RFamide Peptides 43RFa and 26RFa Both Promote Survival of Pancreatic -Cells and Human Pancreatic Islets but Exert Opposite Effects on Insulin Secretion

Riccarda Granata, Fabio Settanni, Letizia Trovato, Davide Gallo, Iacopo Gesmundo, Rita Nano, Maria Pia Gallo, Loredana Bergandi, Marco Volante, Giuseppe Alloatti, et al.

Riccarda Granata,[1] Fabio Settanni,[1] Letizia Trovato,[1] Davide Gallo,[1] Iacopo Gesmundo,[1] Rita Nano,[2] Maria Pia Gallo,[3] Loredana Bergandi,[4] Marco Volante,[4] Giuseppe Alloatti,[3] Lorenzo Piemonti,[2] Jérôme Leprince,[5] Mauro Papotti,[4] Hubert Vaudry,[5] Huy Ong,[6] and Ezio Ghigo[1]

RFamide Peptides 43RFa and 26RFa Both Promote Survival of Pancreatic β-Cells and Human Pancreatic Islets but Exert Opposite Effects on Insulin Secretion

Diabetes 2014;63:2380–2393 | DOI: 10.2337/db13-1522

ISLET STUDIES

RFamide peptides 43RFa and 26RFa have been shown to promote food intake and to exert different peripheral actions through G-protein–coupled receptor 103 (GPR103) binding. Moreover, 26RFa was found to inhibit pancreatic insulin secretion, whereas the role of 43RFa on β-cell function is unknown, as well as the effects of both peptides on β-cell survival. Herein, we investigated the effects of 43RFa and 26RFa on survival and apoptosis of pancreatic β-cells and human pancreatic islets. In addition, we explored the role of these peptides on insulin secretion and the underlying signaling mechanisms. Our results show that in INS-1E β-cells and human pancreatic islets both 43RFa and 26RFa prevented cell death and apoptosis induced by serum starvation, cytokine synergism, and glucolipotoxicity, through phosphatidylinositol 3-kinase/Akt- and extracellular signal–related kinase 1/2-mediated signaling. Moreover, 43RFa promoted, whereas 26RFa inhibited, glucose- and exendin-4–induced insulin secretion, through $G\alpha_s$ and $G\alpha_{i/o}$ proteins, respectively. Inhibition of GPR103 expression by small interfering RNA blocked 43RFa insulinotropic effect, but not the insulinostatic action of 26RFa. Finally, 43RFa, but not 26RFa, induced cAMP increase and glucose uptake. In conclusion, because of their survival effects along with the effects on insulin secretion, these findings suggest potential for 43RFa and 26RFa as therapeutic targets in the treatment of diabetes.

Pancreatic β-cell mass plays an essential role in glucose homeostasis. The reduced capacity of the endocrine pancreas to maintain an adequate insulin secretion, due to decreased β-cell mass and function, underlies both type 1 and type 2 diabetes (1). In type 1 diabetes, immune-mediated release of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β) has been implicated in β-cell apoptosis (2). In type 2 diabetes, β-cell apoptosis results from the combined action of increased plasma glucose/free fatty acid levels (glucolipotoxicity) (3) and cytokines (4). Therefore, identifying molecules capable of increasing pancreatic β-cell survival may be crucial for the treatment and prevention of diabetes.

RFamide-related peptides constitute a family of biologically active peptides terminating in arginine-phenylalanine-amide (Arg-Phe-NH_2) at their C-terminus. They include a 26–amino acid RFamide peptide (26RFa), which was isolated

[1]Division of Endocrinology, Diabetes, and Metabolism, Department of Medical Sciences, University of Torino, Torino, Italy
[2]Diabetes Research Institute, Division of Immunology, Transplantation, and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy
[3]Department of Life Sciences and Systems Biology, University of Torino, Torino, Italy
[4]Department of Oncology, University of Torino, Torino, Italy
[5]Laboratory of Neuronal and Neuroendocrine Differentiation and Communication, International Associated Laboratory Samuel de Champlain, Institute for Research and Innovation in Biomedicine, INSERM U-982, University of Rouen, Rouen, France
[6]Faculty of Pharmacy, University of Montréal, Montréal, Québec, Canada
Corresponding author: Riccarda Granata, riccarda.granata@unito.it.

Received 3 October 2013 and accepted 5 March 2014.

from the frog brain (5), and a longer form of 26RFa, a 43–amino acid peptide with a pyroglutamylated RFamide (termed QRFP-43 or 43RFa). Both peptides were identified as the cognate ligands of the orphan G-protein–coupled receptor 103 (GPR103) (6,7). In humans and rodents, 43RFa, 26RFa, and GPR103 are mainly expressed in brain, particularly in the hypothalamus (8), as well as in peripheral tissues, including the eye, testis, thyroid, adipose tissue, and macrophages (6,9–11). 43RFa and 26RFa have been implicated in many physiological functions, including stimulation of food intake (5,12–17), regulation of gonadotropic axis (18,19), aldosterone secretion (7,20), bone formation (21), adipogenesis and inflammation (10,11), blood pressure (12), and prostate cancer differentiation and migration (22).

Recently, 26RFa has been shown to regulate peripheral glucose metabolism through inhibition of glucose-induced insulin secretion in the perfused rat pancreas, without affecting glucagon secretion. This effect involved inhibition of the adenylyl cyclase/cAMP system via a pertussin toxin (PTX)-sensitive G_i protein (23). On the other hand, to our knowledge, the role of 43RFa on insulin secretion is unknown, as well as the effect of both peptides on pancreatic β-cell survival. Therefore, in the current study, we sought to determine the role of both 43RFa and 26RFa on survival and apoptosis of pancreatic β-cells and human pancreatic islets, particularly in stress conditions such as serum starvation, cytokine synergism, or glucolipotoxicity. Furthermore, we investigated the effects of the peptides on insulin secretion and the underlying signaling mechanisms.

RESEARCH DESIGN AND METHODS

Reagents

Rat QRFP-43 (43RFa), QRFP-26 (26RFa), and exendin-4 (Ex-4) were from Phoenix Pharmaceuticals (Karlsruhe, Germany). PD-98059, wortmannin, PTX, NF449, Hoechst-33258, 3-isobutyl-1-methylxanthine (IBMX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), forskolin, glucose, palmitate, and mammalian cell lysis kit were from Sigma-Aldrich (Milano, Italy). Cytokines (TNF-α, INF-γ, and IL-1β) were from Life Technologies (Milan, Italy). [^{3}H]-2-deoxy-D-glucose was from PerkinElmer Life Sciences, Inc. (Boston, MA). Cell culture and RT-PCR reagents were from Life Technologies. P-Akt (Ser473), P-extracellular signal–related kinase 1/2 (P-ERK1/2), ERK1/2, and Akt antibodies were from Cell Signaling Technology (Euroclone, Milan, Italy). β-Actin antibody was from Santa Cruz Biotechnology (Italia, Milan, Italy). GPR103 antibody was from Abcam (Prodotti Gianni, Milano, Italy) and QRFP from Phoenix Pharmaceuticals (Burlingame, CA) for either immunohistochemistry, immunofluorescence, or Western blot experiments. Primers for RT-PCR were from IDT (Tema Ricerca, Bologna, Italy).

Cell Culture

INS-1E rat β-cells were provided by Claes B. Wollheim (Department of Cell Physiology and Metabolism, University of Geneva, Geneva, Switzerland) and cultured as previously described (24).

Human Islet Isolation

Human islets were obtained from pancreases of multiorgan donors as previously described (24). Islet preparations with purity >70%, not suitable for transplantation, were used after approval by the local ethical committee. Islets (10,000) were cultured in CMRL (Invitrogen) with 10% FBS.

RT-PCR and Real-Time PCR

Total RNA extraction and reversed transcription to cDNA from 3 μg RNA was performed as previously described (24). The following primer sequences were used: rat/mouse/human GPR103, forward 5′-TAGGATCACCCATG TGGCACGT-3′, reverse 5′-AAGAGAGCCACCACTGTCACC ATC-3′ (AB109629.1) (21); human QRFP, forward 5′-ATG GTAAGGCCTTACCCCCTGATCTAC-3′, reverse 5′-CCTG TGGCTGTGAAGCTCT-3′ (BC101127.2); rat QRFP, forward 5′-AGCACACTGGCTTCCGTCTAG-3′, reverse 5′-CG CTGGCCTTCTCTGAGTCA-3′ (NM198200.1) (7); 18S rRNA, forward 5′-GTGGAGCGATTTGTCTGGTT-3′, reverse 5′-CG CTGAGCCAGTTCAGTGTA-3′ (X_01117). cDNA (9 μL) was amplified (GeneAmp PCR System; PerkinElmer, Milan, Italy) in 50 μL under the following conditions: 94°C for 30 s, 60°C for 30 s annealing; 72°C for 60 s, 72°C for 7 min. The final PCR products (318 bp for GPR103, 196 bp for human QRFP, 300 bp for rat QRFP, and 199 bp for 18s rRNA) were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

GPR103 Immunofluorescence

INS-1E β-cells and human pancreatic islets were seeded on glass coverslips in complete medium for 2 days and then washed twice with PBS. INS-1E cells were allowed to grow up to 50–60% confluence. The cells were then fixed with 4% paraformaldehyde and incubated with goat anti-GPR103 antibody overnight at 4°C. After washing three times with PBS, cells were incubated with anti-goat secondary TRITC-conjugated antibody (Life Technologies, Milan, Italy) for 1 h at room temperature. Nuclei were stained with DAPI. Red and blue channels were assigned to GPR103 and nuclei, respectively. Images were taken using a Leica DM2000 fluorescent microscope and a Leica DFC340 FX camera. Adult rat hippocampal cells were used as positive control.

Immunohistochemistry

Human pancreatic tissue (n = 3 samples) was obtained from surgical samples of nonpancreatic diseases; pituitary samples (n = 3 samples) were obtained from autopsies. Tissues were fixed in buffered formalin and embedded in paraffin. Sections (5-μm thick) were deparaffinized and rehydrated in graded alcohols and phosphate buffer, pH 7.5, and endogenous peroxidase activity was blocked by absolute methanol and 0.3% hydrogen peroxide for 15 min. To assess the presence of GPR103 and QRFP

proteins, the following primary antibodies and conditions were used: rabbit polyclonal antibody to GPR103 (diluted 1:150; Abcam, Cambridge, U.K.) and rabbit polyclonal antibody to QRFP-43 (diluted 1:150; Phoenix Pharmaceuticals, Burlingame, CA). Immunoreactions were revealed by a dextran chain (biotin-free) detection system (EnVision; Dako) using 3,3-diaminobenzidine (DAB; Dako) as a chromogen.

Cell Proliferation and Survival

Cell proliferation was assessed by BrdU incorporation ELISA (Roche), as previously described (25). Cells were seeded on 96-well plates at 5×10^3 cells/well in serum-containing medium until 60–70% confluence and serum starved for 24 h before treatments. Cell survival was assessed by MTT as previously described (25).

Hoechst Staining

Morphological changes in the nuclear chromatin of apoptotic cells were detected by Hoechst 33258 staining as previously described (24). Stained nuclei (500) were double counted under a fluorescence microscope (DAPI filter).

Caspase 3 Activity

Caspase 3 activity was assessed by Caspase-3 Colorimetric Kit (Assay Designs, Florence, Italy) in INS-1E β-cells and human islet cell lysates, according to the manufacturer's instructions.

cAMP Assay

Starved 8×10^5 INS-1E β-cells and 1×10^3 human islets were seeded in 100-mm dishes. After incubations, in the presence of IBMX (100 μmol/L), cAMP was measured from lysates, using the Direct Cyclic AMP EIA Kit (Assay Designs) according to the manufacturer's instructions. Forskolin was used as positive control.

Western Blotting

P-ERK and P-Akt (40 μg protein) and GPR103 and QRFP (60 μg) were resolved in 12% SDS-PAGE. Proteins were treated as previously described (24) and incubated with the specific antibody (dilution 1:1,000). Blots were reprobed with the respective total antibodies or with β-actin for normalization. Immunoreactive proteins were visualized with ChemiDoc XRS (Bio-Rad, Milan, Italy), and densitometric analysis was performed with Quantity One software (Bio-Rad).

Insulin Secretion

Insulin secretion in INS-1E β-cells was performed as previously described (24). Human pancreatic islets (n = 3) were incubated for 1 h at 37°C in HEPES-buffered Krebs-Ringer bicarbonate buffer containing 0.5% BSA with 2 mmol/L glucose. The medium was changed, and the cells were incubated again for 1 h in Krebs-Ringer bicarbonate buffer/0.5% BSA containing 1.25, 7.5, 15, or 25 mmol/L glucose, with or without the different peptides or inhibitors. After acid ethanol extraction of the hormone, secreted insulin was quantified by a radioimmunoassay kit (Linco Research, Labodia, Yens, Switzerland) that recognizes human insulin and cross-reacts with rat insulin.

Small Interfering RNA

Rat Qrfpr Silencer Select Predesigned siRNA (s160302) (GPR103 siRNA), Silencer Negative Control siRNA #1 (catalog number AM4611), and Lipofectamine RNAiMAX were purchased from Life Technologies (Monza, Italy). INS-1E cells (2 days postseeding) were transfected in RPMI-1640 serum-free medium (Sigma-Aldrich) without antibiotics, with 50 nmol/L control or GPR103 small interfering RNAs (siRNAs) using Lipofectamine RNAiMAX, according to the manufacturer's instructions. After 24 h, the cells were used for insulin measurement experiments. Efficiency of transfection was assessed by RT-PCR.

[^{3}H]-2-deoxyglucose Uptake

[^{3}H]-2-deoxyglucose uptake was performed in both INS-1E β-cells and human pancreatic islets. Pancreatic islets were dissociated with StemPro Accutase (Gibco, Invitrogen, San Diego, CA) (25). β-Cells were incubated in HEPES buffer without glucose, with either Ex-4, 43RFa, or 26RFa for 20 min, and then with 6 mmol/L 2-deoxyglucose and 1 Ci/mL [^{3}H]-2-deoxyglucose for 5 or 10 min, without HEPES buffer removal. Human islets were incubated in serum-free medium with the peptides for 20 min, and then the media was aspirated and HEPES buffer containing 20 mmol/L 2-deoxyglucose and 1 Ci/mL [^{3}H]-2-deoxyglucose was added for 10 min. The reaction was stopped by washing three times with ice-cold PBS. Cells were solubilized on ice with lysis buffer (NaOH 50 mmol/L). Equal amounts of lysates were mixed with scintillation fluid, and specific activity was counted in a liquid scintillation counter (Beckman Instruments, Fullerton, CA) in duplicate. [^{3}H]-2-deoxyglucose uptake, expressed as pmol/mg, was normalized by using a bicinchoninic acid protein quantification of each sample, as previously described (26).

Statistical Analysis

Results are presented as means ± SE. Results were analyzed using two-tailed Student t test or two-way ANOVA followed by Tukey honestly significant difference for post-ANOVA comparisons (GraphPad Prism 5.0 software, San Diego, CA). Significance was established when $P < 0.05$.

RESULTS

GPR103 and QRFP Are Expressed in INS-1E β-Cells and Human Pancreatic Islets

RT-PCR analysis showed mRNA expression of both GPR103 and QRFP, the precursor of 43RFa and 26RFa, in pancreatic INS-1E β-cells and human pancreatic islets (Fig. 1*A* and *B*). At the protein level, Western blot analysis showed presence of both GPR103 and QRFP, the precursor peptide of 43RFa and 26RFa, in INS-1E β-cells and human pancreatic islets (Fig. 1*C* and *D*). Furthermore, immunofluorescence analysis revealed GPR103 protein expression in both β-cells (Fig. 1*E*) and human islets, where GPR103 showed partial colocalization with insulin

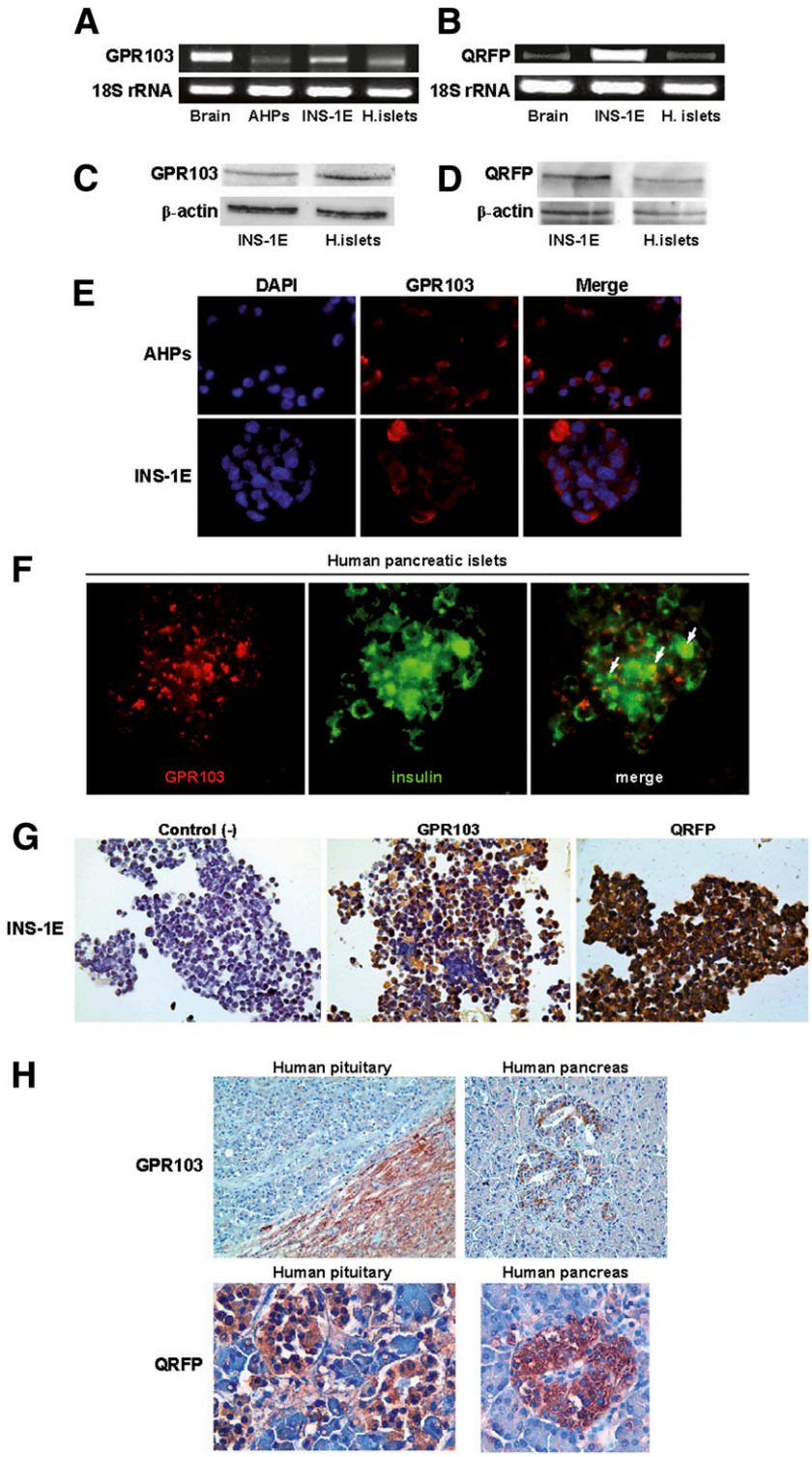

Figure 1— Gene and protein expression of GPR103 and QRFP (the 43RFa and 26RFa precursor) in INS-1E β-cells and human pancreatic islets (H. islets). GPR103 (*A*) and QRFP (*B*) mRNA assessed by RT-PCR. Rat brain (*A* and *B*) and adult hippocampal progenitor cells (AHPs) (*A*) were used as positive control; 18s rRNA was used as internal control. GPR103 (*C*) and QRFP (*D*) protein expression assessed by Western blot on whole lysates from INS-1E β-cells or human pancreatic islets. Equal protein loading was determined by reprobing with antibodies to β-actin. The blots are representative of three independent experiments. *E*: Immunofluorescent staining of GPR103 in INS-1E β-cells. GPR103 is shown in red and nuclei in blue (DAPI); AHPs were used as positive control. Each image is representative of three independent experiments (original magnification ×40). *F*: GPR103 expression in human pancreatic islets assessed by immunofluorescent staining. Insulin is shown in green and GPR103 in red (original magnification ×40) (n = 3). Merge shows colocalization of GPR103 and insulin (yellow), as indicated by arrows. *G*: Immunohistochemical analysis of GPR103 and QRFP expression in INS-1E β-cells. Cells incubated without the primary antibody were used as negative control (−). *H*: Immunohistochemical analysis of GPR103 and QRFP expression in human pancreas; pituitary was used as positive control (n = 3).

in β-cells (Fig. 1*F*). Immunohistochemical studies also confirmed expression of both GPR103 and QRFP in INS-1E β-cells and human pancreatic islets (Fig. 1*G* and *H*). In human pancreas, GPR103 positivity was found in endocrine pancreatic cells, being negative in acinar cells. Similarly, QRFP was positive in pancreatic islets and negative in acinar cells (Fig. 1*H*). Pituitary was used as positive control, showing strong staining for GPR103 in the posterior lobe (neurohypophysis) but almost negative staining in the anterior lobe (adenohypophysis) (Fig. 1*H*). QRFP was negative in the neurohypophysis and weakly positive in isolated cell clusters of the adenohypophysis (corresponding to acydophilic cells) (Fig. 1*H*).

43RFa and 26RFa Both Promote Survival and Proliferation and Inhibit Apoptosis in INS-1E β-Cells

One of the major pathological conditions, responsible for the progression of diabetes, is β-cell loss by apoptosis and decreased β-cell mass. Therefore, we tested the hypothesis as to whether 43RFa and 26RFa may protect β-cells and human pancreatic islets from the harmful effects of stimuli classically involved in diabetes, such as cytokines or glucolipotoxicity.

43RFa and 26RFa effects on survival and proliferation were assessed in INS-1E β-cells cultured in serum-free medium, in either absence or presence of the inflammatory cytokines IFN-γ, TNF-α, and IL-1β. Serum starvation per se is known to reduce survival in many cell types, and cytokine synergism has been implicated in β-cell destruction and apoptosis in both type 1 and type 2 diabetes (4,25). 43RFa and 26RFa were initially tested at different concentrations (1, 10, 25, 50, and 100 nmol/L). Both peptides increased cell survival under serum starvation from 10 to 100 nmol/L, as assessed by MTT assay (Fig. 2*A*). In cytokine-treated cells, they both promoted survival at all the concentrations tested, showing the greatest effect at 100 nmol/L (Fig. 2*B*). Similar results were obtained by performing the Trypan blue assay (data not shown). 43RFa and 26RFa also increased cell proliferation under serum starvation at all the concentrations tested (Fig. 2*C*), whereas with cytokines, they were effective from 25 to 100 nmol/L (Fig. 2*D*). As for cell survival, the best proliferative effect was observed at 100 nmol/L, for both peptides, with a slight dose response under cytokine treatment. Notably, under either serum starvation or cytokines, at 100 nmol/L, the peptides increased survival and proliferation up to levels comparable to those of serum and serum-free medium, respectively.

The glucagon-like peptide 1 receptor (GLP-1R) agonist Ex-4, which exerts antiapoptotic, survival, and proliferative effects in β-cells (27), was used as control peptide. The survival and proliferative effects of Ex-4 were comparable to those of 43RFa and 26RFa, particularly at 100 nmol/L (Fig. 2*A*–*D*).

Glucolipotoxicity, caused by excessive glucose and/or lipids, is a consequence of type 2 diabetes and is a major cause of β-cell dysfunction and death (3). Based on the results obtained in cytokine-treated cells, 43RFa and 26RFa were tested at 100 nmol/L, the concentration that was chosen for the following experiments. The peptides equally increased INS-1E β-cell survival and proliferation, which were both reduced in cells incubated with high glucose (30 mmol/L) and palmitate (0.4 mmol/L), compared with serum-free medium alone (Fig. 2*E* and *F*). These effects were similar to those of Ex-4.

As assessed by Hoechst staining of apoptotic nuclei (Fig. 2*G*) and caspase 3 activation (Fig. 2*H*), apoptosis was increased in cells cultured in serum-free medium, compared with those in serum, and even more with cytokines or high glucose/palmitate. Both 43RFa and 26RFa, at 100 nmol/L and similarly to Ex-4 (100 nmol/L), strongly reduced apoptosis in all experimental conditions (Fig. 2*G* and *H*).

Overall, these results indicate that 43RFa and 26RFa protect pancreatic β-cells against different diabetogenic stimuli.

43RFa and 26RFa Both Promote Survival and Inhibit Apoptosis in Human Pancreatic Islets

Similarly to INS-1E β-cells, 43RFa and 26RFa increased survival and inhibited apoptosis in human pancreatic islets cultured for 72 h under either serum starvation, cytokine synergism, or high glucose/palmitate. The peptides and Ex-4, which was used as positive control, were tested at 1, 10, 25, 50, and 100 nmol/L in islets cultured in either serum-free medium alone or with IFN-γ/TNF-α/IL-1β. Under serum starvation, 43RFa and 26RFa increased survival at 100 nmol/L, having no significant effect at lower concentrations (Fig. 3*A*). In the presence of cytokines, they both increased cell survival from 25 to 100 nmol/L, which was the best concentration tested (Fig. 3*B*). Conversely, Ex-4 was effective at all concentrations in both experimental conditions and in a dose-dependent manner (Fig. 3*A* and *B*). At 100 nmol/L, 43RFa- and 26RFa-induced survival was similar to that of Ex-4, and a dose response was observed under cytokine treatment (Fig. 3*B*). In islets cultured in high glucose/palmitate, 100 nmol/L was also used, where the peptides equally increased islet cell survival (Fig. 3*C*). Apoptosis, assessed by Hoechst staining and caspase 3 activity, was inhibited by both 43RFa and 26RFa in all experimental settings and similarly to Ex-4 (Fig. 3*D* and *E*).

These results indicate that, as for β-cells, in human islets, 43RFa and 26RFa exert protective effects against different detrimental diabetogenic stimuli.

43RFa Survival Effects Involve Activation of Phosphoinositide 3-Kinase/Akt and ERK1/2, Whereas Only ERK1/2 Is Required for 26RFa-Induced Cell Survival

We next investigated the signaling pathways involved in the survival effects of the peptides, and specifically, phosphatidylinositol 3-kinase (PI3K)/Akt and ERK1/2, whose activation plays a key role in β-cell growth and survival (28–30).

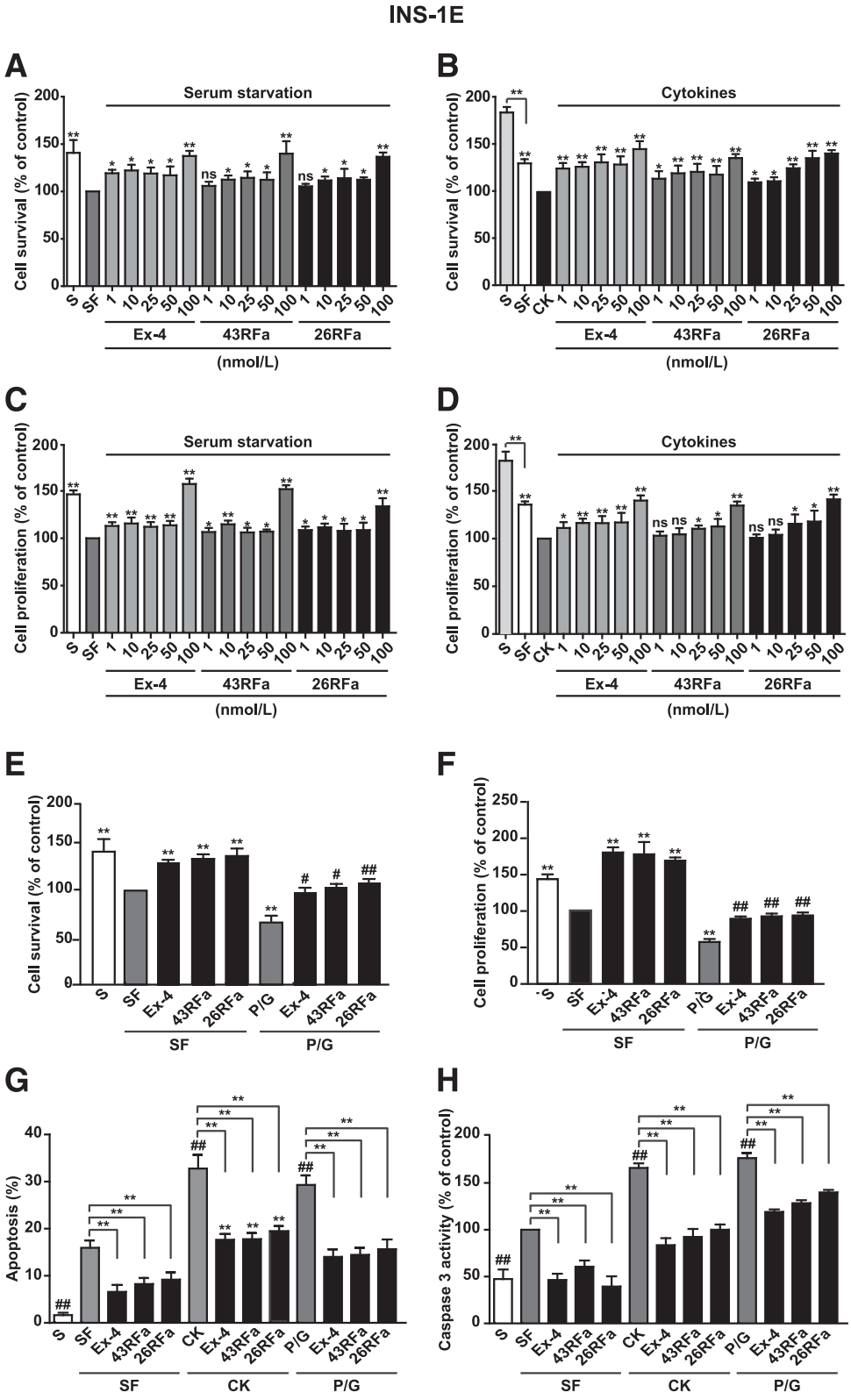

Figure 2—43RFa and 26RFa effects on survival, proliferation, and apoptosis of INS-1E β-cells. The cells were cultured in serum-free medium (SF) for 12 h, and then incubated for a further 24 h without or with the cytokines (CK) TNF-α/IFN-γ/IL-1β (100, 50, and 5 ng/mL, respectively), or with high glucose (30 mmol/L) and palmitate (0.4 mmol/L) (P/G), and with either 43RFa, 26RFa, or Ex-4. The peptides were added 40 min prior to CK or P/G. Cell survival and proliferation (assessed by MTT and BrdU, respectively) in the absence (*A* and *C*) or presence (*B* and *D*) of CK and the peptides, at the indicated concentrations. Data are expressed as percent of control (SF) and are the mean ± SE of five replicates ($n = 3$) (*$P < 0.05$ and **$P < 0.01$ vs. SF; ns, not significant). Cell survival (*E*) and cell proliferation (*F*) in cells cultured in SF medium, alone or with P/G and the indicated peptides (100 nmol/L each). Results are expressed as percent of control (SF); $n = 3$. (**$P < 0.01$ vs. SF; #$P < 0.05$ and ##$P < 0.01$ vs. P/G.) *G*: Apoptosis assessed by counting condensed/fragmented Hoechst-stained

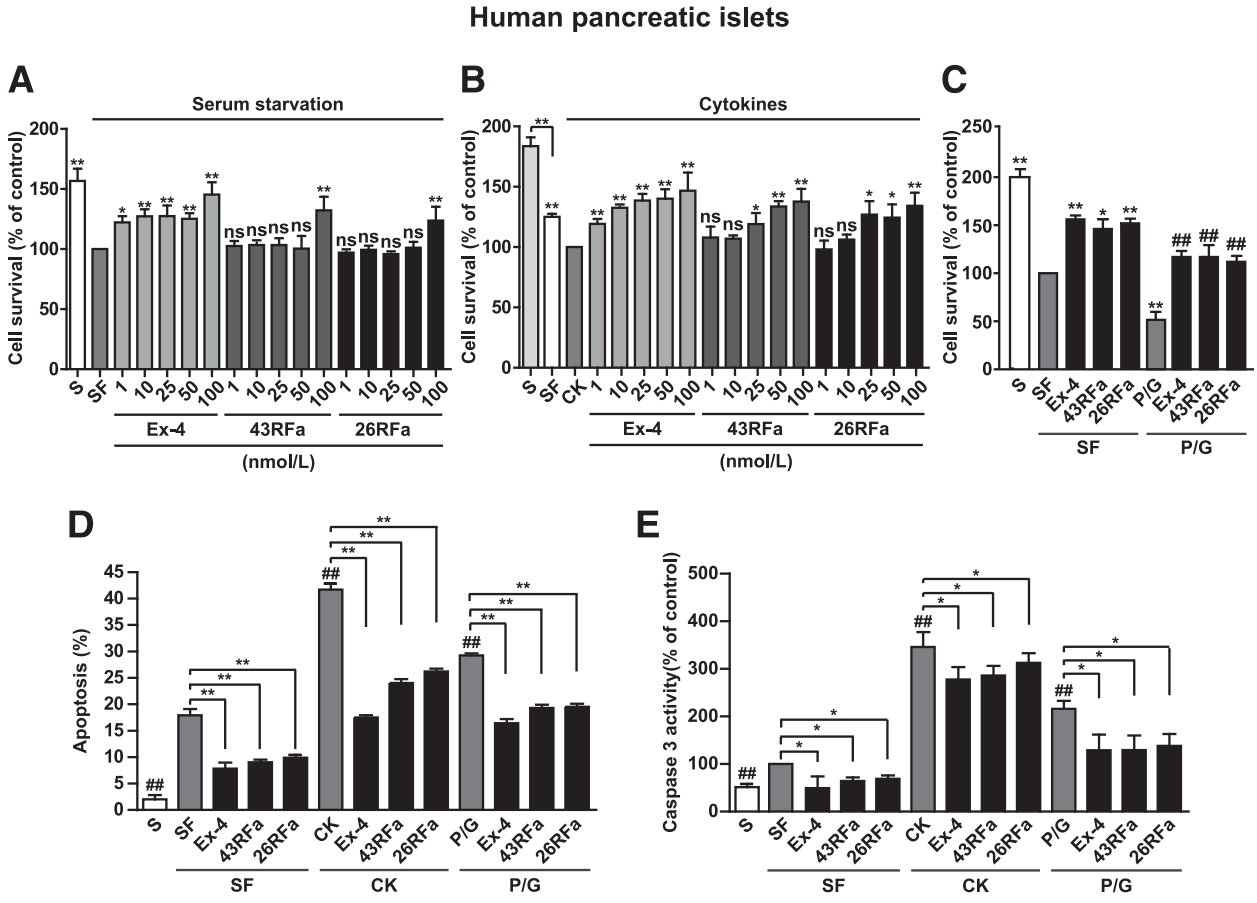

Figure 3—43RFa and 26RFa effects on survival and apoptosis of human pancreatic islets. Islet cells were incubated for 72 h in the presence of serum or in serum-free medium (SF), either alone or with IFN-γ/TNF-α/IL-1β (CK) (5 ng/mL each) and 43RFa or 26RFa. Cell viability assessed by MTT in islets cultured in SF, alone (*A*) or with cytokines (CK) (*B*), and the peptides at the indicated concentrations. Results are expressed as percent of control (SF, $n = 3$) (*$P < 0.05$ and **$P < 0.01$ vs. SF for *A* or vs. CK for *B*; ns, not significant). *C*: Cell survival (MTT) in human islets treated in SF medium alone or with high glucose (30 mmol/L)/palmitate (P/G) (0.4 mmol/L) and the indicated peptides (100 nmol/L each). Results are expressed as percent of control (SF); $n = 3$ (*$P < 0.05$ and **$P < 0.01$ vs. SF; ##$P < 0.01$ vs. P/G). *D*: Apoptosis assessed by Hoechst staining. Values are expressed as percent of apoptotic cells from duplicate determinations (500 cells each, $n = 3$). *E*: Apoptosis assessed by caspase 3 activation. Results are expressed as percent of control (SF). For both *D* and *E*, *$P < 0.05$ and **$P < 0.01$; ##$P < 0.01$ vs. SF; $n = 3$. Each peptide was used at 100 nmol/L.

In INS-1E cells, 43RFa increased the phosphorylation of both Akt (at 15 min) and ERK1/2 (5–30 min, compared with basal time point) (Fig. 4*A* and *B*). 26RFa displayed no effect on Akt, at any time point (Fig. 4*C*), whereas it promoted ERK1/2 increase, from 5 to 60 min compared with basal (Fig. 4*D*). Similar effects were observed in human pancreatic islets, where 43RFa induced Akt and ERK1/2 phosphorylation (both at 5–30 min) (Fig. 4*G* and *H*), whereas 26RFa promoted ERK1/2 (5 and 15 min) (Fig. 4*J*), but not Akt activation (Fig. 4*I*). Accordingly, in both INS-1E and human islets cultured in serum-free medium (Fig. 4*E* and *K*) or in the presence of cytokines (Fig. 4*F* and *L*), preincubation with the inhibitors for either Akt (wortmannin) or ERK1/2 (PD98059) blocked the survival action of 43RFa, whereas that of 26RFa was reduced only by PD98059 and not by wortmannin. These results suggest that both PI3K/Akt and ERK1/2 are involved in the survival effects of 43RFa, whereas for 26RFa, only ERK1/2 is engaged.

43RFa Promotes Glucose-Stimulated Insulin Secretion and Potentiates Ex-4 Insulinotropic Action in β-Cells and Human Islets, Whereas 26RFa Inhibits Insulin Release and Reduces Ex-4 Effects

We next sought to determine 43RFa and 26RFa effects on glucose-stimulated insulin secretion (GSIS) in both INS-1E and human pancreatic islets. The peptides were tested at different glucose concentrations either alone or in combination with Ex-4, which was used as positive

apoptotic nuclei. Values are expressed as percent of apoptotic cells and are the mean ± SE of duplicate determinations (500 cells each) from three independent experiments. *H*: Apoptosis assessed as caspase 3 activation and expressed as percent of control (SF). For both *G* and *H*, **$P < 0.01$; ##$P < 0.01$ vs. SF; $n = 3$. Each peptide was used at 100 nmol/L.

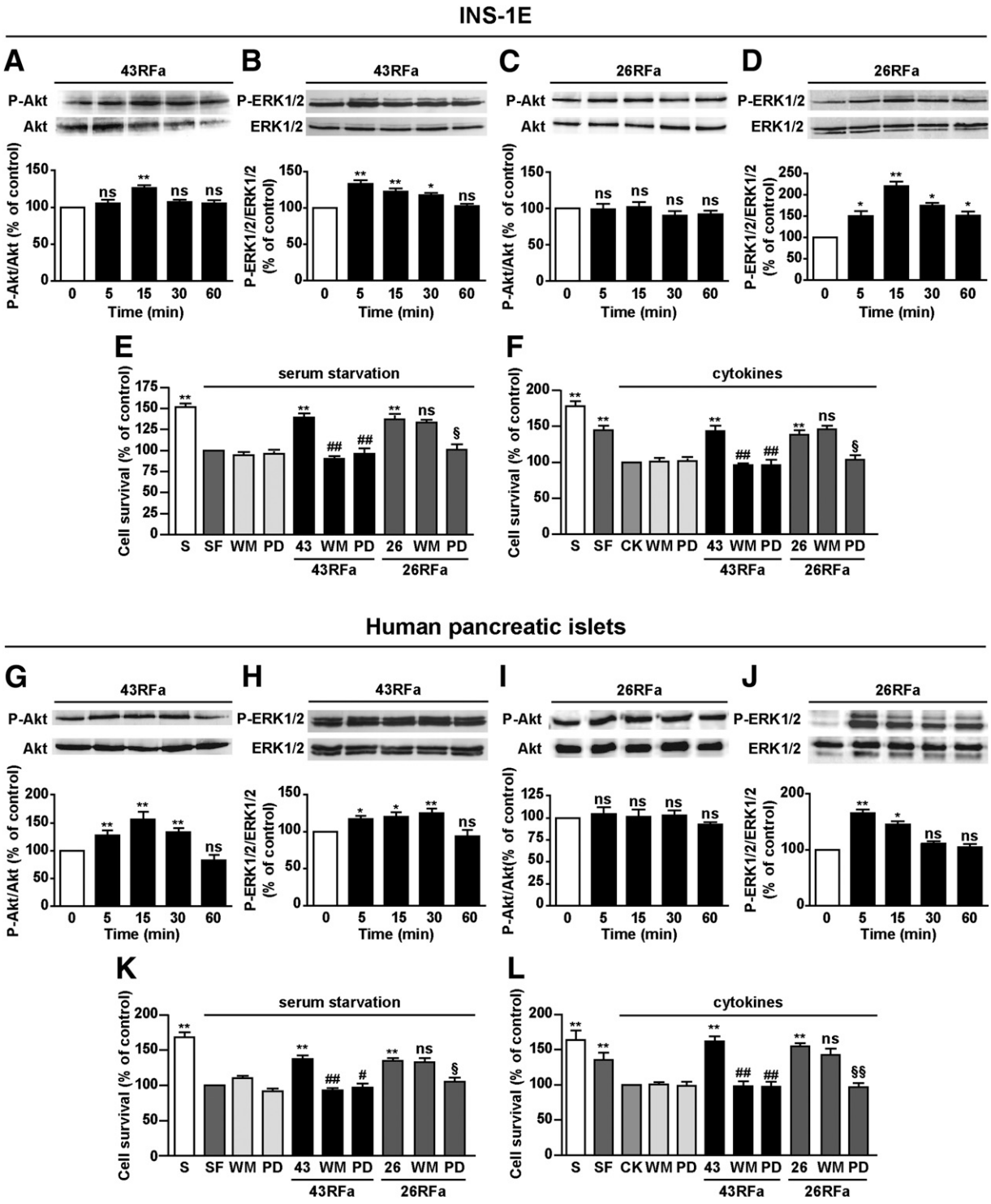

Figure 4—43RFa and 26RFa effects on activation of PI3K/Akt and ERK1/2. Akt and ERK1/2 phosphorylation was assessed by Western blot on whole lysates from INS-1E cells or human pancreatic islets stimulated with either 100 nmol/L 43RFa (*A*, *B*, *G*, and *H*) or 100 nmol/L 26RFa (*C*, *D*, *I*, and *J*) for the indicated times (*top panels*). Equal protein loading was determined by reprobing with antibodies to the respective total proteins (*bottom panels*). Blots are representative of three independent experiments. Graphs show the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as percent of basal (*$P < 0.05$ and **$P < 0.01$; ns, not significant). Cell survival assessed by MTT in INS-1E cells that were starved for 12 h and then incubated for a further 24 h in the absence or presence of either 43RFa (43) or 26RFa (26) (100 nmol/L each), and either without serum-free medium (SF) (*E*) or with TNF-α/IFN-γ/IL-1β (cytokines, CK) (100, 50, and 5 ng/mL, respectively) (*F*), and wortmannin (WM) (100 nmol/L) or PD98059 (40 μmol/L). Inhibitors were added 30 and 60 min before the peptides and cytokines, respectively. **$P < 0.01$ vs. SF (*E*) or vs. CK (*F*); ##$P < 0.01$ vs. 43; §$P < 0.05$ vs. 26; ns, not significant; $n = 3$. Cell survival assessed by MTT in human pancreatic islets incubated for 72 h in the presence of serum or in serum-free medium (SF), either alone (*K*) or with IFN-γ/TNF-α/IL-1β (CK) (5 ng/mL each) (*L*), and 43RFa or 26RFa (100 nmol/L each). Inhibitors were added as for *E* and *F*. **$P < 0.01$ vs. SF (*K*) or vs. CK (*L*); #$P < 0.05$ and ##$P < 0.01$ vs. 43; §$P < 0.05$ and §§$P < 0.01$ vs. 26; ns, not significant; $n = 3$.

control. As expected, Ex-4 increased GSIS at all glucose concentrations, in both β-cells and islets. Similarly, 43RFa increased insulin release at all glucose concentrations in β-cells and islets, to an extent comparable to that of Ex-4. 43RFa even increased basal insulin levels, without glucose stimulation. Moreover, Ex-4– and 43RFa-induced insulin secretion was further potentiated when Ex-4 and 43RFa were given together, at 15 and 25 mmol/L glucose in INS-1E cells, and at 7.5, 15, and 25 mmol/L glucose in human islets (Fig. 5*A* and *B*). On the other hand, 26RFa strongly inhibited both basal and GSIS and dramatically reduced Ex-4 insulinotropic effects at all glucose concentrations, in either INS-1E β-cells or human islets (Fig. 5*A* and *B*).

To investigate the signaling pathways involved in the insulinotropic and insulinostatic effects of 43RFa and 26RFa, respectively, INS-1E β-cells and human islet cells were preincubated with either PTX, an inhibitor of $G\alpha_{i/o}$-coupled receptor (31), or NF449, a selective $G\alpha_s$ protein–coupled receptor antagonist (32). 43RFa-induced increase of insulin secretion was unaffected by pretreatment with PTX, whereas it was blocked by NF449, in both β-cells and islets. Conversely, PTX restored up to control levels GSIS, which was inhibited by 26RFa, whereas NF449 had no effect on the insulinostatic action of the peptide (Fig. 5*C–F*). These results suggest that the opposite effects of 43RFa and 26RFa on insulin secretion involve signaling through $G\alpha_s$ and $G\alpha_{i/o}$, respectively. Furthermore, the role of GPR103 in 43RFa and 26RFa effects on GSIS was determined by transfecting INS-1E β-cells with siRNA targeted to GPR103. As assessed by RT-PCR, at 24 after transfection, GPR103 mRNA expression was strongly reduced by siGPR103, compared with scrambled (control) siRNA (Fig. 5*G*). The insulinotropic effect of 43RFa, assessed at both 0 and 7.5 mmol/L glucose, was completely blocked in siGPR103 cells, as compared with siControl, whereas 26RFa-induced inhibition of GSIS was unchanged in siGPR103-transfected cells (Fig. 5*H*). These findings suggest that 43RFa promotes insulin secretion through binding to GPR103, whereas 26RFa inhibits insulin release likely through a different receptor.

43RFa, but Not 26RFa, Promotes cAMP Increase and Glucose Uptake in β-Cells and Human Pancreatic Islets

cAMP elevation is linked to increased insulin secretion in pancreatic β-cells and protection from apoptosis (33,34). Therefore, we investigated the effects of 43RFa and 26RFa on cAMP levels in both INS-1E β-cells and human pancreatic islets. In INS-1E β-cells, 43RFa elevated intracellular cAMP at 5–30 min with respect to basal (time 0), but not at 60 min, where cAMP returned to basal levels (Fig. 6*A*). Conversely, 26RFa reduced cAMP levels, at the same time points (Fig. 6*B*). Similarly, in human pancreatic islets, cAMP was increased by 43RFa at 15 and 30 min and inhibited by 26RFa, at 5–30 min (Fig. 6*C* and *D*). The cAMP-increasing agent forskolin was used as positive control.

Glucose uptake by pancreatic β-cells, through the glucose transporter GLUT2 and glucokinase activity, is essential for the normal insulin secretory response to hyperglycemia (35,36). To further ascertain the stimulatory and inhibitory roles of 43RFa and 26RFa, respectively, on GSIS, glucose uptake was determined in INS-1E β-cells and human pancreatic islets. In INS-1E β-cells, at 10 min incubation, 43RFa increased glucose 2-[^{3}H]-deoxyglucose uptake at 10 nmol/L and showed a remarkable effect at 100 nmol/L, similarly to Ex-4 and to the cAMP analog 8-Br-cAMP. At 5 min, 43RFa had no effect at 10 nmol/L but stimulated glucose uptake at 100 nmol/L. Conversely, 26RFa had no effect at any concentration and incubation time tested (Fig. 6*E*). Similarly, in human pancreatic islets that were tested for 10 min with 100 nmol/L of each peptide, 43RFa, but not 26RFa, increased 2-deoxyglucose uptake (Fig. 6*F*).

DISCUSSION

The current study shows that the RFamide peptides 43RFa and 26RFa both display survival and antiapoptotic effects in pancreatic β-cells and human pancreatic islets. Furthermore, 43RFa stimulated, whereas 26RFa inhibited, the insulin response to glucose and Ex-4 in both β-cells and human pancreatic islets. These effects involved activation of distinct G proteins and different regulation of cAMP levels and glucose uptake.

In mammals, the mRNAs encoding for the 43RFa and 26RFa precursor (QRFP) and for its receptor GPR103 are mainly expressed in the brain, particularly in the hypothalamus, and in different peripheral organs (6,9). QRFP mRNA has been found expressed in mouse and rat pancreas, but not in human pancreas (37). GPR103 was reported to be absent in human pancreas and expressed at a very low level in mouse pancreas (6,7,37). Here we show QRFP and GPR103 expression in both rat INS-1E β-cells and human pancreatic islets, either at mRNA or protein level. Interestingly, in human islets, GPR103 protein colocalized with insulin, suggesting direct interaction of 43RFa and 26RFa with their receptor in β-cells. Furthermore, QRFP expression in β-cells and human islets may suggest autocrine/paracrine effects of the peptides. Accordingly, 26RFa autocrine/paracrine effects have been recently proposed in prostate cancer cells, where the peptide induced cell migration and neuroendocrine differentiation, and in macrophages and adipocytes, where both 43RFa and 26RFa promoted adipogenesis (10,11,22). Future studies will help elucidate whether autocrine/paracrine mechanisms may also occur in pancreatic islets.

We firstly report here the survival and antiapoptotic effects of 43RFa and 26RFa. Pancreatic β-cells and human pancreatic islets were chosen as in vitro models because, besides the survival actions, we were interested in the metabolic effects of these peptides. Indeed, a role in adipogenesis, lipid metabolism, and inflammation, as well as an association between GPR103 and QRFP expression and obesity, has been recently described (10,11). Moreover, as

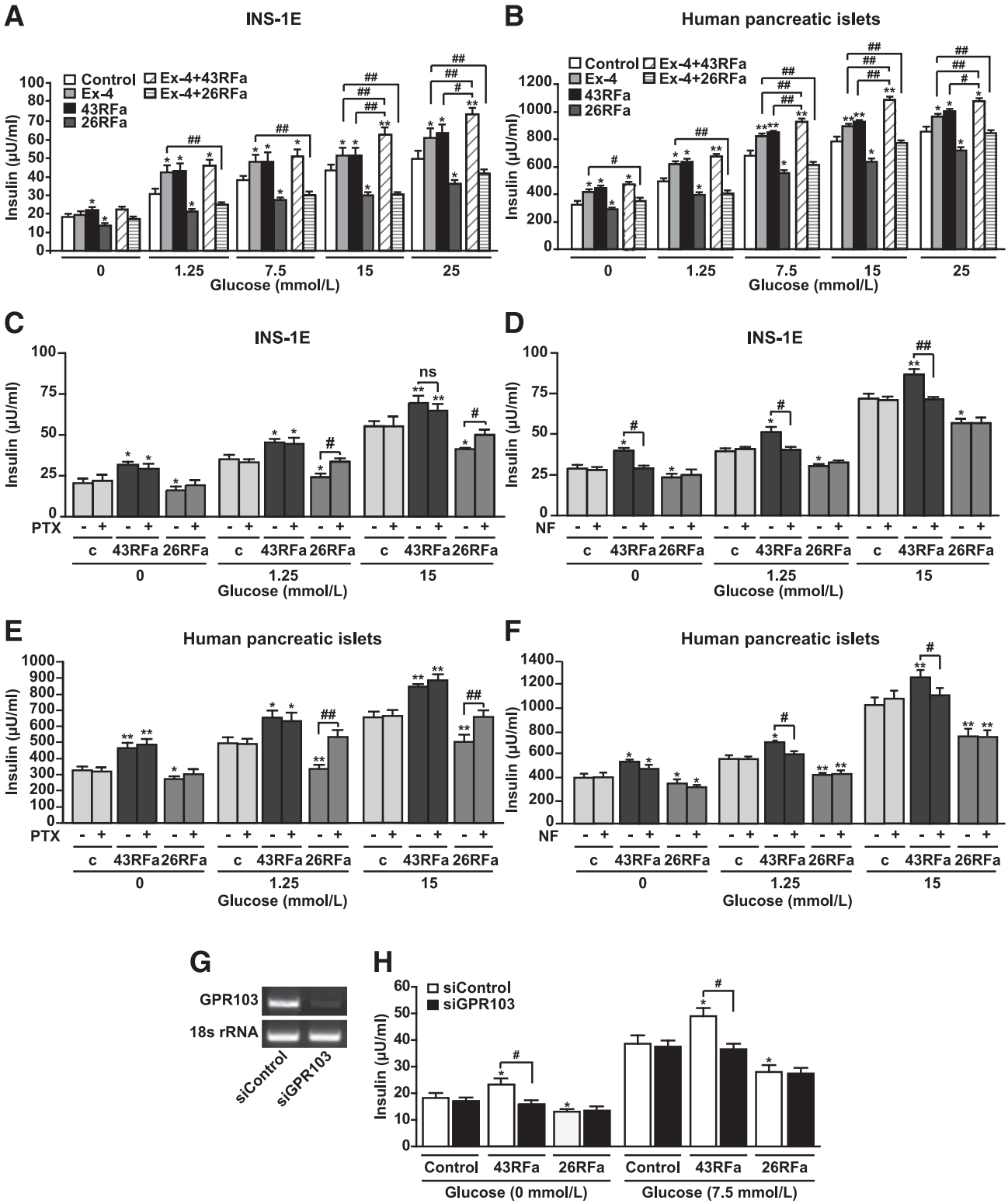

Figure 5—43RFa and 26RFa regulate insulin secretion in INS-1E β-cells and human pancreatic islets. Insulin release was assessed by radioimmunoassay in conditioned medium from INS-1E β-cells or islets, incubated alone or with 2 mmol/L glucose for 1 h and then for a further 1 h with the indicated concentrations of glucose, in the presence or absence of either 43RFa, 26RFa, Ex-4, or a combination of Ex-4/43RFa or Ex-4/26RFa (100 nmol/L each). *A*: Insulin secretion in INS-1E β-cells. *B*: Insulin secretion in human pancreatic islets. For *A* and *B*, values are the means ± SE of triplicate determinations from at least three independent experiments (*$P < 0.05$ and **$P < 0.01$ vs. control at each glucose concentration; #$P < 0.05$ and ##$P < 0.01$). Insulin secretion in INS-1E cells (*C* and *D*) and human pancreatic islets (*E* and *F*) in either absence or presence of PTX (50 ng/mL) or NF449 (10 μmol/L). *$P < 0.05$ and **$P < 0.01$ vs. control (c) at each glucose concentration; #$P < 0.05$ and ##$P < 0.01$; ns, not significant. *G*: GPR103 mRNA assessed by RT-PCR in INS-1E β-cells after 24 h transfection with either nonsilencing siRNA (siControl) or siRNA to GPR103 (siGPR103). As expected, the amplified products corresponded

for QRFP, other orexigenic peptides such as ghrelin exert antiapoptotic and metabolic effects in different cell types, including pancreatic β-cells and human islets (24,38,39). Here, both 43RFa and 26RFa increased survival and inhibited apoptosis of INS-1E β-cells and human pancreatic islets. They also increased proliferation of INS-1E β-cells, which was not investigated in human islets due to the low proliferative rate of human β-cells in vitro (40,41). Besides serum starvation, which induces β-cell death (24,42), β-cells and human islets were challenged with diabetogenic stimuli, such as cytokine synergism and glucolipotoxicity, which are major causes of β-cell dysfunction and death in both type 1 and 2 diabetes (1–4). Interestingly, 43RFa and 26RFa both displayed proliferative, survival, and antiapoptotic actions, comparable to those of the long-acting GLP-1 agonist Ex-4 (27).

Phosphorylation of either PI3K/Akt or ERK1/2 has been shown to mediate β-cell proliferation and survival in response to different external signals, including incretin hormones (24,27,30,43). Here, 43RFa and 26RFa, although displaying similar survival effect in β-cells and human islets, differently regulated these signaling pathways. In fact, whereas 43RFa increased the phosphorylation of both PI3K/Akt and ERK1/2, 26RFa increased ERK1/2, but not PI3K/Akt. Accordingly, inhibition of PI3K and ERK1/2 phosphorylation with wortmannin and PD98059, respectively, blocked 43RFa-induced survival, whereas only ERK1/2 inhibitor decreased 26RFa-induced survival. These results indicate that ERK1/2, and not PI3K/Akt, is required for 26RFa survival effect, whereas 43RFa uses both pathways. Notably, a cross-talk between ERK1/2 and cAMP in pancreatic β-cell survival has been previously described (29). However, here only 43RFa elevated cAMP levels, suggesting that 26RFa-induced activation of ERK1/2 is independent of the cAMP pathway.

To further understand the effects of 43RFa and 26RFa in β-cells, we next determined their role on insulin secretion. To our knowledge, 43RFa effect on insulin secretion has never been studied. Instead, 26RFa was recently shown to inhibit glucose- as well as arginine- and Ex-4–induced insulin secretion in perfused rat pancreas (23). Here, we firstly show that 43RFa promoted insulin secretion in INS-1E β-cells and human islets, both in basal conditions and in the presence of either different glucose concentrations or Ex-4. Conversely, 26RFa showed opposite effects and inhibited both glucose- and Ex-4–induced insulin secretion, in agreement with the previous findings (23). 26RFa was used here at 100 nmol/L, which was also the best concentration to promote survival and prevent apoptosis. Conversely, 10 nmol/L was the concentration used in the study by Egido et al. (23), where the experiments were performed in perfused rat pancreas, at variance with our in vitro models.

Interestingly, either 43RFa or Ex-4 alone showed comparable insulinotropic action in both β-cells and human islets. Moreover, insulin secretion was markedly increased when 43RFa and Ex-4 were administered together, particularly at the highest concentrations of glucose. On the other hand, besides exerting inhibitory action per se, 26RFa hampered Ex-4 insulinotropic effect. These results imply that 43RFa may either potentiate Ex-4 action or act synergistically with Ex-4 to increase insulin secretion, whereas 26RFa behaves in an opposite manner. They also suggest clinical implications in diabetes, as 43RFa may become a therapeutic target for enhancing β-cell function, alone or in combination with incretin hormones, whereas inhibition of 26RFa effect may prevent loss of β-cell competence.

Activation of G-protein–coupled receptors by metabolites and hormones can modulate glucose-induced insulin secretion through different pathways. $G\alpha_s$-coupled receptors increase insulin secretion through stimulation of adenylyl cyclase and activation of protein kinase A (PKA) by cAMP. In turn, $G\alpha_{i/o}$-coupled receptors reduce insulin release by inhibition of adenylyl cyclase and cAMP formation and also through inhibition of voltage-dependent Ca^{2+} channels involving the βγ subunits of $G\alpha_{i/o}$ (27,44). We found here that the $G\alpha_s$ antagonist NF449 blocked the insulinotropic effect of 43RFa, but not the insulinostatic action of 26RFa. Conversely, the $G\alpha_{i/o}$ inhibitor PTX did not affect 43RFa-induced insulin release but blocked the inhibitory effect of 26RFa, in both β-cells and human islets. This in agreement with the findings by Egido et al. (23), except for the fact that here PTX was used in combination with glucose only, and not with Ex-4.

Of note, PTX alone had no effect here on GSIS, whereas with 26RFa, insulin levels were found increased. This is likely because glucose alone, in the absence of G-protein–coupled receptor ligands, causes inhibition of ATP-regulated K^+ channels, opening of voltage-dependent Ca^{2+} channels, and increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), which triggers exocytosis of insulin-containing vesicles (45). Conversely, in the presence of 26RFa, PTX may restore cAMP levels, which are blunted by the peptide, as well as ($[Ca^{2+}]i$), and may thus contribute to increased insulin release.

Our results indicate that the opposite effects of 43RFa and 26RFa on insulin secretion are due to activation of distinct pathways. In fact, 43RFa promotes insulin secretion, at least in part, through $G\alpha_s$ and adenylyl cyclase/cAMP increase, whereas 26RFa inhibits insulin

to 318 bp for rat GPR103 and 199 bp for 18s rRNA, which was used as internal control. *H*: Insulin secretion in siControl- or siGPR103-transfected INS-1E β-cells, incubated for 1 h with the indicated concentrations of glucose, and with 43RFa or 26RFa (100 nmol/L each). Values are the means ± SE of triplicate determinations from at least three independent experiments (*$P < 0.05$ vs. control at each glucose concentration; #$P < 0.05$).

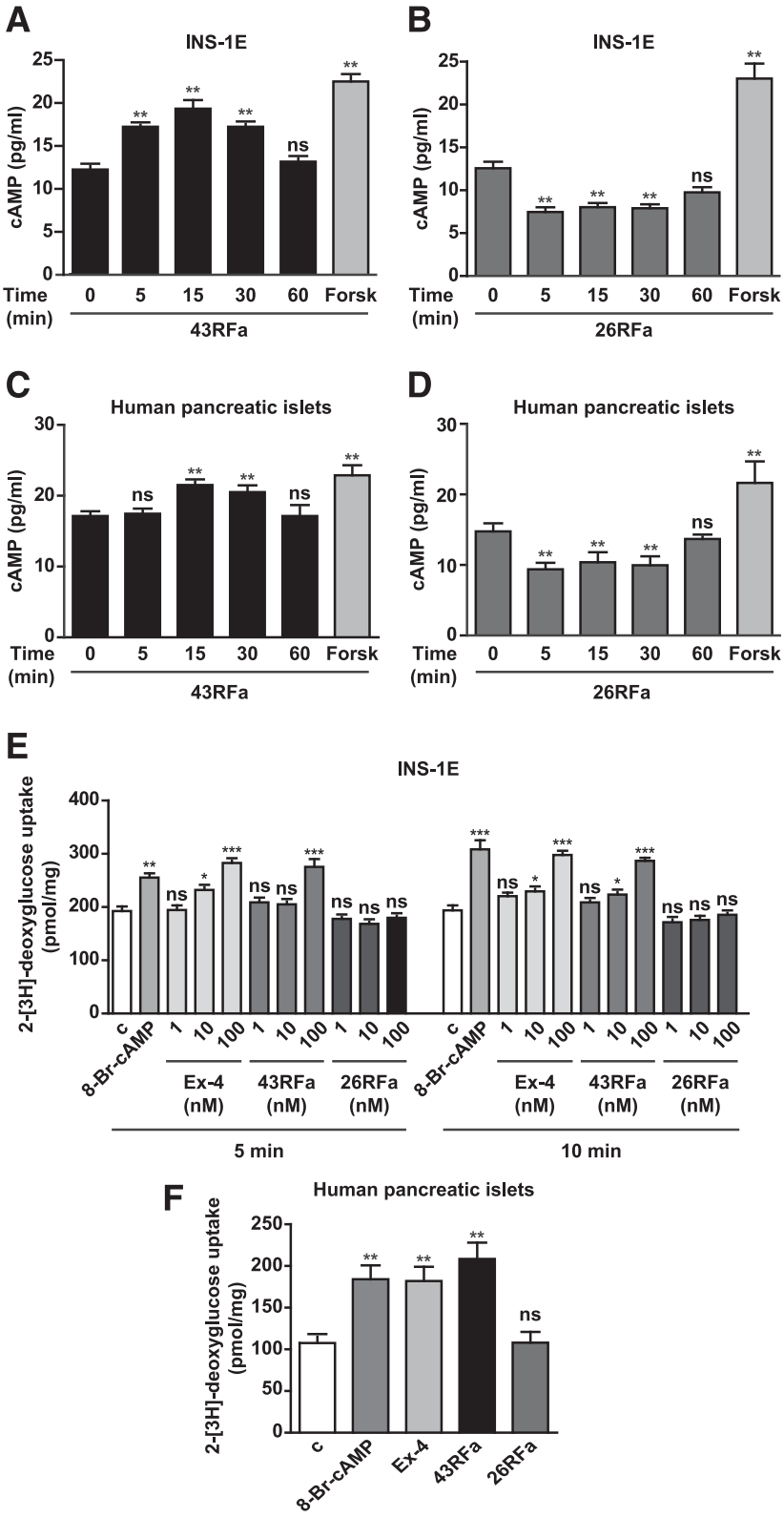

Figure 6—43RFa and 26RFa effects on intracellular cAMP and glucose uptake. *A–D*: Serum-starved INS-1E cells and human islets were cultured for the indicated times with either 100 nmol/L 43RFa or 100 nmol/L 26RFa, in the presence of the phosphodiesterase inhibitor IBMX (100 μmol/L), which was added 30 min before stimulation. Forskolin (Forsk) (50 μmol/L for 1 min) was used as positive control. Results are the mean ± SE of three independent experiments performed in triplicate (**$P < 0.005$ vs. basal time point; ns, not significant). *E*: 2-[^{3}H]-deoxyglucose uptake assessed in serum-starved INS-1E cells cultured for 5 or 10 min with either Ex-4, 43RFa, or 26RFa, at the indicated concentrations. *F*: 2-[^{3}H]-deoxyglucose uptake in human pancreatic islets cultured for 10 min with the peptides that were used at 100 nmol/L each. 8-Br-cAMP was used at 1 mmol/L. For *E* and *F*, $n = 3$; *$P < 0.05$, **$P < 0.005$, and ***$P < 0.001$; ns, not significant vs. control.

release through $G\alpha_{i/o}$ and inhibition of cAMP formation. This assumption is further sustained by our results showing that 43RFa increased, whereas 26RFa inhibited, intracellular cAMP levels in both INS-1E β-cells and human pancreatic islets. At variance with these findings, 43RFa has been recently found to decrease forskolin-induced cAMP in human adrenocortical cells, likely through G_q-mediated mechanisms (20). 26RFa, instead, in line with our results, was shown to decrease cAMP production in CHO- or human embryonic kidney (HEK293)–GPR103 transfected cells, suggesting GPR103 coupling to $G_{i/o}$ and G_q (7). In addition, we found here that similarly to the cAMP analog 8-Br-cAMP and to Ex-4, 43RFa, but not 26RFa, increased glucose uptake, which is required in the initial step of GSIS by pancreatic β-cells and has been shown to be impaired in both type 1 and type 2 diabetes (35,46). Collectively, these findings further support the positive role of 43RFa on β-cell function, which is opposed to that of 26RFa.

An open question during the study was why two peptides that likely bind the same receptor exert opposite effects on insulin secretion. To solve this issue, we knocked down GPR103 expression in INS-1E β-cells using specific siRNA and found complete inhibition of 43RFa insulinotropic action but no effect on 26RFa-induced insulin inhibition. This finding suggests that in β-cells, only 43RFa signals through GPR103, whereas 26RFa interacts with a different receptor. Accordingly, 26RFa was found to display moderate affinity and selectivity for NPFF2, the receptor of neuropeptide FF, another member of the RFamide family (47). Moreover, NPFF and prolactin-releasing peptide (PrRP), both with high affinity for NPFF2 receptor (9), displaced [^{125}I]-26RFa binding in brain regions enriched with NPFF2 receptor (48). Interestingly, NPFF2 receptor is coupled to $G_{i/o}$ protein, in agreement with our finding showing loss of 26RFa insulinostatic effect in β-cells and human islets treated with PTX.

In conclusion, this study shows a new role for 43RFa and 26RFa in promoting β-cell and human islet cell survival, while exerting opposite effects on insulin secretion, via either $G\alpha_s$/cAMP elevation or $G_{i/o}$/cAMP inhibition, respectively. The effects on insulin secretion require GPR103 binding for 43RFa but not for 26RFa. These findings provide new perspectives and comprehension on the role of these peptides, whose functions, particularly at the peripheral level, are still quite unknown. In addition, 43RFa and 26RFa may become novel therapeutic targets for increasing β-cell mass and function in metabolic diseases such as diabetes and obesity.

Acknowledgments. The authors are grateful to Eleonora Gargantini, Jessica Giorcelli, Cristina Grande, Ida Rapa, and Marina Taliano (all from the University of Torino) for their technical support and to the Neuroscience Institute of Turin and the Italian Group of Neuroendocrine Sciences.

Funding. This study was supported by the University of Torino (Ex-60% 2008 to R.G.); by the Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica e Tecnologica (Italian Ministry of Instruction and Research [MIUR: PRIN 2008EFHJ5H_02 to R.G., PRIN 2010B5B2NL to E.G.]); by Compagnia di San Paolo (2011 to R.G. and 2007 to E.G.); and by the Studio delle Malattie Endocrino Metaboliche Foundation (Turin, Italy). Human islets were provided through Juvenile Diabetes Research Foundation Award 31-2008-416 (ECIT, Islet for Research Program).

Duality of Interest. This study was supported by Sanofi S.p.A., Italy. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. R.G. contributed to study design and interpretation and wrote the manuscript. F.S., L.T., D.G., I.G., and L.B. researched data and reviewed and edited the manuscript. R.N. and L.P. provided the human islets and reviewed and edited the manuscript. M.P.G. and G.A. researched data on Ca^{2+} transients and reviewed and edited the manuscript. M.V. and M.P. contributed with the immunohistochemical studies and reviewed and edited the manuscript. J.L., H.V., H.O., and E.G. critically revised the manuscript and helped with the discussion. R.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Muoio DM, Newgard CB. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat Rev Mol Cell Biol 2008;9:193–205

2. Eizirik DL, Colli ML, Ortis F. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. Nat Rev Endocrinol 2009;5:219–226

3. Poitout V, Robertson RP. Glucolipotoxicity: fuel excess and beta-cell dysfunction. Endocr Rev 2008;29:351–366

4. Donath MY, Størling J, Berchtold LA, Billestrup N, Mandrup-Poulsen T. Cytokines and beta-cell biology: from concept to clinical translation. Endocr Rev 2008;29:334–350

5. Chartrel N, Dujardin C, Anouar Y, et al. Identification of 26RFa, a hypothalamic neuropeptide of the RFamide peptide family with orexigenic activity. Proc Natl Acad Sci U S A 2003;100:15247–15252

6. Jiang Y, Luo L, Gustafson EL, et al. Identification and characterization of a novel RF-amide peptide ligand for orphan G-protein-coupled receptor SP9155. J Biol Chem 2003;278:27652–27657

7. Fukusumi S, Yoshida H, Fujii R, et al. A new peptidic ligand and its receptor regulating adrenal function in rats. J Biol Chem 2003;278:46387–46395

8. Bruzzone F, Lectez B, Tollemer H, et al. Anatomical distribution and biochemical characterization of the novel RFamide peptide 26RFa in the human hypothalamus and spinal cord. J Neurochem 2006;99:616–627

9. Chartrel N, Alonzeau J, Alexandre D, et al. The RFamide neuropeptide 26RFa and its role in the control of neuroendocrine functions. Front Neuroendocrinol 2011;32:387–397

10. Mulumba M, Jossart C, Granata R, et al. GPR103b functions in the peripheral regulation of adipogenesis. Mol Endocrinol 2010;24:1615–1625

11. Jossart C, Mulumba M, Granata R, et al. Pyroglutamylated RF-amide peptide (QRFP) gene is regulated by metabolic endotoxemia. Mol Endocrinol 2014;28:65–79

12. Takayasu S, Sakurai T, Iwasaki S, et al. A neuropeptide ligand of the G protein-coupled receptor GPR103 regulates feeding, behavioral arousal, and blood pressure in mice. Proc Natl Acad Sci U S A 2006;103:7438–7443

13. Moriya R, Sano H, Umeda T, et al. RFamide peptide QRFP43 causes obesity with hyperphagia and reduced thermogenesis in mice. Endocrinology 2006;147:2916–2922

14. Lectez B, Jeandel L, El-Yamani FZ, et al. The orexigenic activity of the hypothalamic neuropeptide 26RFa is mediated by the neuropeptide Y and proopiomelanocortin neurons of the arcuate nucleus. Endocrinology 2009;150:2342–2350

15. Beck B, Richy S. Suppression of QRFP 43 in the hypothalamic ventromedial nucleus of Long-Evans rats fed a high-fat diet. Biochem Biophys Res Commun 2009;383:78–82

16. Kampe J, Wiedmer P, Pfluger PT, et al. Effect of central administration of QRFP(26) peptide on energy balance and characterization of a second QRFP receptor in rat. Brain Res 2006;1119:133–149
17. Ukena K, Tachibana T, Iwakoshi-Ukena E, et al. Identification, localization, and function of a novel avian hypothalamic neuropeptide, 26RFa, and its cognate receptor, G protein-coupled receptor-103. Endocrinology 2010;151: 2255–2264
18. Navarro VM, Fernández-Fernández R, Nogueiras R, et al. Novel role of 26RFa, a hypothalamic RFamide orexigenic peptide, as putative regulator of the gonadotropic axis. J Physiol 2006;573:237–249
19. Patel SR, Murphy KG, Thompson EL, et al. Pyroglutamylated RFamide peptide 43 stimulates the hypothalamic-pituitary-gonadal axis via gonadotropin-releasing hormone in rats. Endocrinology 2008;149:4747–4754
20. Ramanjaneya M, Karteris E, Chen J, et al. QRFP induces aldosterone production via PKC and T-type calcium channel-mediated pathways in human adrenocortical cells: evidence for a novel role of GPR103. Am J Physiol Endocrinol Metab 2013;305:E1049–E1058
21. Baribault H, Danao J, Gupte J, et al. The G-protein-coupled receptor GPR103 regulates bone formation. Mol Cell Biol 2006;26:709–717
22. Alonzeau J, Alexandre D, Jeandel L, et al. The neuropeptide 26RFa is expressed in human prostate cancer and stimulates the neuroendocrine differentiation and the migration of androgeno-independent prostate cancer cells. Eur J Cancer 2013;49:511–519
23. Egido EM, Hernández R, Leprince J, et al. 26RFa, a novel orexigenic neuropeptide, inhibits insulin secretion in the rat pancreas. Peptides 2007;28:725–730
24. Granata R, Settanni F, Biancone L, et al. Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: involvement of 3′,5′-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidyl inositol 3-Kinase/Akt signaling. Endocrinology 2007;148:512–529
25. Granata R, Settanni F, Gallo D, et al. Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. Diabetes 2008;57:967–979
26. Granata R, Gallo D, Luque RM, et al. Obestatin regulates adipocyte function and protects against diet-induced insulin resistance and inflammation. FASEB J 2012;26:3393–3411
27. Campbell JE, Drucker DJ. Pharmacology, physiology, and mechanisms of incretin hormone action. Cell Metab 2013;17:819–837
28. Park S, Dong X, Fisher TL, et al. Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function. J Biol Chem 2006;281:1159–1168
29. Costes S, Broca C, Bertrand G, et al. ERK1/2 control phosphorylation and protein level of cAMP-responsive element-binding protein: a key role in glucose-mediated pancreatic beta-cell survival. Diabetes 2006;55:2220–2230
30. Størling J, Juntti-Berggren L, Olivecrona G, Prause MC, Berggren PO, Mandrup-Poulsen T. Apolipoprotein CIII reduces proinflammatory cytokine-induced apoptosis in rat pancreatic islets via the Akt prosurvival pathway. Endocrinology 2011;152:3040–3048
31. Bokoch GM, Katada T, Northup JK, Hewlett EL, Gilman AG. Identification of the predominant substrate for ADP-ribosylation by islet activating protein. J Biol Chem 1983;258:2072–2075
32. Hohenegger M, Waldhoer M, Beindl W, et al. Gsalpha-selective G protein antagonists. Proc Natl Acad Sci U S A 1998;95:346–351
33. Dyachok O, Isakov Y, Sågetorp J, Tengholm A. Oscillations of cyclic AMP in hormone-stimulated insulin-secreting beta-cells. Nature 2006;439: 349–352
34. Kwon G, Pappan KL, Marshall CA, Schaffer JE, McDaniel ML. cAMP dose-dependently prevents palmitate-induced apoptosis by both protein kinase A- and cAMP-guanine nucleotide exchange factor-dependent pathways in beta-cells. J Biol Chem 2004;279:8938–8945
35. Unger RH. Diabetic hyperglycemia: link to impaired glucose transport in pancreatic beta cells. Science 1991;251:1200–1205
36. Park JH, Kim SJ, Park SH, et al. Glucagon-like peptide-1 enhances glucokinase activity in pancreatic β-cells through the association of Epac2 with Rim2 and Rab3A. Endocrinology 2012;153:574–582
37. Zhang Q, Qiu P, Arreaza MG, et al. P518/Qrfp sequence polymorphisms in SAMP6 osteopenic mouse. Genomics 2007;90:629–635
38. Irako T, Akamizu T, Hosoda H, et al. Ghrelin prevents development of diabetes at adult age in streptozotocin-treated newborn rats. Diabetologia 2006;49: 1264–1273
39. Kim MS, Yoon CY, Jang PG, et al. The mitogenic and antiapoptotic actions of ghrelin in 3T3-L1 adipocytes. Mol Endocrinol 2004;18:2291–2301
40. Rutti S, Sauter NS, Bouzakri K, Prazak R, Halban PA, Donath MY. In vitro proliferation of adult human beta-cells. PLoS ONE 2012;7:e35801
41. Thorens B. The required beta cell research for improving treatment of type 2 diabetes. J Intern Med 2013;274:203–214
42. Maestre I, Jordán J, Calvo S, et al. Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the beta-cell line INS-1. Endocrinology 2003;144:335–345
43. Youl E, Bardy G, Magous R, et al. Quercetin potentiates insulin secretion and protects INS-1 pancreatic β-cells against oxidative damage via the ERK1/2 pathway. Br J Pharmacol 2010;161:799–814
44. Blad CC, Tang C, Offermanns S. G protein-coupled receptors for energy metabolites as new therapeutic targets. Nat Rev Drug Discov 2012;11:603–619
45. Fu Z, Gilbert ER, Liu D. Regulation of insulin synthesis and secretion and pancreatic beta-cell dysfunction in diabetes. Curr Diabetes Rev 2013;9:25–53
46. Guillam MT, Dupraz P, Thorens B. Glucose uptake, utilization, and signaling in GLUT2-null islets. Diabetes 2000;49:1485–1491
47. Gouardères C, Mazarguil H, Mollereau C, et al. Functional differences between NPFF1 and NPFF2 receptor coupling: high intrinsic activities of RFamide-related peptides on stimulation of [35S]GTPgammaS binding. Neuropharmacology 2007;52:376–386
48. Bruzzone F, Lectez B, Alexandre D, et al. Distribution of 26RFa binding sites and GPR103 mRNA in the central nervous system of the rat. J Comp Neurol 2007;503:573–591