A novel mechanism for the suppression of a voltage-gated potassium channel by glucose-dependent insulinotropic polypeptide (GIP): Protein kinase A (PKA)-dependent endocytosis.

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Abbreviations list
GIP, glucose-dependent insulinotropic polypeptide; GIPR, GIP receptor; PKA, protein kinase A; KATP, ATP-sensitive K⁺; KCA, calcium-activated K⁺; Kv, voltage gated K⁺; HEK, human embryonic kidney; GLP-1, glucagon-like peptide-1; GSIS, glucose-stimulated insulin secretion; VDCCs, voltage-dependent Ca²⁺ channels; PKA, protein kinase A; WT, wild-type; PDE, phosphodiesterase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; TM, transmembrane; GPCR, G protein-coupled receptor; cAMP, cyclic AMP; CMV, cytomegalovirus; mry-DIP, myristoylated dynamin inhibitory peptide; GFP, green fluorescent protein; NMDA, N-methyl-D-aspartate; CaMK, Calcium/calmodulin-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; KRBH, Krebs-Ringer buffer with HEPES; RIA, radioimmunoassay; ANOVA, analysis of variance.
Summary
The mechanisms involved in glucose regulation of insulin secretion by ATP-sensitive (K_{ATP}) and calcium-activated (K_{CA}) potassium channels have been extensively studied, but less is known about the role of voltage-gated (K_v) potassium channels in pancreatic β-cells. The incretin hormone, Glucose-dependent insulinotropic polypeptide (GIP) stimulates insulin secretion by potentiating events underlying membrane depolarization and exerting direct effects on exocytosis. In the present study, we identified a novel role for GIP in regulating K_v1.4 channel endocytosis.

In GIP receptor expressing HEK-293 cells, GIP reduced A-type peak ionic current amplitude of K_v1.4 via activation of protein kinase A (PKA). Using mutant forms of K_v1.4 with Ala-Ser/Thr substitutions in a potential PKA phosphorylation site, C-terminal phosphorylation was shown to be linked to GIP-mediated current amplitude decreases. Proteinase K digestion and immunocytochemical studies on mutant K_v1.4 localization following GIP stimulation demonstrated phosphorylation-dependent rapid endocytosis of K_v1.4. Expression of K_v1.4 protein was also demonstrated in human β-cells; GIP treatment resulting in similar decreases in A-type potassium current peak amplitude to those in HEK-293 cells. Transient overexpression in INS-1 β-cells (clone 832/13) of wild-type (WT) K_v1.4, or a T601A mutant form resistant to PKA phosphorylation, resulted in reduced glucose-stimulated insulin secretion; WT K_v1.4 overexpression potentiated GIP-induced insulin secretion, whereas this response was absent in T601A cells. These results strongly support an important novel role for GIP in regulating K_v1.4 cell surface expression and modulation of A-type potassium currents, that is likely to be critically important for its insulinotropic action.

Introduction
Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are the two major intestinal hormones (incretins) involved in the stimulation of insulin secretion during a meal (1, 2). Glucose stimulated insulin secretion (GSIS) is mediated via closure of ATP-sensitive K^+ (K_{ATP}) channels resulting in membrane depolarization, activation of voltage-dependent Ca^{2+}-channels (VDCCs) and increases in intracellular Ca^{2+}, followed by membrane repolarization by voltage-dependent K^+ (K_v) and Ca^{2+}-sensitive K (K_{CA}) channels (3-5). Incretins act by potentiating the events underlying membrane depolarization in addition to exerting direct effects on exocytosis. These events ultimately depend upon incretin interaction with its cognate 7-transmembrane (TM) G protein-coupled receptor and activation of proximal signal transduction pathways. In the case of GIP, these include stimulation of the adenyl cyclase/cyclic AMP (cAMP)/protein kinase A (PKA) module, and activation of phospholipase A_2 (PLA_2) (6), protein kinase B (PKB) (7) and mitogen-activated protein kinases (MAPK) (8). There is little known regarding the effect of incretins on membrane repolarization of the β-cells (5).

Voltage-gated potassium channels (K_v channels) belong to the six-TM family of K^+ channels consisting of K_v1 to K_v11 subfamilies (9) and are involved in repolarization of excitable cells (10). They are of interest as potential therapeutic
targets in diabetes because blockade of K<sub>V</sub> channels would be expected to prolong the pancreatic β-cell action potential, sustain the opening of VDCCs and thereby potentiate glucose-induced insulin release (5). Mammalian K<sub>V</sub>1.Xs have been cloned and characterized in heterologous expression systems and they generate at least two different types of outward potassium currents classified on the basis of their inactivation properties: delayed rectifier steady-state currents, which do not rapidly inactivate, and A-type transient currents which inactivate rapidly by N-type inactivation (11, 12). Delayed rectifier current is produced by K<sub>V</sub>1.1, K<sub>V</sub>1.2, K<sub>V</sub>1.3, K<sub>V</sub>1.5 and K<sub>V</sub>1.6 and A-type current is produced by K<sub>V</sub>1.4 (13, 14). Although both types of currents are present in pancreatic β-cells, most of the studies have concentrated on the delayed-rectifier K<sub>V</sub> channels. The current study focused on a potential role for GIP in the regulation of K<sub>V</sub>1.4, a prominent member of the K<sub>V</sub>1 family and one of several channel proteins giving rise to potassium currents proposed to be involved in the regulation of insulin secretion. Using GIPR-expressing human embryonic kidney (HEK)-293 cells, we demonstrated that GIP decreases peak current amplitude of K<sub>V</sub>1.4 via a process involving PKA activation. In parallel studies, K<sub>V</sub>1.4 was shown to be expressed in human pancreatic β-cells and GIP decreased A-type potassium current amplitude in these cells. Additionally, GIP was shown to induce phosphorylation and dynamin-dependent endocytosis of K<sub>V</sub>1.4 and transient over-expression in INS-1 (832/13) β-cells of wild-type (WT) K<sub>V</sub>1.4, or a T601A mutant form resistant to PKA phosphorylation, resulted in reduced GSIS; WT overexpression potentiated GIP-induced insulin secretion, whereas the loss of PKA-dependent phosphorylation (T601A cells) ablated this effect. These results therefore provide compelling evidence for a role for GIP-induced down-regulation of K<sub>V</sub>1.4, via phosphorylation-dependent endocytosis of the channel protein, in the modulation of insulin secretion.

### Experimental Procedures

**Generation of a GIPR-HEK-293 cell line**— HEK-293 cells were grown in DMEM (Invitrogen Inc, Burlington, ON), supplemented with 5 % FBS (Sigma Aldrich, Oakville, ON) and penicillin-streptomycin (50 IU/ml-50 µg/ml; Invitrogen), and transfected with GIPR/pcDNA3 plasmid, expressing rat pancreatic islet GIP receptor cDNA under control of the CMV promoter. Transfections were performed using LIPOFECTAMINE 2000<sup>TM</sup> reagent (Invitrogen) for 4 h according to the manufacturer’s instructions. Stably transfected cells were selected with G418 (Invitrogen) and GIPR-HEK 293 cell clones were analyzed by quantitative real-time RT-PCR to check GIPR mRNA expression levels and by Western blotting to confirm GIPR protein expression respectively.

**cDNA constructions of K<sub>V</sub>1.4 plasmids and transient transfections in GIPR-HEK-293 cells**— K<sub>V</sub>1.4 cDNA was cloned into the pEGFP-N2 vector (Clontech Laboratories, Inc., Palo Alto, CA) and various constructs, as detailed in the Results, were prepared by PCR with HindIII and EcoRI insertions for directed cloning. Site-directed mutant constructs were prepared using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). All transfection plasmids were prepared using the Qiagen Plasmid Midi Kit (Qiagen Inc, Valencia, CA). GIPR-HEK-293 cells were plated at a density of 2 X 10<sup>5</sup> cells/glass coverslip in 35-mm dishes. On the following day,
transfection was performed with 1.5 µg of the indicated Kv1.4 plasmids using LIPOFECTAMINE 2000™ transfection reagent (Invitrogen), according to the manufacturer’s instructions.

**Enzyme Activity Assay of PKA**—PKA activity was measured using a PKA kinase activity assay kit (Stressgen, Mississauga, ON) according to the manufacturer’s protocol. The enzyme activity was normalized to protein concentration and shown as the relative activity to Control.

**Kv1.4 Protein purification and in vitro phosphorylation**—Kv1.4 cDNA was prepared by PCR and subcloned into the pGex4T3 vector (Amersham Pharmacia Biotech, Piscataway, NJ). Glutathione-S-Transferase (GST)-Kv1.4 fusion protein was purified from BL21 (DE3) E. coli expressing pGex4T3/ Kv1.4. GST-fusion proteins were induced by treatment with 1 mM IPTG (Isopropyl-B-D-thiogalactopyranoside, Sigma) for 4 h and the bacteria were harvested by centrifugation, resuspended in PBST-100, pH 7.4 (phosphate-buffered saline, 1% Triton-X100, 1 mmol/L EDTA) and sonicated (Branson Ultrasonic Corp, Brandury, CT) to release the proteins. GST-Kv1.4 fusion protein was affinity purified using a glutathione Sepharose 4B (Amersham Pharmacia Biotech) column and bound protein was eluted with 100 mmol/L NaCl solution containing 25 mmol/L reduced glutathione (Sigma). After affinity purification, Kv1.4 proteins were released from GST fusion protein through thrombin digestion. In vitro phosphorylation reactions were performed at 30°C in a volume of 40 µl of MES buffer (50 mM MES, pH 6.9, 10 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol) and initiated with the addition of 5 µCi of [γ-32P]ATP and unlabeled ATP to a final concentration of 100 µM. For determination of the time course of phosphorylation of target protein, 50 µg of Kv1.4 protein were incubated with 5 µg/ml of recombinant PKA catalytic subunit (active PKA; Sigma) or GIP-treated GIPR-HEK-293 cellular extracts for 1 min to 2 hr. Protein phosphorylation was determined by terminating the reactions with 2× SDS sample buffer, resolving the samples on 12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels, and drying the gels for autoradiography.

**Western blot Analysis**—Proteins (25 µg protein/well) from each sample were separated on a 12.5% SDS-PAGE gel and transferred onto nitrocellulose (Bio-Rad Laboratories, Mississauga, ON) membranes. Probing of the membranes was performed with antibodies against Kv1.4 and β-tubulin. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia) using horseradish peroxidase-conjugated IgG secondary antibodies.

**Islet isolation and cell culture**—Human islets were isolated from the pancreas of adult organ donors using collagenase duct perfusion, gentle dissociation and density gradient purification at the Ike Barber Islet Transplantation Laboratory (Vancouver General Hospital, Vancouver, Canada) (15). The Research Ethics Board of the University of British Columbia provided ethics approval. Islets were dispersed to single cells and plated on laminin-coated glass coverslips in 35-mm dishes in CMRL media supplemented with 10% fetal bovine serum and penicillin-streptomycin (50 IU/ml-50 µg/ml; Invitrogen).

**Electrophysiological studies**—To record ionic current, we used a superfusion solution containing the following (in mM): NaCl, 135; KCl, 5; MgCl2, 1; sodium acetate, 2.8; HEPES, 10; CaCl2, 1; adjusted to pH 7.4 using NaOH. The patch pipettes were filled with the pipette solution containing (in mM): KCl, 130;
EGTA, 5; MgCl₂, 1; HEPES, 10; Na₂ATP, 4; GTP, 0.1; adjusted to pH 7.2 with KOH. All chemicals were from Sigma. Whole cell current recording and data analysis were done using an Axopatch 200B amplifier and pClamp 8 software (Axon Instruments, Foster City, CA) and pipettes with a resistance of 1-3 MΩ were used. HEK-293 cells were depolarized to +60 mV for 900 ms from a holding potential of −100 mV. All whole cell recordings were performed at room temperature (20-23 °C).

Proteinase K digestion experiments— For proteinase K digestion (16, 17), GIPR-HEK-293 cells were transfected with a cDNA coding for Kv1.4 bearing a Green Fluorescent Protein (GFP) tag at the C-terminus (Kv1.4-EGFP). Cells incubated with 100 nM GIP for indicated periods of time, were washed three times with ice-cold PBS and incubated with 10 mM HEPES, 150 mM NaCl, and 2 mM CaCl₂ (pH 7.4) with 200 µg/ml proteinase K at 37 °C for 30 min. The cells were then harvested and proteinase K digestion was quenched by adding ice-cold PBS containing 6 mM phenylmethylsulfonylfluoride and 25 mM EDTA. This was followed by SDS-PAGE and immunoblotting and probing of the membranes was performed with antibodies against GFP and β-tubulin. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia) using horseradish peroxidase-conjugated IgG secondary antibodies.

Confocal microscopy— GIPR-HEK 293 cells were transfected with WT or mutant forms of Kv1.4-EGFP plasmids and treated for 10 mins with GIP (100 nM). Cells were then fixed and proteinase K digested and Alexa fluor® 488 dye conjugated anti-rabbit antibody. Cell nuclei were counterstained with DAPI (4’, 6-diamino-2-phenylindole) and transfected cells were imaged using a Zeiss laser scanning confocal microscope (Axioskop2).

Islet embedding in agar and confocal microscopy— Human islets were fixed in 4% paraformaldehyde in PBS for 30 mins at room temperature. After spinning down, islets were resuspended in PBS, mixed with an equal volume of 2% agar solution and added to a mold. Agarose embedded sections were processed for a double immunostaining for Kv1.4 and insulin. The sections were incubated with Kv1.4 and insulin antibodies and visualized with Alexa fluor® 488 conjugated anti-mouse secondary antibody and Texas Red® dye conjugated anti-rabbit antibody. Cell nuclei were counterstained with DAPI and imaged using a Zeiss laser scanning confocal microscope (Axioskop2). All imaging data were analyzed using the Northern Eclipse program (ver.6).

Insulin secretion— β-INS-1 cells (clone 832/13) were kindly provided by Dr. C. B. Newgard (Duke University Medical Center, Durham, North Carolina). Cells were cultured in 11 mM glucose RPMI 1640 (Sigma) supplemented with 2 mM glutamine, 50 µM ß-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 10% fetal bovine serum, 100 unit/ml penicillin G-sodium and 100 µg/ml streptomycin sulfate. INS-1 cells were plated at a density of 1 × 10⁶ cells/well. On the following day, transfection was performed with 2 µg of control vector, pEGFP-N2, mutant T601A Kv1.4-EGFP or WT Kv1.4-EGFP. Transfections were performed using LIPOFECTAMINE 2000™ reagent (Invitrogen) for 4 h according to the manufacturer’s instructions. On the following day, cells were treated with GIP and incubated for 2 h at 37 °C in KRBH-buffer containing low (2.5 mM) or high glucose (25 mM). Insulin release into the
medium was determined using a radioimmunoassay (RIA) kit (Linco Research Inc. St. Charles, MO).

**Statistical Analysis**—Data are expressed as means ± Standard Errors of the Mean (SEM) with the number of individual experiments presented in the figure legend. Data were analyzed using the non-linear regression analysis program PRISM (GraphPad, San Diego, CA) and significance was tested using Student’s t-test or analysis of variance (ANOVA) with a student Newman-Keuls post hoc test (P < 0.05) as indicated in figure legends.

**Results**

1. GIP decreases peak current amplitude of KV1.4 in GIPR-HEK-293 cells.

GIPR expressing HEK-293 cells (GIPR-HEK-293) were used as an in vitro model system to characterize the modulation of KV1.4 currents by GIP treatment. HEK-293 cells were stably transfected with a rat islet GIPR cDNA construct under control of the cytomegalovirus (CMV) promoter. The resulting GIPR-HEK 293 cell clones transiently transfected with KV1.4-EGFP cDNA (GIPR-HEK-293-KV) demonstrated voltage-activated, rapidly inactivating outward currents (Fig. 1A) with properties similar to KV1.4 currents previously reported in insulin-secreting cells (5). Administration of GIP (1-42) to GIPR-HEK-293-KV cells resulted in a decrease in peak current amplitude that was initiated within 1 min and decreased by 44.0 ± 5.7 % at 5 min (Fig. 1 A and B). The decrease in peak current amplitude was not observed in GIP (1-42)-treated HEK-293 cells, which do not express the GIPR (Control-I), or in GIPR-HEK-293-KV cells treated with the non-insulinotropic truncated form of GIP (GIP (19-30)) (Control-II). The effect of GIP on the decrease of peak current amplitude was concentration-dependent, with 1 nM GIP producing a significant reduction (Fig. 1C). Other macroscopic properties of KV1.4 were next studied to determine whether GIP affects the gating properties of KV1.4. As shown in Fig 1D-F, GIP did not alter the properties of activation (V0.5 Control = -27.6 ± 2.5 mV vs. V0.5 GIP = -25.1 ± 1.1 mV), inactivation (V0.5 Control = -54.5 ± 2.7 mV vs. V0.5 GIP = -52.9 ± 1.7 mV) or recovery from inactivation (\( \tau \) Control = 4.6 ± 0.6 s vs. \( \tau \) GIP = 4.9 ± 0.3 s), indicating that the effect of GIP is not mediated by changes in the macroscopic gating properties of KV1.4.

2. GIP activates Protein Kinase A (PKA) in GIPR-HEK-293-KV cells and GIP-stimulated PKA activation is involved in the decrease of KV1.4 peak current amplitude.

Mechanisms involved in the decrease of KV1.4 peak current amplitude by GIP treatment were next studied. The possibility that changes in KV1.4 peak current amplitude were linked to activation of adenylyl cyclase and PKA was first examined, since this a well established GIP signaling module involved in the regulation of insulin secretion. Treatment of GIPR-HEK-293-KV cells with 100 nM GIP resulted in increased PKA activity (Fig. 2A), apparent 10 min after initiation of GIP treatment, thereafter quickly decreasing with time. GIP stimulation was concentration-dependent, with an EC50 value of 2.10 ± 0.22 nM (Fig. 2B). To establish that GIP-induced activation of PKA was associated with decreases in KV1.4 peak current amplitude, H-89 and Rp-cAMP, selective inhibitors of PKA were applied during electrophysiological
recordings. The inhibitor H-89 significantly blocked GIP-stimulated PKA activation (Fig. 2A and B) and the decreases of Kv1.4 peak current amplitude (Fig. 2C). Similarly, the cAMP antagonist Rp-cAMP eliminated the effect of GIP on Kv1.4 peak current amplitude (Fig. 2C). Taken together, these results demonstrate that GIP-stimulated PKA activation is involved in the regulation of Kv1.4.

3. GIP-stimulated PKA activation resulted in the phosphorylation of Kv1.4 and decreases in Kv1.4 peak current amplitude.

In order to determine whether PKA could phosphorylate Kv1.4, purified recombinant Kv1.4 protein was incubated with the catalytic subunit of PKA and 32P-ATP in vitro. PKA-induced phosphorylation of Kv1.4 protein was apparent 1 min after the initiation of PKA treatment and it was sustained for 2 h (Fig. 3A). Similar rapid onset and sustained phosphorylation of Kv1.4 was observed with GIP-treated GIPR-HEK-293-Kv cellular extracts (Fig. 3B), indicating the involvement of GIP-stimulated PKA activation for the phosphorylation of Kv1.4. To identify the functional region of Kv1.4 involved in GIP-mediated phosphorylation, electrophysiological responses to GIP were recorded in Kv1.4 mutants with deletions at the N- or C-termini. As shown in Fig 3C, △N147 Kv1.4, with 147 amino acids deleted from the N-terminus, showed delayed inactivation compared to WT Kv1.4. The delayed inactivation has been previously shown to result from deletion of an amino-terminal inactivating “ball” domain, that acts as an intracellular tethered blocker of the open channel (12). GIP was found to retain the ability to reduce peak current amplitude expressed by △N147 Kv1.4. On the other hand, the mutant △C55 Kv1.4, with 55 amino acids deleted from C-terminus, demonstrated no responsiveness to GIP (Fig. 3C). These results therefore indicate that the C-terminus in Kv1.4 is responsible for GIP-mediated effects. Inspection of the amino acid composition of the C-terminus of Kv1.4 revealed several motifs that are potentially involved in regulating its subcellular localization and interaction with other proteins. The VXXSL motif is critical for glycosylation and surface membrane localization of the channel (18) and the sequence ETDV is the binding site for PDZ domain proteins, such as PSD-95 and SAP-97 (19, 20). Additionally, we found a potential PKA phosphorylation site, SSTSSS, in the C-terminus of Kv1.4. To determine whether phosphorylation of Kv1.4 is linked to GIP-mediated decreases in current amplitude, studies were performed on the effect of substituting alanine for each of the phosphorylatable serine and threonine residues: S599A, S600A, T601A, S602A, S603A and S604A. When in vitro phosphorylation of the purified alanine-substitution mutants was examined, all proteins, except T601A, were phosphorylated by PKA (Fig. 3D). Electrophysiological recordings showed that GIP was able to reduce peak current amplitude with GIPR-HEK-293 cells expressing S599A, S602A, S603A and S604A, but not T601A (Fig. 3E). The mutant S600A was not functionally expressed in the plasma membrane (Data not shown). Taken together, these results indicate that phosphorylation of Kv1.4 by GIP-stimulated PKA activation is linked to the decrease in peak current amplitude and phosphorylation of T601 in the C-terminus is a critical step in this process.
4. GIP-induced decreases in Kv1.4 peak current amplitude are a result of channel endocytosis.

Although phosphorylation of ion channels has been reported in several systems, in most cases it is associated with changes in inactivation rate and recovery from inactivation, rather than changes in peak current amplitude. Membrane protein phosphorylation directly affects interactions with intracellular proteins involved in many cellular pathways, and it was hypothesized that GIP-mediated decreases in Kv1.4 peak current amplitude were mediated by phosphorylation-associated endocytosis. To examine this proposal, the cellular localization of Kv1.4 during treatment with GIP was studied using proteinase K digestion. The GFP tag on Kv1.4 is located intracellularly at the C-terminus. Proteinase K is able to randomly digest extracellular regions of Kv1.4 channels in the plasma membrane, thus producing a digested form, whereas intracellular channel is protected. As shown in Fig. 4A, surface membrane expression levels of Kv1.4 (S) decreased with GIP treatment and this was evident at 5 min following initiation of treatment. Confocal microscopy also revealed that Kv1.4 channels present in the plasma membrane were greatly reduced by treatment with GIP (Fig. 4B). To determine whether GIP-mediated decreases in Kv1.4 peak current amplitude were mediated by dynamin-dependent endocytosis, electrophysiological recordings were performed on GIPR-HEK-293 cells treated with GIP (100 nM) in the presence or absence of myristoylated dynamin inhibitory peptide (mry-DIP: myr-Gln-Val-Pro-Ser-Arg-Pro-Asn-Arg-Ala-Pro-NH₂) (21). Dynamin is a large GTPase implicated in the budding and scission of nascent vesicles from parent membranes (22). It has been extensively studied in the context of clathrin-coated vesicle budding from the plasma membrane, but it is also involved in the budding of clathrin-coated vesicle from other compartments and budding of caveoli, phagocytosis and vesicle cycling at synapses (23, 24). As shown in Fig. 4C, mry-DIP completely abolished the effect of GIP on Kv1.4. These results therefore strongly suggest that GIP induces the endocytosis of Kv1.4 and that these processes are responsible for the GIP-mediated decrease in Kv1.4 peak current amplitude.

5. Phosphorylation is involved in GIP-induced retrograde trafficking of Kv1.4.

Next, we investigated the potential relationships between phosphorylation and endocytosis of Kv1.4 in response to treatment with GIP. The mutant channels S599A, T601A, S602A, S603A and S604A were transiently expressed in GIPR-HEK-293 cells and confocal microscopy was performed on fixed cells following treatment with GIP. Endocytosis was observed with S599A, S602A, S603A and S604A-transfected GIPR-HEK-293 cells with GIP treatment, but not with T601A-transfected cells (Fig. 5). These results correlate well with the electrophysiological recordings and the combined data demonstrate that phosphorylation and endocytosis are consecutive processes responsible for GIP’s effects on Kv1.4 channel distribution.

6. Kv1 channel expression in human islets.

Kv channels have been shown to play an important role in the regulation of glucose-dependent insulin secretion in rodent islets (5). As shown in Fig 6, Kv1.4 protein is
also expressed in human islets (A), mainly restricted to insulin-expressing pancreatic β-cells (B). Electrophysiological recordings from human islet cells revealed a typical A-type outward potassium current and 100 nM GIP treatment resulted in a decrease in A-type peak current amplitude with a similar pattern to that observed in Kv1.4 currents in GIPR-HEK-293-Kv (Fig 6 C and D). The effect of GIP on peak current amplitude was reversible by washing-out and by 7 minutes following wash-out responses had almost returned to levels achieved prior to treatment with GIP. In order to determine whether A-type current in human islets is mediated by Kv1.4, 4-aminopyridine (4-AP; 1 mM), a conventional Kv channel blocker, was applied (10). As shown in figure 6E, 4-AP treatment resulted in approximately 50% reduction in A-type potassium current. A-type potassium channels of different types exhibit variable sensitivity to 4-AP. For example with cloned Kv1.4 channels 73% of peak current was found to be blocked by 1 mM 4-AP (25) whereas, in contrast, Kv4.2 was seven-fold less sensitive to 4-AP than Kv1.4 (26) and Kv3.4 exhibited much greater sensitivity to 4-AP (µM range) (27). The sensitivity to 4-AP exhibited by the A-type current in human islets suggests that it is mediated by Kv1.4, and that GIP is able to confer its effect on peak current amplitude in a similar manner to that observed with GIPR-HEK-293-Kv cells.

7. Phosphorylation-dependent internalization of Kv1.4 participates in the effect of GIP on insulin secretion.

To establish that phosphorylation-dependent internalization of Kv1.4 contributes to GIP stimulation of insulin secretion, the T601A mutant form, that is resistant to PKA phosphorylation, or WT Kv1.4 channel, were transiently expressed in insulin-secreting β-INS-1 cells and channel internalization determined by proteinase K treatment. The β-INS-1 cell line (clone 832/13) was chosen because it lacks functional A-type current (data not shown), thereby excluding the involvement of endogenous Kv1.4 current in responses to GIP. As shown in Fig. 7A, GIP treatment did not decrease surface membrane expression of mutant T601A under either low (2.5 mM) or high (25 mM) glucose conditions, whereas WT Kv1.4 was internalized by GIP treatment in the presence of high, but not low, glucose. Expression of either WT or T601A mutant Kv1.4 channels reduced GSIS, compared to vector transfected cells (Fig. 7B), presumably because of a prolonged repolarization phase of the β-INS-1 cell action potential. As expected, GIP treatment did not significantly increase insulin secretion in the presence of low glucose in pEGFP-N2 vector, T601A or WT Kv1.4-transfected groups. However, under high glucose conditions, GIP treatment resulted in increased insulin secretion in all groups. GIP responses of β-INS-1 cells overexpressing WT Kv1.4 channels were potentiated compared to pEGFP-N2-transfected cells, whereas the loss of PKA-dependent phosphorylation (T601A cells) ablated this effect. Taken together, these results strongly suggest that phosphorylation-dependent internalization of Kv1.4 is an important component of GIP-potentiated insulin secretion.

Discussion

The ability of GIP to directly enhance GSIS in pancreatic β-cells has been attributed to GIPR activation leading to enhanced depolarization and increases in the intracellular calcium concentration as
well as direct effects on insulin exocytosis (3, 4, 28). Ion channels are the primary determinants of membrane excitability in most cells and they are regulated to maintain membrane potentials within specific limits. Frequently this occurs through modulation of the ion channel’s functional responses to extracellular stimuli. In pancreatic β-cells, insulin secretion is modulated by the activity of several different ionic currents. Among these are the three main potassium currents: inward rectifying potassium currents, including the ATP-sensitive (K<sub>ATP</sub>) channel and others, calcium-activated (K<sub>Ca</sub>) and voltage-gated (K<sub>V</sub>) currents (29-32). The molecular mechanisms involved in the regulation of K<sub>ATP</sub> and K<sub>Ca</sub> channels in pancreatic β-cells have been extensively studied, but considerably less is known about the K<sub>V</sub> channels. Since these channels are considered to be potential therapeutic targets for type 2 diabetes, it is important to establish their physiological role and mechanisms involved in regulating their activity. The current study was therefore initiated with the objective of identifying potential interactions between the incretin hormone GIP and K<sub>V</sub> channels. GIP transduces its biological effects on pancreatic β-cells by interacting with a 7-TM receptor, GIPR that is a member of the class II G protein-coupled receptor (GPCR) subfamily. The best characterized pathway by which GIP acts on insulin secretion in β-cells involves activation of the adenyl cyclase/cAMP/PKA pathway. Using HEK-293 cells co-expressing the GIPR and Kv1.4 (GIPR-HEK-293-Kv cells), we have now shown that GIP reduces peak current amplitude of Kv1.4 channels via a pathway inhibited by the selective inhibitors of PKA, H-89, and the cAMP antagonist, Rp-cAMP. In parallel experiments, it was shown that recombinant PKA catalytic subunits (Fig. 3A) or cell extracts from GIP-stimulated GIPR-HEK-293-Kv cells (Fig. 3B) increased phosphorylation of Kv1.4, and active PKA phosphorylated Thr601 in the C-terminus of Kv1.4 (Fig. 3D), thus substantiating the involvement of PKA signaling in GIP-induced effects on Kv1.4 current. This was confirmed by experiments showing that mutant T601A Kv1.4 channels could not be phosphorylated by PKA and peak currents in this mutant were resistant to GIP (Fig. 3D and E).

The macroscopic current in cells is regulated by two processes: 1. biophysical and biochemical modulation of surface membrane ion channel activity and 2. biosynthesis and trafficking of channel protein (33). Direct phosphorylation of channel proteins by serine/threonine and tyrosine kinases has been established as a mechanism by which ion channels are regulated. The delayed rectifier potassium channel Kv1.2 was the first example of a voltage-gated ion channel shown to be regulated by Ser/Thr phosphorylation (34, 35) and a range of voltage- and ligand-gated channels have been found to be regulated by tyrosine kinases, including N-methyl-D-aspartate (NMDA) receptors, VDCCs and a variety of potassium channels (36, 37). Previous studies have addressed the effects of Ser/Thr phosphorylation of the N-terminal domain of Kv1.4 on physiological responses. Calcium/calmodulin-dependent protein kinase (CaMK) has been shown to slow the inactivation of Kv1.4 currents by phosphorylating S123 in the cytoplasmic N-terminus (38). Treatment of Kv1.4-expressing Xenopus oocytes with phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, has been shown to lead to a biphasic change in the magnitude of peak current: an initial
increase followed by a later reduction (39). Although in most cases the precise mechanisms underlying the effects of Ser/Thr phosphorylation on channel function are unclear, the most commonly suggested mechanism is that phosphorylation-induced changes in channel structure alter its biophysical properties (40). In the present study, GIP reduced Kv1.4 peak current amplitude, without affecting macroscopic gating properties of Kv1.4 (Fig. 1A-F) and threonine phosphorylation of the C terminus by GIP-stimulated PKA activation also resulted in a decrease in Kv1.4 peak current amplitude (Fig. 3). These results imply that different mechanisms are involved, compared to previously reported phosphorylation of Kv channels.

The trafficking of ion channels is one of the processes involved in the modulation of plasma membrane macroscopic currents (33). The regulation of expression of Kv channels in the plasma membrane begins at the level of gene transcription and biosynthesis of the channel protein (41), with further control provided during insertion of the channel into the cell surface and by its regulated retrieval and degradation. Endocytosis was initially defined as a process by which substances are taken into the cell, but it is now recognized as an essential mechanism for the regulation of a variety of membrane proteins. Endocytosis is a first-order mechanism of internalization of membrane-bound proteins undergoing recycling or retrograde trafficking to be degraded. Endocytosis initiated by phosphorylation of Kv channels results in decreased ionic current density (35). In the present study, it was demonstrated that direct phosphorylation of Kv1.4 by GIP-stimulated PKA activation is involved in endocytosis of the channel protein (Fig. 3 and 5). Retrograde trafficking of Kv1.4 resulting in decreased peak current amplitude was observed following treatment with GIP (Fig. 4A and B) and dynamin-dependent endocytosis was involved in this process (Fig. 4C). In contrast, the non-phosphorylatable mutant T601A Kv1.4 was incapable of undergoing endocytosis, demonstrating the critical role played by phosphorylation in GIP-induced endocytosis of Kv1.4 (Fig. 5). The underlying molecular mechanism by which PKA-dependent phosphorylation is linked to endocytosis of Kv1.4 is not clear at the present time. Post-translational modifications of channel proteins by signaling molecules and resulting structural changes of channel proteins may affect protein-protein interactions between channel proteins and proteins involved in the endocytotic pathway.

The Kv1.4 channel was also demonstrated to be present in human pancreatic β-cells and GIP treatment decreased A-type ionic current amplitude (Fig. 6). There have been controversial reports regarding the expression patterns of Kv1 family channels in human islets. It has been reported that Kv1.1, Kv1.2 and Kv1.4 are not found by RT-PCR in human islets, while Kv1.5 and Kv1.6 are present (5). On the other hand, Yan et al. (42) reported that only Kv1.3 and Kv1.6 were detected by RT-PCR, with a very weak indication for Kv1.7. In the current study, we have shown that Kv1.4 protein is expressed in human pancreatic β-cells (Fig. 6B). These discrepant results might arise from phenotypic differences in channel composition between races or individuals. What is the likely effect of A-type potassium current down-regulation on pancreatic β-cell function? A-type current has an important role in the early repolarization phase of action potentials (10, 43). 4-AP potentiates insulin secretion
from rat islets and insulinoma cells stimulated by sulphonylureas, even in the absence of glucose (5). Therefore, it is reasonable to predict that the down-regulation of A-type current mediated by GIP-stimulated Kv1.4 endocytosis in pancreatic β-cells would enhance the duration and amplitude of action potentials, resulting in the prolongation of insulin secretion. This is supported by the demonstration that transient over-expression of WT Kv1.4 channels in INS-1 (832/13) β-cells resulted in potentiated insulin secretion in response to GIP, and that loss of a PKA phosphorylation site ablated this effect (Fig. 7B).

In summary, GIP-induced phosphorylation of Kv1.4 channel protein, resulting in endocytosis and decreases of ionic peak current amplitude, is likely to be an important pathway by which GIP acts as an insulinotropic hormone. This appears to be the first example of a physiological pathway directly linking hormone signaling to endocytosis of Kv channels. The combined effects of GIP and GLP-1 account for approximately 50% of the total insulin response to a meal and it is therefore clear that a deeper understanding of its mechanism of action is an important issue, with strong implications for the development of therapeutic agents for type 2 diabetes.

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Figure legends

Fig. 1. GIP decreases peak ionic current amplitude of Kv1.4 in GIPR-HEK 293 cells. A. Examples of Kv1.4 currents responses to GIP measured in GIPR-HEK-293-Kv cells. Scale bar indicates 10 nA. B. Time course of decreases in Kv1.4 peak current amplitude in response to GIP. GIPR-expressing HEK-293 cells were transfected with Kv1.4-EGFP plasmid and treated for the indicated periods of time with 100 nM of GIP. Currents were recorded in GIP-treated GIPR-HEK-293- Kv cells (GIP), GIP-treated HEK-293 cells not expressing GIPR (Control-I) and GIPR-HEK-293- Kv cells treated with an inactive form of GIP (19-30)-treated (Control-II). C. Concentration dependence of decreases in Kv1.4 peak current amplitude in response to GIP. Cells were prepared as described above and incubated with different concentration of GIP (in nM: 0, 1, 10, and 100). Currents were recorded at 5 min after GIP treatment. Peak current amplitudes at +60 mV were normalized to cell capacitance and represented as percentage ratio to 0 nM GIP group (max.=1). D-F. Effect of GIP on macroscopic Kv1.4 current kinetics. Activation properties (D), inactivation properties (E) and recovery time from inactivation (F) of Kv1.4 were determined during treatment with GIP. All data represent the mean ± S.E.M. from three to five independent experiments and significance was tested using Student’s t-test or ANOVA with a Newman-Keuls post hoc test, where * represents p<0.05 vs Basal.

Fig. 2. GIP activates PKA in GIPR-HEK-293-Kv cells and PKA activation is involved in the decrease in Kv1.4 ionic currents. A. Time course of PKA activation responses to GIP and effect of H-89. GIPR-HEK-293-Kv cells were stimulated for the indicated periods of time with 100 nM GIP in the presence or absence of H-89. H-89 (10 µM) was added to cells during a 30 min preincubation as well as during GIP stimulation. PKA activity assays were performed as described in Materials and Methods. B. Concentration-response effect of GIP on PKA activation and effect of H-89. GIPR-HEK-293-Kv cells were stimulated for 10 min with the indicated concentrations of GIP in the presence or absence of PKA inhibitor, H-89 or cAMP antagonist, Rp-cAMP. Current was recorded at zero time (Control) and 5 min after initiating GIP treatment (GIP). Peak current amplitudes at +60 mV were normalized to cell capacitance and represented as percentage ratio to Control (max.=1). All data represent the mean ± S.E.M. from four to six independent experiments and significance was tested using Student’s t-test or ANOVA with a Newman-Keuls post hoc test, where * represents p<0.05 vs Basal.

Fig. 3. GIP-stimulated PKA activation resulted in the phosphorylation of Kv1.4 and decreases in ionic current. A-B. In vitro phosphorylation of Kv1.4 by PKA. 50 µg of purified Kv1.4 protein were incubated for the indicated periods of time at 30 °C in kinase buffer containing 5 µCi of [γ-32P]ATP in the presence of recombinant protein kinase A catalytic subunit (A) and GIP-stimulated GIPR-HEK-293 cellular extracts (B). The
reactions were terminated by boiling in SDS sample buffer, and proteins were separated by SDS-PAGE. The gels were Coomassie-stained to visualize protein, dried, and exposed to X-ray film to visualize incorporated $^{32}$P. C. Electrophysiological recordings. Schematic diagram of the Kv1.4 serial deletion constructs and their functional elements are presented. Peak current amplitudes are shown at the far right, for comparison between WT and N-terminally deleted (ΔN147) or C-terminally deleted mutants (ΔC55) of Kv1.4. Currents were recorded at zero time (black bar) and 5 minutes after GIP-treatment (white bar) and peak current amplitudes at +60 mV were normalized to cell capacitance and represented as percentage ratio (max.=1). In the right panel, examples of current traces of WT, ΔN147 and ΔC55 of Kv1.4 ± GIP were shown and scale bar indicates 10 nA.. D. In vitro phosphorylation of mutant Kv1.4 by PKA. 50 µg of purified mutant Kv1.4 protein was incubated for 1 hr at 30 °C in kinase buffer containing 5 µCi of [$\gamma$-$^{32}$P]ATP in the presence of recombinant protein kinase A catalytic subunit. Reactions were performed as described in A-B. E. Electrophysiological recordings. Peak current amplitudes are shown at the far right, for comparison between WT and the site-directed mutants (S599A, T601A, S602A, S603A and S604A) of Kv1.4. Currents were recorded at zero time (black bar) and 5 minutes after GIP-treatment (white bar) and peak current amplitudes at +60 mV were normalized to cell capacitance and represented as percentage ratio (max.=1). On the right panel, examples of current traces of WT and the mutants of Kv1.4 ± GIP were shown and scale bar indicates 10 nA. All data represent the mean ± S.E.M. from four to six independent experiments and significance was tested using Student’s t-test, where * represents p<0.05 vs Basal.

Fig. 4. GIP treatment resulted in the endocytosis of Kv1.4 protein and decrease in ionic current. A. Determination of Kv1.4 subcellular distribution by proteinase K treatment. GIPR-expressing HEK-293 cells were transfected with Kv1.4-EGFP plasmid and treated for the indicated periods of time with 100 nM GIP. Proteinase K was applied for 30 min and cell lysates were analyzed for Kv1.4 by SDS-PAGE and immunoblotting. The arrow labeled S indicates the immunoblot position of surface Kv1.4, and the arrow labeled I indicates the immunoblot position of intracellular Kv1.4. B. Determination of Kv1.4 subcellular distribution by confocal microscopy. GIPR-expressing HEK-293 cells were transfected with Kv1.4-EGFP and treated for 10 min ± 100 nM GIP. Control GIPR-HEK-293-Kv cells (-GIP) and GIP-treated GIPR-HEK-293-Kv cells (+GIP) are shown. Immunocytochemical staining was performed using α-GFP antibody and imaged using a Zeiss laser scanning confocal microscope (Axioskop2). All imaging data were analyzed using the Northern Eclipse program (ver.6) and scale bar indicates 10 µm. C. Electrophysiological recordings. GIPR-expressing HEK-293 cells were transfected with Kv1.4-EGFP and treated for the indicated periods of time with 100 nM of GIP in the presence or absence of mry-DIP. Peak current magnitude at each time point was normalized to the peak current from zero time and example traces from mry-DIP treated cells are shown in the inset and scale bar indicates 10 nA. All data represent the mean ± S.E.M. from four to six independent experiments and significance was tested using Student’s t-test, where * represents p<0.05 vs Basal.

Fig. 5. Phosphorylation is involved in GIP-induced retrograde trafficking of Kv1.4. GIPR-expressing HEK-293 cells were transfected with the indicated mutant Kv1.4-EGFP
plasmids and treated for 10 min ± 100 nM GIP. Mutant K\textsubscript{V}1.4-EGFP transfected cells without GIP treatment are shown in the left panels (-GIP) and cells with GIP treatment are shown in the right panels (+GIP), respectively. Immunocytochemical staining was performed using α-GFP antibody and imaged using a Zeiss laser scanning confocal microscope (Axioskop2). All imaging data were analyzed using the Northern Eclipse program (ver.6) and scale bar indicates 10 μm.

**Fig. 6. K\textsubscript{V} channel expression in human islets.** **A. Determination of K\textsubscript{V}1.4 expression in human islets by Western blot analysis.** Total cellular extracts were prepared from human islets and Western blot analyses were performed using antibodies against K\textsubscript{V}1.4 and β-tubulin. Total cellular extracts from GIPR-HEK 293 cells and K\textsubscript{V}1.4-transfected GIPR-HEK 293 cells (GIPR-HEK 293 + K\textsubscript{V}1.4) were used as negative and positive controls for Western blotting, respectively. **B. Determination of K\textsubscript{V}1.4 expression in human pancreatic β-cells by confocal microscopy.** Human islets were embedded in agar and immunohistochemical staining was performed using K\textsubscript{V}1.4 and insulin antibody and imaging using a Zeiss laser scanning confocal microscope (Axioskop2). All imaging data were analyzed using the Northern Eclipse program (ver.6) and scale bar indicates 50 μm. C-E. Cells from human islets were briefly hyperpolarized to -100 mV followed by depolarization to +60 mV for 900 ms from a holding potential of −80 mV. All whole cell recordings were performed at room temperature (20-23 °C). **C. Example of current traces measured in human islet in response to treatment with GIP.** Scale bar indicates 2 nA. **D. Time course of decreases in A-type peak current amplitude in response to 100 nM GIP.** Peak current magnitude at each time point was normalized to the peak current from zero time. **E. Effect of 1 mM 4-AP on A-type current in human islet.** All data represent the mean ± S.E.M. from three to six independent experiments and significance was tested using Student’s t-test, where * represents p<0.05 vs Basal. Scale bar indicates 2 nA.

**Fig. 7. Phosphorylation-dependent internalization participates in the effect of GIP on insulin secretion.** **A. Determination of subcellular K\textsubscript{V}1.4 distribution by proteinase K treatment.** β-INS-1 cells (clone 832/13) were transfected with either mutant T601A K\textsubscript{V}1.4-EGFP or WT K\textsubscript{V}1.4-EGFP and incubated with 100 nM GIP for 10 mins. Proteinase K was applied for 30 min and cell lysates were analyzed for K\textsubscript{V}1.4 by SDS-PAGE and immunoblotting. The arrow labeled S indicates the immunoblot position of surface K\textsubscript{V}1.4, and the arrow labeled I indicates the immunoblot position of intracellular K\textsubscript{V}1.4. **B. Insulin secretory responses.** β-INS-1 cells (clone 832/13) were transfected with control vector, pEGFP-N2, mutant T601A K\textsubscript{V}1.4-EGFP or WT K\textsubscript{V}1.4-EGFP, treated with GIP and incubated for 90 min at 37 °C in KRBH-buffer containing low (2.5 mM) or high glucose (25 mM). Insulin release into the medium was determined by radioimmunoassay (RIA). All data represent the mean ± S.E.M. and significance was tested using ANOVA with a Newman-Keuls post hoc test, where * represents p<0.05 vs High glucose, -GIP, WT K\textsubscript{V}1.4, # represents p<0.05 vs High glucose, +GIP, T601A and & represents p<0.05 vs High glucose, +GIP, pEGFP-N2.
Figure 1.

A. 

Time (s)  
0 5 10 15 20 25 30 35  
Fraction recovery  
0.0 0.2 0.4 0.6 0.8 1.0 1.2 
Contol GIP

B. 

Voltage (mV)  
-150 -100 -50 0 50 100  
Normalized peak current  
0.0 0.2 0.4 0.6 0.8 1.0 1.2  
Control GIP

C. 

GIP concentration  
0 nM 1 nM 10 nM 100 nM  
Normalized peak current  
0.0 0.2 0.4 0.6 0.8 1.0 1.2  
0 nM 1 nM 10 nM 100 nM

D. 

Voltage (mV)  
-100 -80 -60 -40 -20 0 20 40 60 80 100  
Normalized peak current  
0.0 0.2 0.4 0.6 0.8 1.0 1.2  
Control I Control II GIP (100 nM)

E. 

Voltage (mV)  
-150 -100 -50 0 50 100  
Normalized peak current  
0.0 0.2 0.4 0.6 0.8 1.0  
Control GIP

F. 

Time (s)  
0 5 10 15 20 25 30 35  
Fraction recovery  
0.0 0.2 0.4 0.6 0.8 1.0 
Control GIP
Figure 2.

A. 

![Graph showing the relative PKA activity over time (mins). The graph compares GIP and GIP + H-89.](image)

B. 

![Graph showing the relative PKA activity with Log10[GIP] on the x-axis. The graph compares GIP and GIP + H-89.](image)

C. 

![Graph showing the normalized peak current for WT, H-89, and Rp-cAMP.](image)
Figure 3.

A.

B.

C.
Figure 3.

D.

E.

|        | WT : SSTSSS | S599A: ASTSSS | T601A: SSASSS | S602A: SSTASS | S603A: SSTSAS | S604A: SSTSSA |
|--------|-------------|---------------|---------------|---------------|---------------|---------------|
| Normalized peak current | | | | | | |
Figure 4.

A. 

B. 

C. 

Normalized peak current

Time (min)
Figure 5.

- GIP

+ GIP

S599A

T601A

S602A

S603A

S604A
Figure 6.

A.  

B.  

C.  

D.  

E.
Figure 7.

A. Tubulin: I → S

| Glucose | GIP | DNA   |
|---------|-----|-------|
| Low     | -   | T601A |
| Low     | +   | WT K_v1.4 |
| High    | -   |       |
| High    | +   |       |
| Low     | -   |       |
| Low     | +   |       |
| High    | -   |       |
| High    | +   |       |

B. Secreted insulin (ng/ml)

| Glucose | GIP | DNA   |
|---------|-----|-------|
| Low     | -   | pEGFP-N2 |
| Low     | +   |       |
| High    | -   | T601A  |
| High    | +   |       |
| Low     | -   |       |
| Low     | +   |       |
| High    | -   |       |
| High    | +   | WT K_v1.4 |

&

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*
A novel mechanism for the suppression of a voltage-gated potassium channel by glucose-dependent insulinotropic polypeptide (GIP): Protein kinase A (PKA)-dependent endocytosis

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