Endothelial Differentiation Gene Receptors in Pancreatic Islets and INS-1 Cells

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The endothelial differentiation gene (EDG) receptors are a class of G protein–coupled receptors. EDG-1, -3, -5, -6, and -8 bind the bioactive lipid sphingosine-1-phosphate (SPP) as the primary signaling ligand. EDG-2, -4, and -7 bind the ligand lysophosphatidic acid. EDG-1, -2, -3, -5, -6, and -7, but not -8, mRNAs were expressed in isolated rat pancreatic islets, whereas INS-1 insulinoma cells expressed only EDG-1, -2, -3, and -5 mRNAs. EDG-4 mRNA was expressed in mouse islets. EDG-1 mRNA but not EDG-3 mRNA was rapidly induced relative to 18S rRNA after stimulation of isolated islets with phorbol 12-myristate 13-acetate (PMA) or cholecystokinin-8S for 2 h. The protein kinase C inhibitor GF 109203X blocked the EDG-1 induction by PMA. Similarly, in islets stimulated for 2 h with 17 mmol/l glucose, the relative EDG-1 mRNA levels increased almost twofold compared with levels in control islets at 5.5 mmol/l glucose. In contrast, after 11 mmol/l glucose stimulation for 7 days, the relative levels of rat islet EDG-1 mRNA were significantly reduced to 54% below that of islets cultured at 5.5 mmol/l glucose. There was no change in relative EDG-3 mRNA levels. Stimulation of EDG receptors in islets and INS-1 cells with SPP inhibited glucagon-like peptide 1 (GLP-1)-stimulated cAMP production and insulin secretion in a concentration-dependent manner. Pertussis toxin antagonized the SPP effects on insulin release. Thus, EDG receptors are expressed in pancreatic islet β-cells and G_i seems to mediate the inhibition by SPP of adenylyl cyclase and cAMP formation and inhibition of the stimulation of insulin secretion by GLP-1. *Diabetes* 52:1986–1993, 2003

Phospholipids and their metabolites, including arachidonic acid, eicosanoids, inositol phosphates, and diacylglycerol, play important roles in β-cell function and insulin secretion (1,2). A unique bioactive phospholipid, spingosine-1-phosphate (SPP), is a metabolite of sphingosine and has been described as a potent stimulus for DNA synthesis, Ca^{2+} mobilization, and mitogen-activated protein kinase pathway activation (3,4). SPP can act both as an intracellular second messenger and as a receptor ligand. The endothelial differentiation gene (EDG) receptor class includes the EDG-1 family of receptor isoforms (EDG-1, -3, -5, -6, and -8) that bind SPP with high affinity and specificity (3–5). Another family of EDG receptors (EDG-2, -4, and -7) recognizes lysophosphatidic acid as the signaling ligand (6).

EDG receptors are G protein–coupled cell surface receptors that are differentially expressed in various tissues (7,8). EDG-coupled G proteins regulate diverse signal transduction pathways and elicit pleiotropic responses unique to the cell type and relative expression of EDG receptors. SPP responses mediated through EDG receptors include platelet activation (9), inhibition of cell motility (10), activation of G_i in myocytes (11), and neurite retraction and cell rounding (12,13). EDG-1 has been characterized as an immediate-early gene (7,14) that inhibits adenylyl cyclase (5) and activates extracellular signal–regulated kinase (ERK) (8) through G_i. The importance of cAMP as a second messenger in β-cells that potentiates glucose-stimulated insulin secretion (1) underscores the importance of understanding the role of EDG receptor activation. EDG-3 and -5 are coupled predominantly to G_q and activate phospholipase C to induce Ca^{2+} mobilization through the production of inositol 1,4,5-trisphosphate (15–17) and induce activation of ERK, stress-activated protein kinase (SAPK)/c-Jun NH_2-terminal kinase (JNK), and p38 mitogen-activated protein (18). EDG-1, -3, -5, -6, and -8 are widely expressed especially in tissues of the cardiovascular and nervous systems. EDG-3 has been identified in pancreas (4). However, EDG receptors have not been identified previously in rodent pancreatic islets or β-cells. SPP also has intracellular sites of action. SPP mobilizes intracellular Ca^{2+} from internal stores independent of inositol trisphosphate generation and activates ERK but inhibits SAPK/JNK (4). Given the important signal transduction pathways that SPP potentially impacts and their recognized roles in insulin secretion (1), characterizing SPP actions in the β-cell is imperative. This study was undertaken to test the hypothesis that EDG receptors are expressed in the islet β-cell and play a potentially important role(s) in the regulation of insulin secretion.

RESEARCH DESIGN AND METHODS

Materials. Collagenase was obtained from Serva (Crescent Chemical Co., Islandia, NY). CMRL-1066 and RPMI-1640 culture media, *Taq* DNA polymerase, random hexamer, Superscript II RNase H^-, glycogen, RNaseOUT, DNase 1 amplification grade, deoxynucleotide triphosphates (dNTPs), and TRIzol were from Invitrogen/Life Technologies (Grand Island, NY). QuantumRNA

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dNTP, deoxynucleotide triphosphate; EDG, endothelial differentiation gene; ERK, extracellular signal–regulated kinase; GLP-1, glucagon-like peptide 1; IBMX, 3-Isobutyl-1-methylxanthine; KRBH, Krebs Ringer bicarbonate HEPES; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RIA, radioimmunoassay; SAPK, stress-activated protein kinase; SPP, sphingosine-1-phosphate.

Classic 18S standards (488 bp) were from Ambion (Austin, TX). Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). Rat insulin for radioimmunoassay (RIA) standard was a gift from Eli Lilly & Co. (Indianapolis, IN). [^{125}I]Insulin (porcine) and adenosine 3′,5′-cyclic phosphoric acid 2′-O-succinyl[^{125}I]iodotyrosine methyl ester were obtained from NEN Life Science Products (Boston, MA). 3-Isobutyl-1-methylxanthine (IBMX), BSA fraction V (insulin-free and fatty acid free), glucagon-like peptide (7-36) amide (GLP-1), cholecystokinin fragment 26–33 sulfated amide (CCK-8), and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Antibody to cAMP was a gift from Dr. David L. Garbers (Howard Hughes Medical Institute, Dallas, TX). INS-1 E cells were a gift from Dr. Claes Wollheim (Geneva, Switzerland). All other chemicals were reagent grade.

Isolation of rat and mouse islets and culture of cells and tissue.

Pancreatic islets were isolated from pancreata of adult male Sprague Dawley rats using collagenase, essentially as described previously (19); islets from adult C57BL/6 mice were isolated in a similar manner. All animal procedures were approved by the institutional animal care and use committee. Freshly isolated islets (fresh) were either used immediately or cultured in CMRL-1066 for up to 2 h in the presence or absence of various stimuli or inhibitors as described in the text. Some groups of islets were cultured in CMRL-1066 containing 5.5 mmol/l glucose (basal) or 11 mmol/l glucose for up to 7 days. RNA from fresh islets paired with 7-day cultured islets was extracted immediately after islet isolation. CMRL-1066 medium contained penicillin (100 units/ml), streptomycin (100 μg/ml), and the absence or presence of 10% serum as indicated in the text. Culture conditions were 5% CO_2/95% air and 37°C. INS-1 cells were cultured in RPMI-1640, as described previously (20).

Insulin secretion. Insulin secretion from isolated islets was determined essentially as described previously (19). Isolated islets (10/sample) were either freshly isolated or cultured and then washed in oxygenated Krebs Ringer bicarbonate HEPES (KRBH) incubation buffer (pH 7.4) containing 5.5 mmol/l glucose, 0.01% fatty acid free BSA, and HEPES (16 mmol/l). The islets were preincubated in the KRBH buffer for 60 min in the absence or presence of pertussis toxin (0.5 μg/ml final concentration). Then, the islets were washed once and fresh KRBH buffer was added in the absence (controls) or presence of pertussis toxin and a stimulatory concentration of glucose and other agents as indicated in the text. An aliquot of incubation medium was removed to determine zero-time insulin levels. Islets were then incubated for 60 min at 37°C in a shaking water bath in an atmosphere of O_2/CO_2 (95:5). An aliquot of the incubation buffer was removed to determine insulin release. Insulin was quantified by RIA, and zero-time insulin levels were subtracted from 60-min values.

RNA isolation and RT-PCR analysis. Total RNA was extracted from isolated rat or mouse islets (12–15 islets/sample) or INS-1 cells (0.2×10^6 cells) using TRIzol plus glycogen (40 μg) to facilitate recovery. The RNA pellet was dissolved in 8.5 μl of diethyl pyrocarbonate–treated water, 1 μl of DNase 1, and 1 μl of reaction buffer and allowed to react for 15 min at room temperature, with subsequent addition of 1 μl of 25 mmol/l EDTA and incubation for 15 min at 65°C. Treatment with DNase effectively removed any DNA in the samples because no PCR bands were observed when samples were reverse-transcribed in the absence of reverse transcriptase. cDNA was reverse-transcribed from 540 ± 45 ng of total RNA. Reverse transcription was carried out using a mixture of random hexamer, 5 mmol/l dNTPs, and RNA, which was allowed to react for 5 min at 65°C, with subsequent addition of 50 mmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol, RNaseOUT (recombinant ribonuclease inhibitor), 100 units of Superscript II RNase H^- reverse transcriptase, and first-strand buffer in a final volume of 20 μl. Reactions were incubated for 1 h at 42°C and then heated to 70°C for 15 min. Preliminary studies were conducted to determine the optimal amount of cDNA and number of PCR cycles required to maintain reactions in the exponential phase of the amplification. PCR was carried out using a Hybaid Sprint thermalcycler. Each sample was analyzed by biplex PCR using primers for 18S rRNA (Ambion) and primers specific for EDG receptor isoforms. PCR was carried out in a 25-μl reaction volume containing 0.8 mmol/l dNTPs, 10 pmol each oligonucleotide primer, 2 μl of QuantumRNA Classic 18S rRNA primers (diluted 4–10× depending on sample RNA content), 0.625 units of *Taq* DNA polymerase, 1.5 mmol/l $MgCl_2$ for EDG-1 and -3 primer pairs, and 2 mmol/l $MgCl_2$ for all other EDG primer pairs, as well as PCR buffer. Approximately 0.5 μl cDNA was used per PCR, with the actual amount based on the level of 18S rRNA detected. Amplification conditions after the initial denaturation period of 2 min at 94°C were denatured for 30 s at 94°C, annealing for 30 s at 55°C for EDG-1 and -3, and 60°C for all other EDG amplification reactions, with extension for 45 s at 72°C, and a final extension for 7 min. Typically, 28–32 cycles were sufficient for 18S rRNA and EDG primer exponential amplification. The sequences of primer pairs for rat EDG receptors used in this study and the sizes of the products generated are EDG-1 sense 5′-caggtaccaaccccatcatc-3′, antisense 5′-ttgcagcagtggagaaagag-3′ (327 bp); EDG-2 sense 5′-gaggaaggtggagtctctgtgg-3′, antisense 5′-tccaagatgcagag gagtcagg-3′ (346 bp); EDG-3 sense 5′-tcaacattctgatgtccggtag-3′, antisense 5′-gtgaagatgctgatgagaaagg-3′ (334 bp); EDG-5 sense 5′-atgtacctgttcctcg gcaacc-3′, antisense 5′-tccagatggtccagacaattcc-3′ (329 bp); EDG-6 sense 5′-tgctcaactcagccatcaatcc-3′, antisense 5′-accggctggttcaagctctagg-3′ (284 bp); EDG-7 sense 5′-attgcctctgcaacatctcagc-3′, antisense 5′-tgctgcacattacactgct tgc-3′ (316 bp); EDG-8 sense 5′-caaggcctatgtgctcttctg-3′, antisense 5′-taggat tcagcagcgagttagc-3′ (344 bp). Primer pairs for mouse are EDG-4 sense 5′-ggcctctgatcctgtaggttgc-3′, antisense 5′-gtgccagaagtgtgcaggtagc-3′ (628 bp). The amplimers were separated by electrophoresis in a 1.5% agarose gel in Tris-borate-EDTA buffer. The gel was stained by ethidium bromide and viewed by Gel Doc 1000 (BioRad Laboratories) with density analysis of each PCR fragment by Molecular Analyst software (BioRad Laboratories). The image densities of coamplified 18S rRNA and EDG receptor isoforms were compared to determine the ratio of expression in samples. Values are expressed as relative levels of EDG receptor mRNA/18s rRNA.

EDG mRNA stability was determined with actinomycin D (10 μmol/l). Islets were cultured in the presence of 5.5 or 11 mmol/l glucose for 7 days and then actinomycin D was added to islets at time 0. RNA was extracted from islets beginning at 2 h after inhibitor addition and up to 6 h. Relative levels of EDG mRNA were quantified at each time point.

cDNA sequencing. The PCR products were sequenced directly by Model 373 DNA sequencing system (Applied Biosystems, Foster City, CA). Each of the amplified products of the RT-PCR was found to be >95% identical to the published sequence in GENBANK.

cAMP determination. Islets were isolated and washed three times in KRBH buffer (pH 7.4) containing 2.8 mmol/l glucose (basal) and 0.01% fatty acid free BSA, as described previously (19,21). Islets were incubated in KRBH buffer for 30–60 min at 37°C in an atmosphere of 95% O_2/5% CO_2 in a shaking water bath. INS-1 cells (~1×10^6 cells/well of a 24-well culture plate) were treated essentially in the same way as islets, except that they were cultured in RPMI-1640 medium at 2.8 mmol/l glucose in the absence of serum. Islet and INS-1 samples were pretreated with the phosphodiesterase inhibitor IBMX (0.2 mmol/l) 20 min before time 0 stimulation with agents described in the text. cAMP levels were determined at time 0 and after 20 min of incubation. All treatments were performed in duplicate. The reaction was stopped by the addition of ice-cold 0.1N HCl (final concentration), and samples were immediately frozen at −80°C. The samples were freeze-thawed and sonicated, and cAMP levels were determined by RIA, as described previously (21,22). Protein content of INS-1 cell samples was determined by Bio-Rad assay.

Statistical analysis. Significant differences between samples were determined by Student's t test (paired, two-tailed) or one-way ANOVA with Student/Newman-Keuls multiple comparison test. $P \leq 0.05$ was accepted as significant. Where percentage of basal or control values are shown, statistical comparisons between the samples were performed on actual values for parent data.

RESULTS

EDG receptor mRNA expression in islets and β-cells. To determine the potential for bioactive lipid signaling in the islet, we investigated expression of mRNA for EDG receptors and SPHK. Total RNA was extracted from rat islet or mouse islet and INS-1 insulinoma cells and analyzed by RT-PCR. The results showed that rat islets contain the mRNA for EDG-1, -2, -3, -5, -6, and -7 receptors (Fig. 1*A*). EDG-8 receptor mRNA was not detected in rat islet RNA extracts; however, rat heart RNA extract showed a band for EDG-8 using these primers (data not shown). Mouse islets expressed EDG-4 mRNA (Fig. 1*C*). INS-1 cells showed mRNA expression for EDG-1, -2, -3, -5, and -6 (Fig. 1*B*) but not EDG-7 or -8 (data not shown).

Phorbol ester and cholecystokinin effects on EDG receptor mRNA expression. EDG-1 has been reported to be an immediate-early response gene induced by phorbol esters (7,14). To test the hypothesis that EDG receptors are regulated in pancreatic islets, we cultured isolated rat islets in the presence and absence of PMA for 2 h. The results indicated that EDG-1 mRNA levels were significantly increased relative to 18S rRNA levels after exposure to PMA (190 ± 14% of paired basal values; Fig. 2*A*). In

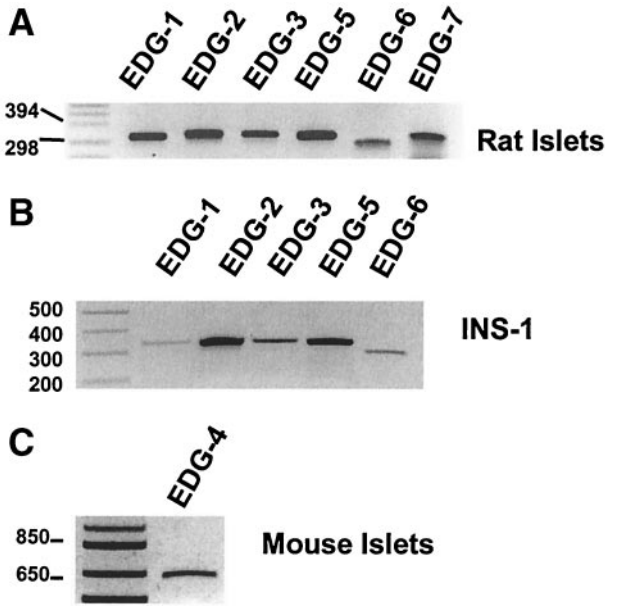

FIG. 1. EDG receptor mRNA expression in islets and INS-1 cells. Total RNA was extracted from rat or mouse isolated pancreatic islets or from INS-1 cells. Primers for EDG receptors 1–7 were used to determine expression of mRNA transcripts by RT-PCR in rat islets (*A*), INS-1 cells (*B*), and mouse islets (*C*). Primers for EDG-4 were specific for the mouse gene. Amplified transcripts were identified by ethidium bromide staining in agarose gels. Numbers on the left identify size markers.

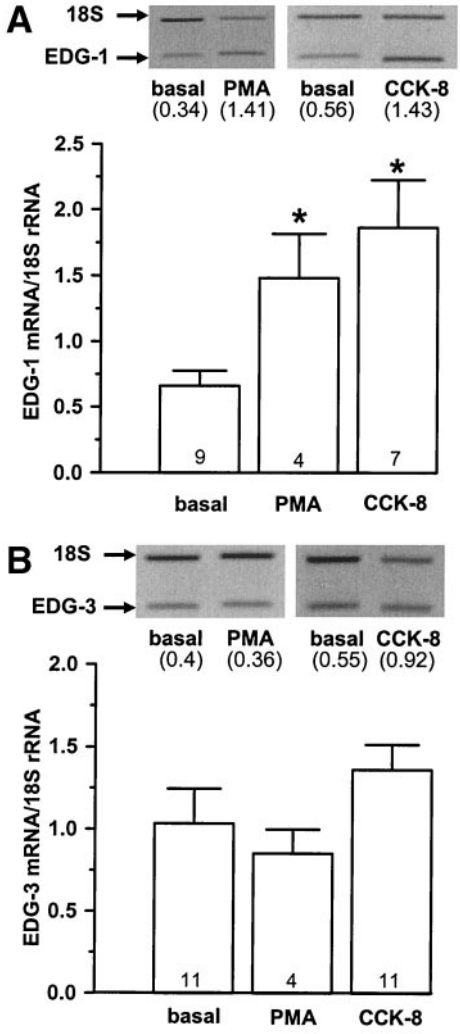

FIG. 2. Induced EDG mRNA expression in isolated islets. Rat islets were cultured in complete CMRL-1066 medium containing 5.5 mmol/l glucose in the absence (basal) or presence of PMA (0.5 μmol/l) or CCK-8 (0.4 μmol/l) for 2 h. The islets were washed, and total RNA was extracted. EDG-1 (*A*) and EDG-3 (*B*) mRNA levels relative to 18s rRNA were determined after biplex RT-PCR. *Inset*: Representative ethidium bromide–stained PCR product bands are shown above each graph; numbers in parentheses are the ratio of EDG mRNA/18S rRNA for each sample. Graph values are means ± SE for the number of independent experiments shown at the base of each bar. *$P < 0.02$ vs. basal values for each set, as determined by one-way ANOVA and multiple comparison test.

contrast, relative EDG-3 mRNA expression levels were not significantly affected by PMA exposure (88 ± 12% of paired basal values; Fig. 2*B*). Shorter times of exposure to PMA (30 and 60 min) did not significantly increase EDG-1 mRNA levels (108 ± 20% of paired basal values) or EDG-3 mRNA levels (95 ± 23% of paired basal values). To determine whether the activation of protein kinase C (PKC) mediated the effects of PMA on EDG expression, we cultured islets with an inhibitor of PKC, GF 109203X. In the presence of GF 109203X, PMA failed to significantly increase relative to EDG-1 mRNA expression levels (Fig. 3*A*), and EDG-1 mRNA levels were 34 ± 10% below levels in islets treated with PMA alone. GF 109203X alone did not significantly affect expression levels of EDG-1 or EDG-3 mRNAs compared with basal values (Fig. 3).

Because PMA activates PKC, CCK-8 that activates polyphosphatidylinositol hydrolysis and PKC through the generation of diacylglycerol (23,24) was investigated for effects on EDG receptor expression. CCK-8 stimulation of islets for 2 h increased EDG-1 mRNA relative levels by 319 ± 66% of basal (Fig. 2*A*). In contrast, CCK-8 did not significantly affect the relative expression level of islet EDG-3 mRNA, although there was a trend to increase this receptor mRNA (Fig. 2*B*).

Glucose effects on EDG receptor mRNA expression. With the use of biplex PCR, relative expression levels of EDG-1 mRNA and 18S rRNA were determined for freshly isolated islets or islets cultured for 2 h or 7 days at different glucose concentrations. Freshly isolated islets cultured for only 2 h in the presence of a maximally secretagogic concentration of glucose (17 mmol/l) showed a significant almost twofold increase in relative expression levels of EDG-1 mRNA/18S rRNA (1.06 ± 0.12) compared with paired fresh control islets (0.60 ± 0.11) cultured for 2 h at 5.5 mmol/l glucose (Fig. 4). In contrast, relative expression levels of EDG-3 mRNA after 2 h of glucose (17 mmol/l) stimulation (0.66 ± 0.12) were not significantly different from levels in paired fresh control islets (0.64 ± 0.14; $P > 0.05$; $n = 5$).

The effects of chronic glucose stimulation on EDG receptor mRNA expression was also investigated. Islets were cultured in the presence of 5.5 or 11 mmol/l glucose for up to 7 days because islets cultured at a hyperglycemic concentration of glucose (11 mmol/l) display signs of glucose desensitization, with reduced insulin secretory responsiveness and signal transduction activity (19,21,25). The relative levels of EDG-1 mRNA/18S rRNA in islets that were cultured for 7 days at 5.5 mmol/l glucose (1.20 ± 0.22) were reduced by ~30% in comparison with paired groups of fresh islets (1.66 ± 0.19; Fig. 4*B*). More pronounced, however, was the significant 74 ± 5% reduction

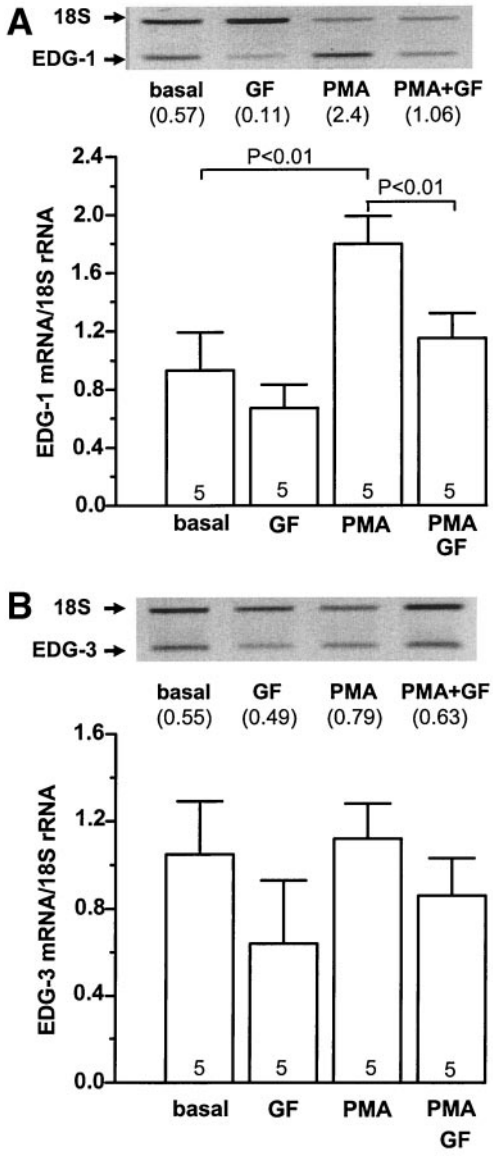

FIG. 3. Inhibition of PKC modulates EDG-1 expression. Rat islets were cultured in complete CMRL-1066 medium containing 5.5 mmol/l glucose in the absence (basal) or presence of PMA (0.5 μmol/l) and/or GF 109203X (GF; 0.5 μmol/l) for 2 h. The islets were washed, and total RNA was extracted. EDG-1 (*A*) and EDG-3 (*B*) mRNA levels relative to 18S rRNA were determined after biplex RT-PCR. *Inset*: Representative ethidium bromide–stained PCR product bands are shown above each graph; numbers in parentheses are the ratio of EDG mRNA/18S rRNA for each sample. Graph values are means ± SE for the number of independent experiments shown at the base of each bar. *P* values were determined by one-way ANOVA and multiple comparison test.

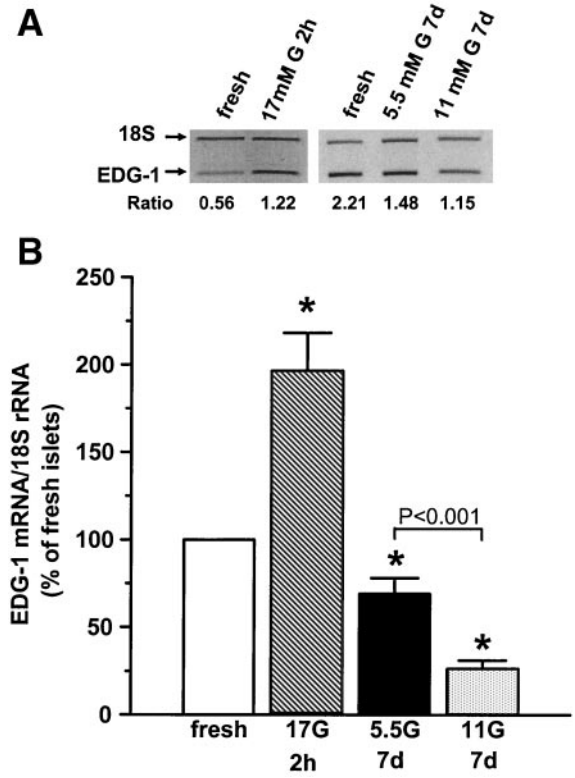

FIG. 4. Chronic glucose stimulation regulates EDG-1 mRNA expression. Rat islets either were freshly isolated (fresh) and cultured for only 2 h at 17 mmol/l glucose (G) in complete CMRL-1066 medium or were cultured for 7 days at 5.5 or 11 mmol/l G, as indicated. After culture, the islets were washed and total RNA was extracted in TRIzol. *A*: Biplex RT-PCR products of EDG-1 mRNA and 18S rRNA (18S) in representative experiments. The ratio of the two bands is shown under the gel. *B*: Isolated rat islets were cultured as described above at 5.5 (5.5G), 11 (11G), or 17 (17G) mmol/l G, as indicated. Fresh islets paired with 7-day islets were extracted immediately, and fresh islets paired with 17G islets were cultured at 5.5 mmol/l G for 2 h before extraction. Values are the mean ± SE for five independent experiments for 2-h samples or 8–10 independent experiments for 7-day samples. *P* values were determined by paired *t* test for 2-h samples and one-way ANOVA with multiple comparison test for 7-day samples. **P* < 0.01 vs. fresh islet values.

in relative EDG-1 mRNA levels in islets that were cultured for 7 days at 11 mmol/l glucose (0.46 ± 0.12) compared with levels in freshly isolated islets (Fig. 4*B*). The relative EDG-1 mRNA expression levels in islets that were cultured for 7 days at 11 mmol/l glucose were also reduced by 54 ± 10% below basal EDG-1 levels of islets that were cultured for 7 days at 5.5 mmol/l glucose (Fig. 4). In contrast to EDG-1 mRNA, relative EDG-3 mRNA/18S rRNA levels after 7 days of 11 mmol/l glucose stimulation (0.68 ± 0.10) were not significantly different ($P > 0.05$; $n = 4$) from the levels in paired groups of fresh islets (0.77 ± 0.09).

The EDG-1 mRNA levels in islets that were cultured for 7 days at 11 mmol/l glucose were investigated for mRNA stability changes. After culture for 7 days, islets that were cultured at 5.5 or 11 mmol/l glucose were incubated in the presence of actinomycin D (10 μmol/l) with islet mRNA sampling over a 6-h period. At time 0, relative levels of EDG-1 mRNA in islets that were cultured at 5.5 and 11 mmol/l glucose were 2.04 ± 0.36 and 1.2 ± 0.20, respectively. After 6 h in the presence of actinomycin D, relative EDG-1 mRNA/18S rRNA levels in islets that were treated with 5.5 (0.33 ± 0.07) and 11 mmol/l glucose (0.50 ± 0.06) were significantly ($P < 0.001$) reduced compared with paired time-0 values (Fig. 5). After 6 h, the rate of decline in relative EDG-1 mRNA levels in islets that were cultured at 5.5 mmol/l glucose (slope, −0.071 ± 0.003) was significantly greater ($P < 0.05$) than the rate of decline observed in islet EDG-1 mRNA in islets that were cultured at 11 mmol/l glucose (slope, −0.090 ± 0.006) in six experiments.

SPP effects on cAMP production in islets and INS-1 cells. To determine whether SPP affects G_i-mediated responses to EDG-1 in β-cells, we incubated isolated islets and INS-1 cells in the absence and presence of GLP-1 (100 nmol/l) and various concentrations of SPP. GLP-1 receptor stimulation activates G_s and stimulates adenylyl cyclase

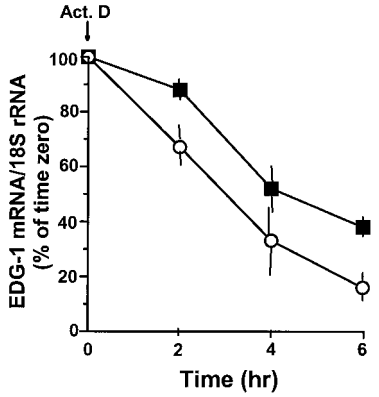

FIG. 5. EDG-1 mRNA degradation. Isolated islets were cultured for 7 days in CMRL-1066 containing 5.5 (○) or 11 (■) mmol/l glucose. Then at time 0, the islets were cultured with actinomycin D (Act.D; 10 μmol/l) and relative expression of EDG-1 mRNA/18s rRNA levels were determined between time 0 and 6 h. Relative EDG-1 mRNA levels are expressed as percentage of time 0. Standard errors are shown for three to five independent determinations.

and cAMP production in islets by 37-fold (Fig. 6*A*) and in INS-1 cells by 7-fold (Fig. 6*B*). In the presence of SPP (0.01–1 μmol/l), there was a concentration-dependent inhibition of cAMP formation in islets and INS-1 cells (Fig.

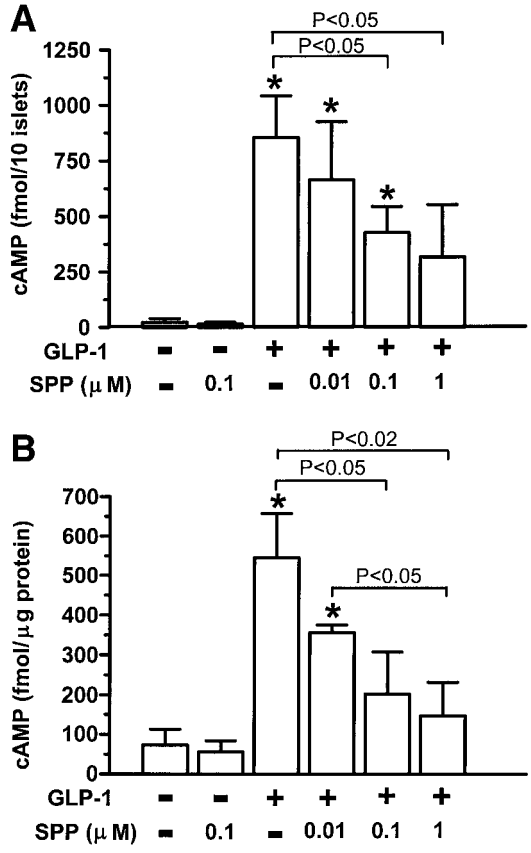

FIG. 6. Effects of SPP on islet and INS-1 cell cAMP production. Islets were incubated in KRBH buffer (*A*) or INS-1 cells in RPMI-1640 medium (*B*) containing 2.8 mmol/l glucose (basal) and the absence (−) or presence (+) of GLP-1 (0.1 μmol/l) and SPP (0.01–1 μmol/l), as indicated, for 20 min. All samples contained 0.2 mmol/l IBMX to inhibit phosphodiesterase activity. cAMP levels were determined by RIA. Values are means ± SE for three to five (*A*) or three (*B*) independent experiments. *P* values were determined by one-way ANOVA with multiple comparison test. **P* < 0.01 vs. basal values.

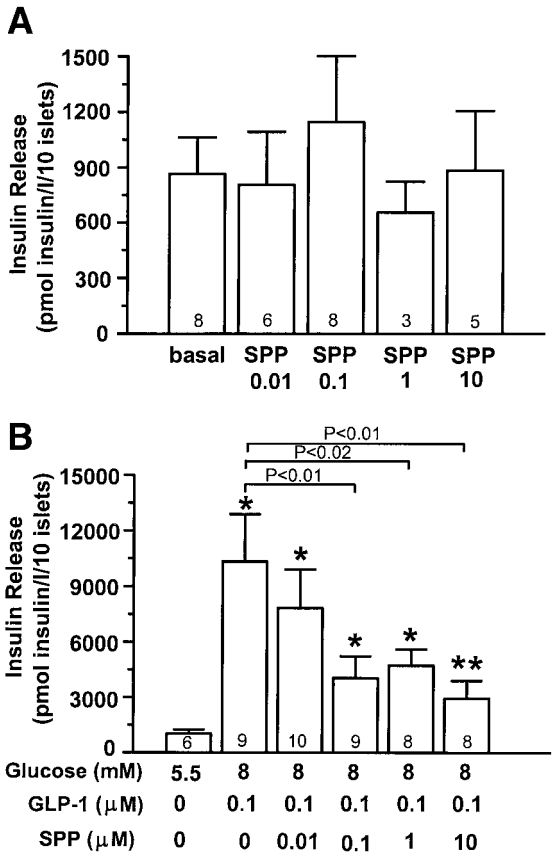

FIG. 7. Effects of SPP on insulin release from rat islets. Isolated islets were incubated in KRBH buffer containing 5.5 mmol/l glucose in the absence or presence of increasing concentrations of SPP (0.01–10 μmol/l; *A*) or 5.5 or 8 mmol/l glucose in the absence or presence of GLP-1 or SPP (*B*) at the concentrations indicated, for 60 min. Insulin release was determined by RIA. Values are means ± SE for the number of independent experiments shown at the base of each bar. *P* values were determined by one-way ANOVA with multiple comparison test. **P* < 0.05, ***P* < 0.01 vs. basal 5.5 mmol/l glucose values.

6). SPP (0.1 μmol/l) did not significantly affect basal cAMP levels in islets or INS-1 cells (Fig. 6).

SPP effects on G_s-mediated islet insulin release. The effects of EDG receptor stimulation by SPP on insulin secretion was investigated. Islets that were incubated with 5.5 mmol/l glucose (basal) did not show a significant change in insulin release in the presence of 0.01–10 μmol/l SPP (Fig. 7*A*). To induce G_s-mediated insulin secretion, we combined a threshold secretagogic concentration of glucose (8 mmol/l) with GLP-1 because GLP-1 is a weak secretagogic stimulus in the absence of glucose. In islets that were stimulated by glucose (8 mmol/l) and GLP-1 (0.1 μmol/l), insulin secretion was increased 10-fold compared with nonstimulated islet (5.5 mmol/l glucose) insulin release (Fig. 7*B*). However, the presence of SPP (10 nmol/l to 10 μmol/l) induced a concentration-dependent decrease in glucose plus GLP-1–stimulated insulin secretion (Fig. 7*B*). Although insulin release with the highest concentration of SPP (10 μmol/l) tested was reduced by 69 ± 9% below basal, that insulin release was not totally inhibited suggests that a component of the glucose plus GLP-1–stimulated secretion was not affected by SPP. Thus, even though GLP-1–stimulated cAMP levels in the presence of SPP (1 μmol/l) were reduced to near basal levels (Fig. 6*A*), insulin release was still elevated by 53 ± 5% above basal, support-

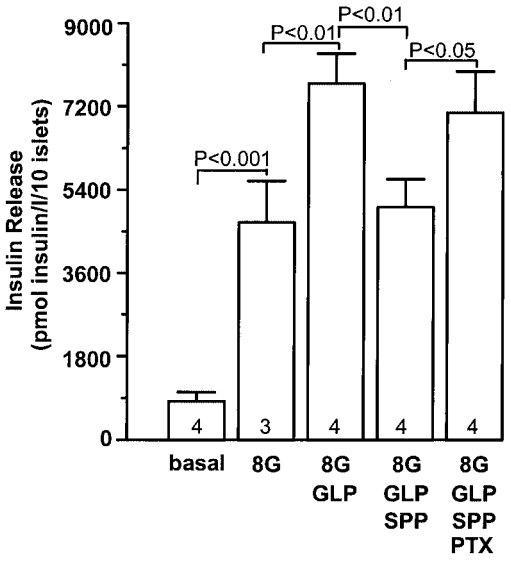

FIG. 8. Effects of pertussis toxin on insulin release with SPP. Isolated islets were incubated in KRBH buffer containing 5.5 (basal) or 8 (8G) mmol/l glucose in the absence or presence of GLP-1 (GLP; 0.1 μmol/l), SPP (0.1 μmol/l), and pertussis toxin (PTX; 0.5 μg/ml). Insulin release was determined by RIA. Values are means ± SE for the number of independent determinations shown at the base of each bar. *P* values were determined by one-way ANOVA with multiple comparison test. Each 8G-treated sample set had significantly ($P < 0.01$) higher insulin release levels versus basal values.

ing the argument that SPP did not inhibit a component of glucose plus GLP-1–stimulated secretion.

To test the hypothesis that the inhibition of insulin release with SPP was due to interaction with G_i, we incubated islets with pertussis toxin. As expected, GLP-1 potentiated glucose-induced insulin secretion by approximately twofold, and SPP prevented the potentiation allowing only the glucose-stimulated component to persist (Fig. 8). However, in the presence of pertussis toxin and SPP, there was almost complete restoration of the GLP-1 potentiation of glucose-induced insulin release (Fig. 8).

DISCUSSION

The EDG receptor family shows evidence of growing importance in cell signaling paradigms as a result of EDG receptor participation in diverse cellular functions. This is the first study to demonstrate the expression of EDG receptors in pancreatic islets and β-cells and to characterize their role in major signal transduction pathways and insulin secretion. The subfamily of G protein–coupled EDG receptors that binds SPP with high affinity, EDG-1, -3, -5, and -6, are expressed in rat islets and the β-cell line INS-1. EDG-1 receptors have previously been shown to dominate in endothelial cells and to be expressed in lower amounts in epithelial, neural, and myocytic cells, whereas EDG-3 and EDG-5 are widely expressed in epithelial cells and fibroblasts, although EDG-3 is also found in brain neuronal cells (26). EDG-1, -3, and -5 are also expressed in HEK-293 cells (27). EDG-6 is an orphan that has been isolated from dendritic cells and lymphoid and hematopoietic tissue and lung (26,28). EDG-8 mRNA was not detected in islets or INS-1 cells, although the primers detected EDG-8 mRNA in rat heart RNA extracts. The presence of EDG-1 (that signals through G_i) in β-cells suggested that SPP ligand binding has the potential to inhibit G_i-mediated processes, such as adenylyl cyclase. The presence of EDG-3 and EDG-5 suggested that G_q-mediated pathways are transduced in response to SPP, such as phospholipase C activation, inositol trisphosphate and diacylglycerol production, and PKC activation (4). EDG receptors that bind lysophosphatidic acid with high affinity (EDG-2, -4, and -7) are expressed in rat and/or mouse islets, whereas EDG-2 but not EDG-7 was detected in INS-1 cells. Lysophosphatidic acid–binding EDG receptors have also been reported for cardiomyocytes (EDG-2 and -4), developing neurons and oligodendrocytes and brain white matter (EDG-2), ovarian cancer cell lines and T-cells, and brain neuronal cells (EDG-4) (6,26). EDG-7 is highly expressed in prostate (29).

The expression levels indicated that EDG-1 mRNA was highly expressed in islets but was barely detectable in INS-1 cells. It is possible that the endothelial cells of the islet contributed in part to the expression level of EDG-1, although there may be other contributing factors (as discussed below). EDG-2, -3, and -5 mRNAs are highly expressed in islets and INS-1 cells. Among the receptors, EDG-6 and EDG-7 mRNAs were the least prevalent in islets, requiring reamplification RT-PCR for adequate detection in islet extracts by ethidium bromide, and EDG-7 mRNA was not detected in INS-1 cells. The results suggest that EDG-6 and -7 in islets are expressed either in primary endocrine cells or in the case of EDG-7 in other cell types present in islets of Langerhans. Fibroblasts also express transcripts for EDG-1, -3, and -5 but not for EDG-6 and may contribute to the islet receptor profile.

The regulation of EDG receptors has been primarily associated with immediate-early gene induction of EDG-1 transcript by PMA (30). In the present study, effects of PMA on relative islet EDG-1 mRNA expression was confirmed after acute exposure to phorbol ester. In addition, the intestinal hormone analog CCK-8 that stimulates phospholipase C and PKC activities in islets at concentrations similar to those used in this study (23,24) increased the expression of EDG-1 mRNA in islets. A role for PKC in the regulation of EDG-1 expression was confirmed by the ability of GF 109203X to inhibit the PMA-induced increase in expression. Thus, islet EDG-1 mRNA is acutely regulated by pharmacologic and physiologic stimuli. In these experiments, we were not able to elicit quantitative increases in EDG-1 mRNA by PMA in INS-1 cells (data not shown). EDG-1 mRNA was barely detectable in these cells using RT-PCR, and semiquantitative analysis against 18S rRNA was not reliable.

EDG receptor mRNA regulation was also investigated in islets that were cultured short-term and long-term at elevated glucose concentrations. Short-term glucose stimulation increased islet relative EDG-1 mRNA levels. The latter might be expected because glucose stimulates phospholipase C activation, diacylglycerol formation, and PKC activation (31), although other actions of glucose have not been ruled out. In contrast, the long-term culture of islets at 11 mmol/l glucose resulted in a marked suppression in relative EDG-1 mRNA levels. This suppression was not due to increased instability of the mRNA, because the rate of mRNA degradation with actinomycin D present was slower in islets that were stimulated at 11 mmol/l glucose than in islets that were cultured at 5.5 mmol/l glucose. It is

likely, therefore, that the decline in EDG-1 mRNA with 7-day glucose stimulation was due to a reduction in transcription of the EDG-1 gene. It has been reported previously that 4- to 7-day glucose stimulation similar to the conditions in this study results in a decrease in rat islet inositol 1,4,5-trisphosphate receptor II mRNA expression levels (32); impaired cAMP responsiveness (21); *myo*-inositol transport; and Na^+-K^+-ATPase (19), ATP generation, and phosphoinositide turnover (25). These signal transduction and metabolic consequences of long-term glucose stimulation likely contribute to desensitization of glucose-stimulated insulin secretion (19,33,34). It is interesting to consider that INS-1 cells, which are maintained at 11 mmol/l glucose in RPMI-1640 medium, show low levels of EDG-1 mRNA expression. Perhaps the chronic glucose stimulation of these cells contributes to the reduced expression of EDG-1 receptor mRNA. Unfortunately, islet EDG-1 receptor protein expression levels were not quantified by Western blotting because commercially available antibodies to rodent EDG-1 lack sufficient specificity to be useful in this regard at physiologic levels of the receptor found in islets.

A role for EDG-1 receptors in the regulation of β-cell adenylyl cyclase regulation and insulin secretion was investigated. Because EDG-1 is characterized as a G_i-coupled receptor (4), an effect of the stimulation of these receptors on cAMP responses in β-cells was investigated. The results show that increasing concentrations of the EDG receptor ligand SPP resulted in diminished ability of GLP-1 to stimulate β-cell adenylyl cyclase and cAMP production. The concentrations of SPP studied were physiologically relevant because the constitutive plasma level of SPP is in the high nanomolar range (5). There was also a correlation between the cAMP responses and insulin secretion in isolated islets. Whereas GLP-1 markedly potentiated a threshold glucose concentration effect on insulin secretion, the presence of SPP induced a concentration-dependent reduction in the GLP-1 component of the secretory response. A concentration of 0.1 μmol/l SPP seemed to induce maximal effects on both cAMP production and insulin release, because higher concentrations of the bioactive lipid did not induce greater inhibitory effects. Additional evidence that SPP evoked responses mediated by G_i was obtained with pertussis toxin, a G_i inhibitor that blocked the SPP-induced inhibitory response in islets. We have not ruled out the possibility that pertussis toxin might also be affecting other G proteins in these cells that might impact secretion. These results provide convincing evidence that SPP and EDG-1 receptors have the potential to play an important role in the regulation of insulin secretion. In addition to inhibition of adenylyl cyclase, EDG-1 stimulation has been reported to activate mitogen-activated protein kinase ERK and Rho signal transduction pathways (4).

We have not ruled out the possibility that extracellular SPP might have effects on intracellular mechanisms; however, micromolar concentrations of SPP are usually associated with intracellular effects of this phosphorylated agent not mediated by the EDG receptors. In addition, we have not determined the role that EDG-3 and EDG-5 that are linked to G_q might be having on the secretory responses. However, G_q-mediated responses would be expected to be antagonistic to the G_i-mediated response, because G_q-coupled phospholipase C increases polyphosphoinositide hydrolysis and inositol trisphosphate and diacylglycerol production. The inositol trisphosphate triggers Ca^{2+} mobilization, whereas diacylglycerol increases PKC activation; thus, both Ca^{2+} and diacylglycerol would be expected to increase insulin secretion rather than inhibit it in response to SPP, yet SPP did antagonize the GLP-1 stimulation of insulin secretion, probably because cAMP production is the major signal transduction pathway of this agonist.

In summary, these results demonstrate that EDG receptor mRNA is transcribed in isolated islets and the β-cell line INS-1. The EDG-1 gene transcription seems to be regulated by PKC activation in response to physiologic and pharmacologic stimuli in islets. The profound downregulation of EDG-1 mRNA in chronic glucose-stimulated islets suggests that the EDG-mediated responses may be lost during hyperglycemia and diabetes. These data suggest that EDG receptors and constitutive circulating levels of SPP act as a "brake" on insulin secretion.

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