and rGPR120 (**b**). The indicated concentrations of fatty acids (OA, C8, *open triangle*; lauric acid, C12, *closed triangle*; linoleic acid, C18:2, *open circle*; α-LA, C18:3, *closed circle*) were added, and $[Ca^{2+}]_i$ response was measured as described under "Materials and methods"

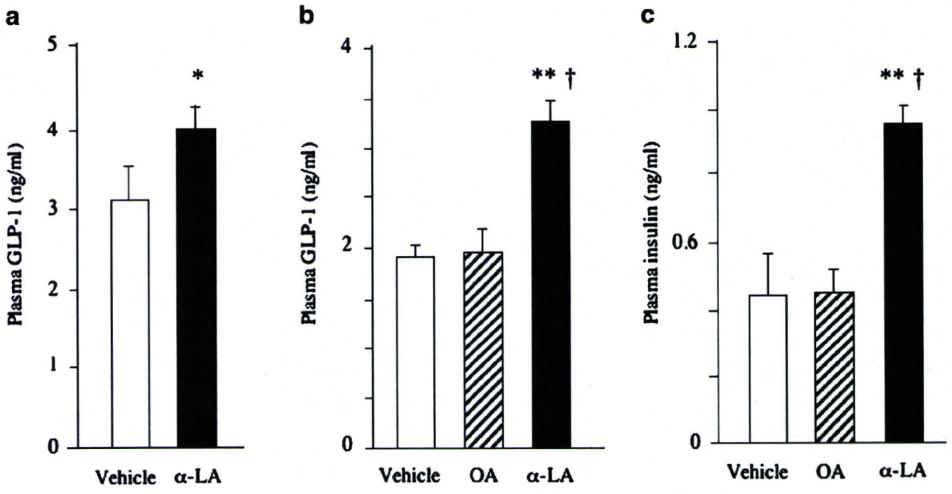

Fig. 4 Plasma GLP-1 and insulin levels after acute (**a**) and long-term (**b**, **c**) oral administration of free fatty acids in the rat. **a** Fifteen minutes after administration of α-LA (3 μmol/100 μl) or vehicle (PEG400, 100 μl; $n=12$ each), blood samples were collected, and the plasma obtained was used for the measurement of GLP-1 levels. **b**, **c** Oral administration of fatty acids (α-LA, OA, 3 μmol/100 μl) or vehicle (PEG400, 100 μl; $n=8$ each) was performed everyday for 4 weeks. After 4 weeks, the animals were fasted for at least 18 h, and blood samples were collected, and the plasma obtained was used for the measurement of GLP-1 and insulin levels. Data are shown as mean±SEM. *$p<0.05$, **$p<0.01$ compared with vehicle administration. †$p<0.01$ compared with OA administration

proliferation of pancreatic β cell in rats that were fed a high-fat diet (Figs. 4 and 5). Although there were no differences in body weight or fasting plasma glucose levels among α-LA, OA, and vehicle-treated rats, plasma GLP-1 and insulin levels were significantly increased in rats treated with α-LA. Proliferation of pancreatic β cells is known to be regulated by many factors including hypoglycemia (Kassem et al. 2000), growth hormones (Nielsen et al. 1989), IGF-1 (Hugl et al. 1998), placental lactogens (Vasavada et al. 2000), prolactin (Brelje et al. 1994), and insulin-resistant states (Pick et al. 1998). Recently, GLP-1 was added to this list when exendin-4, a long-acting GLP-1 agonist, was found to stimulate proliferation of β cells in the rat (Xu et al. 1999). Furthermore, potentiation of endogenous postprandial levels of GLP-1 by inhibiting dipeptidyl peptidase-IV (DPPIV), which rapidly degrades GLP-1, has also been shown to expand β cell mass (Baggio and Drucker 2006). Although the present study did not clearly show the detailed mechanism for proliferation of pancreatic β cells after long-term treatment with α-LA, it would be plausible to hypothesize that enhancement of the GPR120-GLP-1 cascade by α-LA may lead to this result,

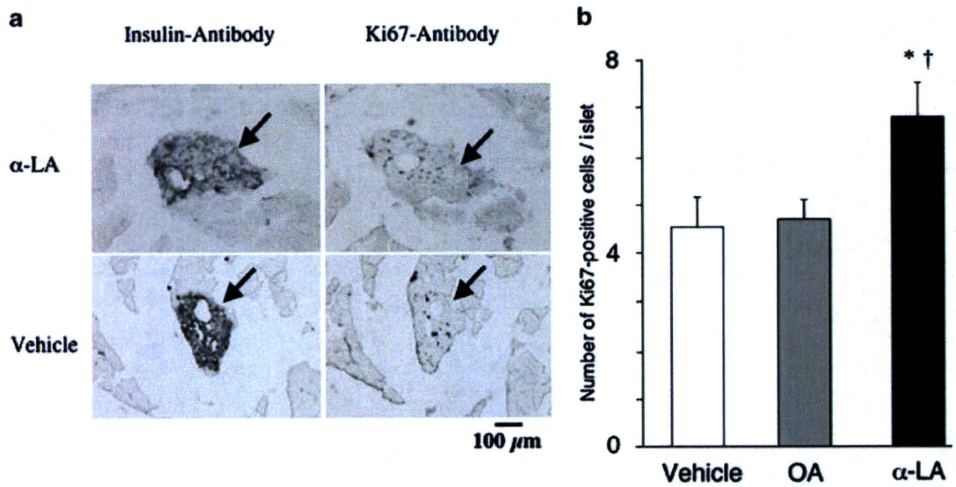

Fig. 5 Effect of long-term administration of free fatty acids on the proliferation of pancreatic β cells. **a** Pancreatic islet histology. Immunohistochemisty of pancreatic secretion from rats orally administered α-LA (3 μmol/100 μl, *top row*) or vehicle (PEG400, 100 μl, *bottom row*) for 4 weeks, with insulin antibody (*left column*) or ki67 antibody (*right column*). *Arrows* show pancreatic islets. **b** Number of ki67-positive cells in pancreatic islets. In a series of random fields from ten regions, ki67-positive cells in pancreatic islets were counted. Data are shown as mean±SEM ($n=5$). *$p<0.01$ compared with vehicle administration. †$p<0.01$ compared with OA administration

as we confirmed that long-term treatment with α-LA did not affect the DPPIV activity at all in the present study (data not shown). Clearly, further studies using genetically modified animals or specific pharmacological tools would be required to test this important hypothesis.

In summary, we have, for the first time, cloned and characterized rat GPR120. Enhanced GLP-1 secretion was found to be induced by the oral administration of α-LA, a natural ligand for GPR120. Moreover, the long-term administration of α-LA leads to enhanced insulin secretion and proliferation of pancreatic β cells. This study provides an important therapeutic suggestion that GPR120-mediated GLP-1 secretion, induced by either food or drugs, may be important not only to enhance the endogenous postprandial levels of GLP-1 but also in regenerating β cells in diabetes.

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References

Adachi T, Tanaka T, Takemoto K, Koshimizu TA, Hirasawa A, Tsujimoto G (2006) Free fatty acids administered into the colon promote the secretion of glucagon-like peptide-1 and insulin. Biochem Biophys Res Commun 340:332–337

Baggio LL, Drucker DJ (2006) Therapeutic approaches to preserve islet mass in type 2 diabetes. Annu Rev Med 57:265–281

Brelje TC, Parsons JA, Sorenson RL (1994) Regulation of islet beta-cell proliferation by prolactin in rat islets. Diabetes 43:263–273

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR Jr., Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 278:11303–11311

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM, Wise A, Dowell SJ (2003) The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 278:11312–11319

Brubaker PL, Drucker DJ (2004) Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. Endocrinology 145:2653–2659

de la Tour D, Halvorsen T, Demeterco C, Tyrberg B, Itkin-Ansari P, Loy M, Yoo SJ, Hao E, Bossie S, Levine F (2001) Beta-cell differentiation from a human pancreatic cell line in vitro and in vivo. Mol Endocrinol 15:476–483

Dupre J, Behme MT, Hramiak IM, McFarlane P, Williamson MP, Zabel P, McDonald TJ (1995) Glucagon-like peptide I reduces postprandial glycemic excursions in IDDM. Diabetes 44:626–630

Feng DD, Luo Z, Roh SG, Hernandez M, Tawadros N, Keating DJ, Chen C (2006) Reduction in voltage-gated K currents in primary cultured rat pancreatic beta-cells by linoleic acids. Endocrinology 147:674–682

Fujiwara K, Maekawa F, Yada T (2005) Oleic acid interacts with GPR40 to induce Ca^2 signaling in rat islet beta-cells: mediation by PLC and L-type Ca^2 channel and link to insulin release. Am J Physiol Endocrinol Metab 289:E670–E677

Ghanekar D, Hadac EM, Holicky EL, Miller LJ (1997) Differences in partial agonist action at cholecystokinin receptors of mouse and rat are dependent on parameters extrinsic to receptor structure: molecular cloning, expression and functional characterization of the mouse type A cholecystokinin receptor. J Pharmacol Exp Ther 282:1206–1212

Herrmann C, Goke R, Richter G, Fehmann HC, Arnold R, Goke B (1995) Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients. Digestion 56:117–126

Hirai K, Shiwaku K, Tsuboi T, Torii M, Nishida H, Yamane Y (1983) Biological effects of Spirometra erinacei plerocercoids in several species of rodents. Z Parasitenkd 69:489–499

Hirasawa A, Shibata K, Kotosai K, Tsujimoto G (1994) Cloning, functional expression and tissue distribution of human cDNA for the vascular-type vasopressin receptor. Biochem Biophys Res Commun 203:72–79

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 11:90–94

Hugl SR, White MF, Rhodes CJ (1998) Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucose-dependent. Synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. J Biol Chem 273:17771–17779

Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H, Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 422:173–176

Kassem SA, Ariel I, Thornton PS, Scheimberg I, Glaser B (2000) Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. Diabetes 49:1325–1333

Katsuma S, Hatae N, Yano T, Ruike Y, Kimura M, Hirasawa A, Tsujimoto G (2005) Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1. J Biol Chem 280:19507–19515

Kushiro M, Takahashi Y, Ide T (2004) Species differences in the physiological activity of dietary lignan (sesamin and episesamin) in affecting hepatic fatty acid metabolism. Br J Nutr 91:377–386

Latour MG, Alquier T, Oseid E, Tremblay C, Jetton TL, Luo J, Lin DC, Poitout V (2007) GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo. Diabetes 56:1087–1094

Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, Parmentier M, Detheux M (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 278:25481–25489

Nauck MA, Kleine N, Orskov C, Holst JJ, Willms B, Creutzfeldt W (1993) Normalization of fasting hyperglycaemia by exogenous

glucagon-like peptide 1 (7-36 amide) in type 2 (non-insulin-dependent) diabetic patients. Diabetologia 36:741–744

Nielsen JH, Linde S, Welinder BS, Billestrup N, Madsen OD (1989) Growth hormone is a growth factor for the differentiated pancreatic beta-cell. Mol Endocrinol 3:165–173

Oksenberg D, Marsters SA, O'Dowd BF, Jin H, Havlik S, Peroutka SJ, Ashkenazi A (1992) A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT1B receptors. Nature 360:161–163

Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, Polonsky KS (1998) Role of apoptosis in failure of beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat. Diabetes 47:358–364

Rocca AS, Brubaker PL (1995) Stereospecific effects of fatty acids on proglucagon-derived peptide secretion in fetal rat intestinal cultures. Endocrinology 136:5593–5599

Schmidt WE, Siegel EG, Creutzfeldt W (1985) Glucagon-like peptide-1 but not glucagon-like peptide-2 stimulates insulin release from isolated rat pancreatic islets. Diabetologia 28:704–707

Shapiro H, Shachar S, Sekler I, Hershfinkel M, Walker MD (2005) Role of GPR40 in fatty acid action on the beta cell line INS-1E. Biochem Biophys Res Commun 335:97–104

Shibata K, Foglar R, Horie K, Obika K, Sakamoto A, Ogawa S, Tsujimoto G (1995) KMD-3213, a novel, potent, alpha 1a-adrenoceptor-selective antagonist: characterization using recombinant human alpha 1-adrenoceptors and native tissues. Mol Pharmacol 48:250–258

Stoddart LA, Brown AJ, Milligan G (2007) Uncovering the pharmacology of the G protein-coupled receptor GPR40: high apparent constitutive activity in guanosine 5′-*O*-(3-[35S]thio) triphosphate binding studies reflects binding of an endogenous agonist. Mol Pharmacol 71:994–1005

Tanaka T, Katsuma S, Adachi T, Koshimizu TA, Hirasawa A, Tsujimoto G (2007) Free fatty acids induce cholecystokinin secretion through GPR120. Naunyn-Schmiedeberg's Archives of Pharmacology (Oct 30, in press)

Vasavada RC, Garcia-Ocana A, Zawalich WS, Sorenson RL, Dann P, Syed M, Ogren L, Talamantes F, Stewart AF (2000) Targeted expression of placental lactogen in the beta cells of transgenic mice results in beta cell proliferation, islet mass augmentation, and hypoglycemia. J Biol Chem 275:15399–15406

Wang Q, Brubaker PL (2002) Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. Diabetologia 45:1263–1273

Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, Yanagisawa M (2004) Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 101:1045–1050

Xu G, Stoffers DA, Habener JF, Bonner-Weir S (1999) Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48:2270–2276

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ORIGINAL ARTICLE

Free fatty acids induce cholecystokinin secretion through GPR120

Toshiki Tanaka · Susumu Katsuma · Tetsuya Adachi · Taka-aki Koshimizu · Akira Hirasawa · Gozoh Tsujimoto

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Abstract The ingestion of fat induces secretion of the gut peptide hormone cholecystokinin (CCK); however, the mechanism responsible for lipid-induced CCK release remains unknown. Recently, a group of free fatty acid (FFA) receptors, which includes the long-chain FFA receptors GPR120 and GPR40, has been identified. In this study, we examined whether these FFA receptors mediate lipid-induced CCK release in the mouse. We first observed that intra-gastric administration of long-chain FFAs increased plasma CCK levels. Using mouse enteroendocrine STC-1 cells as a model system, we further studied the mechanism of this FFA-induced CCK secretion. Long-chain FFAs promoted CCK secretion from STC-1 cells, which was abolished either by removal of extracellular Ca^{2+} or by the L-type Ca^{2+} channel blocker nicardipine. Furthermore, this FFA-induced CCK secretion was specifically inhibited by transfection of GPR120-specific, but not GPR40-specific, short hairpin RNA. These results indicate that long-chain FFAs induce CCK secretion through GPR120-coupled Ca^{2+} signaling.

T. Tanaka · S. Katsuma · T. Adachi · T.-a. Koshimizu · A. Hirasawa · G. Tsujimoto (✉)
Department of Genomic Drug Discovery Science,
Graduate School of Pharmaceutical Sciences, Kyoto University,
46-29 Yoshida Shimoadachi-cho,
Sakyo-ku, Kyoto 606-8501, Japan
e-mail: gtsuji@pharm.kyoto-u.ac.jp

Present address:
S. Katsuma
Graduate School of Agricultural and Life Sciences,
Department of Agricultural and Environmental Biology,
The University of Tokyo,
Yayoi 1-1-1,
Bunkyo-ku, Tokyo 113-8657, Japan

Keywords Cholecystokinin · Free fatty acid · GPR120 · GPR40 · STC-1 cells · shRNA

Introduction

The presence of ingested nutrients, particularly fat, in the proximal small intestine induces secretion of the gut hormone cholecystokinin (CCK) from specialized enteroendocrine cells (I-cells) (Liddle 1997). CCK is important in digestion because it plays a key role in regulating a range of intestinal responses, which include stimulation of pancreatic secretion, gall bladder emptying, and inhibition of gastric motility (Higham et al. 1997; Hopman et al. 1985; Liddle 1997; Liddle et al. 1986; Smith and Gibbs 1994). Collectively, these responses help to integrate and optimize the digestion of fat.

The signal transduction pathway for free fatty acid (FFA)-induced CCK secretion in enteroendocrine cells involves an increase in intracellular Ca^{2+} concentration (McLaughlin et al. 1998; Sidhu et al. 2000), but it is not known whether signal transduction begins at the cell surface or intracellularly. Because fatty acids rapidly cross the cell membrane, an intracellular receptor or channel stimulated by FFAs or FFA metabolites cannot be ruled out (Sidhu et al. 2000). Interestingly, fatty acid-induced CCK secretion has been shown to be dependent on chain length; only test meals containing fatty acids with a chain length of greater than 11 carbon atoms induce CCK secretion in human volunteers (McLaughlin et al. 1999). This chain length dependency is also observed in enteroendocrine cell line models, namely, STC-1 cells (McLaughlin et al. 1998; Sidhu et al. 2000) and GLUTag (Sidhu et al. 2000) cells. This dependency suggests that the detection mechanism for fatty acid is sensitive to the chemical structure of the molecule, and

hence, may rely on one or more specific fatty acid receptors.

Recently, a group of G-protein-coupled fatty acid receptors mediating a variety of FFA-induced regulatory functions has been described (Brown et al. 2003; Hirasawa et al. 2005; Itoh et al. 2003; Wang et al. 2006). GPR40 and GPR120 are activated by medium and long-chain FFAs, whereas GPR41 and GPR43 can be activated by short-chain FFAs (Brown et al. 2003). GPR40, which is preferentially expressed in pancreatic beta-cells, mediates the majority of the effects of FFAs on insulin secretion (Itoh et al. 2003). On the other hand, GPR120, which is abundantly expressed in the intestine, functions as a receptor for unsaturated long-chain FFAs and promotes the secretion of glucagon-like peptide-1 (GLP-1) (Hirasawa et al. 2005). In addition to the similar pharmacological properties, long-chain FFA receptors GPR40 and GPR120 are similar in the subcellular localization. Regarding the cellular localization, we had previously reported that GPR120-GFP fusion protein was observed on the cell surface in the HEK293 cells when stably (and also transiently) expressed (Hirasawa et al. 2005). Also, GPR40 was reported to be localized on the cell surface in the HeLa cells when stably expressed (Salehi et al. 2005). In addition to these observations on cloned receptors, GPR40 was reported to be located on the cell surface in pancreatic beta cells by using specific antibody (Salehi et al. 2005). Regarding GPR120, so far, no report is available on its subcellular localization in native cells. Our preliminary study with specific antibody indicated that GPR120 are localized on the cell surface in mouse adipocytes (manuscript in preparation). In the present study, we investigated the molecular mechanism of FFA-induced CCK secretion, with particular emphasis on the FFA receptors GPR40 and GPR120.

Materials and methods

Chemicals

Thapsigargin was purchased from Nacalai Tesque (Kyoto, Japan). Bombesin was from Peptide Institute (Minoh, Japan). H-89 was from BIOMOL (Plymouth Meeting, PA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

In vivo experiments

Animals

Male C57BL/6J mice (8 weeks old) were purchased from SLC Japan (Hamamatsu, Japan). The animals were maintained in a temperature-controlled room (20~23°C) subjected to a 12-h light/dark cycle. The animals were fed a standard rodent chow diet (MF, Oriental Yeast, Osaka, Japan) and had free access to food and water. This study was approved by the Kyoto University Animal Care and Use Committee.

In vivo administration study was performed according to the previous study (Adachi et al. 2006; Hirasawa et al. 2005). Briefly, the animals fasted for at least 18 h before experiments and were anesthetized with sodium pentobarbital (60 mg/kg body weight). α-Linolenic acid, octanoic acid (100 nmol/g body weight) in PEG, or PEG was administered directly into gastro with gastric needle or into jejunum with cannula. Blood were collected from the portal vein 5 min after administration. Plasma was obtained from trunk blood to measure CCK.

In vitro experiments

Cell culture

Murine enteroendocrine STC-1 cells were maintained in Dulbecco's modified Eagle's medium containing 15% (*v/v*) horse serum and 5% (*v/v*) fetal bovine serum (Sidhu et al. 2000).

Construction and transfection of short hairpin RNA (shRNA)

Short hairpin RNA (shRNA) against mouse GPR40 and GPR120 were constructed and transfected as previously described (Katsuma et al. 2005). The sequences of shRNA expression vectors pSi-GPR120 or pSi-GPR40 are 5′-GGGCGACCACCGGTTGGTGTTCAAGAGACAC CAACCGGTGGTCGCCCTTTTTT-3′ and 5′-CGCCA GTTGTGACATTCTTTTCAAGAGAAAGAATGTCA CAACTGGCGTTTTTT-3′, respectively. Briefly, DNA oligonucleotides targeting GPR120 and GPR40 were first synthesized and inserted into the short interfering RNA expression vector pSilencer1.0 (Applied Biosystems Japan, Tokyo, Japan), and plasmids were transfected into STC-1 cells with Lipofectamine 2000 (Invitrogen Japan, Tokyo, Japan).

Real-time RT-PCR

Total RNA was extracted, and reverse-transcription reactions were performed using the Superscript-II enzyme (Invitrogen Japan). Quantitative detection of specific mRNA transcripts was carried out by real-time polymerase chain reaction (PCR) using DNA Engine Opticon2 System and DyNAmo HS SYBR Green qPCR kit (BIO-RAD Japan, Tokyo, Japan).

FFA stimulation

FFA stimulation study was performed as described previously (Hirasawa et al. 2005). Measurements of CCK were

carried out 24–48 h after seeding STC-1 cells at 1×10^5 cells/cm^2 on 12-well plates. We washed STC-1 cells three times with Hank's balanced salt solution, transferred them to growth medium and incubated them for 60 min at 37°C in Hank's balanced salt solution containing fatty acid and other reagents. After incubation, conditioned medium was collected to measure CCK.

Quantification of CCK

CCK concentration in the plasma and conditioned medium was measured by specific cholecystokinin octapeptide (26–33) enzyme immunoassay (Phoenix Pharmaceuticals, Belmont, CA).

Monitoring of [Ca^{2+}]i in STC-1 cells

To confirm the depletion of intracellular Ca^{2+}stores by the treatment of thapsigargin, [Ca^{2+}]i was measured as previously described (Sidhu et al. 2000). Briefly, STC-1 cells in cover-glass-bottom culture dish were incubated with 4 μM fura-2 AM dissolved in the buffer (mM: 140 NaCl, 4.5 KCl, 10 Hepes acid, 10 Hepes salt, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 glucose) containing Pluronic acid (0.025%) with or without 1 μM thapsigargin for 45 min. After the cells were washed twice with buffer, changes in [Ca^{2+}]i were monitored every 4 s by fluorescence spectrophotometer (OLYMPUS, Tokyo, Japan) at the excitation wavelengths of 340 and 380 nm. All studies were carried out at room temperature.

Statistical analysis

We used one-way or two-way analysis of variance (ANOVA) to evaluate treatment effects. $p<0.05$ was considered significant. All statistics were run with R (R Development Core Team (2005). R: A language and environment for statistical computing. These analyses were performed using R, which is available at the Comprehensive R Archive Network site (http://www.cran.r-project.org)).

Results and discussion

FFA promotes CCK secretion in vivo and in STC-1 cells

We first examined the effect of α-linolenic acid (C18:3) on CCK secretion in mice. Five minutes after intra-gastric administration of α-linolenic acid (100 nmol/g body weight) in C57/BL6 mice, the plasma concentrations of CCK were significantly higher than in mice treated with octanoic acid (C8) or vehicle (Fig. 1). Intra-gastric administration (but not direct administration of α-linolenic acid into the jejunum via a cannula) stimulated CCK secretion (Fig. 1). This difference

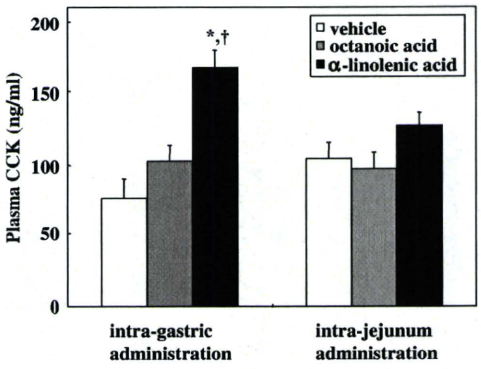

Fig. 1 Effect of FFA administration on plasma CCK concentrations in mice. Plasma CCK concentrations were monitored after intra-gastric administration or intra-jejunum administration of vehicle (PEG), octanoic acid, and α-linolenic acid (100 nmol/g body weight) in C57/BL6 mice. Data are shown as means±SEM (n=5). *$p<0.01$ vs vehicle, †$p<0.005$ vs octanoic acid

may reflect variability in the regional distribution of cells containing CCK (I-cells), as CCK-immunoreactive cells are reported to be located in higher numbers in the pylorus and upper small intestine than in the duodenum or jejunum in C57BL/6 mice (Ku et al. 2003). This in vivo study indicates that the chain length dependency of fatty acid on CCK secretion, which was observed in human as mentioned in the Introduction section (McLaughlin et al. 1999), was also noted in mouse.

We next examined the mechanism of FFA-stimulated CCK secretion using mouse enteroendocrine STC-1 cells as a model system. Sixty minutes after administration, 10 μM α-linolenic acid, 10 μM palmitoleic acid (C16:1), and octanoic acid (at either 10 or 100 μM) had no significant effect on CCK secretion, but 100 μM of α-linolenic acid or palmitoleic acid potently promoted CCK secretion in STC-1 cells (Fig. 2). This result confirmed the chain length dependency of fatty acids on CCK secretion in STC-1 cells, as observed in in vivo study.

FFA promotes CCK secretion through GPR120 in STC-1 cells

To determine whether the medium- and long-chain FFA receptors, GPR120 and GPR40, were involved in α-linolenic acid-stimulated CCK secretion in STC-1 cells, we examined the inhibitory effect of short hairpin RNAs (shRNAs) on α-linolenic acid-stimulated CCK secretion. First, we examined the effect of shRNA on either GPR40 or GPR120 mRNA expression. Real-time reverse transcriptase (RT)-PCR of the STC-1 cells showed that transfection with GPR120-specific shRNA reduced GPR120 mRNA expression by 27±4% (n=3) with little effect on GPR40 mRNA expression; on the other hand, GPR40-specific shRNA reduced GPR40 mRNA expression by 23±0.1% (n=3) with little effect on GPR120 mRNA expression. Furthermore, transfection with GPR120-

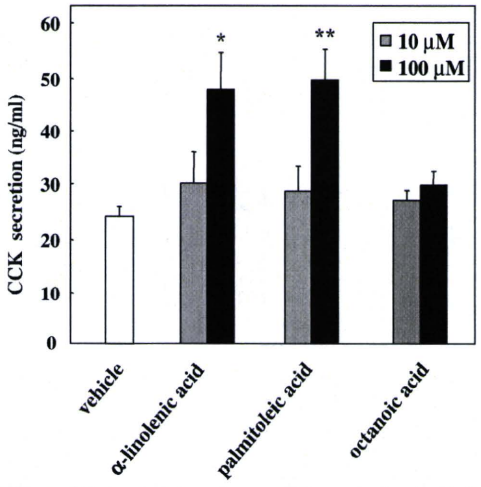

Fig. 2 Effect of FFA administration on CCK secretion in STC-1 cells. CCK secretion from STC-1 cells was measured after administration of 100 or 10 μM α-linolenic acid, oleic acid, palmitoleic acid, octanoic acid, and vehicle (DMSO). Data are shown as means±SEM (n=3). *p< 0.01, **p<0.005 vs vehicle

specific shRNA, but not with GPR40-specific shRNA, significantly (~30%) suppressed α-linolenic acid-stimulated CCK secretion (Fig. 3). This result indicated that long-chain FFA-stimulated CCK secretion in STC-1 cells occurs mainly through GPR120 but not through GPR40.

FFA-promoted CCK secretion is dependent on extracellular Ca^{2+}entry via L-type Ca^{2+}channels

We next examined the signal pathway for FFA-stimulated CCK secretion in STC-1 cells using an extracellular Ca^{2+}-free medium, nicardipine (L-type Ca^{2+}channel blocker), or thapsigargin (an agent that selectively inhibits endoplasmic reticulum Ca^{2+}ATPase). A high concentration of K^+(50 mM) potently stimulated CCK secretion, which was completely suppressed by either removal of extracellular Ca^{2+}or addition

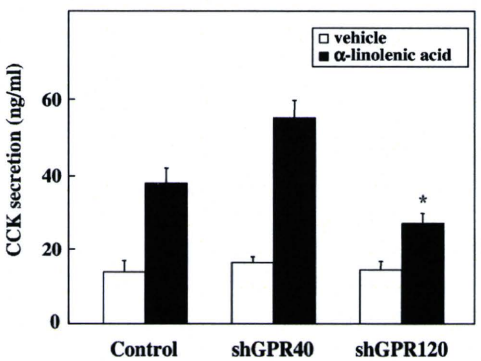

Fig. 3 Effect of GPR40- or GPR120-specific shRNA on FFA-induced CCK secretion in STC-1cells. CCK secretion was measured after administration of vehicle or 100 μM α-linolenic acid in STC-1 cells transfected with GPR40- or GPR120-specific shRNA. Data are shown as means±SEM (n=6). *p<0.05 vs control

of 1 μM nicardipine; however, treatment with 1 μM thapsigargin had no inhibitory effect on K^+-induced CCK secretion (Fig. 4). Similarly, Ca^{2+}-free medium or nicardipine almost abolished α-linolenic acid-stimulated CCK secretion, but treatment of thapsigargin (1 μM, 45 min) did not affect α-linolenic acid-stimulated CCK secretion at all (Fig. 4). The depletion of intracellular Ca^{2+}stores by thapsigargin was confirmed by observing that 1 μM bombesin-induced [Ca^{2+}] i response was abolished in STC-1 cells (data not shown). These results show that FFA-induced CCK secretion in STC-1 cells may be mainly dependent on Ca^{2+}influx through L-type Ca^{2+}channels, rather than Ca^{2+}mobilization from intracellular Ca^{2+}stores. Furthermore, 50 μM protein kinase A (PKA) inhibitor H-89 abolished α-linolenic acid-stimulated CCK secretion (Fig. 4), indicating that PKA is involved in pathway for GPR120-mediated L-type Ca^{2+}channel activation in STC-1 cells. Similar activation of depolarization-induced L-type Ca^{2+}channel by protein kinase A was recently observed in GH3 cells (Vela et al. 2007).

Taken together, the present study indicates that long-chain FFAs induce CCK secretion through GPR120-coupled Ca^{2+}signaling and that this mechanism may underlie the physiological regulation of CCK secretion in response to the ingestion of fat. Also, following from our previous study (Hirasawa et al. 2005), the present study shows that FFAs promote the secretion of gut peptides (GLP-1 and CCK) by an increase in [Ca^{2+}]i; this may be coupled to GPR120, but not GPR40, in vivo as well as in

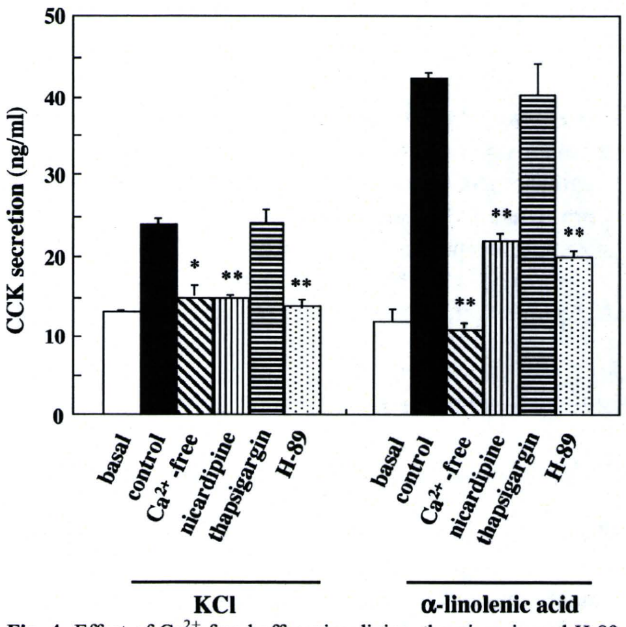

Fig. 4 Effect of Ca^{2+} free buffer, nicardipine, thapsigargin and H-89 on FFA-induced CCK secretion in STC-1 cells. CCK secretion from STC-1 cells was measured after administration of 50 mM KCl or 100 μM α-linolenic acid with treatment of Ca^{2+}-free buffer, 1 μM nicardipine, 1 μM thapsigargin, 50 μM H-89. Data are shown as means±SEM (n=3). *p<0.01, **p<0.001 vs control