

Kir6.2 Polymorphisms Sensitize β -Cell ATP-Sensitive Potassium Channels to Activation by Acyl CoAs

A Possible Cellular Mechanism for Increased Susceptibility to Type 2 Diabetes?

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The commonly occurring E23K and I337V Kir6.2 polymorphisms in the ATP-sensitive potassium (K_{ATP}) channel are more frequent in Caucasian type 2 diabetic populations. However, the underlying cellular mechanisms contributing to the pathogenesis of type 2 diabetes remain uncharacterized. Chronic elevation of plasma free fatty acids observed in obese and type 2 diabetic subjects leads to cytosolic accumulation of long-chain acyl CoAs (LC-CoAs) in pancreatic β -cells. We postulated that the documented stimulatory effects of LC-CoAs on K_{ATP} channels might be enhanced in polymorphic K_{ATP} channels. Patch-clamp experiments were performed on inside-out patches containing recombinant K_{ATP} channels (Kir6.2/SUR1) to record macroscopic currents. K_{ATP} channels containing Kir6.2 (E23K/I337V) showed significantly increased activity in response to physiological palmitoyl-CoA concentrations (100–1,000 nmol/l) compared with wild-type K_{ATP} channels. At physiological intracellular ATP concentrations (mmol/l), E23K/I337V polymorphic K_{ATP} channels demonstrated significantly enhanced activity in response to palmitoyl-CoA. The observed increase in K_{ATP} channel activity may result in multiple defects in glucose homeostasis, including impaired insulin and glucagon-like peptide-1 secretion and increased glucagon release. In summary, these results suggest that the E23K/I337V polymorphism may have a diabetogenic effect via increased K_{ATP} channel activity in response to endogenous levels of LC-CoAs in tissues involved in the maintenance of glucose homeostasis. *Diabetes* 52: 2630–2635, 2003

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FFA, free fatty acid; GLP-1, glucagon-like peptide-1; IC_{50} , half-maximal inhibitory concentration; K_{ATP} , ATP-sensitive potassium; LC-CoA, long-chain acyl CoA.

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Type 2 diabetes is a multifactorial disease with both genetic and environmental components contributing to its development. Despite the investigation of polymorphic variations in genes encoding for key components in pathways controlling insulin secretion, their precise roles in the etiology of type 2 diabetes are not well understood.

Glucose homeostasis is maintained through the coordinated release of several hormones, including insulin, glucagon, and glucagon-like peptide-1 (GLP-1). A key component regulating the release of these hormones is the ATP-sensitive potassium (K_{ATP}) channel (1–3). Hormone secretion in the pancreatic β - and α -cell and in the intestinal L-cell is controlled through metabolic regulation of electrical activity, a process critically linked to glucose and fatty acid metabolism, which in turn regulates the activity of K_{ATP} channels that control membrane potential (1–4).

The K_{ATP} channel is a hetero-octameric protein complex comprised of the pore-forming inward-rectifier Kir6.2 subunit coupled to the high-affinity sulfonylurea receptor SUR1 subunit (5,6) in a stoichiometry of (Kir6.2)₄/(SUR1)₄. Mutations that reduce K_{ATP} channel activity can lead to the increased β -cell excitability and excessive insulin secretion that underlies the congenital disorder of persistent hyperinsulinemic hypoglycemia of infancy (7). In addition, transgenic animal models demonstrate that targeted overactivity of K_{ATP} channels severely impairs insulin secretion and leads to a severe neonatal diabetic phenotype (8). Therefore, enhanced β -cell K_{ATP} channel activity reduces the magnitude of the glucose-stimulated insulin secretory response in β -cells and can lead to the development of diabetes.

A recently discovered set of three commonly occurring single nucleotide polymorphisms (E23K, L270V, and I337V) in the Caucasian population has been found within the Kir6.2 gene (9,10). Both the E23K and I337V polymorphisms appear to be tightly linked, with >72% of those possessing E23K also possessing I337V (9,11). Recent clinical studies have indicated that homozygous polymorphic individuals are at an increased risk of developing type 2 diabetes compared with individuals with either heterozy-

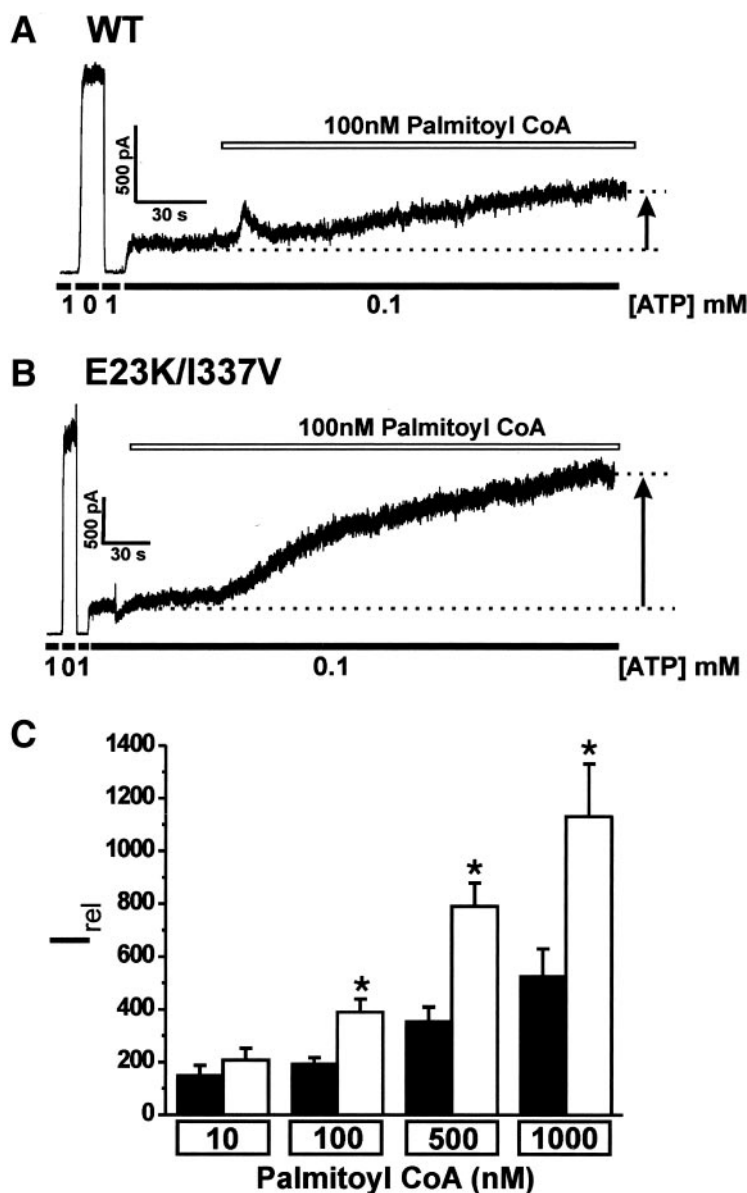


FIG. 1. The Kir6.2(E23K/I337V) polymorphism increases K_{ATP} channel sensitivity to LC-CoAs. *A* and *B*: Representative wild-type (WT) Kir6.2/SUR1 (*A*) and Kir6.2(E23K/I337V)/SUR1 (*B*) channel recordings in response to 100 nmol/l palmitoyl-CoA. K_{ATP} currents were recorded at a holding potential of -60 mV under symmetrical K^+ conditions in excised inside-out membrane patches from transiently transfected tsA201 cells (see RESEARCH DESIGN AND METHODS). Dashed lines represent steady-state current levels at 0.1 mmol/l ATP before and after application of palmitoyl-CoA to the inside of the membrane patch. *C*: Histogram plot of pooled data. Values were calculated as steady-state current reached after application of palmitoyl-CoA over steady-state current at 0.1 mmol/l ATP and expressed as mean \pm SE. ■, wild-type Kir6.2/SUR1 channel population; □, polymorphic Kir6.2(E23K/I337V)/SUR1 channel population. * $P < 0.05$ vs. wild-type pair ($n = 6-36$ patches).

gous or wild-type K_{ATP} channels (9,11–13). Homozygous subjects also possess impaired glucose-stimulated insulin secretion (upon oral glucose challenge), and the prevalence of the Kir6.2 polymorphisms may be linked to an increase in BMI (14). In addition, recent evidence suggests that patients homozygous for the E23K polymorphism have a reduced glucagon secretion response during hyperglycemia (15) that could result from increased K_{ATP} channel activity (2). Taken together, the available evidence suggests that the homozygous polymorphic population may be especially susceptible to impairment of glucose homeostasis if they are overweight or obese, and/or that these polymorphisms may predispose patients to diabetes and excessive weight gain. However, the cellular mechanisms underlying these associations are still poorly understood.

At the cellular mechanistic level, it has been shown that the E23K polymorphism can decrease ATP sensitivity, rendering K_{ATP} channels slightly more active at higher ATP concentrations (16). In addition, it is known that plasma free fatty acid (FFAs) levels are elevated in obese (17) and

diabetic (18) individuals with chronic elevation leading to an accumulation of long-chain acyl CoAs (LC-CoAs) in the cytosol, which can then directly activate β -cell K_{ATP} channels (19,20). Due to the cytosolic location of the polymorphisms in the Kir6.2 gene (Fig. 2D) and their possible effects on ATP and LC-CoA sensitivity, we hypothesized that binding of LC-CoAs may further potentiate polymorphic K_{ATP} channel activity relative to wild-type channels, thus reducing cellular excitability and contributing to altered nutrient-stimulated hormone release, such as reduced insulin secretion, that is observed in homozygous polymorphic individuals with type 2 diabetes (14).

Due to the coupling of the E23K and I337V polymorphisms in vivo, we have examined the sensitivity of the E23K/I337V double-polymorphic K_{ATP} channel to activation by the LC-CoA palmitoyl-CoA relative to wild-type (Kir6.2/SUR1) K_{ATP} channels in a recombinant expression system. Application of palmitoyl-CoA resulted in a dose-dependent increase in current of both wild-type and polymorphic K_{ATP} channels (Fig. 1). At palmitoyl-CoA concentrations of 100–1,000 nmol/l, polymorphic K_{ATP} channel current was

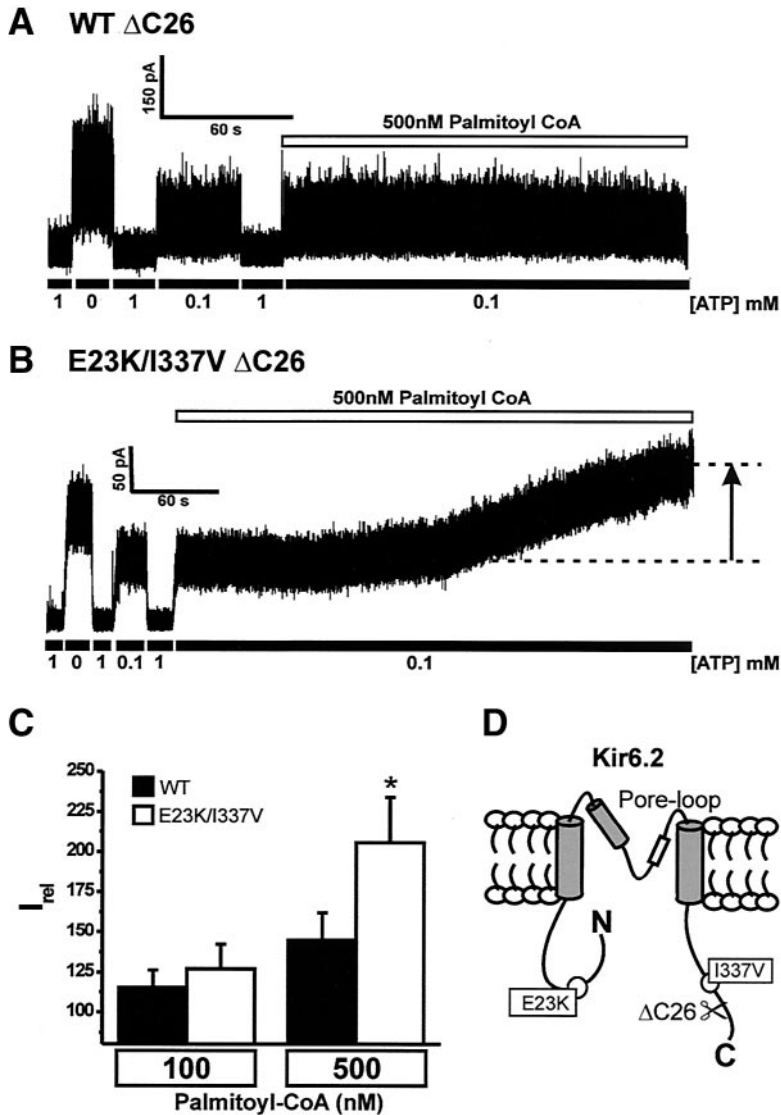


FIG. 2. The increase in acyl CoA sensitivity of Kir6.2 (E23K/I337V) polymorphic K_{ATP} channels resides primarily in the Kir6.2 subunit. Representative wild-type (WT) Kir6.2 Δ C26 (**A**) and polymorphic Kir6.2(E23K/I337V) Δ C26 (**B**) channel recordings in response to 500 nmol/l palmitoyl-CoA. K_{ATP} currents were recorded at a holding potential of -60 mV from excised inside-out membrane patches as described previously (Fig. 1). Dashed lines represent steady-state current levels at 0.1 mmol/l ATP before and after application of palmitoyl-CoA to the inside of the membrane patch. **C:** Histogram plot of pooled data. Values were calculated as steady-state current reached after application of palmitoyl-CoA over the steady-state current at 0.1 mmol/l ATP and expressed as mean \pm SE. \blacksquare , wild-type Kir6.2 Δ C26 channel population; \square , polymorphic Kir6.2(E23K/I337V) Δ C26 channel population. * $P < 0.05$ vs. wild-type pair ($n = 7$ –10 patches). **D:** Schematic of pore-forming Kir6.2 subunit highlighting the cytosolic location of the E23K and I337V single nucleotide polymorphisms. Removal of the COOH-terminal 26 amino acids results in the functional expression of Kir6.2 tetrameric K_{ATP} channels.

significantly increased (approximately twofold) over wild-type at the same dose (Fig. 1C). For example, K_{ATP} channel current was increased by $389.69 \pm 48.62\%$ compared with $196.82 \pm 20.30\%$ in wild-type channels ($P < 0.05$) upon application of 100 nmol/l palmitoyl-CoA. With estimates of β -cell cytosolic free LC-CoA in the range of 0.5–1 μ mol/l and binding affinities for various LC-CoA-utilizing enzymes in the nanomolar to micromolar range (21), our results show that in the presence of physiological concentrations of palmitoyl-CoA, polymorphic K_{ATP} channels are significantly more active than wild-type K_{ATP} channels.

To investigate the mechanisms responsible for the observed increase in sensitivity to LC-CoAs in the polymorphic K_{ATP} channel population, we utilized the Kir6.2 Δ C26 truncation mutant to assess the contribution of the SUR1 subunit (Fig. 2D). The Kir6.2 Δ C26 mutant can be expressed to yield functional ATP-sensitive potassium currents in the absence of the accessory SUR1 subunit (22). Although higher concentrations of palmitoyl-CoA were required to activate the Kir6.2 Δ C26 channel, a similar significant increase in current magnitude in response to 500 nmol/l palmitoyl-CoA was observed between polymorphic ($219.67 \pm 37.27\%$) and wild-type ($144.80 \pm$

17.20%) Kir6.2 Δ C26 channels, as was noted in K_{ATP} channels comprised of both the Kir6.2 and SUR1 subunits ($789.91 \pm 89.30\%$ in polymorphic K_{ATP} channels vs. $355.80 \pm 52.56\%$ in wild-type K_{ATP} channels) (Fig. 1C). These results, together with the observation that sulfonylurea sensitivity is unaffected in the presence of the E23K polymorphism (23), suggest that the principal mechanism by which the E23K/I337V polymorphism alters LC-CoA sensitivity lies primarily within the Kir6.2 subunit.

Increases in both open probability and single-channel amplitude could result in the observed increase in macroscopic polymorphic K_{ATP} channel activity in response to palmitoyl-CoA. To test which of these parameters is altered, we performed single-channel experiments on recombinant K_{ATP} channels expressed in tsA201 cells. Our results indicate that at 1 mmol/l ATP, application of 100 nmol/l palmitoyl-CoA to wild-type or polymorphic K_{ATP} channels does not significantly alter single-channel amplitude (Fig. 3). At a holding potential of -60 mV, wild-type channels had a measured conductance of 60.24 ± 0.97 pS versus polymorphic channels at 56.67 ± 2.70 pS (not significant [NS], $n = 7$), suggesting that the observed

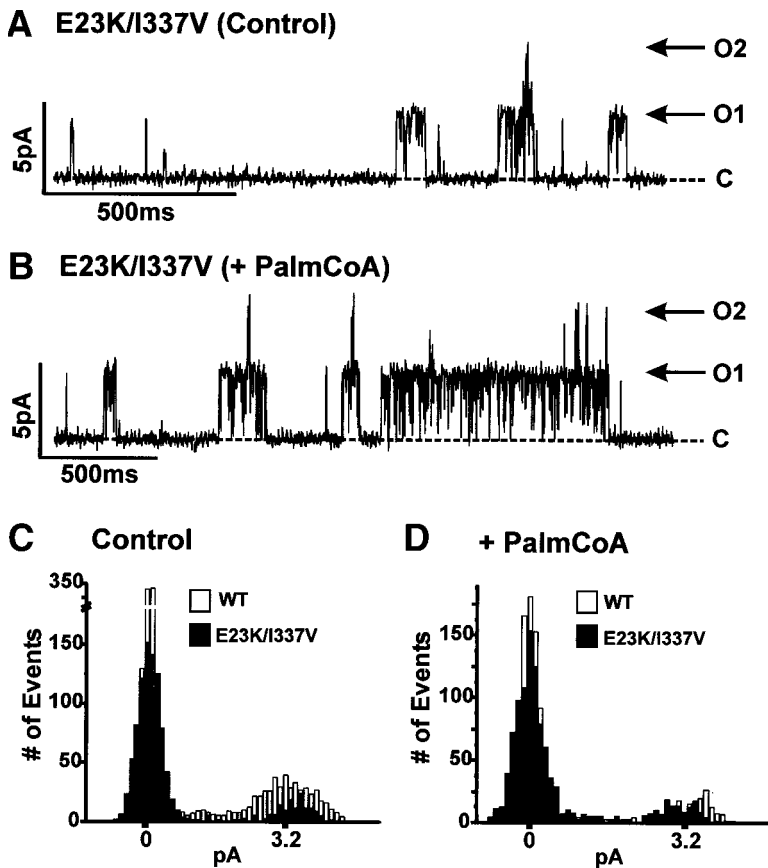


FIG. 3. E23K/I337V does not alter single-channel amplitude in the absence or presence of LC-CoAs. **A:** Representative single-channel current recording from excised inside-out patches of recombinant polymorphic E23K/I337V K_{ATP} channels in the presence of 1 mmol/l ATP before (**A**) and after application of 100 nmol/l palmitoyl-CoA (**B**). **C**, closed channel level; **O1** and **O2**, open channel levels with one or two channels open simultaneously, respectively. **B:** All points histograms generated from representative single-channel recordings, indicating that single-channel amplitude is unaffected by the E23K/I337V polymorphism (**C**), or by the presence of 100 nmol/l palmitoyl-CoA (**D**). ■, E23K/I337V polymorphic K_{ATP} channels; □, wild-type (WT) channels. Histograms were plotted on the *x*-axis such that the 0-pA level represents peak closed-event number.

increase in K_{ATP} channel activity must occur through changes in open probability.

To gain insights into the physiological significance of these findings, we have examined ATP sensitivity in wild-type and polymorphic K_{ATP} channels in the absence and presence of palmitoyl-CoA. We found that 100 nmol/l palmitoyl-CoA decreased ATP sensitivity in both wild-type and polymorphic K_{ATP} channel populations (Fig. 4A and B), shifting the half-maximal inhibitory concentration (IC_{50}) to the right 1.88- and 1.90-fold, respectively. However, our results indicate that ATP sensitivity is slightly increased at the respective IC_{50} concentrations versus wild-type channels in both the absence and presence of palmitoyl-CoA. This appears to be in contrast to the findings of Schwanstecher et al. (16), who noted a rightward shift in the ATP concentration-inhibition curve at all tested ATP concentrations. The study by Schwanstecher et al. noted an increased spontaneous open probability in K_{ATP} channels containing the single Kir6.2 E23K polymorphism in the absence of ATP. In the present study, we examined the single-channel properties of the E23K/I337V double polymorphism in tsA201 cells. In this recombinant expression system, we observed no significant difference between the spontaneous open probability of wild-type versus polymorphic K_{ATP} channels (wild-type 0.837 ± 0.08 vs. E23K/I337V 0.829 ± 0.04 ; NS, $n = 7$) as measured by noise analysis in the absence of ATP.

As physiological ATP concentrations in resting β -cells are estimated to be in the low millimolar range (24,25), we examined this portion of the concentration-response curve in greater detail (Fig. 4C and D). At 1 mmol/l ATP in the presence of 100 nmol/l palmitoyl-CoA, the polymorphic

K_{ATP} channel population exhibited a significant increase in current ($6.42 \pm 1.21\%$ of maximal K_{ATP} current vs. $3.61 \pm 0.40\%$ in wild-type). The difference in effect at low versus high ATP concentrations occurs due to a reduction in the Hill coefficient of ATP binding from 2.28 in wild-type to 1.58 in polymorphic K_{ATP} channels (Fig. 4F). After application of palmitoyl-CoA, the Hill coefficients were further reduced to 1.41 in the wild-type and 1.24 in the polymorphic K_{ATP} channel population. This reduction in Hill coefficient suggests that the ability of ATP to reach its binding site(s) may be impaired in the presence the E23K/I337V polymorphism. A further impairment of ATP binding after exposure to palmitoyl-CoA may occur due to competition at the ATP/LC-CoA binding site. A similar effect on ATP inhibition has been noted in cardiac sarcolemmal K_{ATP} channels in response to protein kinase C (26). In the presence of palmitoyl-CoA, the calculated crossover concentration was 100 μ mol/l ATP, suggesting that at physiological $[ATP]_i$ the E23K/I337V polymorphism will render K_{ATP} channels more active than wild-type (Fig. 4D). From the ATP concentration-inhibition curves, it was calculated that the residual K_{ATP} channel activity at 1 mmol/l ATP was $\sim 40\%$ greater in polymorphic K_{ATP} channels compared with wild-type K_{ATP} channels (Fig. 4E). Using a mathematical model of β -cell electrical activity, we found that increases in K_{ATP} channel conductance on the order of $\sim 25\%$ are adequate to significantly reduce the bursting behavior characteristic of glucose-stimulated β -cells (27), suggesting that the observed increase in K_{ATP} channel activity in this study may be sufficient to alter insulin secretion. Taken together, the results and simulations indicate that physiological palmitoyl-CoA concentra-

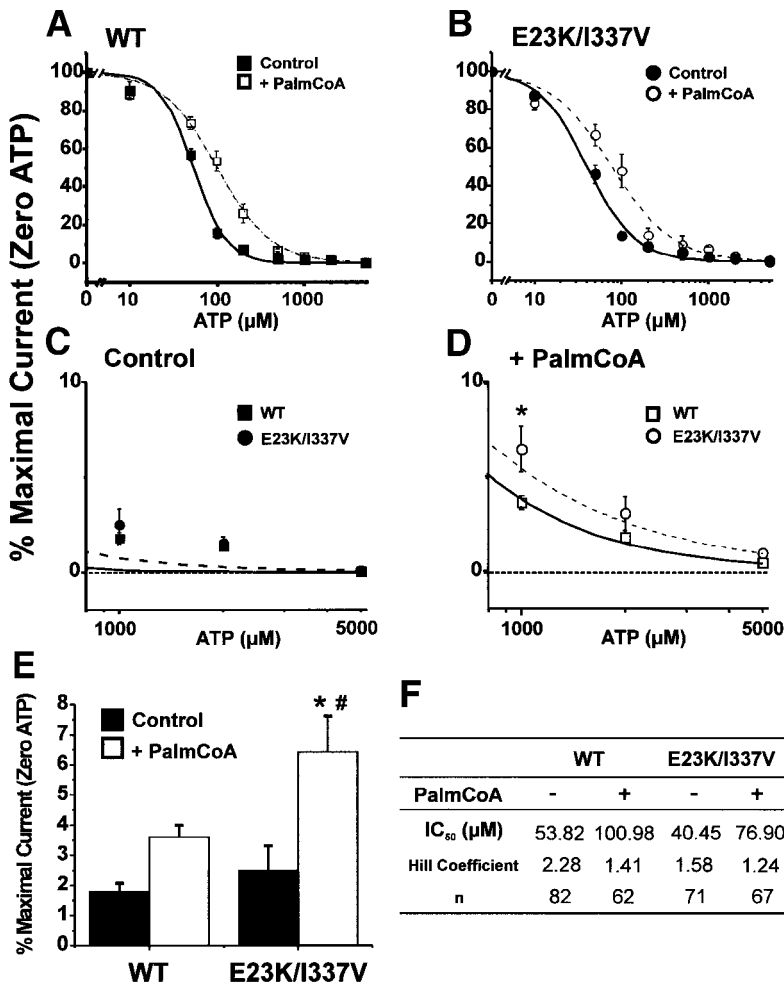


FIG. 4. The Kir6.2(E23K/I337V) polymorphism decreases ATP sensitivity of K_{ATP} channels at physiological ATP concentrations. **A** and **B**: ATP sensitivity of wild-type (WT) Kir6.2/SUR1 (**A**) and polymorphic Kir6.2 (E23K/I337V)/SUR1 (**B**) channels in the absence (control) and presence of 100 nmol/l palmitoyl-CoA (+ palmCoA). Results are expressed as a percent of maximum current obtained in patches exposed to ATP-free solution. Currents at 5 mmol/l were estimated using Hill function and IC₅₀ values reported. **C** and **D**: Extrapolated ATP concentration inhibition curves highlighting physiological range of intracellular ATP. **P* < 0.05 vs. wild-type at 1 mmol/l ATP. **E**: Pooled data from ATP concentration-inhibition curves at 1 mmol/l ATP. ■, absence of palmitoyl-CoA; □, patches treated with 100 nmol/l palmitoyl-CoA. Values are expressed as mean ± SE. **P* < 0.05 vs. wild-type patches treated with palmitoyl-CoA; #*P* < 0.05 vs. polymorphic channels treated with control solution. **F**: Table of results from fitting data in **A** and **B** to the equation $I_{rel} = 1/[1 + ([ATP]/IC_{50})^h]$, where *I*_{rel} is the current observed relative to maximal current in ATP-free solution and *h* is the Hill coefficient; *n* values ranged from 8 to 21 patches for each ATP concentration.

tions could lead to an increased basal polymorphic K_{ATP} channel activity resulting in dysfunctional glucose-stimulated hormone secretion.

Glucose homeostasis is properly maintained through a balanced secretion of hormones from multiple tissues. Interestingly, the K_{ATP} channel is involved in appropriate secretion of insulin (1), glucagon (2), and GLP-1 (3,28). Neuronal control of food intake and hormone secretion via glucose-sensing neurons in the hypothalamus represents an additional potentially impaired process in homozygous polymorphic individuals, as K_{ATP} channels are highly expressed in the central nervous system (29). It is therefore highly plausible that in addition to impaired insulin secretion from β-cells, the E23K/I337V polymorphism may result in inappropriate secretion of glucagon and GLP-1 and dysfunctional regulation of food intake.

In summary, we propose that increased polymorphic K_{ATP} channel activity in the presence of normal or elevated levels of intracellular acyl CoAs may result in decreased insulin and GLP-1 secretion coupled with increased glucagon release. Together, the inappropriate secretion of these hormones may contribute to the association of Kir6.2 polymorphisms with type 2 diabetes. Additional studies will be required to directly assess altered hormone secretion in cell lines expressing a homozygous population of polymorphic K_{ATP} channels. This study is the first to propose a mechanistic link between increased BMI and plasma FFA levels and the observed

high prevalence of type 2 diabetes in the K_{ATP} channel homozygous polymorphic population.

RESEARCH DESIGN AND METHODS

Cell culture and transfection. tsA201 cells (an SV40-transformed variant of the HEK293 human embryonic kidney cell line) were maintained in Dulbecco's modified Eagle's medium supplemented with 25 mmol/l glucose, 2 mmol/l L-glutamine, 10% FCS, and 0.1% penicillin/streptomycin. Cells were kept at 37°C with 5% CO₂. The K_{ATP} channel Kir6.2 subunit clone from mouse was generously provided by Dr. S. Seino (5). The SUR1 subunit clone from hamster was generously provided by Drs. L. Aguilar Bryan and J. Bryan (6). Clones were inserted into the mammalian expression vector pCDNA3. tsA201 cells were plated at 50–70% confluency on 35-mm culture dishes 4 h before transfection. Clones were then transfected into tsA201 cells using the calcium phosphate precipitation technique. Transfected cells were identified using fluorescence optics in combination with coexpression of the green fluorescent protein plasmid (pGreenLantern; Life Technologies, Gaithersburg, MD). Single-channel recordings were made from cells 24 h after transfection and macroscopic recordings 48–72 h after transfection. In contrast to cells expressing SUR1/Kir6.2, nontransfected cells did not exhibit any ATP-sensitive potassium current (data not shown).

Molecular biology. The E23K and I337V mutations were introduced into the Kir6.2 gene using the protocol outlined in the QuikChange site-directed mutagenesis kit (Stratagene) and the generation of the mutations confirmed through sequence analysis. The ΔC26Kir6.2 truncation mutant was constructed as previously described (23).

Patch-clamp experiments. The inside-out patch technique was used to measure macroscopic K_{ATP} channel currents in transfected tsA201 cells. Pipettes were back-filled with solution containing the following (in millimoles per liter): 134 KCl, 10 HEPES, 1.4 MgCl₂, 1 EGTA, 6 KOH, and 10 glucose. The pH of the solution was adjusted to 7.4 with KOH. Macroscopic recordings were performed using patch pipettes pulled from borosilicate glass (G85150T;

Warner Instrument, Hamden, CT) to yield pipettes with a resistance of 2–6 M Ω . Patch pipettes used for single-channel recordings were pulled from borosilicate glass (PG52151-4; World Precision Instruments, Sarasota, FL) to yield pipette resistances of 20–25 M Ω when filled with pipette solution. Once a G Ω seal was formed, the membrane patch was excised from the cell and positioned in the path of a multi-input perfusion pipette. Membrane patches were directly exposed to test solutions under symmetrical K⁺ conditions through this perfusion pipette (time to change solution at the tip of the recording pipette was <2 s). All patch clamp experiments were performed at room temperature (20–22°C). An Axopatch 200B patch-clamp amplifier and Clampex 8.0 software (Axon Instruments, Foster City, CA) were used for data acquisition and analysis.

Single-channel amplitude measurements were analyzed using Fetch software version 6.0 (Axon Instruments). For measurement of Po by noise analysis, the following equation was used: $P_o = 1 - (\sigma^2/[i \times I])$, where σ^2 is the variance measured in the absence of ATP minus the variance measured in between channel opening in the presence of 1 mmol/l ATP (i.e., baseline noise when all channels closed), i is the single channel amplitude, and I is the macroscopic mean current measured in the absence of ATP.

Experimental compounds. MgATP (Sigma, St. Louis, MO) was prepared from a 10-mmol/l stock and stored at –20°C until use. Palmitoyl CoA (Sigma) was dissolved in distilled water as 5-mmol/l stocks. Stock solutions were diluted in pipette solution to concentrations indicated in the text.

Statistical analysis. Recombinant macroscopic K_{ATP} currents were normalized to yield I_{rel}, where I_{rel} is the current under test conditions relative to the maximal control current observed and expressed as a percentage (i.e., I_{rel} = I_{test}/I_{control} × 100). Statistical significance was assessed using the unpaired Student's *t* test, with *P* values <0.05 considered significant. Data are expressed as the mean ± SE (NS, not significant).

Mathematical modeling. Simulations of pancreatic β -cell electrical activity were generated using a mathematical model modified from that described by Bertram et al. (30). In this model, K_{ATP} channel activity as denoted by conductance is a variable and can thus be modified to reflect the experimentally observed changes in K_{ATP} channel activity.

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