The Common Single Nucleotide Polymorphism E23K in $K_{IR}6.2$ Sensitizes Pancreatic β-Cell ATP-Sensitive Potassium Channels Toward Activation Through Nucleoside Diphosphates

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E23K, a common polymorphism in the pore-forming subunit $K_{IR}6.2$ of pancreatic β-cell ATP-sensitive K^+ (K_{ATP}) channels, is functionally relevant and thus might play a major role in the pathophysiology of common type 2 diabetes. In this study, we show that in the simultaneous presence of activatory and inhibitory nucleotides, the polymorphism exerts opposite effects on the potencies of these modulators: channel opening through nucleoside diphosphates is facilitated, whereas sensitivity toward inhibition through ATP is slightly decreased. The results support the conclusion that E23K predisposes to type 2 diabetes by changing the channel's response to physiological variation of cytosolic nucleotides, resulting in K_{ATP} overactivity and discrete inhibition of insulin release. *Diabetes* 51 (Suppl. 3): S363–S367, 2002

Type 2 diabetes is generally perceived as a polygenic disorder, with disease development being influenced by both hereditary and environmental factors (1). Genes encoding for key components of insulin secretion and glucose metabolism pathways have been widely considered as targets for defects in type 2 diabetes (2). One of these key proteins is the ATP-sensitive K^+ channel (K_{ATP} channel) in pancreatic β-cells. This channel critically controls insulin secretion by coupling metabolism to electrical activity (3). The β-cell channel is assembled with a tetradimeric stoichiometry from two structurally distinct subunits: the inwardly rectifying potassium channel subunit ($K_{IR}6.2$), which forms the pore, and the regulatory sulfonylurea receptor subunit 1 (SUR1) (4,5). While hypoglycemic sulfonylureas (e.g., glibenclamide) exert their effects on channel activity by interacting with SUR1, there is strong evidence that the receptor site for inhibitory ATP is located on $K_{IR}6.2$.

E23K (substitution of a lysine [K] for a glutamic acid [E] in position 23) is one of three common missense single nucleotide polymorphisms (SNPs) that have been observed in $K_{IR}6.2$ (E23K, L270V, I337V) (6–10). Recently, we presented evidence that this polymorphism predisposes to type 2 diabetes by inducing overactivity of K_{ATP} channels in the pancreatic β-cell (11). In particular, E23K markedly affects channel gating, significantly reducing the time spent in long interburst closed states and thereby producing an evident increase of spontaneous open probability (P_O). Consistent with the idea that nucleotide-induced channel inhibition results from interaction with the interburst closed states (12,13), sensitivity toward inhibitory ATP^{4-} was found to be decreased (11).

In intact cells, however, other factors besides inhibitory ATP contribute to regulation of channel activity, with ADP presumably representing the most important of these additional parameters (14). ADP has at least three distinct effects on channel activity: *1*) Free ADP^{3-} inhibits channel activity in isolated patches with a potency slightly lower than that of ATP^{4-} (half-maximal inhibitory concentration value [IC_{50}] 49 vs. 8.9 μmol/l, respectively) (11,15). *2*) The Mg^{2+} complex of ADP (MgADP) per se potently activates the channels by interaction with a separate nucleotide site residing on SUR1 (16). *3*) MgADP strongly reduces sensitivity toward inhibitory nucleotides (17).

In this study, we analyzed the impact of the E23K polymorphism of $K_{IR}6.2$ on the regulation of channel activity by activating nucleoside diphosphates. The results support the conclusion that the polymorphism predisposes to type 2 diabetes by inducing overactivity of pancreatic β-cell K_{ATP} channels.

RESEARCH DESIGN AND METHODS

Materials. All chemicals were obtained from the sources described elsewhere (18).

Molecular biology. $K_{IR}6.2$ mutants were constructed as previously described (11). Briefly, point mutations were introduced into human (GenBank Q14654) or mouse (GenBank D50581) $K_{IR}6.2$ by standard molecular biology techniques, and constructs were sequenced to verify PCR fidelity before transfection. For analysis of combinations of the polymorphisms (Figs. 1*C* and 2*C*), mutations were sequentially inferred into the same $K_{IR}6.2$ cDNA.

Electrophysiology. Transfections were performed as previously described (11,18). COS-1 cells cultured in Dulbecco's modified Eagle's medium (DMEM)–high glucose (HG) (10 mmol/l glucose) supplemented with 10% FCS were plated at a density of 8×10^4 cells per dish (35 mm) and allowed to attach overnight. Next, 20 μg of pECE-human SUR1 (GenBank NP_000343) comple-

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DMEM, Dulbecco's modified Eagle's medium; EC_{50}, half-maximal effective concentration; E_{max}, maximal effect; GDP, guanosine 5′-diphosphate; HG, high glucose; IC_{50}, half-maximal inhibitory concentration value; K_{ATP} channel, ATP-sensitive K^+ channel; Kir channel, inwardly rectifying potassium channel; NDPK, nucleoside diphosphate kinase; P_O, open probability; SNP, single nucleotide polymorphism; SUR, sulfonylurea receptor.

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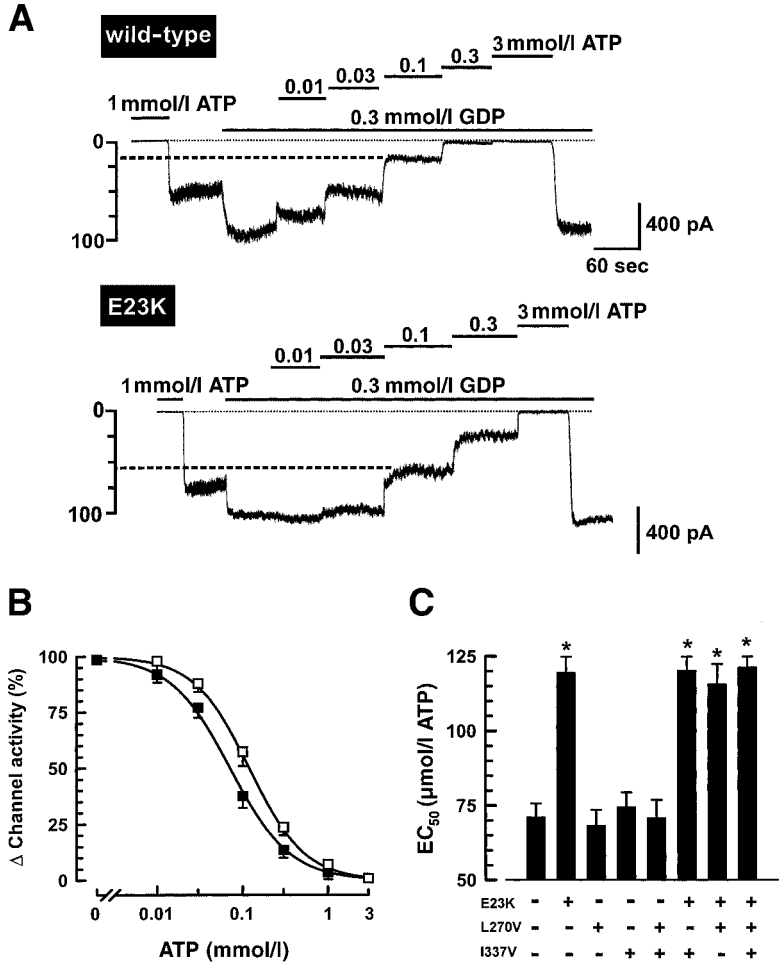

FIG. 1. Effect of E23K on ATP potency in the presence of GDP. *A*: ATP sensitivity of channels reconstituted in COS-1 cells by co-expression of human SUR1 with human $K_{IR}6.2_{wt}$ or $K_{IR}6.2_{E23K}$, as indicated. Representative currents recorded at −50 mV from inside-out patches exposed to ATP and GDP (0.3 mmol/l), as shown by the lines above the records. Inward currents are depicted as downward deflections. Dashed lines indicate residual channel activity (percent) in the presence of 100 μmol/l ATP (plus 0.3 mmol/l GDP). *B*: Potencies of ATP in the presence of 0.3 mmol/l GDP. Channel inhibition was recorded in inside-out patches as shown in *A*. Results are given as means ± SE of 10–15 independent experiments. EC_{50} values and Hill coefficients are as follows: wild-type (■), 71 ± 4.5 μmol/l and 1.22; E23K (□), 120 ± 5.2 μmol/l and 1.34. $P < 0.01$ for comparison of the EC_{50} values. *C*: The effect of E23K on the EC_{50} value for ATP was not altered by the additional introduction of L270V and/or I337V into human $K_{IR}6.2$. *$P < 0.05$ vs. wild-type (n = 8–10).

mentary DNA and 20 μg of pECE-human $K_{IR}6.2$ complementary DNA were mixed and used to transfect six 35-mm plates. In control experiments, we used pECE-hamster SUR1 (GenBank A56248) and pECE-mouse $K_{IR}6.2$ instead of the human isoforms. For transfection, the cells were incubated for 4 h in a Tris-buffered salt solution containing DNA (5–10 μg/ml) plus DEAE-dextran (1 mg/ml), 2 min in HEPES-buffered salt solution plus dimethyl sulfoxide (10%), and 4 h in DMEM-HG plus chloroquine (100 μmol/l). Cells were then returned to DMEM-HG plus 10% FCS. Experiments in the inside-out configuration of the patch-clamp technique were performed 1–2 days after transfection at room temperature, as previously described (11,18). Membrane patches were clamped at −50 mV. The intracellular bath solution contained (in mmol/l) 140 KCl, 2 $CaCl_2$ (free $[Ca^{2+}]$ = 50 nmol/l), 0.7 free Mg^{2+}, 10 EGTA, and 5 HEPES (pH 7.3), and the pipette solution contained (in mmol/l) 140 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, and 10 HEPES (pH 7.4). Test media were applied directly to the patch via a PF-8 rapid perfusion system (E.S.F. Electronic, Göttingen, Germany; diameter of the application pipette 200 μm, flux rate 1 ml/min with the tip of the patch pipette positioned in the middle of the media stream).

The stability of a patch was analyzed in a 3-min test period before the start of the experiments (presence of 1 μmol/l MgATP). Only patches with little "run-down" over this period (<20% loss of channel activity) were chosen. E23K did not affect the percent of patches complying with this condition (wild-type 73%, 95% CI 62–84%; E23K 76%, 95% CI 65–87%; n = 60 each). Patches were corrected for "run-down" over the measuring period by use of linear interpolation. Artifacts from incomplete wash-out of nucleotides or slow reversibility were excluded by ensuring that experiments with stepwise decrease of nucleotide concentrations yielded half-maximal effective concentration (EC_{50}) values and slope factors identical to those from experiments with a stepwise increase of the concentration. The density of K_{ATP} channels per patch ranged from 100 to 700. Variations of channel densities did not affect the percent of sensitive channels, EC_{50} values, or Hill coefficients.

Statistical analysis. Data analysis and statistics were performed as previously described (11,18). Results are shown as records from representative single experiments or as means ± SE (n = 8–15). EC_{50} values and Hill coefficients (n) were estimated by fitting the function $\Delta P_O = 1/\{1+([\text{nucleotide}]/EC_{50})^n\}$ to the data of each single experiment, where ΔP_O is the maximal nucleotide-induced change of the P_O. P values were calculated by the Mann-Whitney U test with correction for multiple comparisons.

RESULTS

Modulation of ATP action. Both ADP and guanosine 5′-diphosphate (GDP) are thought to be involved in the regulation of K_{ATP} channel activity, with resting cytosolic concentrations of ~300 and ~60 μmol/l, respectively (19,20). Each of these nucleotides is a potent activator of channel activity, but GDP (up to 2 mmol/l), in contrast to ADP, does not induce channel inhibition through interaction with the $K_{IR}6.2$ site (21). Hence, to facilitate interpretation of results, GDP was used in the experiments.

Pure wild-type channels (SUR1/$K_{IR}6.2$) transiently expressed in COS cells and analyzed in inside-out patches were strongly activated by the addition of 300 μmol/l GDP (Fig. 1*A*). Exposure to increasing concentrations of ATP led to complete suppression of this activity, with an IC_{50} of 71 ± 4.5 μmol/l (n = 15; Fig. 1*A* and *B*). E23K significantly weakened this effect, inducing a 1.7-fold rightwards shift of the concentration inhibition curve (IC_{50} = 120 ± 5.2 μmol/l; n = 10; $P < 0.01$) (Fig. 1*A* and *B*). This effect was neither reproduced by L270V or I337V nor altered by additional introduction of these polymorphisms into the $K_{IR}6.2_{E23K}$ cDNA (Fig. 1*C*).

Modulation of GDP action. Spontaneous channel activity of pure wild-type channels was partially suppressed by the addition of 10 μmol/l ATP (Fig. 2*A*). Exposure to

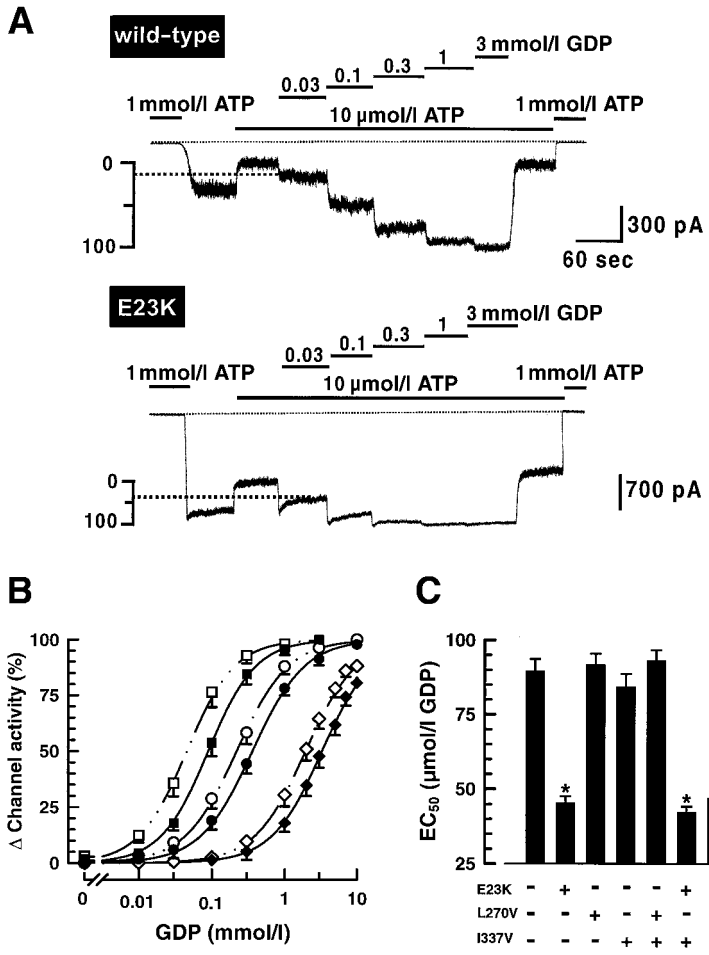

FIG. 2. Effect of E23K on GDP potency in the presence of ATP. *A*: GDP sensitivity of channels reconstituted in COS-1 cells by co-expression of human SUR1 with human $K_{IR}6.2_{wt}$ or $K_{IR}6.2_{E23K}$, as indicated. Representative currents recorded at −50 mV from inside-out patches exposed to GDP and ATP (10 µmol/l), as shown by the lines above the records. Dashed lines indicate channel activation (percent) by 30 µmol/l GDP. *B*: Potencies of GDP in the simultaneous presence of various ATP concentrations (10 and 100 µmol/l and 1 mmol/l). Channel activation was recorded in inside-out patches as shown in *A*. Results are given as means ± SE of 10 independent experiments. EC_{50} values and Hill coefficients are as follows. With 10 µmol/l ATP: wild-type (■), 89 ± 4.1 µmol/l and 1.33, and E23K (□), 45 ± 2.2 µmol/l and 1.32; with 100 µmol/l ATP: wild-type (●), 362 ± 18 µmol/l and 1.21, and E23K (○) 219 ± 7.2 µmol/l and 1.23; with 1 mmol/l ATP: wild-type (♦) 3.29 ± 0.15 mmol/l and 1.24, and E23K (◇) 1.95 ± 0.08 mmol/l and 1.23. $P < 0.01$ for comparison of the EC_{50} values for wild-type and E23K each. *C*: The effect of E23K on the EC_{50} value for GDP was not altered by the additional introduction of L270V and/or I337V into human $K_{IR}6.2$. *$P < 0.05$ vs. wild-type ($n = 8$–10).

increasing concentrations of GDP led to reactivation, with an EC_{50} of 89 ± 4.1 µmol/l ($n = 10$) (Fig. 2*A* and *B*). E23K significantly enhanced this effect, inducing a twofold leftwards shift of the concentration activation curve for GDP ($IC_{50} = 45 \pm 2.2$ µmol/l; $n = 10$; $P < 0.01$) (Fig. 2*A* and *B*). Equivalent effects were observed when the concentration of ATP was increased to 100 µmol/l or 1 mmol/l ($P < 0.01$ each) (Fig. 2*B*). Similar to the results presented above, the effect of E23K on GDP potency was neither reproduced by L270V or I337V nor altered by the additional introduction of these polymorphisms into the $K_{IR}6.2_{E23K}$ cDNA (Fig. 2*C*). All results obtained with the human isoforms were confirmed in channels reconstituted from mouse $K_{IR}6.2$ plus hamster SUR1 (results not shown).

DISCUSSION

The results showed that besides reducing sensitivity toward inhibitory ATP, E23K in $K_{IR}6.2$ increases sensitivity of pancreatic β-cell K_{ATP} channels for activation through nucleoside diphosphates. Experiments were performed in the simultaneous presence of nucleoside di- and triphosphates, because in intact cells both types of nucleotides interact in a complex manner in channel control.

Which of the two effects is physiologically relevant in regulating insulin release—either inhibition of channel activity through interaction of nucleotides with their site on $K_{IR}6.2$ or channel activation through binding to the SUR1 subunit, or both? Actually, in concurrence with the conclusion that insulin secretion might be controlled by the cytosolic ATP/ADP ratio (22), there is evidence in favor of both sites. First, in resting pancreatic β-cells, estimates of the cytosolic concentration of ATP range from 0.6 to 3 mmol/l (19,22–24) and those of ADP, from 0.3 to 1.2 mmol/l (19,22). Consistent with previous reports (25), our experiments show modulation of channel activity if at 300 µmol/l GDP the ATP concentration varies ~1 mmol/l (Fig. 1*B*) and, vice versa, at 1 mmol/l ATP the GDP concentration fluctuates ~300 µmol/l (Fig. 2*B*). Thus, it seems plausible to assume that effects at both the inhibitory and the activatory nucleotide site are involved in physiological channel control. Second, in agreement with this conclusion, loss of the activatory effect of nucleoside diphosphates results in permanently closed channels and hypersecretion of insulin, despite severe hypoglycemia (as is seen in persistent hyperinsulinemic hypoglycemia of infancy) (4,5). Third, on the other hand, the importance of the inhibitory site is underlined by the observation that reduction of ATP sensitivity by no more than 3.6-fold has been shown to induce severe neonatal diabetes in transgenic mice (3). Hence, presumably both effects of E23K—reduced ATP sensitivity and increased nucleoside diphosphate potency—are physiologically relevant.

How does the initial observation of increased spontaneous P_O relate to these effects (11)? In the absence of nucleotides, E23K reduces the time spent in interburst closed states by affecting as well the frequency and mean duration of these states (11). Thus, equilibrium between interburst and burst times seems shifted toward the latter

by both, stabilizing the intraburst states (open state plus closed state) and destabilizing interburst closure. Interestingly, similar changes have been observed in nucleoside diphosphate–induced channel activation (26), which suggests that E23K exerts its effect by facilitating the conformational change that in the physiological channel control results from SUR-K_{IR} interaction. Hence, based on channel kinetics, we would expect E23K to increase the probability that nucleoside diphosphate binding results in channel opening and thus to induce a leftwards shift of the concentration activation curve.

What about ATP? In contrast to nucleoside diphosphates, ATP does not affect burst duration, but solely increases the average time spent in interburst closed states (27,28), suggesting exclusive binding to these states. E23K-induced reduction of the time spent in interburst closure should thus lower the amount of bound ATP. Higher ATP concentrations would be expected to compensate this effect by accelerated association and, accordingly, E23K should induce a rightwards shift of the concentration-inhibition curve. Thus, the effects of the polymorphism on spontaneous channel kinetics (11) appear consistent with the changes in nucleotide sensitivities observed in this study.

Considerable nucleoside diphosphate kinase (NDPK) activity has been demonstrated in pancreatic β-cells (29). Specifically, the effect of GDP on glibenclamide binding in particulate and solubilized β-cell membranes (30,31) suggests that this enzyme is closely linked to β-cell K_{ATP} channels, and therefore NDPK activity in excised patches might have led to a distortion of the results obtained in this study. Yet there are clear arguments against this deduction. First, Hill coefficients of the concentration inhibition curves for ATP in the presence of GDP were 1.22 and 1.34 for wild-type and E23K channels, respectively (Fig. 1*B*), which is consistent with values expected in a medium void of ADP (18). Second, a rough calculation reveals that because of rapid and direct application of the test media (see RESEARCH DESIGN AND METHODS), enzymatically formed nucleotides (ADP or GTP) would at no time be expected to reach concentrations at the excised patch higher than 0.1% of educt (ATP or GDP) concentrations.

In conclusion, in the presence of physiological nucleotide concentrations, E23K in $K_{IR}6.2$ sensitizes pancreatic K_{ATP} channels toward activatory nucleoside diphosphates, whereas the inhibitory effect of ATP is reduced. Results support the inference that the polymorphism predisposes to type 2 diabetes through changing the channel's response to physiological variations of cytosolic nucleotides, resulting in K_{ATP} overactivity and discrete inhibition of insulin release (11).

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