

A Potent and Highly Selective VPAC2 Agonist Enhances Glucose-Induced Insulin Release and Glucose Disposal

A Potential Therapy for Type 2 Diabetes

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Pituitary adenylate cyclase-activating peptide (PACAP) and vasoactive intestinal peptide (VIP) activate two shared receptors, VPAC1 and VPAC2. Activation of VPAC1 has been implicated in elevating glucose output, whereas activation of VPAC2 may be involved in insulin secretion. A hypothesis that a VPAC2-selective agonist would enhance glucose disposal by stimulating insulin secretion without causing increased hepatic glucose production was tested using a novel selective agonist of VPAC2. This agonist, BAY 55-9837, was generated through site-directed mutagenesis based on sequence alignments of PACAP, VIP, and related analogs. The peptide bound to VPAC2 with a dissociation constant (K_d) of 0.65 nmol/l and displayed >100-fold selectivity over VPAC1. BAY 55-9837 stimulated glucose-dependent insulin secretion in isolated rat and human pancreatic islets, increased insulin synthesis in purified rat islets, and caused a dose-dependent increase in plasma insulin levels in fasted rats, with a half-maximal stimulatory concentration of 3 pmol/kg. Continuous intravenous or subcutaneous infusion of the peptide reduced the glucose area under the curve following an intraperitoneal glucose tolerance test. The peptide had effects on intestinal water retention and mean arterial blood pressure in rats, but only at much higher doses. BAY 55-9837 may be a useful therapy for the treatment of type 2 diabetes. *Diabetes* 51:1453–1460, 2002

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the superfamily of metabolic, neuroendocrine, and neurotransmitter peptide hormones (1). PACAP exists as either a 38-amino acid (aa) peptide (PACAP38) or as a 27-aa peptide (PACAP27) with an amidated carboxyl (2). The shorter form has 68% structural homology to vasoactive

intestinal peptide (VIP), a 28-aa peptide (3). The effects of PACAP are mediated by three G-protein-coupled receptors that exert their action through the cAMP-mediated and other Ca^{2+} -mediated signal transduction pathways: the PACAP-preferring type 1 (PAC1) and two VIP-shared type 2 receptors (VPAC1 and VPAC2) (4). PACAP has comparable activities toward all three receptors, while VIP selectively activates the two VPAC receptors.

Both PACAP and VIP stimulate insulin secretion from insulinoma cells, mouse pancreatic islets, and perfused rat pancreas (5–8). The cloning of VPAC2 from the mouse β -cell line, MIN6, and its expression in insulin-secreting cells suggests that activation of this receptor may contribute to the increase in insulin secretion (9). On the other hand, the expression pattern of VPAC1, coupled with the glycogenolytic activity of PACAP, suggests that activation of this receptor contributes to the increase in hepatic glucose production (10–12). The administration of PACAP27 to either mice (13) or humans (14) has been reported to increase plasma insulin levels without affecting plasma glucose levels. Because PACAP27 activates both VPAC1 and VPAC2, the increase in glucose production may have offset the increase in insulin secretion. Therefore, we have postulated that a VPAC2-selective agonist would enhance pancreatic β -cell insulin release without causing increased glucose production by the liver, and would thereby lead to increased glucose disposal. To test this hypothesis, VPAC2-selective agonists were engineered through several rounds of site-directed mutagenesis based on sequence alignments of PACAP, VIP, and known PACAP receptor-selective analogs (data not shown). In this article, the *in vitro* and *in vivo* properties of BAY 55-9837, a potent and highly selective VPAC2 agonist, are described.

RESEARCH DESIGN AND METHODS

Peptide synthesis. BAY 55-9837, [K15, R16, L27]VIP(1-7)/GRF(8-27) (15), and PG 97-269 (16) were synthesized by Sigma Genosys (The Woodlands, TX) and characterized using reverse-phase analytical high-performance liquid chromatography and mass spectrometry to >95% purity. BAY 55-9837 has the following amino acid sequence: HSDAVFTDNYTRLRKQVAACKYLQSIKNKRY, with a molecular weight of 3,743.

Cell culture. The human PAC1 was cloned via PCR from human brain quick-clone cDNA (Clontech, Palo Alto, CA). The human VPAC1 and VPAC2 were cloned via RT-PCR from human heart mRNA and human testis mRNA, respectively, using TaqPlus Precision PCR System (Stratagene, La Jolla, CA). The PCR products were subcloned into pCDNA3.1 (Invitrogen, Carlsbad, CA) for *in vitro* translation and mammalian expression. The cell line chosen for

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aa, amino acid; AUC, area under the curve; B_{max} , maximum binding capacity; EC_{50} , half-maximal stimulatory concentration; GLP, glucagon-like peptide; IC_{50} , half-maximal inhibitory concentration; IPGTT, intraperitoneal glucose tolerance test; K_d , dissociation constant; MAP, mean arterial pressure; PACAP, pituitary adenylate cyclase-activating polypeptide; SPA, scintillation proximity assay; VIP, vasoactive intestinal peptide.

expression was the CHOcreluc line already expressing a cAMP response element-luciferase reporter along with $G\alpha_{16}$. These cells were grown under hygromycin selection at 0.4 mg/ml. On the day of transfection, CHOcreluc cells at 70% confluency were washed with serum-free medium and transfected using Lipofectamine Plus Reagent (Gibco, Grand Island, NY). Stable pools were selected in the presence of 0.4 mg/ml hygromycin and 1.5 mg/ml G418. Once viably frozen stocks had been made from these pools, they were cloned by limiting dilution. Expression and functionality of the receptors were confirmed by treatment of the cells with PACAP27 and VIP peptides and luciferase assay.

Competition binding assay. PACAP receptor binding assay was performed using a modification of a previously described protocol (17). Membranes were prepared from CHO cells transfected with PAC1, VPAC1, or VPAC2. Cells were washed with PBS, scraped in homogenization buffer (10 mmol/l Tris, pH 7.4, 2 mmol/l EDTA, 5 mmol/l $MgCl_2$, and 1 mmol/l phenylmethylsulfonyl fluoride), and centrifuged at 4,000g for 10 min at 4°C. The cell pellet was resuspended in homogenization buffer and homogenized using a Polytron. Membranes were collected by centrifugation at 30,000g for 30 min at 4°C, resuspended in homogenization buffer, and stored at -80°C until use. To measure binding of PACAP peptides, 10 μ g membrane was incubated with 0.1 nmol/l [125 I]PACAP27 (NEN Life Science Products, Boston, MA) in the presence of increasing concentrations of peptide, in a total volume of 100 μ l of 20 mmol/l HEPES (pH 7.4), 150 mmol/l NaCl, 0.5% BSA, 2 mmol/l $MgCl_2$, and 0.1 mg/ml bacitracin. After incubating at 37°C for 20 min, bound ligand was collected on GF/C filters pretreated with 0.1% polyethylenimine. The filters were washed with cold 25 mmol/l $NaPO_4$ containing 1% BSA and counted in a gamma counter. All reagents were purchased from Sigma unless otherwise indicated. [125 I]BAY 55-9837 was custom iodinated by NEN Life Science Products (iodination with chloramine-T, specific activity 2,200 Ci/mmol, in a solution containing 50 mmol/l sodium phosphate, pH 6.0, with 150 mmol/l sodium chloride, 1,000 mmol/l glycine, 0.1% BSA, 50 mmol/l *N*-acetylmethionine, and 50 mmol/l ascorbic acid, >95% radiochemically pure).

cAMP scintillation proximity assay. For the cAMP scintillation proximity assay (SPA), CHO cells transfected with human PAC1, VPAC1, or VPAC2 were plated in Costar 96-well plates at 8×10^4 cells/well and grown at 37°C for 24 h in α -minimal essential medium with nucleosides and glutamine (Gibco), 10% FCS, 100 μ g/ml penicillin/streptomycin, 0.3 mg/ml glutamine, 1 mmol/l HEPES, and 0.5 mg/ml geneticin (Gibco). The RINm5F rat insulinoma cell line (ATCC, Rockville, MD) was grown in RPMI 1640 (Gibco) supplemented with 5% FCS (Gibco), penicillin (100 U/ml), and streptomycin (100 μ g/ml). The medium was removed, and the plates were washed with PBS. The cells were incubated with compounds in HEPES-PBS-BSA (1%) with 0.4 mg/ml soybean trypsin inhibitor, 0.5 mg/ml bacitracin, and 100 μ mol/l isomethyl butyl xanthine for 15 min at 37°C. cAMP in the cell extracts was quantitated using a commercial cAMP SPA screening assay system (Amersham Pharmacia, Piscataway, NJ).

Insulin release and biosynthesis in isolated pancreatic islets. Pancreatic islets were isolated from Wistar rats (275–320 g) using collagenase digestion and harvested after Ficoll gradient procedure (18). The effect of BAY 55-9837 on insulin release was tested in static islet incubation as described previously (19). Briefly, a group of five islets was loaded in each incubation well. After 30 min preincubation, islets were transferred to incubation buffer (HEPES-buffered Krebs-Ringer buffer, pH 7.4) containing different concentrations of glucose and indicated peptides. Both preincubation and incubation were performed at 37°C in a water bath shaker with an atmosphere of 95% O_2 , 5% CO_2 . Samples of incubation buffer were collected at 60 min of incubation for insulin determination using enzyme-linked immunosorbent assay (ELISA) (ALPCO, Windham, NH).

Purified human islets were obtained from the Diabetes Institute at the University of Minnesota and cultured overnight in 10% FCS/RPMI 1640 medium containing 11 mmol/l glucose. Twenty islets were loaded in each incubation well and preincubated in 10% FCS/RPMI 1640 containing 3 mmol/l glucose for 30 min. Next, these islets were incubated in RPMI 1640 containing 8 mmol/l glucose with either the vehicle or the specified peptide for 2 h. The conditioned medium was collected, and the insulin concentration was measured using the SPA assay kit (Amersham Pharmacia).

Insulin biosynthesis was studied in isolated rat islets using a modification of a previously described protocol (20). After an overnight culture, groups of 20 islets were preincubated in RPMI 1640 containing 3 mmol/l glucose for 30 min. The islets were incubated for 90 min at 37°C with 100 nmol/l glucagon-like peptide (GLP)-1 or BAY 55-9837 in 100 μ l Krebs-Ringer bicarbonate buffer that contained 8 mmol/l glucose, 5 mg/ml BSA, and 4 μ mol/l L -[4- 3 H]leucine (0.1 mCi/ml). Islets were then washed three times with the same buffer containing 1 mmol/l unlabeled L -leucine. Islets were lysed in 2 mol/l acetic acid and neutralized by adding 10N NaOH. Tris-HCl (50 mmol/l, pH 8.0) with 0.1% Triton X-100 was then added to bring the volume to 1 ml. Protein A bead

solution and 1:10 diluted anti-insulin antibody (I-8510; Sigma) were added and incubated overnight at 4°C. The beads were washed once with the solution containing 0.5 mol/l LiCl, 50 mmol/l Tris, pH 7.5, and 0.1% Triton X-100 and twice with the solution containing 0.1 mol/l NaCl, 50 mmol/l Tris, pH 7.5, and 0.1% Triton X-100. Finally, the beads were resuspended in 0.5 ml Tris-Triton buffer. Scintillation fluid was added, and the samples were counted in a Microbeta counter (Wallac Instruments; Perkin-Elmer, Boston, MA). Insulin biosynthesis was expressed as [3 H]leucine incorporation (% of basal) after subtracting the nonspecific counts, i.e., background in islets incubated in the same buffer with 3 mmol/l glucose (2,952 cpm).

Insulin levels following an intravenous glucose tolerance test in rats. Wistar rats (250–300 g) were fasted overnight (17 h) and then anesthetized with pentobarbital (65 mg/kg i.p.). Glucose (0.4 g/kg dissolved in 0.9% saline with 1% human plasma albumin) with or without peptide was injected intravenously into the tail vein. The rats were eye-bled 1 min after the injection, and 50–100 μ l of the plasma was assayed for insulin level with a rat radioimmunoassay kit (Linco Research, St. Charles, MO).

Continuous intravenous infusion and intraperitoneal glucose tolerance test. Wistar rats were continuously infused for 3 days with saline with or without BAY 55-9837 via the external jugular vein using a no. 2002 Alzet minipump implanted subcutaneously in the midscapular region. Before the intraperitoneal glucose tolerance test (IPGTT), the rats were fasted overnight and anesthetized with pentobarbital (65 mg/kg i.p.), and a blood sample was taken. Then 1 g glucose/kg body wt was given intraperitoneally, and blood samples were taken after 15, 30, and 60 min. Plasma glucose levels were determined using the Technicon Axon autoanalyzer (Bayer Corporation, Tarrytown, NY), and the glucose area under the curve (AUC) was determined for each rat. The AUC was calculated using the following formula: $AUC = (PG_0 + PG_{60})/2 + PG_{15} + PG_{30}$, where PG $_x$ is the plasma glucose level at 0, 15, 30, or 60 min.

Continuous subcutaneous infusion and IPGTT. Wistar rats were continuously infused for 3 days with saline with or without BAY 55-9837 at 3, 30, or 300 pmol \cdot kg $^{-1}$ \cdot min $^{-1}$ via a no. 2002 Alzet minipump implanted subcutaneously in the midscapular region. Before the IPGTT, the rats were fasted overnight and anesthetized, and a blood sample was taken. Then 1 g/kg glucose was injected intraperitoneally. Blood samples were taken after 15, 30, 60, and 90 min, and glucose levels were determined. The glucose AUC was determined by the formula above.

Measurement of intestinal water retention in rats. Male Wistar rats were fasted for 24 h, and water was removed for 2–3 h before the start of the experiment. Peptide or vehicle (0.9% saline and 1% human serum albumin) was injected into the tail vein of conscious rats. Rats were killed with CO_2 10 min after dosing, and the small intestine was dissected out and weighed. The intestine was cut open, and the water in the lumen was absorbed with filter paper. The intestine was weighed again. The amount of water retained by the intestine was the difference between the initial and final intestinal weights.

Blood pressure measurement in rats. Wistar rats were anesthetized with pentobarbital (65 mg/kg i.p.), and the carotid artery and external jugular vein were cannulated. A pressure transducer was attached to the arterial cannula. Vehicle (0.2 ml saline) was injected into the jugular vein, and the changes in blood pressure and heart rate were measured for 10 min. Increasing doses of BAY 55-9837 were then injected at about 10-min intervals, and blood pressure and heart rate were measured using a Biopac Data Acquisition system (Biopac Systems, Santa Barbara, CA). The data reported are the maximum changes in mean arterial pressure (MAP) at any given dose.

Metabolic profile. Wistar rats were implanted with intravenous minipumps, and BAY 55-9837 (13 pmol \cdot kg $^{-1}$ \cdot min $^{-1}$) was continuously infused for 3 days. Blood was collected after 3 days of treatment and analyzed for levels of following hormones and metabolites: free fatty acids (Waco Chemicals, Richmond, VA), lactate and triglycerides (Sigma), and C-peptide and insulin (Linco Research). Glucagon, prolactin, cholesterol, epinephrine, and corticosterone measurements were outsourced to Analytix, Gaithersburg, MD.

Statistical analysis. ANOVA or *t* test was used to evaluate compound effects using InStat (GraphPad Software, San Diego, CA). Significance was achieved with $P < 0.05$.

All studies were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care following protocols approved by Bayer's Institutional Animal Care and Use Committee. The procedures were performed in accordance with the principles and guidelines established by the National Institutes of Health for the care and use of laboratory animals.

RESULTS

Binding of BAY 55-9837 to PACAP receptors. Competition binding of [125 I]PACAP27 on membranes purified

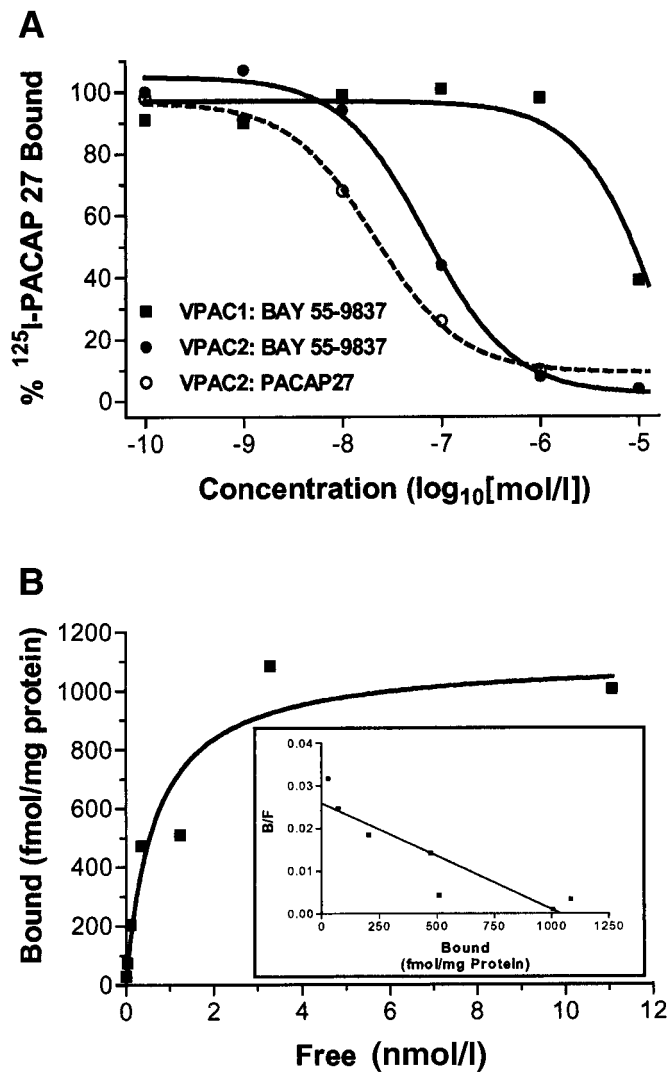


FIG. 1. Binding of BAY 55-9837 to PACAP receptors. **A:** Displacement of [^{125}I]PACAP27 by BAY 55-9837 or PACAP27 in membranes purified from CHO cells expressing human VPAC1 and VPAC2. The results are expressed as % of maximum binding by [^{125}I]PACAP27. The data are representative of at least three separate experiments. **B:** Saturation binding of [^{125}I]BAY 55-9837 in membranes purified from CHO cells expressing the human VPAC2. Specific binding is shown on the y -axis, with a B_{max} value of 1,100 fmol/mg protein. A Scatchard plot is shown in the inset. The estimated K_d was 0.65 nmol/l.

from CHO cells expressing each of the three subtypes of human PACAP receptors identified BAY 55-9837 as a VPAC2-selective peptide (Fig. 1A). BAY 55-9837 competitively displaced [^{125}I]PACAP27 from VPAC2, with a half-maximal inhibitory concentration (IC_{50}) of 60 nmol/l, whereas PACAP27 had an IC_{50} of 21 nmol/l. The IC_{50} for BAY 55-9837 at human VPAC1 was 8,700 nmol/l, whereas no competition was observed up to 10 $\mu\text{mol/l}$ at PAC1 (data not shown). Binding of [^{125}I]BAY 55-9837 to human VPAC2 was saturable, with a dissociation constant (K_d) of 0.65 nmol/l (Fig. 1B), which is similar to the 1 nmol/l value reported for PACAP27 (21).

PACAP receptor activation by BAY 55-9837. Accumulation of cAMP in human PACAP receptor-transfected cells was used as an index of agonist activity. BAY 55-9837 was a potent, full agonist of the human VPAC2, with a half-maximal stimulatory concentration (EC_{50}) of 0.4

nmol/l (Fig. 2). It was 250-fold less potent at human VPAC1 (EC_{50} of 100 nmol/l) and had no activity toward the human PAC1. BAY 55-9837 was also a full agonist, with an EC_{50} of 8.4 nmol/l in the RINm5F cell line that endogenously expresses the VPAC2 receptor and with a maximum binding capacity (B_{max}) of 52 fmol/mg protein (data not shown).

Effect of BAY 55-9837 on insulin secretion and biosynthesis in isolated pancreatic islets. BAY 55-9837 caused a concentration-dependent increase in insulin secretion from isolated rat and human islets incubated in a medium containing 8 mmol/l glucose (Fig. 3A and B). This effect was similar to that of PACAP27 and GLP-1, known insulin secretagogues (22). No polypeptide-induced increase in insulin secretion was observed at 3 mmol/l glucose (Fig. 3A), demonstrating that the effect of BAY 55-9837 on insulin secretion is glucose dependent. BAY 55-9837 also stimulated insulin biosynthesis in rat islets (Fig. 3C). Treatment of islets for 90 min with either 100 nmol/l BAY 55-9837 or 100 nmol/l GLP-1 in medium containing 8 mmol/l glucose markedly increased the incorporation of [^3H]leucine into insulin compared with 8 mmol/l glucose alone.

In vivo efficacy of intravenously administered BAY 55-9837. The ability of BAY 55-9837 to enhance glucose-induced insulin secretion in vivo was tested in fasted rats given 0.4 g/kg glucose intravenously. Intravenous injection of the peptide caused a dose-dependent increase in plasma insulin, and the peptide was equipotent with GLP-1 (Fig. 4A). Both peptides had ED_{50} values of ~ 3 pmol/kg. The peptide also was given to rats by continuous intravenous infusion for 3 days, and then the rats were challenged with an IPGTT (Fig. 4B). BAY 55-9837 caused a dose-dependent decrease in the glucose AUC after the IPGTT, and the dose-response curve and magnitude of the effect were similar to that for intravenous infusion of GLP-1. The magnitude of the effect on glucose disposal was the same whether the infusion was for 1 hour or 6 days, with no sign of desensitization over the treatment period (data not shown). Furthermore, no hypoglycemia was observed in fasted rats infused with BAY 55-9837 at any dose. Fasting

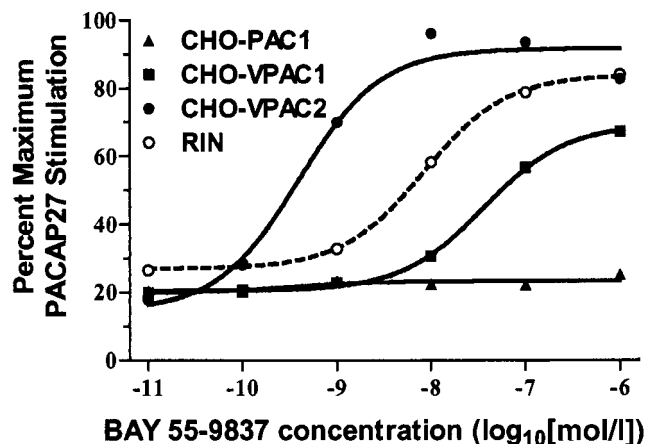


FIG. 2. BAY 55-9837-induced cAMP accumulation in CHO cells expressing human PAC1, VPAC1, and VPAC2 and rat insulinoma cells (RIN). The results are expressed as percent of maximum cAMP accumulation by PACAP27. Basal and maximum stimulated cAMP levels generally were 1 and 5 pmol per well. The data shown are representative of at least three similar experiments.

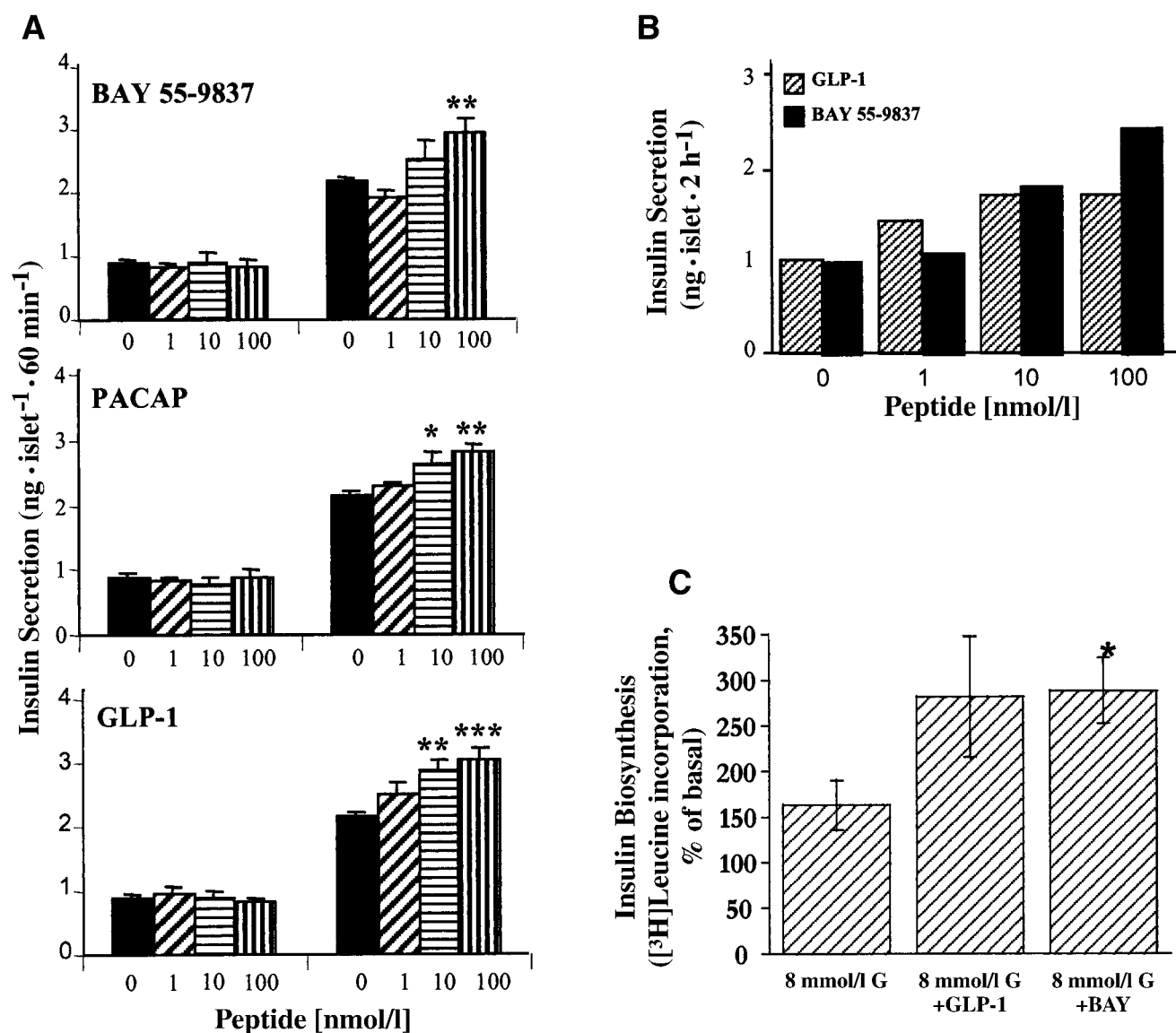


FIG. 3. Effect of BAY 55-9837 on insulin release and biosynthesis in isolated pancreatic islets. *A*: Rat islets were statically incubated in buffer containing 3 or 8 mmol/l glucose and indicated concentrations of peptides for 60 min. Each bar is the means \pm SE ($n = 5$). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (one-way ANOVA). *B*: Human islets (one experiment with triplicates) were statically incubated in buffer containing 8 mmol/l glucose and indicated concentrations of peptides for 2 h. *C*: Insulin biosynthesis in isolated rat islets incubated in buffer containing 8 mmol/l glucose with 100 nmol/l of either GLP-1 or BAY 55-9837 for 90 min. The data are expressed as % of basal [3 H]leucine incorporation (3 mmol/l glucose). Each bar is the mean \pm SE ($n = 3$). $*P \leq 0.05$ (paired t test).

plasma glucose levels were ~ 70 mg/dl and were not different from the vehicle control (data not shown).

In vivo efficacy of subcutaneously administered BAY 55-9837. BAY 55-9837 also was effective when administered subcutaneously. The peptide was injected into fasted rats 5 min before an IPGTT, and glucose levels were determined 30 min later. Plasma glucose levels were reduced by 17% (262 ± 11 mg/dl; $P < 0.01$) and 32% (214 ± 12 mg/dl; $P < 0.001$) after injection of 1 and 10 nmol/kg, respectively, compared with rats that received only glucose (315 ± 12 mg/dl). Continuous subcutaneous infusion of BAY 55-9837 into rats for 3 days decreased plasma glucose levels following an IPGTT (Fig. 5A) and caused a significant decrease in the glucose AUC at 3, 30, and 300 pmol \cdot kg $^{-1}$ \cdot min $^{-1}$ (Fig. 5B). No fasting hypoglycemia was observed at any dose.

Effect of BAY 55-9837 on plasma level of metabolites.

Continuous intravenous infusion of BAY 55-9837 at 13 pmol \cdot kg $^{-1}$ \cdot min $^{-1}$ for 3 days did not significantly alter the level of any of the following metabolites: glucagon, prolactin, cholesterol, epinephrine, corticosterone, free fatty acids, lactate, triglycerides, C-peptide, and insulin (Table 1).

Effect of BAY 55-9837 on watery diarrhea. Because watery diarrhea is associated with elevated VIP levels (23–25), the effects of BAY 55-9837 and VIP on intestinal water retention were investigated in vivo (Fig. 6). VIP dose-dependently increased water retention, with the maximum effect (3.8-fold increase) obtained at 20 nmol/kg; the estimated ED $_{50}$ value was 2 nmol/kg. Identical results were obtained with the VPAC1-selective agonist [K15, R16,L27]VIP(1-7)/GRF(8-27) (15). On the other hand, BAY

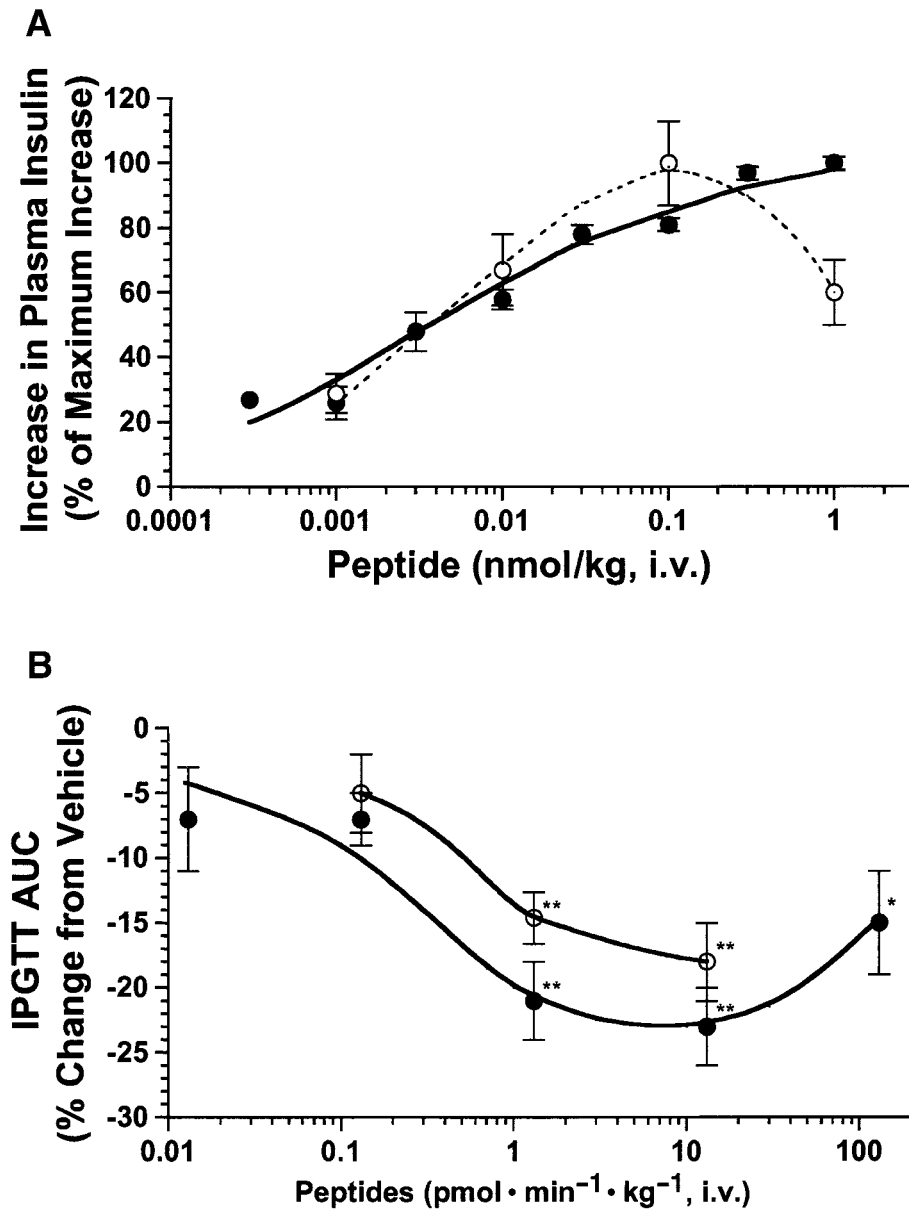


FIG. 4. In vivo efficacy of intravenously administered BAY 55-9837. **A:** Effect of BAY 55-9837 (●) and GLP-1 (○) on insulin secretion in fasted rats. Fasted rats were injected intravenously with glucose along with saline or the indicated peptide. Blood was collected 1 min later and analyzed for insulin as described in RESEARCH DESIGN AND METHODS. Values are the means \pm SE (n for vehicle, 39; 0.0003 and 0.001 mg/kg, 12; 0.003 mg/kg, 24; 0.01 mg/kg, 46; 0.03 and 0.1 mg/kg, 56; 0.3 mg/kg, 34; 1.0 mg/kg, 21). Nontreated, basal (glucose alone), and maximal values for insulin were 0.31 ± 0.06 , 3.3 ± 0.2 , and 10.6 ± 0.5 ng/ml, respectively. **B:** Effect of continuous intravenous infusion of BAY 55-9837 (●) and GLP-1 (○) for 3 days on the glucose AUC following an IPGTT in fasted rats. The vehicle AUC was 563 ± 22 and 567 ± 21 mg/dl for the BAY 55-9837 and GLP-1 curves, respectively. Each point is the mean \pm SE ($n = 12$). ** $P < 0.01$, * $P < 0.05$ vs. vehicle control.

55-9837 had no effect at 1 nmol/kg and had only 19% of the maximum effect of VIP at 10 nmol/kg. The ED_{50} value was ~ 20 nmol/kg, since that dose of BAY 55-9837 produced $46 \pm 8\%$ of the maximum VIP effect. The VPAC1 antagonist, PG 97-269 (16), had no effect on water retention (Fig. 6), but it completely abolished both the VPAC1- and VPAC2-selective agonist-induced increase in water retention (data not shown).

Cardiovascular effects of BAY 55-9837. Because PACAP27 causes peripheral vasodilation that elicits a compensatory increase in heart rate (14,26,27), the effects of BAY 55-9837 on MAP were determined in rats (Fig. 7). BAY 55-9837 decreased MAP in a dose-dependent fashion, with an ED_{50} of 440 pmol/kg. Heart rate, which is elevated in rats (~ 400 bpm), did not change with vehicle or peptide treatment. Blood pressure and heart rate also were measured in rats that had been continuously infused subcutaneously with $300 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ BAY 55-9837 for 5 days. BAY 55-9837 had no effect on MAP (control, 101 ± 9 mmHg, $n = 3$; BAY 55-9837, 98 ± 6 mmHg, $n = 3$) or heart rate (control, 329 ± 37 bpm; BAY 55-9837, 329 ± 26 bpm).

DISCUSSION

The hallmarks of type 2 diabetes are increased glucose output by the liver, peripheral insulin resistance, and impaired insulin secretion by the pancreas. It is the impaired insulin secretion that actually creates the diabetic state, since adequate insulin secretion can maintain normal blood glucose levels in the face of insulin resistance. One approach to treating the disease is to maintain euglycemia through administration of sulfonylurea drugs that increase insulin levels or by injecting insulin itself. Both therapies produce significant bouts of hypoglycemia, because their onset of action is independent of the prevailing level of glucose. New therapies that retain or enhance glucose-dependent insulin secretion would be a significant advance, since they would reduce the risk of hypoglycemia.

BAY 55-9837 is a VPAC2-selective agonist that displays high selectivity for VPAC2 over VPAC1 and PAC1 in receptor binding and functional assays. This peptide stimulated glucose-dependent insulin secretion in isolated rat

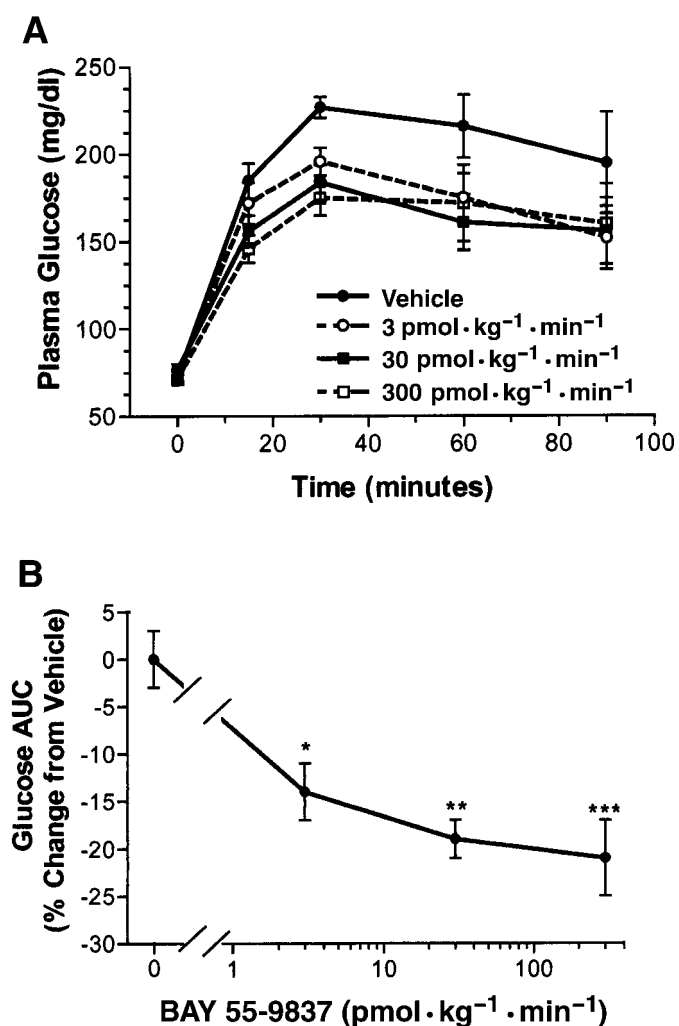


FIG. 5. Effect of 3-day continuous subcutaneous infusion of BAY 55-9837 on glucose disposal after an IPGTT in fasted rats. The rats were fasted for the final 16 h of continuous infusion and then given an IPGTT. **A:** Time course of glucose disposal during the IPGTT. Values are the means \pm SE for $n = 12$. **B:** Dose-dependent effects on glucose AUC after the IPGTT. The AUC of the vehicle-treated group was 762 ± 31 mg/dl. Each point is the mean \pm SE ($n = 12$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. vehicle control.

and human pancreatic islets in a glucose-dependent fashion, increased insulin synthesis in purified rat islets, and caused a dose-dependent increase in plasma insulin levels in rats. Continuous intravenous or subcutaneous infusion of the peptide reduced the glucose AUC following an IPGTT, with an efficacious dose as low as 1.3 or 3 $\text{pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, respectively. Neither hypoglycemia nor receptor desensitization were detected during continuous infusion for as long as 6 days.

Insulin secretion from pancreatic β -cells is regulated by blood glucose (28). cAMP is one of the major regulators of glucose-dependent insulin secretion. Little, if any, effect of cAMP on insulin secretion occurs in the absence of elevated glucose levels (29). Secretagogues such as PACAP and GLP-1 signal through the cAMP system to regulate insulin secretion in a glucose-dependent fashion (30). In addition, GLP-1 (31–34) and PACAP (35) have stimulatory effects on insulin gene expression and biosynthesis through cAMP (36–38). Current findings suggest that the stimulatory effect of PACAP on insulin biosynthe-

TABLE 1
Plasma level of selected hormones and metabolites after 3 days of continuous intravenous infusion of BAY 55-9837 at 13 $\text{pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$

	Vehicle	BAY 55-9837
FFA (mmol/l)	0.49 ± 0.11	0.45 ± 0.10
Lactate (mmol/l)	1.50 ± 0.90	1.54 ± 0.20
Cholesterol (mg/dl)	62 ± 39	72 ± 9
Triglycerides (mg/dl)	56 ± 12	60 ± 11
Insulin (ng/ml)	0.58 ± 0.57	0.80 ± 0.73
C-peptide (pmol/l)	375 ± 157	496 ± 218
Prolactin (ng/ml)	24.7 ± 5.5	34.6 ± 28.6
Epinephrine (ng/ml)	28.8 ± 30.6	9.2 ± 10.6
Corticosterone (ng/ml)	709 ± 88	698 ± 188
Glucagon (pmol/l)*	28.2 ± 10.6	28.4 ± 5.3

Data are for $n = 6$ (vehicle) or $n = 5$ (BAY 55-9837). *Glucagon was measured after 3 days of continuous intravenous infusion of 130 $\text{pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ BAY 55-9837 ($n = 6$).

sis is at least in part mediated by the VPAC2 receptor. However, cAMP elevation may not be sufficient to explain this effect, since glucagon—although it stimulates cAMP and insulin release in pancreatic islets (39)—causes repression of insulin gene transcription (40).

Clinical data with GLP-1 support the beneficial effect of raising cAMP levels in β -cells. Infusion of GLP-1 in poorly controlled patients with type 2 diabetes normalized their fasting blood glucose levels (41), and longer infusions improved their β -cell function to that of normal subjects (42). GLP-1 also improved the β -cell's ability to respond to glucose in subjects with impaired glucose tolerance (43). However, nearly the same doses of GLP-1 caused undesirable gastrointestinal effects (44), and this may limit its usefulness as a treatment for type 2 diabetes.

PACAP27 also increased insulin levels in mice (13) and humans (14), but it did not alter glucose levels. We have hypothesized that this lack of effect on glucose may be due to activation of VPAC1, causing increased hepatic glucose production that counteracts the VPAC2-mediated increase in insulin secretion, and that a VPAC2-selective agonist would both increase insulin secretion and enhance glucose disposal. Indeed, we demonstrate for the first time that a VPAC2-selective agonist increases both insulin levels and glucose disposal (Figs. 4 and 5). Furthermore, when animals are dosed at 100 times the maximum

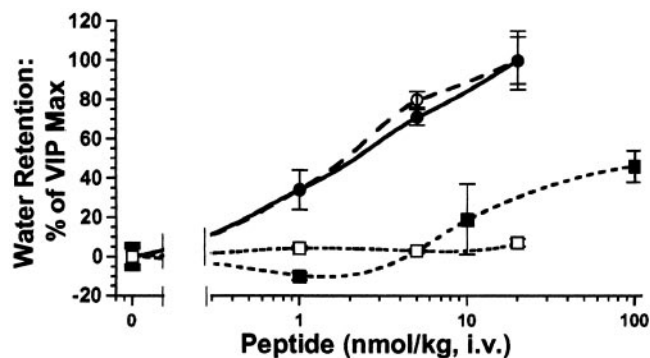


FIG. 6. Effect of intravenously administered peptides on intestinal water retention. Values are the means \pm SE ($n > 4$). The average control value was 0.6 ± 0.18 g, and the maximum effect was 2.3 ± 0.14 g water. ●, VIP; ○, VPAC1 agonist [K15, R16, L27]VIP(1-7)/GRF(8-27); □, VPAC1 antagonist PG 97-269; ■, BAY 55-9837.

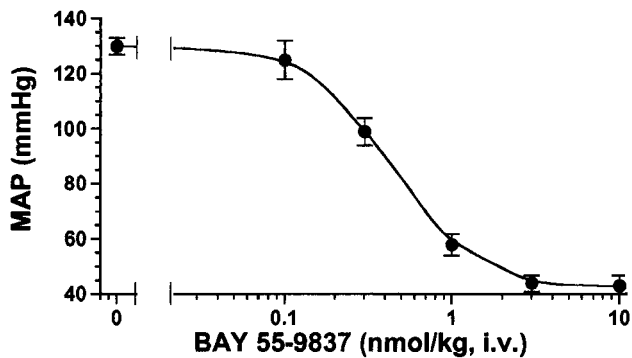


FIG. 7. Effects of intravenous bolus injection of BAY 55-9837 on MAP in fasted Wistar rats. Values are means \pm SE ($n = 3$) for each data point.

effective dose ($100 \text{ pmoles} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) (Fig. 4B), the effect of BAY 55-9837 on glucose is diminished. This effect may be due in part to the cross-activation of the VPAC1 receptor by the peptide at this high dose, causing hepatic glucose output. We also made the observation that at the highest dose of GLP-1 tested (1 nmol/kg), its effect on insulin was diminished compared with a lower dose of 0.1 nmol/kg (Fig. 4A). Other investigators have made similar observations with insulinotropic peptides, including GLP-1 and exendin-4 (45); however, the mechanism underlying this phenomenon is not known at this time.

The role of VPAC1 in insulin secretion is unclear. When rats were given an intravenous injection of 1 nmol/kg of the VPAC1-selective agonist [K15, R16, L27]VIP(1-7)/GRF(8-27), it increased plasma insulin levels by twofold, but, consistent with the role of VPAC1 in enhancing hepatic glucose production, it also increased blood glucose levels by 14% 30 min after an IPGTT (data not shown). The VPAC1-selective agonist displayed dose-dependent effects on intestinal water retention that were identical to those of VIP, whereas the effect of the VPAC2-selective agonist was much less. Moreover, the increased water retention induced by either the VPAC1 or VPAC2 agonist was abolished by coadministration of the VPAC1-selective antagonist PG97-269 (data not shown). These results strongly suggest that VIP-induced watery diarrhea in rats is mediated by VPAC1 receptors. This likely would make VPAC1-selective agonists unsuitable for the treatment of type 2 diabetes.

Activation of the PAC1 receptor also has been implicated in glucose homeostasis, based on the observation that PAC1-knockout mice have impaired glucose tolerance due to a reduced insulinotropic response to glucose (46). However, PAC1 may be the major mediator of the heart rate increase induced by PACAP. In our hands, PACAP27 and the PAC1-selective agonist, maxadilan (47), increased the heart rate in dogs by twofold when the peptides were injected intravenously at 0.1 nmol/kg, whereas VIP and the VPAC1-selective agonist increased heart rate by only 10–20%. The VPAC2-selective agonist had no effect at the same dose (data not shown). The increase in heart rate due to PAC1 activation may be mediated by increased catecholamine release from the adrenal glands (48,49). Consistent with our findings in dogs, the VPAC2-selective agonist BAY 55-9837 did not significantly increase catecholamine levels after 3 days of continuous intravenous

infusion at 10 times the effective dose (Table 1). These results suggest that PAC1-selective agonists also may not be useful for the treatment of type 2 diabetes.

In summary, selective activation of VPAC2 by BAY 55-9837 can result in enhancement of glucose-mediated insulin secretion and concomitant increase in glucose disposal without hypoglycemia. These results suggest that a VPAC2-selective agonist may serve as a therapeutic agent for the treatment of type 2 diabetes.

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