Original Article

Membrane Phosphoinositides Control Insulin Secretion Through Their Effects on ATP-Sensitive K^+ Channel Activity

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ATP-sensitive K^+ channels (K_{ATP} channels) of pancreatic β-cells play key roles in glucose-stimulated insulin secretion by linking metabolic signals to cell excitability. Membrane phosphoinositides, in particular phosphatidylinositol 4,5-bisphosphates (PIP_2), stimulate K_{ATP} channels and decrease channel sensitivity to ATP inhibition; as such, they have been postulated as critical regulators of K_{ATP} channels and hence of insulin secretion in β-cells. Here, we tested this hypothesis by manipulating the interactions between K_{ATP} channels and membrane phospholipids in a β-cell line, INS-1, and assessing how the manipulations affect membrane excitability and insulin secretion. We demonstrate that disruption of channel interactions with PIP_2 by overexpressing PIP_2-insensitive channel subunits leads to membrane depolarization and elevated basal level insulin secretion at low glucose concentrations. By contrast, facilitation of channel interactions with PIP_2 by upregulating PIP_2 levels via overexpression of a lipid kinase, phosphatidylinositol 4-phosphate 5 kinase, decreases the ATP sensitivity of endogenous K_{ATP} channels by ~26-fold and renders INS-1 cells hyperpolarized, unable to secrete insulin properly in the face of high glucose. Our results establish an important role of the interaction between membrane phosphoinositides and K_{ATP} channels in regulating insulin secretion. *Diabetes* 54:2852–2858, 2005

Pancreatic β-cells secrete insulin in response to glucose stimulus. The ATP-sensitive K^+ (K_{ATP}) channel, a complex of four inwardly rectifying K^+ channel Kir6.2 subunits and four sulfonylurea receptor 1 (SUR1) subunits, is a key component in this stimulus-secretion coupling process (1–3). The hallmark features of K_{ATP} channels are their sensitivities to intracellular nucleotides ATP and ADP, the derivatives of glucose metabolism (1,2). ATP inhibits channel activity, whereas ADP, in complex with Mg^{2+}, stimulates channel activity. It is now generally accepted that the physiological activity of K_{ATP} channels is regulated primarily by the relative concentrations of ATP and ADP (1,4,5). As plasma glucose increases, ATP concentration increases and ADP concentration decreases, resulting in K_{ATP} channel closure, membrane depolarization, Ca^{2+} influx, and insulin release. Conversely, when glucose decreases, the concentration ratio of ATP to ADP decreases, leading to K_{ATP} channel opening, membrane hyperpolarization, and termination of insulin secretion. The importance of ATP and ADP in regulating K_{ATP} channels in vivo has been confirmed by the finding that mutations that reduce channel sensitivity to ATP or MgADP are causative in permanent neonatal diabetes or congenital hyperinsulinism, respectively (5–9).

The discovery that membrane phosphoinositides, in particular the most abundant phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP_2) (10), stimulate K_{ATP} channel activity and antagonize the inhibitory effect of ATP in isolated membrane patches (11,12) has led to the proposal that in addition to ATP and ADP, PIP_2 may also be important in controlling the physiological activity of K_{ATP} channels in β-cells (11–14). However, to date, direct evidence for a role of membrane phosphoinositides in regulating K_{ATP} channel activity, hence regulation of insulin secretion, in β-cells is still lacking (15).

In addition to modulating the activity of transporters and ion channels, phosphoinositides play significant roles in membrane trafficking and cytoskeleton organization (16,17). Recent studies show that adequate PIP_2 levels are necessary for cytoskeleton rearrangement and priming of insulin secretory granules (18,19). These studies highlight the complex role of phosphoinositides in insulin secretion and the need to elucidate the physiological relevance of K_{ATP} channel gating by PIP_2 in the context of β-cell function and insulin secretion. In this study, we manipulated the interactions between K_{ATP} channels and membrane PIP_2 in insulin-secreting cells and examined the effects of such manipulations on K_{ATP} channel activity and insulin secretion. We show that disrupting the interaction between K_{ATP} channels and PIP_2 by overexpressing Kir6.2 mutants with decreased sensitivity to PIP_2 causes persistent membrane depolarization and elevated basal level insulin secretion. On the other hand, promoting channel-PIP_2 interactions by expressing a murine type Iβ phosphatidylinositol 4-phosphate 5 kinase (PIP5K) (20,21), which increases PIP_2 levels and thereby K_{ATP} channel activity, renders INS-1 cells less able to couple glucose stimulation to insulin secretion. The results provide direct evidence

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GFP, green fluorescence protein; HA, influenza A virus hemagglutinin; HBSS, HEPES balanced salt solution; K_{ATP} channel, ATP-sensitive K^+ channel; PIP_2, phosphatidylinositol 4,5-bisphosphates; PIP5K, phosphatidylinositol 4-phosphate 5 kinase; RMP, resting membrane potential.

that regulation of K_{ATP} channel activity by phosphoinositides plays an integral role in insulin secretion.

RESEARCH DESIGN AND METHODS

Molecular biology. Kir6.2 containing point mutations or the influenza A virus hemagglutinin (HA)–epitope tag (YPYDVPDYA inserted between amino acid 100 and 101 of Kir6.2; an extra nine amino acids, DLYAYMEKG, inserted between amino acid 98 and 99 to aid accessibility of the epitope) were prepared using the QuickChange site-directed mutagenesis kit (Stratagene) and subcloned into the pCMV6b vector. Recombinant adenoviruses were constructed using the AdEasy system (Stratagene). The cDNAs encoding wild-type PIP5K Iβ (PIP5K:WT) or a deletion mutant lacking the NH_2-terminal 238 amino acids (PIP5K:Δ1–238) (20) or the various rat Kir6.2 wild type and mutants were subcloned into the pCMV shuttle vector (pShuttle). They were then recombined with the pAdEasy vector in the BJ5183 strain of *Escherichia Coli*. Positive recombinants were selected, and pAdEasy plasmids containing the correct insert were used to transfect HEK293 cells for virus production. Single plaques were isolated for further amplification in HEK293 cells. Recombinant viruses were purified on a CsCl gradient and titers determined by spectrometry. All constructs were sequenced to verify the correct mutations.

Infection of INS-1 cells with adenovirus. For protein expression in INS-1 cells, recombinant adenoviruses with desired titers were used for infection as follows. INS-1 cells [clone 832/13, a gift from Dr. Chris Newgard (ref. 22)] were plated at 10^6 cells/35-mm dish and cultured in RPMI-1640 with 11.1 mmol/l D-glucose (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mmol/l HEPES, 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, and 50 μmol/l β-mercaptoethanol. Twenty-four hours later, cells were infected with appropriate titer of each adenovirus diluted in 1 ml OptiMEM at 37°C for 1.5 h. One milliliter of RPMI-1640 with 2× supplement was then added to each dish for overnight incubation. The next day, the infection medium was replaced with regular medium and incubated for another day before the experiment.

Immunoblotting and immunostaining. INS-1 cells were lysed 24 h postinfection in 20 mmol/l HEPES, pH 7.0, 5 mmol/l EDTA,150 mmol/l NaCl, and 1% Nonidet P-40 (IGAPEL) with Complete protease inhibitors (Roche Applied Science, Indianapolis, IN). Proteins were separated by SDS/PAGE, transferred to nitrocellulose membrane, probed with appropriate primary antibodies followed by horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ), and visualized by enhanced chemiluminescence (Super Signal West Femto; Pierce, Rockford, IL). The primary antibodies used were rabbit polyclonal anti-Kir6.2 for Kir6.2 (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-PIP5K, which recognizes both the wild type and the Δ1-238 mutant (kindly provided by Dr. J. Nathan Davis), for PIP5K:WT and PIP5K:Δ1-238. To confirm expression of mutant Kir6.2 at the cell surface, living INS-1 cells infected with a virus carrying HA-tagged R206A Kir6.2 were immunostained for surface HA using protocols described previously (23).

Phospholipids assays. INS-1 cells were grown on 35-mm dishes and infected with PIP5K:WT or PIP5K:Δ1-238 viruses. Twenty-four hours postinfection, cells were preincubated for 1 h in a phosphate-free medium before labeling with 20 μCi ^{32}P for 4 h. Cells were then washed twice with phosphate-free medium and scraped into 1 ml MeOH:HCl (10:1). After scraping, the dish was washed again with 1 ml of 2-mmol/l $AlCl_3$ in H_2O, and the wash was combined with the cell lysate. The lipids were extracted by 2 ml $CHCl_3$. The aqueous phase was transferred to a new tube and extraction repeated twice. The $CHCl_3$ phase from the three extractions was combined and extracted with 2 ml MeOH:HCl (1:1). The final lipid extract was dried and resuspended in 15 μl solvent ($CHCl_3$:MeOH:10 mmol/l HCl [20:10:1]) and analyzed by thin-layer chromatography using oxalate-coated plates. Approximately 30 μg of each lipid standard in the same solvent was run simultaneously. The plates were developed with $CHCl_3$:MeOH:H_2O:NH_4OH (90:90:20:7) and the labeled phospholipids quantified by phosphorimaging. Lipid standards were visualized by iodine staining.

Electrophysiology. Resting membrane potential (RMP) of INS-1 cells was measured at room temperature using whole-cell or perforated patch-clamp recording with the Axopatch 1D amplifier and Clampex 8.1 (Axon, Foster City, CA). For measuring the RMP in 3 or 12 mmol/l glucose, cells were preincubated in indicated glucose concentrations for 3 h before recording. During recording, cells were bathed in Tyrode's solution consisting of (in mmol/l) 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5 HEPES, 3 $NaHCO_3$, and 0.16 NaH_2PO_4, with glucose as specified in the figure legends. Pipette solution contained (in mmol/l) 140 KCl, 10 HEPES, 1 EGTA, 1 EDTA (referred to as K-INT), and 1 ATP plus 1 $MgCl_2$ for experiments shown in Fig. 1. For measuring RMP at 12 mmol/l glucose using whole-cell patch-clamp recording, pipette solution contained (in mmol/l) 10 KCl, 130 Kgluconate, 10 HEPES, 1 EGTA, 3 $MgCl_2$, and 5 ATP. The pH of all solutions was adjusted to ~7.2. To measure RMP without dialyzing internal cellular contents, the perforated patch was adopted. The pipette resistance ranged between 3 and 5 MΩ. The tip solution was first filled up to ~0.5 mm with the clean pipette solution containing (in mmol/l) 76 K_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$, and 5 HEPES, pH~7.2; the pipette was then backfilled with the pipette solution containing ~160–250 μg/ml amphotericin B (Sigma). The stable perforated patch usually followed after 6–10 min of

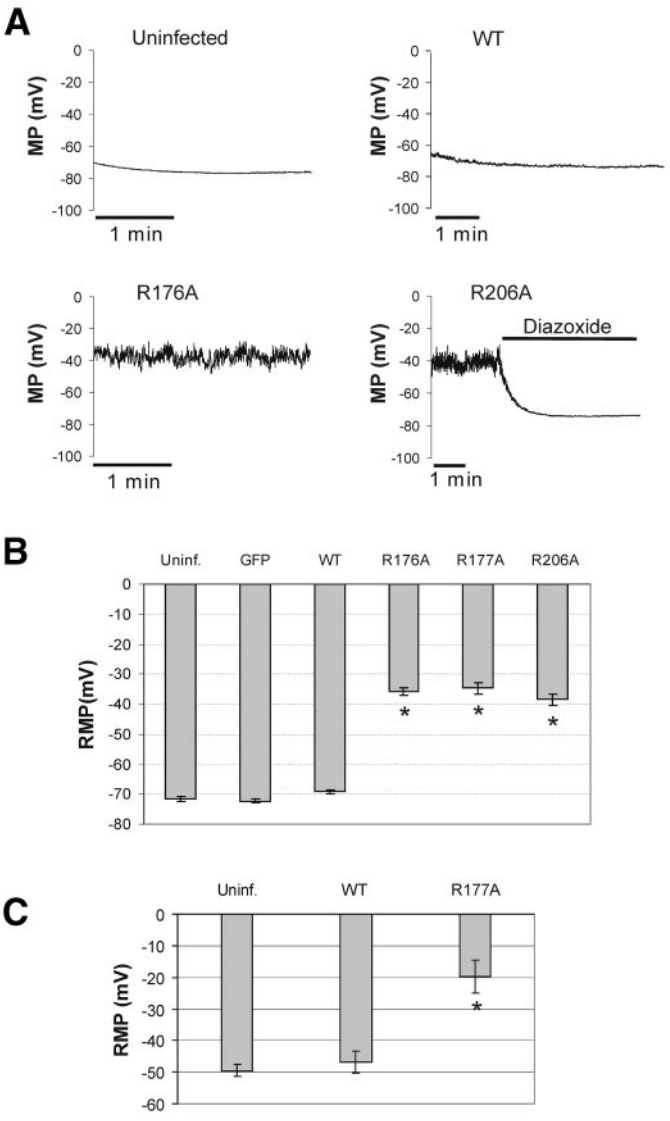

FIG. 1. Effects of PIP_2-insensitive mutants on INS-1 cell membrane potential (MP). *A*: INS-1 cell membrane potential was monitored by whole-cell recordings in current clamp mode. Cells were bathed in Tyrode's solution with 3 mmol/l glucose. The pipette solution contained 1 mmol/l ATP and 1 mmol/l $MgCl_2$. Shown are representative recordings from uninfected cells or cells infected with wild type (WT), R176A, or the R206A-Kir6.2 virus. While the RMP of an uninfected cell or a cell infected with WT Kir6.2 virus hyperpolarized to a steady value of approximatley −70 mV soon after break-in (due to perfusion with 1 mmol/l MgATP, which is likely lower than the intracellular MgATP concentration before break-in), that of a cell infected with the R176A or R206A-Kir6.2 virus was much more depolarized (approximately −40 mV) and remained depolarized even after perfusion with pipette solution containing 1 mmol/l ATP. The depolarized membrane potential could be hyperpolarized to the level close to that seen in uninfected cells by adding 250 μmol/l K_{ATP} channel opener diazoxide to the bath solution. *B*: Averaged steady-state RMP using whole-cell patch clamp recording shown in *A* (n = 18–39). *C*: Averaged membrane potential measured by perforated patch-clamp recording in uninfected cells or cells infected with the wild type or the R177A mutant Kir6.2 virus at 3 mmol/l glucose (n = 8–13). In both *B* and *C*, the error bar is the SE. *P < 0.001 using Student's t test for paired data (the same statistical analysis was used in all subsequent figures).

initial Giga seal. For ATP sensitivity measurement, inside-out patch-clamp recording was used as described previously (21). K-INT was used in both the bath and pipette solution. All currents were measured at a membrane potential of −50 mV (pipette voltage = +50 mV). ATP dose-response curves were fitted by the Hill equation: $I_{rel} = 1/\{1 + [(ATP)/K_i]^H\}$, where I_{rel} = current in [ATP]/current in 0 ATP, H = Hill coefficient, and $K_{1/2}$ = [ATP] causing half-maximal inhibition. All data are presented as means ± SE.

Membrane capacitance measurements were carried out in the standard whole-cell configuration using an EPC-9 amplifier and Pulse software (version 8.4; Heka Elektronik, Lambrecht, Germany). Patch electrodes were made from borosilicate glass capillaries, coated with dental wax (Kerr, Romulus, MI) at the tips, and fire polished. The cells were constantly perfused with prewarmed extracellular solution consisting of (in mmol/l) 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, and 5 HEPES (pH 7.4 with NaOH). The pipette solution contained (in mmol/l) 125 CsMeSO, 25 CsCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 0.05 EGTA, 3 Mg ATP, and 0.01 GTP, pH 7.2. The temperature close to the cell was 32–34°C. Exocytosis was elicited by a train of six (at 1 Hz) 300-ms voltage-clamp depolarization from −70 mV to 0 and detected as changes in cell capacitance estimated by the Lindau-Neher technique ("Sine + DC" feature of the lock-in software module). The amplitude of the sine wave was 20 mV, and the frequency set as 500 Hz.

Insulin secretion assay. INS-1 cells were seeded in 24-well tissue culture plates at ~5×10^5/well, cultured for ~24 h, and infected with viruses as described above. Twenty-four hours postinfection, the culture medium was replaced by RPMI-1640 with 5 mmol/l glucose and cells incubated for at least 18 h. Insulin secretion was assayed in HEPES balanced salt solution (HBSS) consisting of (in mmol/l) 114 NaCl, 4.7 KCl, 1 $MgCl_2$, 1.2 KH_2PO_4, 1.16 $MgSO_4$, 20 HEPES, 2.5 $CaCl_2$, 25.5 $NaHCO_3$, and 0.2% BSA, pH ~7.2 (22). Cells were washed twice with prewarmed (37°C) HBSS buffer with 3 mmol/l glucose followed by 2-h incubation in the same buffer before stimulation with 0.8 ml/well prewarmed HBSS buffer containing 3 or 12 mmol/l glucose for 2 h. The medium was harvested and insulin content determined using Immunochem coated-tube insulin radioimmunoassay from ICN Pharmaceuticals (Costa Mesa, CA). Insulin release in different concentrations of glucose was normalized to that observed at 3 mmol/l glucose and expressed as fold increase.

RESULTS

Disruption of K_{ATP} channel–PIP_2 interactions in INS-1 cells results in depolarized membrane potential and increased basal level insulin secretion. PIP_2 exerts its effect on K_{ATP} channel gating largely via the Kir6.2 subunit (11,12). There is evidence that binding of the negatively charged phosphate groups of membrane PIP_2 to the cytoplasmic domain of Kir6.2 leads to channel opening (11,12,24–27). Several positively charged Kir6.2 residues critical for channel gating by PIP_2 have been identified, including R176, R177, and R206 (27,11). Mutation of these residues to the neutral amino acid alanine either reduces or abolishes channel activity in inside-out membrane patches in the absence of inhibitory ATP (these mutants are referred to as PIP_2-insensitive mutants hereinafter). To elucidate the role of K_{ATP} channel–PIP_2 interactions in β-cell excitability and insulin secretion, we sought to disrupt channel-PIP_2 interactions by overexpressing PIP_2-insensitive Kir6.2 mutants in the rat β-cell line INS-1 clone 832/13, which exhibits robust insulin response to glucose stimulation (22). In COS cells, coexpression of the PIP_2-insensitive Kir6.2 mutant with wild-type Kir6.2 (WT Kir6.2) at 1:1 molar ratio in the presence of SUR1 led to a >50% reduction in total K_{ATP} currents (data not shown). Mutant Kir6.2 expressed in INS-1 cells is therefore expected to compete with endogenous WT Kir6.2 for incorporation into the channel complex and reduces overall channel sensitivity to PIP_2. Reduced K_{ATP} channel activity is in turn expected to cause membrane depolarization even in the absence of glucose stimulation (28). We have chosen the recombinant adenovirus approach for expressing mutant Kir6.2 in INS-1 cells because it is much more efficient than conventional transfection methods (29); infection with adenoviruses carrying the green fluorescence protein (GFP) gene yielded >95% GFP-positive cells 12 h after infection.

As shown in Fig. 1, in cells expressing the R176A, R177A, or the R206A Kir6.2 mutant, the RMP (as assessed by whole-cell patch-clamp recording with pipette solution containing 1 mmol/l ATP and 1 mmol/l $MgCl_2$) in low (3 mmol/l) glucose was indeed significantly more depolarized (approximately −35 to 40 mV) than that observed in uninfected control cells (approximately −72 mV) or cells expressing either GFP (approximately −74 mV) or WT Kir6.2 (approximately −69 mV). To further validate the membrane potential obtained using whole-cell patch-clamp recording, we also measured membrane potential using perforated patch-clamp recording, a procedure that better preserves the intracellular milieu. A substantially more depolarized membrane potential was again observed in cells infected with the PIP_2-insenstive R177A Kir6.2 mutant compared with uninfected cells or cells infected with WT Kir6.2 at low (3 mmol/l) glucose (Fig. 1*C*) but not at high (12 mmol/l) glucose (not shown). Note that the membrane potential obtained using the perforated patch-clamp technique was ~20 mV more depolarized than that obtained by whole-cell recording after dialysis with 1 mmol/l MgATP. The difference likely reflects the higher intracellular MgATP concentration in intact INS-1 cells. The above results are consistent with the notion that K_{ATP} channel–membrane PIP_2 interactions are necessary to sustain K_{ATP} channel activity and maintain the membrane potential at the resting hyperpolarized state in the absence of glucose stimulation. The depolarized membrane potential in cells expressing mutant Kir6.2 could largely be hyperpolarized to the level seen in uninfected cells (R206A, for example) by adding the K_{ATP} channel opener diazoxide in the bath solution, demonstrating that the depolarized membrane potential is not due to adverse effects of virus infection or mutant Kir6.2 expression on the health of the cell. Although PIP_2-insenstive Kir6.2 mutants have been reported to render the channel less responsive to diazoxide (30,31), residual sensitivity of mutant channels to the channel opener as well as endogenous WT Kir6.2 subunits present in some channels may be sufficient to confer the diazoxide sensitivity we observed.

To ensure that the titer of each recombinant Kir6.2 adenovirus used for infection gives rise to similar protein expression level, we analyzed Kir6.2 protein level by immunoblotting. Furthermore, to confirm that the mutant Kir6.2 subunit is incorporated into the channel complex and expressed on the surface of INS-1 cells, we tagged one of the mutant R206A with a HA-epitope tag in the extracellular domain between amino acids 100 and 101 (HA-R206A-Kir6.2). This extracellular epitope tag allows for evaluation of surface expression of the mutant without permeabilizing the cell; the HA-tag does not interfere with the function of the channel (data not shown). As Fig. 2*A* illustrates, the titer of each of the WT-, R176A-, R177A-, or HA-R206A-Kir6.2 viruses could be adjusted to obtain similar protein expression levels (approximately fivefold of endogenous Kir6.2). Immunofluorescent staining of HA-R206A-Kir6.2 also confirmed that the mutant protein is indeed incorporated into the channel complex and expressed at the cell surface (Fig. 2*B*).

Next, we evaluated insulin secretion response in cells expressing the various PIP_2-insensitive Kir6.2 mutants at both low (3 mmol/l) and high (12 mmol/l) glucose concentrations. These glucose concentrations represent basal and maximal insulin response to glucose stimulation,

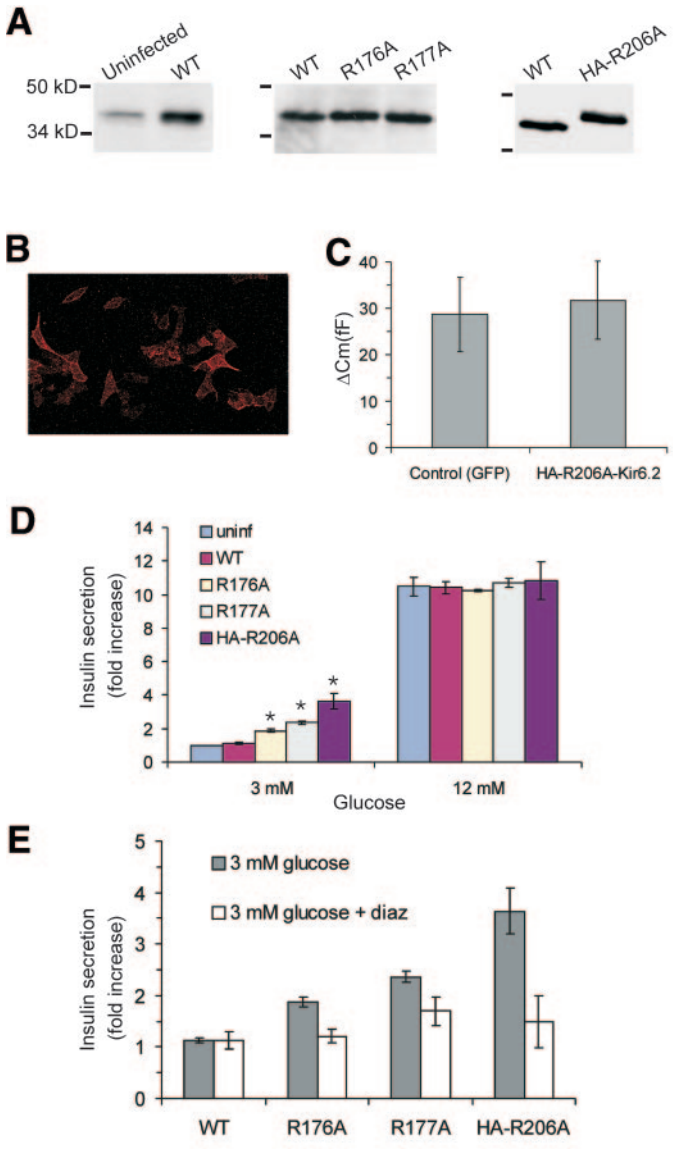

FIG. 2. Overexpression of PIP_2-insensitive mutants causes depolarized membrane potential and elevated insulin secretion at basal glucose. *A*: Western blots of endogenous and exogenous Kir6.2 or HA-R206A-Kir6.2 (which has slightly higher molecular mass) expressed in INS-1 cells using anti-Kir6.2 antibodies. Note at the virus titer used for experiments shown in this study, the exogenous Kir6.2 protein is in approximately fivefold excess (as assessed by densitometry) to endogenous Kir6.2 detected in uninfected cells. Molecular mass markers for all three blots are the same, as shown on the left. *B*: Surface staining of HA-tagged R206A-Kir6.2 using an anti-HA antibody verifies that the mutant Kir6.2 is incorporated into the channel complex and expressed on the cell surface. Staining of uninfected cells using the same antibody did not give rise to detectable fluorescent signal (not shown). *C*: Averaged membrane capacitance changes in response to voltage-clamp depolarization from −70 to 0 mV in control cells (infected with a GFP virus) and cells infected with the HA-R206A-Kir6.2 virus. There is no statistically significant difference between the two groups. *D*: Insulin secretion in response to 3- or 12-mmol/l glucose stimulation. The response is normalized to that observed in uninfected cells at 3 mmol/l glucose. Cells infected with the R176A, R177A, or HA-R206A virus showed significantly (*$P < 0.001$) elevated insulin secretion response at 3 mmol/l glucose (1.9 ± 0.1-, 2.4 ± 0.1-, and 3.6 ± 0.4-fold, respectively, $n = 5–9$) compared with cells infected with the WT Kir6.2 virus (1.1 ± 0.1). By contrast, no significant difference was observed at 12 mmol/l glucose. *E*: Opening of K_{ATP} channels by diazoxide (250 μmol/l) consistently reversed the effect of all three PIP_2-insensitive Kir6.2 mutants on basal insulin secretion, although the extent of reversal varied.

respectively, based on a glucose dose-response curve (0–20 mmol/l glucose) established in INS-1 cells (not shown) (22). To exclude a direct effect of the Kir6.2 mutant on the insulin secretory machinery, we first measured membrane capacitance changes in response to 500 ms voltage-clamp depolarization. In these experiments, effects of channel-PIP_2 interactions on the membrane potential are excluded and the capacitance change represents fusion of insulin granules that are available for immediate release upon the evoked membrane depolarization. Figure 2*C* shows that no significant difference in capacitance increase is detected between GFP virus–infected control cells and cells expressing HA-R206A-Kir6.2. Insulin secretion assays demonstrated that at 3 mmol/l glucose, while cells infected with WT-Kir6.2 virus had insulin secretion response indistinguishable from that observed in uninfected control cells (1.1-fold), cells infected with each of the three PIP_2-insensitive Kir6.2 mutants all exhibited significantly elevated insulin secretion (1.9-, 2.4-, and 3.6-fold that of uninfected cells for R176A, R177A, and HA-R206A, respectively; Fig. 2*D*). These results are consistent with the electrophysiological data (Fig. 1) showing depolarized membrane potential in cells expressing the PIP_2-insensitive mutants. As expected, at a high glucose concentration (12 mmol/l) no significant difference in insulin secretion response could be discerned between cells expressing the various Kir6.2 or uninfected cells because the membrane depolarizing effect caused by the mutant Kir6.2 is now masked by the depolarizing effect caused by high glucose stimulation. Also consistent with the electrophysiological data, treatment of cells with diazoxide reversed the elevated basal level insulin secretion in cells expressing the PIP_2-insensitive Kir6.2 mutants to various extents (Fig. 2*E*). The results described above provide strong evidence that K_{ATP} channel–PIP_2 interactions are necessary to allow channel opening and to prevent insulin secretion when glucose concentrations are low.

Enhancing K_{ATP} channel–PIP_2 interactions reduces channel sensitivity to ATP inhibition and attenuates membrane depolarization and insulin secretion upon glucose stimulation. To assess how increased interactions between K_{ATP} channels and PIP_2 affect β-cell excitability and insulin secretion, we sought to upregulate PIP_2 levels by overexpressing lipid kinase PIP5K:WT (murine type I isoform β), which is involved in the synthesis of phosphatidylinositol 4,5-P_2 (10,20,21). Overexpression of PIP5K:WT in COSm6 cells has previously been shown to decrease the ATP sensitivity of K_{ATP} channels exogenously expressed in those cells (21). As a negative control, we infected cells with viruses carrying a mutant kinase whose NH_2-terminal 238 amino acids have been deleted to remove its enzymatic activity (referred to as PIP5K:Δ1-238) (20,21). Figure 3*A* is a Western blot showing similar expression level of PI4P5K:WT or PIP5K:Δ1-238 in INS-1 cells. To validate that expression of PIP5K:WT increases PIP_2 levels, we metabolically labeled cells with ^{32}P and analyzed the ^{32}P-labeled phospholipids by thin-layer chromatography (Fig. 3*B*). The amount of PIP_2 in cells expressing PIP5K:WT was dramatically increased (~30-fold) compared with uninfected cells or cells expressing PIP5K:Δ1-238.

We next examined how the increased PIP_2 affects K_{ATP} channel activity by inside-out patch-clamp recording. In these experiments, patches were excised into K-INT solution with no ATP and then exposed quickly to K-INT containing various concentrations of ATP (all in the ab-

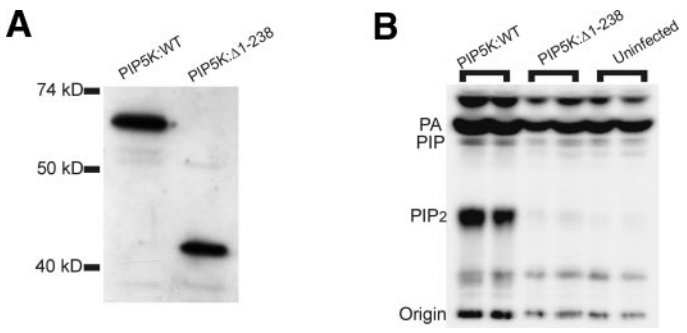

FIG. 3. Infection of INS-1 cells with the PIP5K:WT virus increases cellular PIP_2 level. *A*: Western blots of the PIP5K:WT and the NH_2-terminal deletion mutant kinase (PIP5K:Δ1-238). Titers of the two viruses used to infect cells were adjusted to achieve similar protein expression levels in this and the subsequent two figures. *B*: PIP_2 measured in uninfected cells, cells infected with the PIP5K:WT virus, or the PIP5K:Δ1-238 virus. Standards of phosphatidic acid (PA), phosphatidylinositol 4-P, and phosphatidylinositol 4,5-P_2 were run on the same silica gel and visualized by iodine spray to help identify the different phospholipid species. ^{32}P-labeled PIP_2 was quantified using a PhosphorImager.

sence of Mg^{2+}) before any significant current rundown occurred to ensure accurate measurement of ATP dose response (Fig. 4*A*) (21). As shown in Fig. 4*B*, K_{ATP} channels in cells infected with the PIP5K:WT virus exhibited a

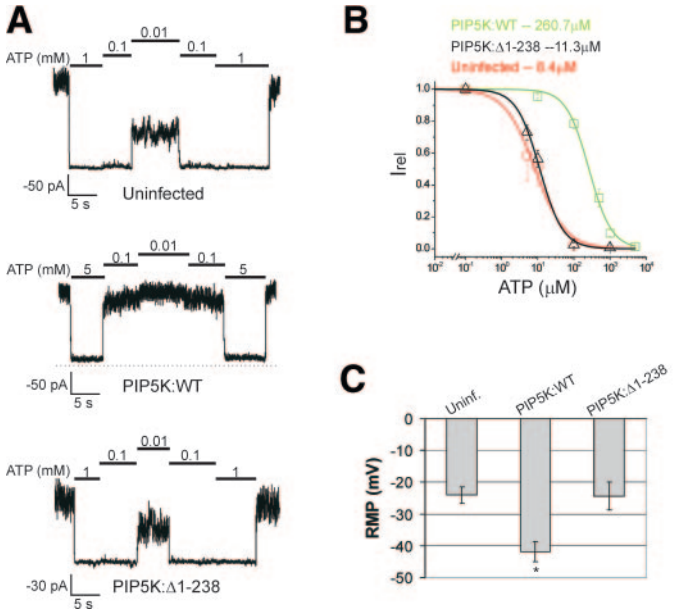

FIG. 4. Overexpression of PIP5K:WT reduces K_{ATP} channel sensitivity to ATP and causes membrane hyperpolarization and attenuated insulin secretion at high glucose. *A*: Representative inside-out patch clamp recordings of K_{ATP} currents from control INS-1 cells or cells overexpressing PIP5K:WT or PIP5K:Δ1-238. Patches were exposed to different concentrations of ATP as indicated by the bars above the recording. Experiments were done in symmetrical K-INT solution at −50 mV. Inward currents were shown as upward deflections. *B*: ATP dose-response curves derived from recordings shown in *A*. The curves were fit by the Hill equation ($I_{rel} = 1/\{1 + [(ATP)/K_{1/2}]^H\}$; I_{rel} = current in [ATP]/current in 0 ATP, where H = Hill coefficient and $K_{1/2}$ = [ATP] causing half-maximal inhibition) to averaged data. Channels of uninfected cells exhibit an expected $K_{1/2}$ of 8.4 μmol/l (H = 1.04). By contrast, channels of cells overexpressing PIP5K:WT have a $K_{1/2}$ of 260.7 μmol/l (H = 1.4), ~26-fold higher than channels of uninfected cells or cells infected with PIP5K:Δ1-238 ($K_{1/2}$ of 11.3 μmol/l, H = 1.4). *C*: Averaged membrane potential of uninfected INS-1 cells and cells infected with the PIP5K:WT or PIP5K:Δ1-238 virus (n = 5–12) at 12 mmol/l glucose monitored using perforated patch-clamp recording. The error bar is the SE. The RMP of cells infected with the PIP5K:WT virus is significantly more hyperpolarized than uninfected cells at high glucose. *P < 0.001.

~26-fold decrease in ATP sensitivity compared with channels in uninfected cells or cells infected with the PIP5K:Δ1-238 virus. The large rightward shift in ATP dose response is predicted to cause K_{ATP} channels to remain active even when the glucose level rises, resulting in hyperpolarized membrane potential and reduced insulin response. To test these, we examined the membrane potential at high glucose using a perforated patch-clamp technique. As mentioned above, the perforated-patch recording permits measurement of membrane potential without disturbing intracellular factors that are involved in regulating K_{ATP} channel activity. Indeed, we found that at 12 mmol/l glucose, the averaged membrane potential is significantly more hyperpolarized in cells infected with the PIP5K:WT virus than in uninfected cells or cells infected with the PIP5K:Δ1-238 virus (Fig. 4*C*). Similar results were obtained using whole-cell recordings with 12 mmol/l glucose in the bath solution and 5 mmol/l ATP plus 3 mmol/l Mg^{2+} in the pipette solution (not shown). Accordingly, while in uninfected cells or cells infected with the PIP5K:Δ1-238 virus increasing the glucose concentration from 3 to 12 mmol/l led to a 9.5- and 8.4-fold increase in insulin release, respectively, in cells infected with the PIP5K:WT virus the same glucose concentration change only caused a 3.5-fold increase in insulin release (Fig. 5*A*). This reduced secretion is not due to an inability of insulin granules to fuse with the membrane because no difference in membrane capacitance increase was found between cells infected with the PIP5K:WT virus and uninfected cells either during the first depolarization or during an entire train of step voltage-clamp depolarizations (Fig. 5*C*). In addition, overexpression of PIP5K did not alter voltage-gated Ca^{2+} currents during these experiments (shown as the charge influx in Fig. 5*D*) (32). These results lead us to conclude that overexpression of PIP_2 reduces insulin secretion at high glucose by rendering K_{ATP} channels insensitive to ATP.

DISCUSSION

Despite the effects of membrane phosphoinositides, especially PIP_2, on K_{ATP} channel activity having been well documented in vitro studies for some time (11,12,33,34), the relevance of channel regulation by PIP_2 to insulin secretion remains controversial (15,35). To address this issue, it is necessary to directly correlate the interactions between K_{ATP} channels and PIP_2 in β-cells to insulin secretion. INS-1 clone 832/13 is a rat insulinoma cell line widely used to study β-cell function; it exhibits robust glucose-stimulated insulin secretion, mediated by both K_{ATP}-dependent and independent pathways (22). Our studies demonstrate that alterations in K_{ATP} channel–PIP_2 interactions have profound effects on insulin secretion, establishing a physiological role of PIP_2 in controlling insulin secretion through K_{ATP} channel regulation.

In altering channel–PIP_2 interactions, we took two approaches: one by manipulating the channel subunit Kir6.2, the other by manipulating cellular PIP_2 levels. While interpretation of experiments in which Kir6.2 subunit is the subject of manipulation is straightforward, interpretation of experiments involving the manipulation of PIP_2 concentrations is more complicated due to its role in diverse cellular processes. For example, recent studies indicate that PIP_2 is required for recruitment of insulin granules to the release sites during the secondary phase of insulin secretion: a decrease in PIP_2 levels reduces, whereas an increase in PIP_2 levels enhances insulin secretion (18,19). In addition, PIP_2 has been shown to control

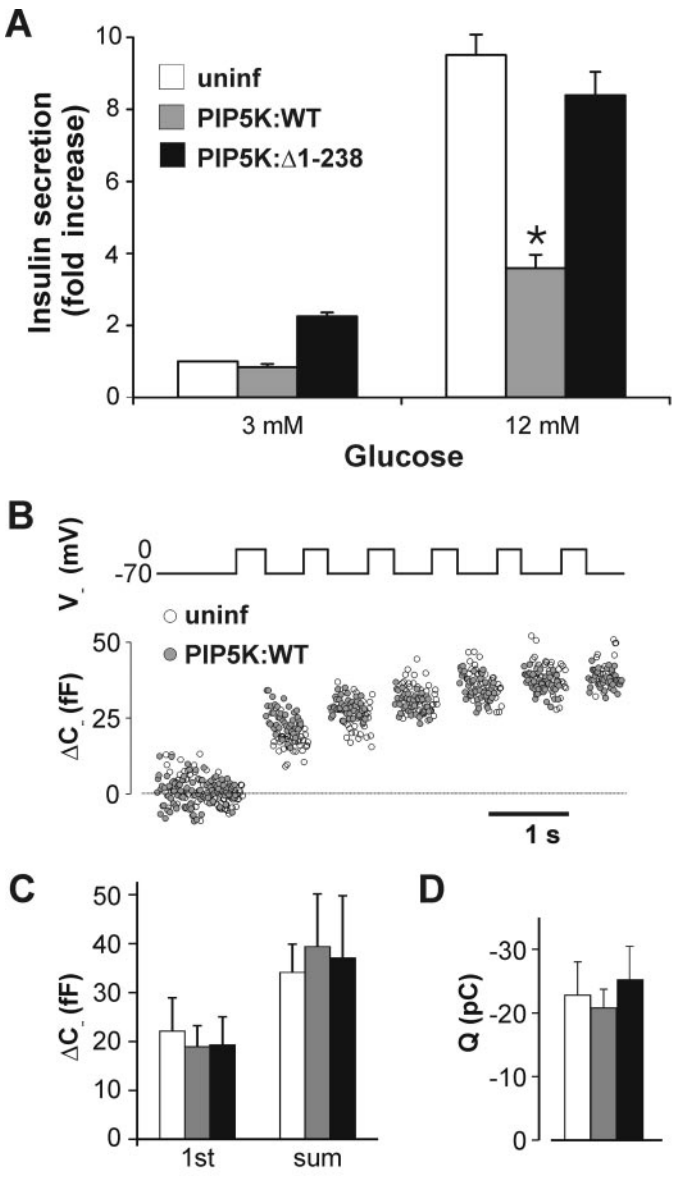

FIG. 5. Overexpression of PIP5K:WT attenuates insulin secretion response to glucose stimulation. *A*: Insulin secretion at 3 or 12 mmol/l glucose was measured in uninfected (uninf) cells or cells infected with the PIP5K:WT or the PIP5K:Δ1-238 virus. The response is normalized to that observed in uninfected cells at 3 mmol/l glucose. Cells infected with the PIP5K:WT virus had significantly reduced insulin secretion response at 12 mmol/l glucose (3.6 ± 0.4-fold) compared with uninfected cells (9.5 ± 0.6-fold) or cells infected with the PIP5K:Δ1-238 virus (8.4 ± 0.7-fold; n = 5–9 in each case). By contrast, no significant difference was observed between cells infected with the PIP5K:WT virus or uninfected control cells at 3 mmol/l glucose. Note that at 3 mmol/l glucose, cells infected with the PIP5K:Δ1-238 virus showed a twofold increase in insulin release; this is likely due to the documented dominant-negative effect of the mutant on wild-type kinase activity (20,21), leading to a slight decrease in PIP_2 levels and elevated basal insulin secretion. *P < 0.001. *B*: Membrane capacitance change in response to voltage-clamp depolarizations. The upper trace represents the voltage-clamp protocol used to evoke exocytosis, and the dotted lower traces the capacitance increase observed in PIP5K:WT overexpressing (gray) and uninfected control cells (white). *C*: Averaged capacitance increase during the first (left) and all six depolarizations (right) in experiments as in *B* (n = 8–12; color code as in A). *D*: Charge influx during the first depolarization from the same set of cells shown in *C*. With Cs in the pipette (see RESEARCH DESIGN AND METHODS), the vast majority of the current is through voltage-gated Ca channels (32).

the rate of endocytosis (36–38). Curiously, unlike that reported in primary β-cells and chromaffin cells (18,39), we did not detect a significantly larger increase in membrane capacitance change upon direct voltage-clamp membrane depolarization in INS-1 cells with elevated PIP_2 (Fig. 5*B* and *C*). The discrepancy might be due to differences in the level of PIP_2 overexpression; the prolonged high level of PIP_2 seen in PIP5K:WT virus–infected INS-1 cells might lead to increased membrane endocytosis, masking the capacitance increase expected from enhanced exocytosis. Regardless, even if there is an effect of PIP_2 that acts directly on the exocytotic machinery, this effect is likely to be small compared with the effect of PIP_2 via K_{ATP} channels, as the net result of PIP_2 overexpression in our experiments is a decrease in glucose-induced insulin secretion (Fig. 5*A*). Increased endocytosis due to elevated PIP_2 might also affect the level of membrane proteins involved in stimulation-secretion coupling, such as K_{ATP} channels. If true, this would lead to fewer K_{ATP} channels at the cell surface and increased insulin secretion, making our observed impact of enhanced channel-PIP_2 interaction on insulin secretion an underestimate. Taken together, despite the multiple mechanisms by which PIP_2 may affect insulin secretion, our observation that elevated PIP_2 dramatically reduces the ATP sensitivity of endogenous K_{ATP} channels (Fig. 4) provides strong consistency with a critical role of membrane PIP_2 in controlling β-cell K_{ATP} channel activity and hence β-cell excitability and insulin secretion.

In conclusion, our study demonstrates that membrane phosphoinositides play an integral role in determining the activity of K_{ATP} channels in β-cells. Many hormones, neurotransmitters, and nutrients affect the metabolism of phosphoinositides in β-cells (40–44). Our finding raises the possibility that variation in membrane phosphoinositides under normal and pathological conditions may affect insulin secretion by altering K_{ATP} channel activity. Mutations in other Kir channels such as Kir2.1 that impair channel sensitivity to PIP_2 have been shown to underlie Anderson syndromes (45). Our study also raises the possibility that naturally occurring mutations in Kir6.2 that alter channel-PIP_2 interactions may cause insulin secretion diseases.

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