Brief Genetics Report

Single Nucleotide Polymorphisms in K_{ATP} Channels

Muscular Impact on Type 2 Diabetes

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ATP-sensitive K^+ channels (K_{ATP} channels) play an important role in glucose homeostasis. A single nucleotide polymorphism (SNP) in the Kir6.2 subunit causes a point mutation of Glu23 to lysine and reduces the ATP sensitivity of pancreatic K_{ATP} channels. The SNP found in 58% of Caucasians accounts for 15% of type 2 diabetes. Here we show evidence for dysregulations of muscular K_{ATP} channels with the E23K variation. We were particularly interested in the channel modulation by intracellular protons, as pH changes widely and frequently in skeletal muscles. Surprisingly, we found that the defect of the E23K variant was more related to pH than ATP. A level of intracellular acidification seen during exercise not only activated the E23K channel more readily than the wild type, but also relieved the channel inhibition by ATP, leading to a vast increase in the channel open-state probability by approximately sevenfold at pH 6.8 over the wild-type channel at pH 7.4. Considering the reduction in sarcolemmal excitability, muscle fatigue, and impairment of muscular glucose uptake found previously by genetically disrupting K_{ATP} channels, it is likely that the E23K variant in muscular K_{ATP} channels affects systemic glucose homeostasis and poses an important risk factor for type 2 diabetes and obesity. *Diabetes* 54:1592–1597, 2005

Type 2 diabetes is a challenge to modern medicine. Although defects in glucose homeostasis of the disease have been recognized for decades, molecular mechanisms underlying the defects remain poorly understood. Recent discovery of a single nucleotide polymorphism (SNP) in the *Kcnj11* gene provides an important genetic link to type 2 diabetes (1–3). This SNP results in a point mutation of Glu23 to lysine (E23K) in the pore-forming Kir6.2 subunit of the ATP-sensitive K^+ channels (K_{ATP} channels). Genotypic frequencies average 42% for E/E (homozygous at residue 23), 47% for E/K (heterozygous), and 11% for K/K (4). Risk and frequency estimates have shown that ~15% of type 2 diabetic cases in Caucasians are attributable to the K/K and E/K genotypes (4). Similar allelic frequencies of E23K (34%) have been reported in Japanese populations (5).

The K_{ATP} channels are composed of four pore-forming Kir6 subunits (Kir6.1 or Kir6.2) and four regulatory subunits of sulfonylurea receptor (SUR1, SUR2A, or SUR2B). The channels are normally inhibited by physiological concentrations of ATP and activated when the ATP level drops, allowing them to couple the cellular metabolism with membrane excitability (6,7). Experimental evidence suggests that the E23K mutation augments pancreatic K_{ATP} channels and reduces insulin secretion from β-cells (8,9). In addition to β-cells, the Kir6.2 subunit is expressed in cardiac and skeletal muscles (6,7). The muscular isoform of K_{ATP} channels consisting of Kir6.2 and SUR2A subunits regulates muscular excitability and plays a role in glucose uptake (10–13). Therefore, genetic variations in the muscular K_{ATP} channels may contribute to abnormalities of systemic glucose homeostasis. Since proton is a potent regulator of the K_{ATP} channels (14–16), and since intracellular pH levels change widely in the skeletal muscles during the exercise (14), alterations in the pH sensitivity of the E23K variant may affect K_{ATP} channel activity, membrane excitability, and muscular glucose uptake, contributing a risk factor for type 2 diabetes and obesity. To test this hypothesis, we studied the pH sensitivity and the effect of pH on the ATP sensitivity of the wild-type and E23K variant Kir6.2 expressed with SUR2A using inside-out patches with symmetric concentrations of K^+ (145 mmol/l) applied to both sides of patch membranes.

When the internal surface of patch membranes was exposed to a perfusate with various pH levels in the presence of 0.3 mmol/l ATP, the wild-type Kir6.2/SUR2A currents increased with moderate acidification, reached a maximum activation at pH 6.2, and were inhibited at extremely acidic pH, consistent with the Kir6.2/SUR1 currents shown in our previous studies (16,17) (Fig. 1*A*, online appendix [available at http://diabetes.diabetesjournals.org]). Such a biphasic response was seen in the presence of 1.0 mmol/l ATP, although the peak activation took place at pH 5.9 (Fig. 1*B*, online appendix). Since the inhibition or channel rundown was not seen in whole-cell recording (16), further studies focused on pH-dependent channel activation. The pH-current relationship was described

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K_{ATP} channel, ATP-sensitive K^+ channel; SNP, single nucleotide polymorphism.

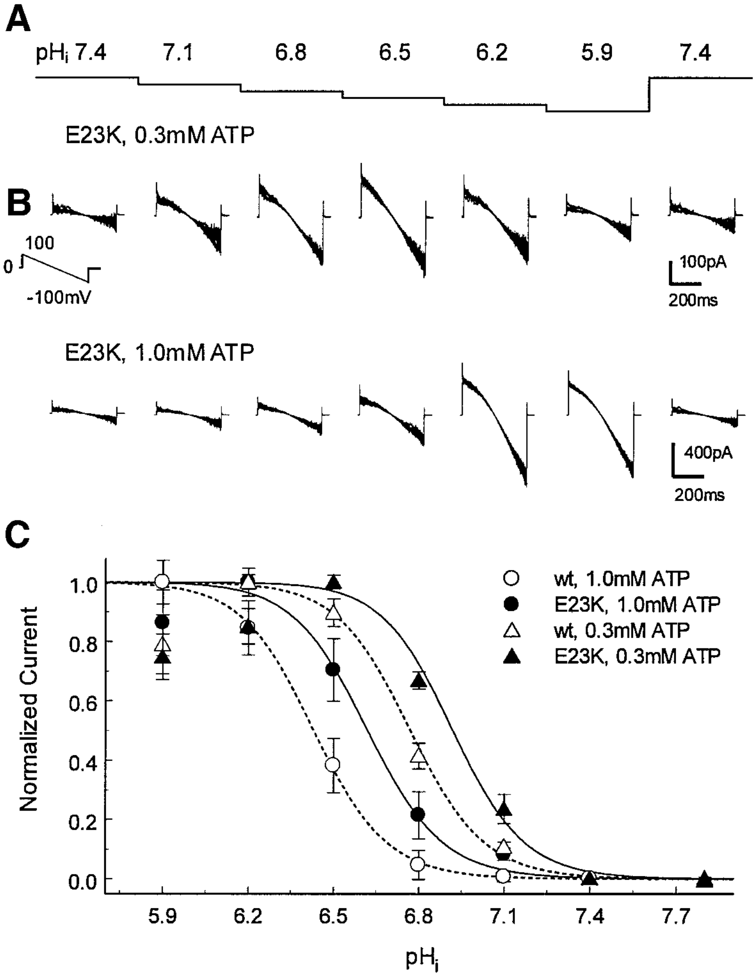

FIG. 1. Increase in the pH sensitivity of the E23K variant. Kir6.2 with the E23K mutation was coexpressed with SUR2A in HEK293 cells and studied in inside-out patches. *A* and *B*: Currents were activated when the internal membranes were exposed to acidic pH levels. Peak activation was reached at pH 6.5 and 6.2 in the presence of 0.3 mmol/l ATP and 1.0 mmol/l ATP, respectively. Note that eight superimposed traces are shown in each panel. *C*: The pH-current relationship is plotted and described with the Hill equation. With 0.3 mmol/l ATP, the midpoint pH for channel activation or pK$_a$ is pH 6.77 for the wild-type and pH 6.92 for the E23K variant. The pK$_a$ values changed to pH 6.43 for the wild-type and pH 6.62 for the E23K variant in the presence of 1.0 mmol/l ATP.

using the Hill equation. In the presence of 0.3 mmol/l ATP, the pH level for 50% current activation (pK$_a$) was 6.77 ± 0.02 (n = 10) with the Hill coefficient (h) 3.5 (Fig. 1*C*). The pH-current relationship shifted toward lower pH levels when 1.0 mmol/l ATP was part of the internal solution (pK$_a$ = 6.43 ± 0.01, h = 3.4, n = 7).

Exposures of the internal patch membranes to ATP produced a concentration-dependent inhibition of the Kir6.2/SUR2A currents (Fig. 1*C* and *D*, online appendix). At pH 7.4, the ATP concentration for 50% current inhibition (IC$_{50}$) was 32 ± 2.0 μmol/l (n = 10) (Fig. 2*C*). The ATP sensitivity, however, was markedly reduced when the internal solution became acidic. At pH 6.8, the currents were half inhibited by 75 ± 4.0 μmol/l ATP (n = 6) (Fig. 2*C*).

Like the wild-type channel, the E23K variant displayed a biphasic response to acidic pH with peak activation at pH 6.5 and 6.2 in the presence of 0.3 and 1.0 mmol/l ATP, respectively (Fig. 1*A* and *B*). As a result, its pH-current relationship clearly differed from that of the wild-type channel. With 0.3 mmol/l ATP, the E23K variant showed a pK$_a$ of 6.92 ± 0.03 (n = 8) and an h of 3.7 (Fig. 1*C*). Such a change was even more obvious in the presence of 1.0 mmol/l ATP (pK$_a$ 6.62 ± 0.03, h = 3.4, n = 8).

We have previously shown pH sensitivity of the truncated Kir6.2 channel (Kir6.2ΔC36) expressed without SUR (16,17). For a comparison purpose, we studied the Kir6.2ΔC36-E23K in the absence of ATP and fitted the pH-current relationship using a sum of two Hill equations as described previously (16,17). The E23K mutation enhanced the pH sensitivity by 0.34 pH units (Fig. 2*A–C*, online appendix). Such pH sensitivity was also seen in the wild type and E23K coexpressed with SUR2A, although the pH-current relationship shifted toward more alkaline pH (Fig. 2*C*, online appendix).

The ATP sensitivity of the E23K polymorphic Kir6.2/SUR2A decreased modestly at pH 7.4 compared with the wild-type channel (Fig. 2*A*). The ATP-current relationship plot showed an IC$_{50}$ of 68 ± 4.0 μmol/l (n = 8) for the E23K variant (Fig. 2*C*). The ATP sensitivity dropped greatly at pH 6.8, with an IC$_{50}$ of 225 ± 5.0 μmol/l (n = 7), three times as high as that for the wild-type channel at the same pH level (Fig. 2*B* and *C*). Consistent with these observations, a reduction in the ATP sensitivity and augmentation of the pH sensitivity were also found in the E23K mutant of rodent Kir6.2/SUR2A channels (Fig. 3*A* and *B*, online appendix).

In addition to ATP and protons, K$_{ATP}$ channels are modulated by ADP. The channels are activated with micromolar concentrations of ADP in the presence of Mg^{2+} and inhibited with millimolar concentrations (18). Thereby, the ATP-current relationship was studied with 0.3 mmol/l ADP and 1.0 mmol/l free Mg^{2+} in the internal solution. Under this condition, the E23K currents continued to be activated by ADP and inhibited by ATP. However, the ATP-current relationship curves shifted in

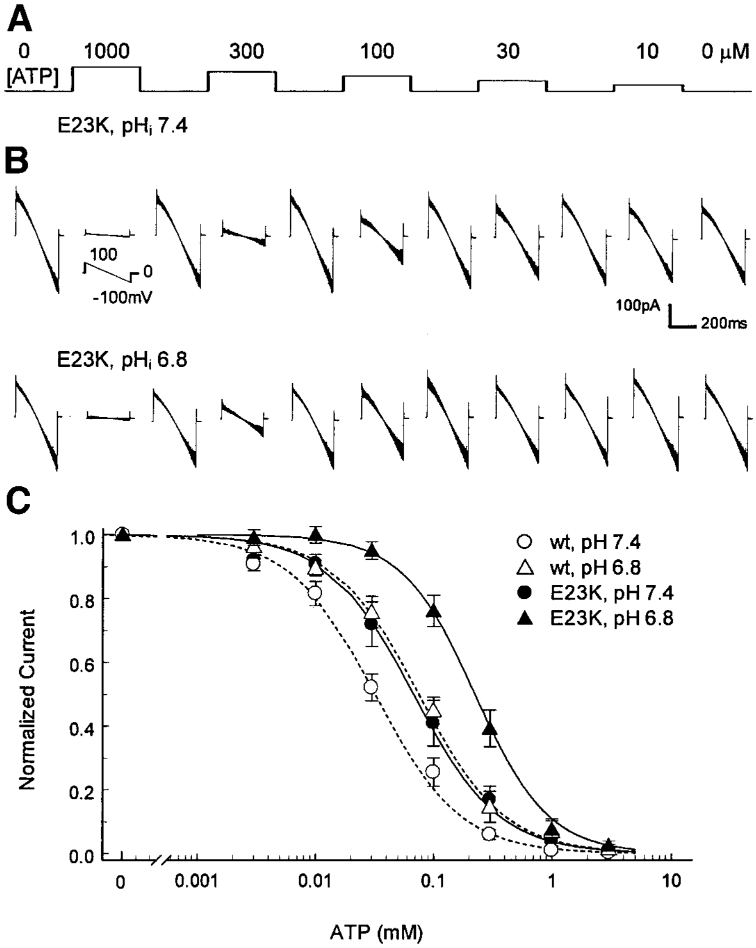

FIG. 2. Dose-dependent inhibition of the E23K currents by ATP. *A* and *B*: Current responses to different ATP concentrations were measured at pH 7.4 and 6.8. The midpoint inhibition of the E23K currents was reached at ~100 μmol/l and 300 μmol/l ATP concentrations at pH 7.4 (*A*) and pH 6.8 (*B*), respectively. Note that eight superimposed traces are shown in each panel. *C*: Current amplitude measured at −100 mV is expressed as a function of ATP concentrations using the Hill equation. The IC_{50} was 32 μmol/l at pH 7.4 and 75 μmol/l at pH 6.8 for the wild-type channel. However, ATP sensitivity was much lower in E23K mutant channels with IC_{50} 68 μmol/l at pH 7.4 and 225 μmol/l at pH 6.8. The Hill coefficient (*h*) remained 1.2 in both wild-type and E23K mutant at these pH levels.

parallel toward higher ATP levels in the E23K variant, with an IC_{50} of 150 ± 9.0 μmol/l ($n = 6$) at pH 7.4 and 400 ± 29.0 μmol/l ($n = 7$) at pH 6.8 (Fig. 3*A* and *E*), indicating that the ATP sensitivity of the E23K variant is further reduced in the presence of ADP.

Another polymorphism, I337V in the *Kcnj11* gene, was studied as a control. Both ATP and pH sensitivities remained close to the wild-type channel (Fig. 3*C–F*), indicating that the changes in ATP and proton sensitivities of the E23K polymorphism are specific.

Single-channel studies showed that conductance of the E23K (74 ± 8.4 pS, $n = 27$) remained comparable with that of the wild-type channels (72 ± 6.0 pS, $n = 23$; $P > 0.05$). The channel open-state probability (P_{open}) was measured in inside-out patches at −80 mV membrane potential. To have a pseudo-physiologic condition, 0.3 mmol/l ADP and 1.0 mmol/l ATP were added to the internal solution. At pH 7.4, the P_{open} was low (0.029 ± 0.006, $n = 22$) in the wild-type channel. Although it remained low, the P_{open} (0.079 ± 0.014, $n = 18$) was doubled in the E23K variant over the wild type ($P < 0.001$). At pH 6.8, the P_{open} increased in E23K (0.191 ± 0.015, $n = 26$) compared with wild type (0.112 ± 0.013, $n = 17$; $P < 0.001$). The P_{open} of the E23K at pH 6.8 was approximately sevenfold as high as the wild-type channel at pH 7.4 (Fig. 4). Similar results were obtained in the absence of ATP (Fig. 2*D*, online appendix).

The E23K has been shown to be associated with type 2 diabetes, both alone and in combination with I337V (1,2). Previous studies suggest that the homozygous K/K is much more likely to predispose the carrier to type 2 diabetes than the heterozygous E/K (1–3). The channel activity is increased by 1.4- and 2.2-fold in the E23K heterozygous and K23K homozygous models, respectively (8). The E23K polymorphism enhances baseline open-state probability and reduces ATP sensitivity of the K_{ATP} channels in pancreatic β-cells. The overactivation of β-cell K_{ATP} channels prevents the intracellular Ca^{2+} rise necessary for insulin secretion and thus inhibits insulin release (8,9).

In addition to the β-cells, several lines of evidence suggest that K_{ATP} channels in peripheral tissues are also involved in glucose homeostasis. The effect of the E23K genotype on β-cell function has been inconsistent and even contradictory, as indicated by different research groups (8,9,19,20). The β-cell hypothesis cannot totally explain the disruption of glucose homeostasis in type 2 diabetes, as glucose uptake is problematic in type 2 diabetes (21). As a subunit of muscular K_{ATP} channels, Kir6.2 is expressed in both skeletal and cardiac muscles, which constitute the predominant tissues for glucose utilization in the human body (6,7). K_{ATP} channel openers suppress glucose uptake in human skeletal muscles, and such an effect can be blocked by K_{ATP} channel inhibitors (22). The K_{ATP} channel openers also reduce force development leading to muscle fatigue, whereas they have no effect on action potentials and fatigue kinetics of skeletal muscles in Kir6.2$^{-/-}$ mice (10). Genetical disruption of K_{ATP} channels lowers muscular excitability and enhances

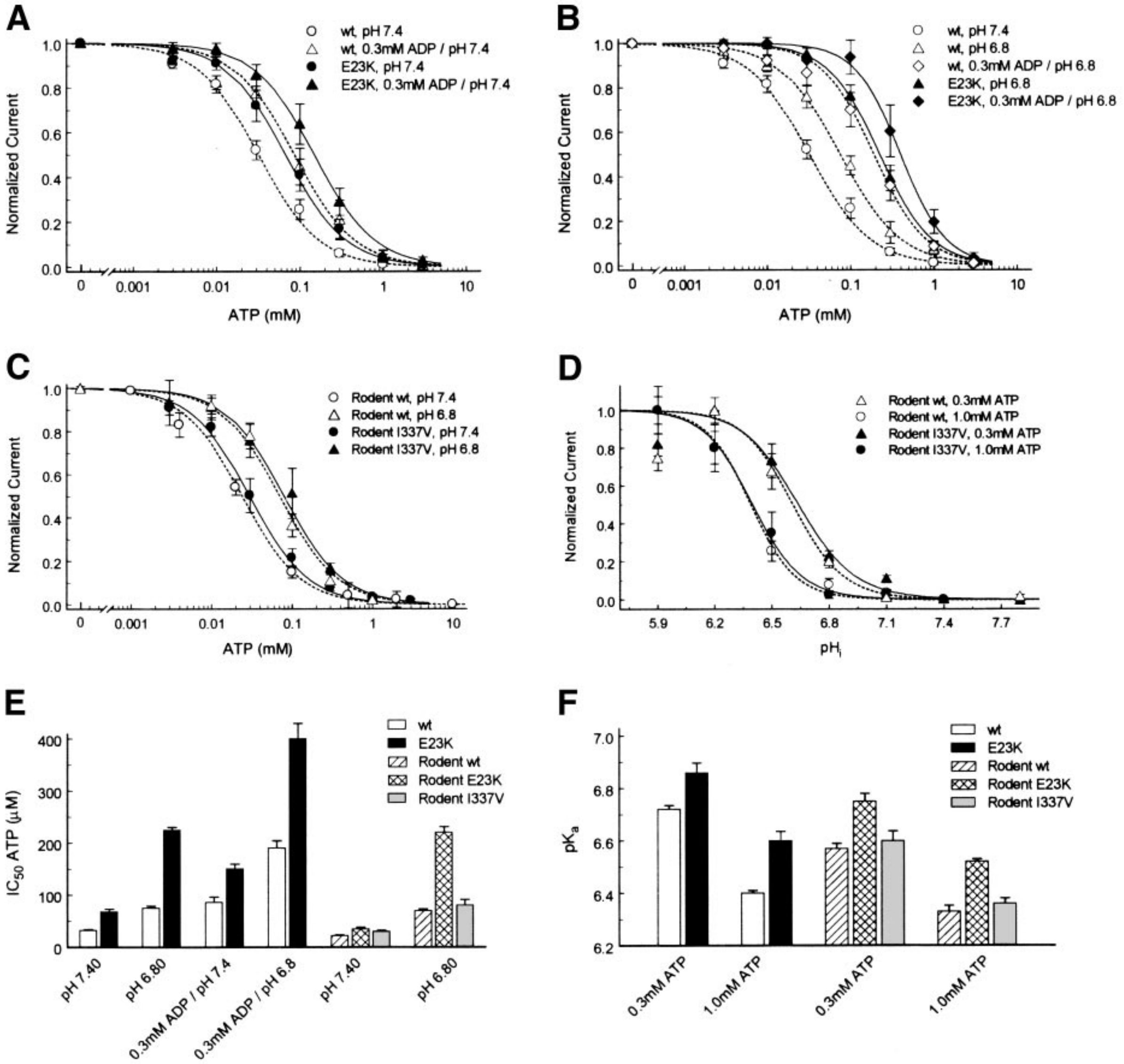

FIG. 3. ATP and pH sensitivities of the E23K and I337V variants. *A*: The ATP-current relationship was studied in the same condition as Fig. 2 with addition of 0.3 mmol/l ADP and 1.0 mmol/l mg^{2+} in the perfusate. The ATP sensitivity of both the wild-type (wt) and E23K variants decreased in the presence of ADP and mg^{2+}, while the decrease in ATP sensitivity was more evident in the E23K variant (IC$_{50}$ = 150 μmol/l) than the wild-type channel (IC$_{50}$ = 86 μmol/l) at pH 7.4. *B*: At pH 6.8, such a reduction in the ATP sensitivity was further enhanced in the E23K variant (IC$_{50}$ 400 μmol/l) compared with the wild-type channels (IC$_{50}$ = 190 μmol/l). *C* and *D*: Control experiments were performed on another polymorphic I337V in the mouse Kir6.2. Note the changes in both pH sensitivity and ATP sensitivity for I337V are insignificant compared with the wild-type channels. In the presence of 0.3 mmol/l ATP, the pK$_a$ was 6.64 (h = 3.0) for the I337V and 6.61 (h = 3.4) for the wild-type channel. With 1.0 mmol/l ATP, the pK$_a$ was 6.40 (h = 3.4) for the I337V and 6.38 (h = 3.4) for the wild type. *E* and *F*: Summary of IC$_{50}$ and pK$_a$ levels of ATP and pH sensitivities of wild type, E23K, and I337V in both human and mouse Kir6.2. Data are shown as means ± SE (n = 6–12).

glucose uptake in skeletal muscles (12,13). In knockout mice lacking both *Kcnj11* and insulin receptor substrate-1 genes, K$_{ATP}$ channel–mediated glucose uptake is found to be independent of insulin receptor substrate-1 and phosphatidylinositol-3 kinase signaling pathway (11).

The E23K variant has been previously shown to reduce ATP sensitivity when expressed with SUR1; at pH 7.15, the IC$_{50}$ for the E23K is about twice as high as the wild type (8), consistent with our observation in the muscular isoform at pH 7.4. It is worth noting that the IC$_{50}$ level of the wild-type channels varies in a similar range depending on experimental conditions (8,17,23). Such a variation might have prevented some previous studies from showing different ATP sensitivity between the wild-type and E23K variant (24).

To our surprise, we found that the E23K defect in muscular K$_{ATP}$ channels was more related to pH than ATP. Intracellular acidification not only activates the E23K channel more readily than the wild type but also relieves the channel inhibition by ATP, leading to a vast activation of the muscular K$_{ATP}$ channels. The activation of K$_{ATP}$ channels can cause hyperpolarization of sarcolemmal membranes, muscle fatigue, and a reduction in glucose uptake, as shown in previous studies in genetically disrupted K$_{ATP}$ channels (10–13). Therefore, it is likely that the enhanced pH sensitivity together with the reduced ATP sensitivity in the E23K variant leads to overactivation of the muscular K$_{ATP}$ channels, impairs glucose uptake during exercise, and contributes to the development of type 2 diabetes.

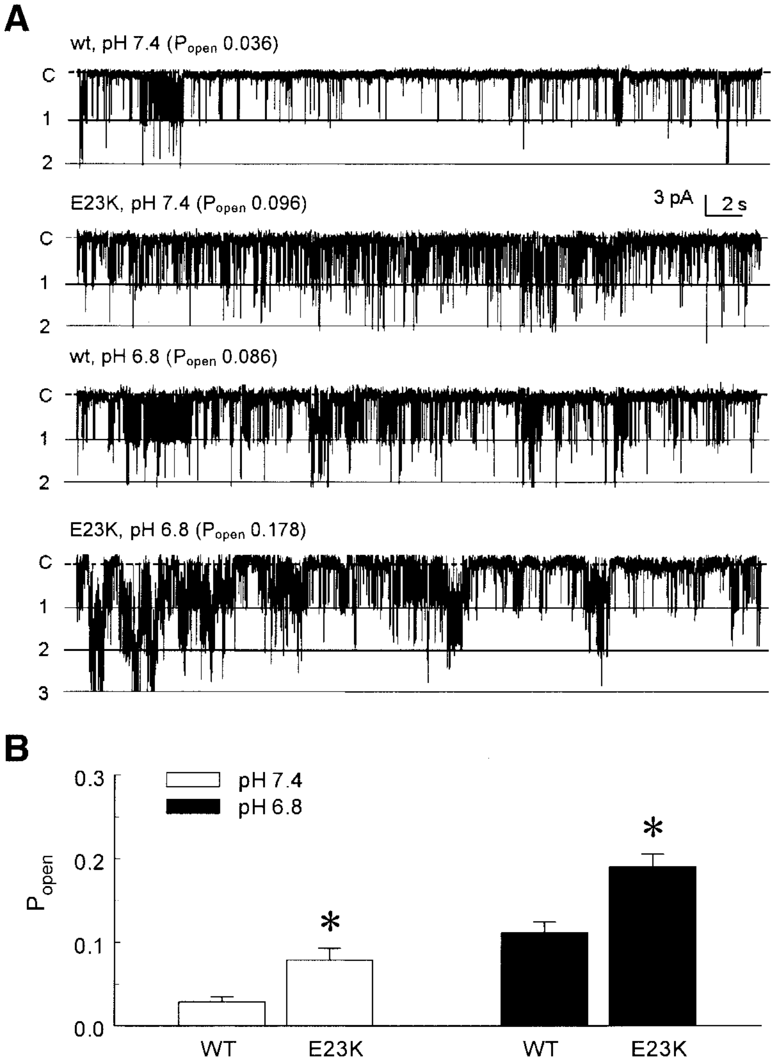

FIG. 4. *A*. Single-channel activity of the E23K and wild-type channels. Currents were recorded at membrane potential −80 mV with 0.3 mmol/l ADP and 1.0 mmol/l ATP added to the internal solution. *A*: Compared with the wild-type (wt) channels, the E23K variant showed a higher P_{open}, while the P_{open} was higher at pH 6.8 than at pH 7.4 in both wild type and E23K. *B*: The difference in P_{open} was statistically significant between wild type and E23K at both pH levels (*$P < 0.001$). Data are presented as means ± SE ($n = 17–26$).

Our results also suggest a link of the genetic factor to the environmental factors, such as sedentary lifestyle and obesity, as the easy activation of polymorphic muscular K_{ATP} channels tends to hyperpolarize muscular cells, leading to muscle fatigue, reduction in glucose uptake, and fat deposition. Then, why is such an adverse genetic variant widely reserved in human populations? We believe that the E23K variant, which potentially results in slow glucose utilization, muscle fatigue, and obesity, may facilitate population survival of the early humans, as their food supplies may be seasonal and discontinuous.

RESEARCH DESIGN AND METHODS

Human Kir6.2 (Genbank no. D50582), human SUR2A (Genbank no. NM_005691), mouse Kir6.2 (Genbank no. D50581), and rat SUR2A (Genbank no. D83598) were used in the present study. The cDNAs were cloned in a eukaryotic expression vector and expressed in mammalian cell line. Site-specific mutations were produced using a site-directed mutagenesis kit based on the *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Correct mutations were confirmed with DNA sequencing. The channels were expressed in HEK293 cells by transfection using Lipofectamine^{2000} (Invitrogen, Carlsbad, CA).

Patch-clamp experiments were performed at room temperature as described previously (16,17). Macropatch and single-channel recordings were performed using solutions containing equal concentrations of K^+ applied to the bath and recording pipettes. The solution contained (in mmol/l): 10 KCl, 105 potassium gluconate, 5 potassium fluoride, 20 potassium pyrophosphate, 0.1 sodium vanadate, 5 EGTA, 5 glucose, and 10 HEPES (pH = 7.4). It is known that pyrophosphate and vanadate prevent channel rundown, and their concentrations in our patch solution have been shown to have no effect on the Kir6.2 channels (16,17).

Low pH exposures were carried out using the same bath solutions, which were titrated to different pH levels as required by experimental protocols. For ATP (K^+ salt) exposures, pH levels were adjusted after appropriate ATP concentrations were made in each solution. To avoid ATP degradation, all ATP-containing solutions were made immediately before experiments and used for no longer than 4 h. The pH-current relationship was described using the Hill equations: $y = 1/[1 + (pH/pK_a)^h]$, where pK_a is the pH level for midpoint channel activation and h is the Hill coefficient. The ATP-current relationship was expressed with the regular Hill equation: $y = 1/(1 + [\{ATP\}/IC_{50}]^h)$, where {ATP } is the ATP concentration and IC_{50} is the {ATP } at midpoint channel inhibition. Differences in means were tested with the ANOVA or Student's *t* test and were accepted as significant if $P \leq 0.05$.

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