FEBS Letters 409 (1997) 232–236 FEBS 18633

Subunit stoichiometry of the pancreatic β-cell ATP-sensitive K^+ channel

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Received 1 April 1997; revised version received 18 April 1997

Abstract **We have investigated the subunit stoichiometry of the pancreatic β-cell ATP-sensitive K^+ (K_{ATP}) channel (SUR1/Kir6.2 channel) by constructing cDNA encoding a single polypeptide (βα polypeptide) consisting of a SUR1 (β) subunit and a Kir6.2 (α) subunit. $^{86}Rb^+$ efflux and single-channel properties of COS1 cells expressing βα polypeptides were similar to those of COS1 cells coexpressing α monomers and β monomers. Coexpression of βα polypeptides with α monomers inhibited the K^+ currents, while coexpression with β monomers did not. We then constructed another single polypeptide ($\beta\alpha_2$) consisting of a β subunit and a dimeric repeat of the α subunit. $^{86}Rb^+$ efflux from COS1 cells expressing $\beta\alpha_2$ polypeptides was small, but was restored by supplementation with β monomers. These results indicate that the activity of K_{ATP} channels is optimized when the α and β subunits are coexpressed with a molar ratio of 1:1. Since inward rectifier K^+ channels are thought to function as homo- or hetero-tetramers, this suggests that the K_{ATP} channel functions as a multimeric protein, most likely a hetero-octamer composed of a tetramer of the Kir6.2 subunit and a tetramer of the SUR1 subunit.**

Key words: Potassium channel; ATP; Inward rectifier; Sulfonylurea receptor; Stoichiometry

1. Introduction

ATP-sensitive potassium (K_{ATP}) channels play an important role in various cellular responses such as secretion and muscle excitability by linking the cell's metabolic state to its membrane potential [1]. We have shown that pancreatic β-cell K_{ATP} channel currents are the product of a complex comprising the inward rectifier K^+ channel member Kir6.2 subunit [2,3] and a sulfonylurea receptor SUR1 subunit [4]. In addition, we have isolated a cDNA encoding an isoform of SUR1, designated SUR2, and have shown that coexpression of the SUR2 subunit and the Kir6.2 subunit in COS1 cells reconstitutes K_{ATP} channel properties similar to those found in cardiac and skeletal muscle [5]. In addition, it has recently been shown that coexpression of the Kir6.1 subunit and the SUR2B subunit, a variant form of SUR2, reconstitutes K^+ currents with properties similar to those of K_{ATP} channels in smooth muscle [6,7]. Thus, differing SUR subunits in combination with Kir6.0 subfamily subunits may account for the functional diversity of K_{ATP} channels. Although it has been proposed that both inward rectifier and voltage-gated K^+ channels function as homomeric or heteromeric multimers [8–15], the subunit stoichiometry of the K_{ATP} channel is unknown.

In the present study we have investigated the subunit stoichiometry of the pancreatic β-cell K_{ATP} (SUR1/Kir6.2) channel by constructing single polypeptides, βα polypeptide and $\beta\alpha_2$ polypeptide, consisting of a SUR1 (β) subunit and a Kir6.2 (α) subunit and a β subunit and a dimeric repeat of the α subunit, respectively. The number of β and α subunits required to form a functional K_{ATP} channel was assessed by coexpressing these single polypeptides with α or β monomers. Our data suggest that the β-cell K_{ATP} channel probably functions as a hetero-octamer comprising a tetramer of the SUR1 subunit and a tetramer of the Kir6.2 subunit.

2. Materials and methods

2.1. Construction of plasmid DNAs

We constructed the expression vector (pCMVβα) containing a DNA fragment encoding a single polypeptide (βα) consisting of a SUR1 subunit (β) and a Kir6.2 subunit (α). To link a β and an α subunit in a head-to-tail fashion, the nucleotide sequence $(CAG)_9$CAAATCGAT encoding a stretch of 10 glutamine residues was introduced between the 3′-end of hamster SUR1 cDNA [4] and the 5′-end of mouse Kir6.2 cDNA [2] (Fig. 1A). As a result, the intervening amino acid sequence $(Q)_{10}$IDFEPGANGA is present between the β and α subunits. We constructed another expression vector (pCMV$\beta\alpha_2$) encoding a single polypeptide ($\beta\alpha_2$) consisting of one β and two α subunits. To link the β subunit and a dimeric repeat of the α subunit in a head-to-tail fashion, the nucleotide sequence $(CAG)_{10}$AATTC encoding a stretch of 10 glutamine residues was introduced between the 3′-end of a βα-coding DNA fragment as described above and the 5′-end of mouse Kir6.2 cDNA (Fig. 4A). As a result, the intervening amino acid sequences, $(Q)_{10}$IDFEPGANGA and $(Q)_{10}$NSSSVPGANGA, are present between the β subunit and the first α subunit and between the first and second α subunits, respectively.

2.2. In vitro mutagenesis

A 4981 bp *Eco*RI fragment of hamster SUR1 cDNA [4] and a 1552 bp *Sma*I/*Eco*RI fragment of mouse Kir6.2 cDNA [2] were subcloned into pALTER (Promega, Madison, WI). Single-strand templates of pALTER SUR1 and pALTER Kir6.2 were prepared using helper phage R408. The K719R mutation in the Walker A motif [4,16] in the first nucleotide-binding fold (NBF)-1 of SUR1 and the G132S mutation in the H5 region of Kir6.2 were introduced using 21-mer oligonucleotides according to the manufacturer's instructions (in vitro mutagenesis system, Promega). An *Eco*RI fragment of mutant SUR1 was subcloned into the mammalian expression vector pCMV6c as described above. The correct construction of the mutant was confirmed by automated DNA sequence analysis (ABI PRISM™ 310 Genetic Analyzer, Perkin Elmer, Foster City, CA).

2.3. Cell culture and transfection

COS1 cells were plated at a density of 3×10^5 per dish (35 mm in diameter) for single-channel analysis or per well (30 mm 6-well dish) for $^{86}Rb^+$ efflux measurements, respectively, and cultured in DMEM (high glucose) (DMEM-HG) supplemented with fetal calf serum (10%). For single-channel analysis, various plasmid constructs in pCMV, with the expression plasmid vector for green fluorescence protein (pSRαGFP, 0.05 μg) as a reporter gene for transfection [5],

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PII S0014-5793(97)00488-2

were transfected into COS1 cells with Lipofectamine and Opti-MEM I reagents (Life Technologies, Inc., Gaitherburg, MD) according to the manufacturer's instructions. For $^{86}Rb^+$ efflux measurements, transfection procedures were as described above except for the exclusion of pSRαGFP. In each experiment, the total amount of plasmid DNA was adjusted to 2 μg with pCMV vector.

2.4. Electrophysiology

After transfection, the cells were cultured for 48–92 h. Single-channel recordings were made in the transfected cells which were selected by green fluorescence under a microscope, as described [5]. Single-channel recordings were made in the excised inside-out patch configurations as described [2]. The bath solution contained 110 mM K-aspartate, 30 mM KCl, 2 mM $MgSO_4$, 1 mM EGTA, 0.084 mM $CaCl_2$, and 10 mM MOPS (pH 7.2). Dipotassium ATP (1 μM) was added to the bath solution unless otherwise noted. The pipette solution contained 140 mM KCl, 2 mM $CaCl_2$, and 5 mM MOPS (pH 7.4). Recordings were made at 20–22°C. Currents were filtered at 2 kHz and digitized at 5 kHz for analysis. The ATP-sensitivity of channels was measured by applying K_2ATP and the % of maximal channel activity was calculated as described previously [2].

2.5. Rb^+ efflux measurements

Two days after transfection, $^{86}RbCl$ (1 μCi/ml) (Amersham International plc., Buckinghamshire, UK) was added in fresh DMEM-HG containing fetal calf serum (10%) and incubated for 12–24 h. The cells were further incubated for 30 min at 37°C in Krebs-Ringer solution containing 1 μCi $^{86}RbCl$/ml with or without oligomycin (2.5 μg/ml) and 1 mM 2-deoxy-D-glucose. After washing the cells once in $^{86}Rb^+$-free Krebs-Ringer solution, with or without added metabolic inhibitors and glibenclamide, $^{86}Rb^+$ efflux was measured at 37°C as previously described [2]. Briefly, portions of the medium from each time were counted, and the values were summed to determine the efflux. The data were expressed as the percentage of the $^{86}Rb^+$ content at the start of the incubation.

3. Results and discussion

Fig. 1B,C show $^{86}Rb^+$ efflux from COS1 cells transfected with the expression vector pCMV alone, pCMV carrying mouse Kir6.2 cDNA (pCMVα) plus pCMV carrying hamster SUR1 cDNA (pCMVβ), or pCMVβα. The efflux from COS1 cells coexpressing α and β monomers was stimulated above the endogenous efflux from COS1 cells transfected with the vector alone as described previously (Fig. 1B) [2]. The efflux from COS1 cells expressing βα polypeptides also was stimulated as much as that from the cells coexpressing α and β monomers (Fig. 1B). Metabolic poisoning with oligomycin (2.5 μg/ml) and 2-deoxyglucose (1 mM) stimulated both the efflux from COS1 cells expressing βα polypeptides and the efflux from COS1 cells coexpressing α and β monomers to a similar degree, despite the simultaneous inhibition of the endogenous COS1 cell efflux (Fig. 1C).

Single-channel recordings of COS1 cells expressing βα polypeptides were performed. Expression of βα polypeptides in COS1 cells elicited inward rectifying K^+ currents with single-channel conductance of 79.2 ± 1.3 pS (mean ± SE, $n = 6$), at a membrane potential of −60 mV with 140 mM K^+ on both sides of the membrane (Fig. 2A). The currents elicited by both βα polypeptides were inhibited by ATP in a dose-dependent manner (IC_{50}: 18.5 ± 3.3 μM (mean ± SE, $n = 11$)) (Fig. 2B,C). These results show that βα polypeptides reconstitute K_{ATP} channels with properties similar to those of β-cell K_{ATP} channels reconstituted from α and β monomers [2] (Fig. 2), suggesting that coexpression of the α and β subunit with a molar ratio of 1:1 is sufficient for functional expression of the K_{ATP} channel. On the assumption that the inward rectifier K^+ channel functions as a homo- or hetero-tetramer [11–15]), the βα polypeptides probably form a tetramer (Fig. 1D).

To further analyze the subunit stoichiometry of the K_{ATP} channel, we coexpressed βα polypeptides with α monomers (Fig. 3). Assuming that four α subunits form the K^+ ion

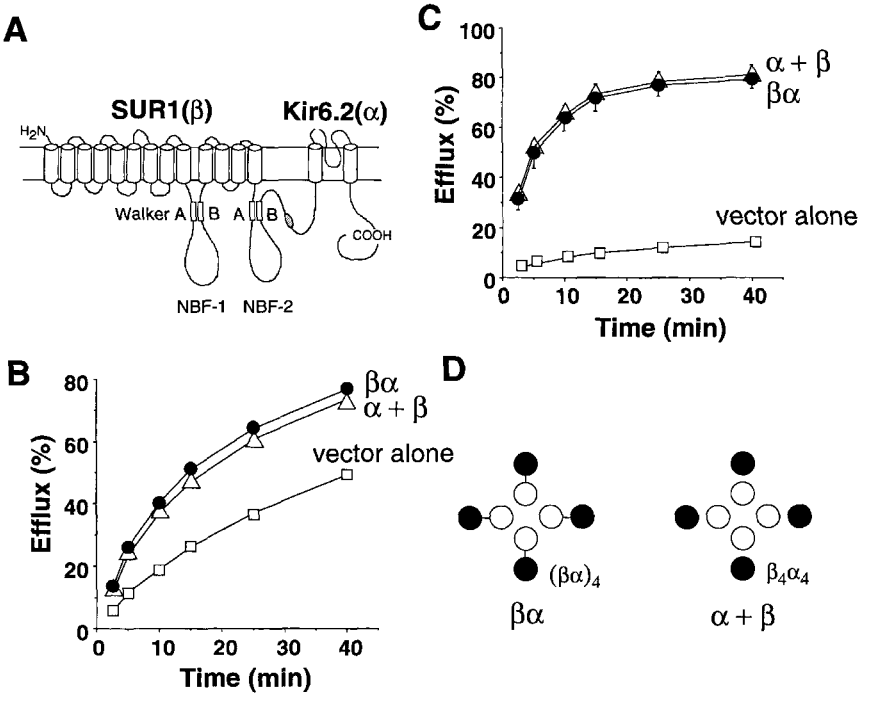

Fig. 1. $^{86}Rb^+$ efflux from COS1 cells reconstituted from βα polypeptides consisting of a SUR1 (β) subunit and a Kir6.2 (α) subunit. A: Schematic representation of the fusion protein consisting of a β and α subunit linked in a head-to-tail fashion. B,C: $^{86}Rb^+$ efflux from COS1 cells transfected with pCMV vector alone (0.5 μg; □), pCMVα plus pCMVβ (0.5 μg each; △), or pCMVβα (0.5 μg; ●) without (B) or with (C) metabolic poisoning. Each curve is the averages of 3–5 independent experiments. The error bars indicate SEM. D: Presumed stoichiometry of the channels reconstituted from βα polypeptides (left) and from α and β monomers (right). ○, α subunit; ●, β subunit.

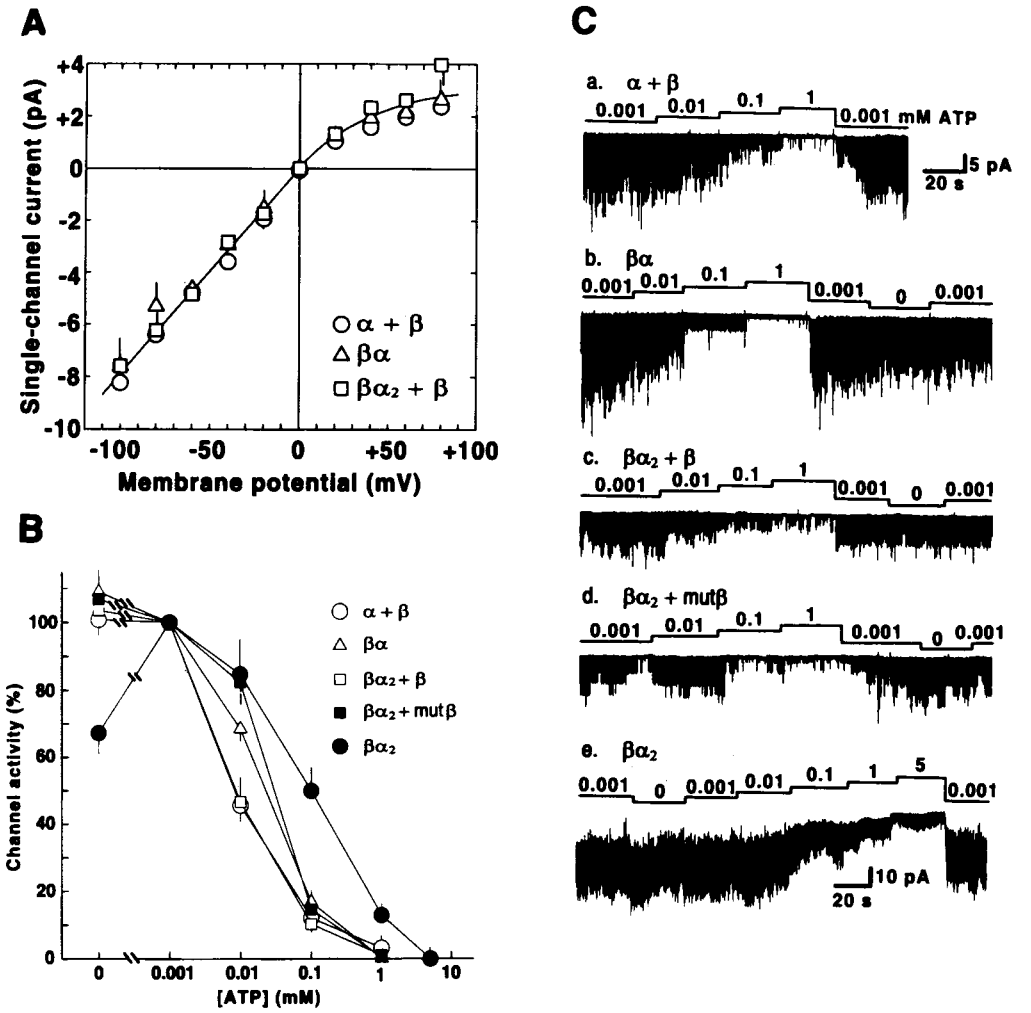

Fig. 2. Electrophysiological recordings from COS1 cells expressing reconstituted K_{ATP} channels. A: Inwardly rectifying properties of single-channel currents reconstituted from α plus β monomers (○), βα polypeptides (△), and $\beta\alpha_2$ polypeptides plus β monomers (□). B,C: Dose-dependent effect of ATP on the reconstituted channels (B) and their representative traces (C). Recordings of channels reconstituted from α plus β monomers (○, a), βα polypeptides (△, b), $\beta\alpha_2$ polypeptides plus β monomers (□, c), $\beta\alpha_2$ polypeptides plus mutant β monomers (■, d), and $\beta\alpha_2$ polypeptides (●, e) are shown. The recordings were made in the inside-out configuration of patch-clamp technique. The horizontal bars and the numbers above them indicate application periods and concentrations (mM) of ATP. Calibrations are the same from (a) to (d). Independent calibrations are given for the panel (e) in the area underneath. Channel activity was calculated as described previously [2] and is expressed as percentage of control (at 0.001 mM ATP). Values are means ± SEM, $n = 5$–15 for each point.

selective pore region, possible molar ratios of the α monomer to βα polypeptide are 0:4, 1:3, 2:2, 3:1, or 4:0 (Fig. 3B). Cotransfection of pCMVβα (0.5 μg) with pCMVα in various amounts (0.01, 0.03, 0.1, or 0.3 μg) suppressed $^{86}Rb^+$ efflux from COS1 cells in a dose-dependent manner in the absence (data not shown) or presence (Fig. 3A) of metabolic inhibitors. These results suggest that coexpression of βα polypeptides with α monomers results in a relative shortage of the β subunit for functional expression of the K_{ATP} channels. This dominant-negative effect of the α monomer on channel activity of βα polypeptides suggests that the K_{ATP} channel reconstituted from four α subunits plus less than four β subunits does not function well.

We next constructed a cDNA (pCMV$\beta\alpha_2$) encoding a single polypeptide ($\beta\alpha_2$ polypeptide) consisting of a β subunit and a dimeric repeat of the α subunit linked in a head-to-tail fashion (Fig. 4A). Although efflux from COS1 cells expressing $\beta\alpha_2$ polypeptides was significantly stimulated above endogenous efflux, it was much less than that from COS1 cells coexpressing α and β monomers or expressing βα polypeptides (compare Fig. 4B with Fig. 1C). Supplementation with β monomers by cotransfection with pCMVβ (1 μg) remarkably stimulated efflux to levels comparable to those found in cells coexpressing α and β monomers (compare Fig. 4B with 4D), suggesting that $\beta\alpha_2$ polypeptides require additional β monomers to form a proper channel complex (Fig. 4C). Single-channel recording analysis revealed that the properties of K_{ATP} channels reconstituted from $\beta\alpha_2$ polypeptides and β monomers were indistinguishable from those reconstituted from α and β monomers [2], including inward rectification, single-channel conductance (79.7 ± 2.1 pS, $n = 7$) and ATP-sensitivity (IC_{50} = 12.4 ± 2.5 μM, $n = 7$) (Fig. 2).

To confirm that the β subunit region of $\beta\alpha_2$ polypeptide is functionally involved in the formation of the channel complex, we performed coexpression experiments with mutant β (mutβ) monomers and $\beta\alpha_2$ polypeptides. The mutant β monomer was prepared by replacing amino acid residue lysine-719 in the NBF-1 of SUR1 with arginine. Coexpression of $\beta\alpha_2$

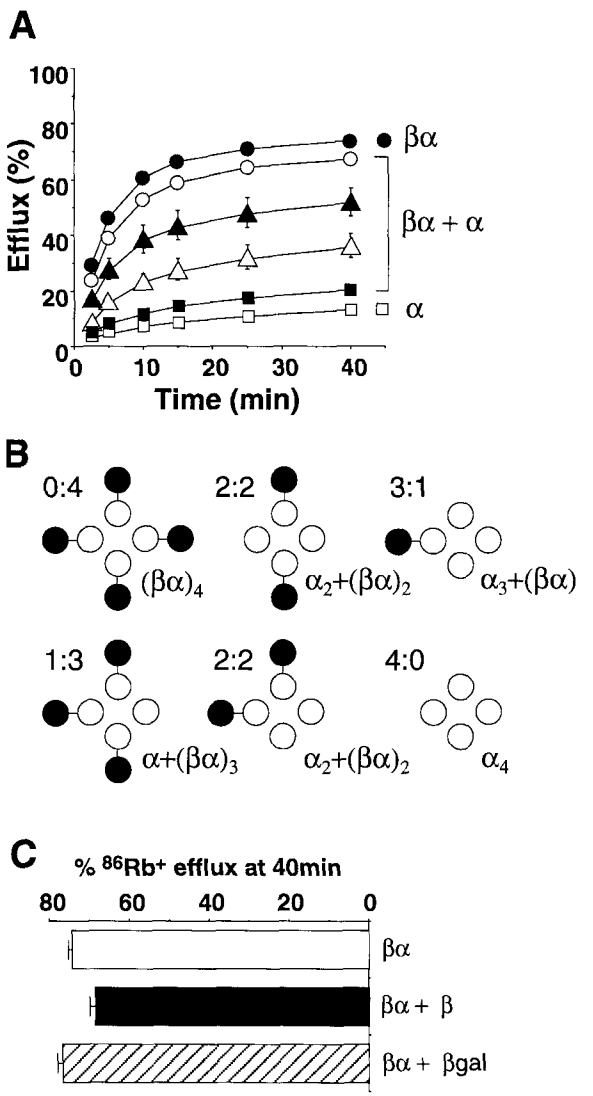

Fig. 3. The effect of α and β monomers on the efflux elicited by βα polypeptides. A: $^{86}Rb^+$ efflux from COS1 cells cotransfected with various amounts of pCMVα (0.01 μg, ○; 0.03 μg, ▲; 0.1 μg, △; and 0.3 μg, ■) and pCMVβα (0.5 μg), in the presence of metabolic inhibitors. $^{86}Rb^+$ efflux from COS1 cells transfected with pCMVβα alone (0.5 μg; ●) or pCMVα alone (0.5 μg; □) also is shown. B: Coexpression of βα polypeptides with α monomers results in the relative shortage of β subunits and formation of a complex composed of α monomers and βα polypeptides at various ratios: 0:4 ((βα)4), 1:3 (α+(βα)3), 2:2 (α2+(βα)2), 3:1 (α3+(βα)), or 4:0 (α4). ○, α subunit; ●, β subunit. C: $^{86}Rb^+$ efflux from COS1 cells cotransfected with pCMVβ (0.5 μg) and pCMVβα (0.5 μg) or cotransfected with pCMVβgal (0.5 μg) and pCMVβα (0.5 μg) is compared to that from COS1 cells transfected with pCMVβα alone (0.5 μg), in the presence of metabolic inhibitors. Each value is expressed as % $^{86}Rb^+$ efflux at 40 min from COS1 cells transfected with pCMVβα alone (0.5 μg). Each value is the average of 3-5 independent experiments. The error bars indicate SEM.

polypeptides and mutant β monomers increased the efflux significantly above the efflux generated by $\beta\alpha_2$ polypeptides, although the increment was less than that elicited by coexpression of $\beta\alpha_2$ polypeptides and β monomers, in the presence of metabolic inhibitors (Fig. 4B). Since coexpression of α monomers and mutant β monomers in COS1 cells elicited no efflux (Fig. 4D), these results indicate that both the β subunit region of $\beta\alpha_2$ polypeptide and the mutant β monomer are functionally involved in the formation of the channel complex (Fig. 4C).

Similarly, to confirm that each α subunit region of $\beta\alpha_2$ polypeptide is functionally involved in the formation of the channel complex, two different mutant $\beta\alpha_2$ polypeptides were prepared: one having an amino acid residue serine-132 instead of glycine in the H5 region of the first α subunit region of the polypeptide (β-mutα-α), the other having the same substitution in the corresponding region of the second α subunit region of the polypeptide (β-α-mutα). The mutant α monomer with serine-132 (mutα) in the H5 region was also prepared. Coexpression of the mutant α (mutα) monomers and β monomers elicited no current (data not shown). The efflux from COS1 cells expressing β-mutα-α polypeptides or β-α-mutα polypeptides was much less than that found for $\beta\alpha_2$ polypeptides (Fig. 4E). Coexpression of either β-mutα-α polypeptides or β-α-mutα polypeptides and β monomers revealed no increase in the efflux above that generated by the respective mutant alone (Fig. 4E). These results show that both of the two α subunit regions of $\beta\alpha_2$ polypeptide are functionally involved in the formation of the channel complex.

We further characterized single-channel properties reconstituted from $\beta\alpha_2$ polypeptides and from $\beta\alpha_2$ polypeptides plus the mutant β monomers. Single-channel conductance of the channels reconstituted from either $\beta\alpha_2$ polypeptides alone or $\beta\alpha_2$ polypeptides plus the mutant β monomers was similar to that reconstituted from α and β monomers (data not shown). However, ATP-sensitivity of the channel reconstituted from $\beta\alpha_2$ or $\beta\alpha_2$ polypeptides plus the mutant β monomers was less than that of the channel reconstituted from α monomers and β monomers (IC_{50}: 115.4 ± 41.3 μM ($n = 17$) for the channel reconstituted from $\beta\alpha_2$ polypeptides and 30.58 ± 5.7 μM ($n = 23$) for the channel reconstituted from $\beta\alpha_2$ polypeptides plus the mutant β monomers) (Fig. 2B,C). This suggests that each of the four β subunit regions in the channel complex is functionally involved in the ATP-sensitivity.

To learn if the number of β subunits required for a functional K_{ATP} channel exceeds four, we coexpressed β monomers with βα polypeptides. If more than four β subunits are required for functional expression of the K_{ATP} channel, coexpression with β monomers could supplement the relative shortage of the β subunit. As a result, the channel currents would be increased. However, the efflux from COS1 cells expressing βα polypeptides was actually somewhat inhibited by coexpression of β monomers, in the presence of metabolic inhibitors (Fig. 3C). To exclude the possibility of a non-specific effect of protein expression on channel activity of βα polypeptide, β-galactosidase protein was coexpressed with βα polypeptides. Coexpression of β-galactosidase protein in COS1 cells did not alter the efflux from cells expressing βα polypeptides (Fig. 3C). In addition, the mutant β monomer did not show a dominant-negative effect on the efflux from COS1 cells expressing βα polypeptides. These results suggest that the number of β subunits required for a functional K_{ATP} channel does not exceed four.

In summary, our results indicate that the activity of the K_{ATP} channel is optimized when the SUR1 subunit and Kir6.2 subunit are coexpressed with a molar ratio of 1:1. Assuming that the inward rectifying K^+ channels function as tetramers, the stoichiometry of the β-cell K_{ATP} channel is most likely a hetero-octamer comprising a tetramer of the SUR1 subunit and a tetramer of the Kir6.2 subunit. Biochem-

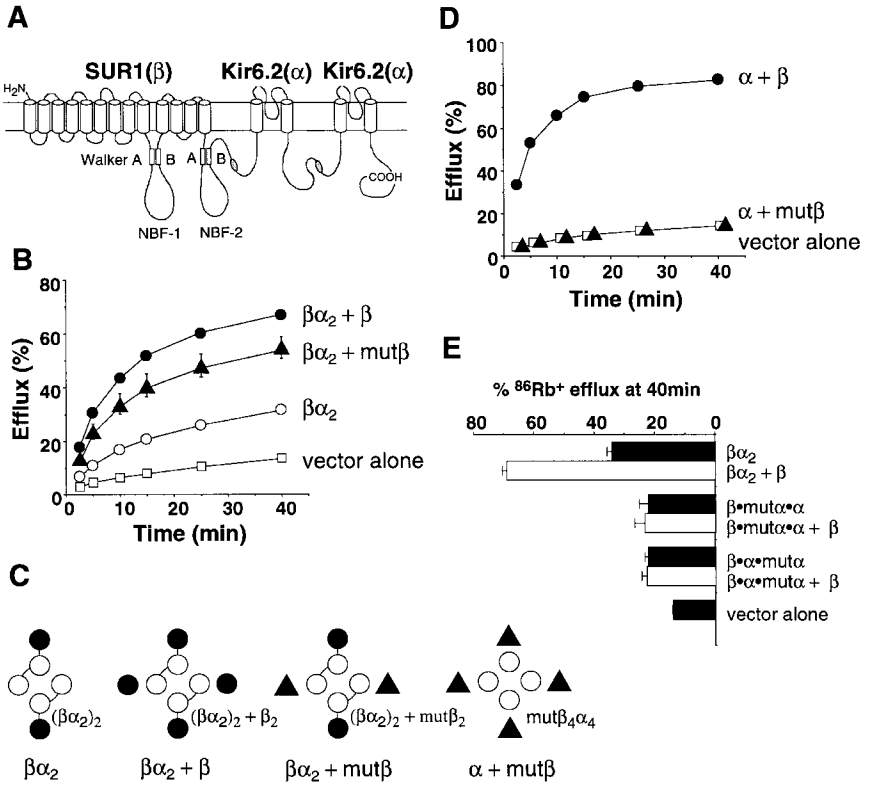

Fig. 4. $^{86}Rb^+$ efflux from COS1 cells expressing $\beta\alpha_2$ polypeptides consisting of a SUR1 (β) subunit and a dimeric repeat of the Kir6.2 (α) subunit. A: Schematic representation of the fusion protein consisting of a β subunit and a dimeric repeat of the α subunit linked in a head-to-tail fashion. B: $^{86}Rb^+$ efflux from COS1 cells transfected with pCMV vector alone (1 μg; □), pCMV$\beta\alpha_2$ (1 μg; ○), pCMV$\beta\alpha$2 plus pCMVmutβ (1 μg each; ▲), or pCMV$\beta\alpha_2$ plus pCMVβ (1 μg each; ●), in the presence of metabolic inhibitors. Mutβ, the mutant β of which amino acid residue lysine-719 was mutated to arginine. C: Presumed stoichiometry of the channels reconstituted from $\beta\alpha_2$ polypeptides, from $\beta\alpha_2$ polypeptides and β monomers, from $\beta\alpha_2$ polypeptides and mutβ monomers, and from α monomers and mutβ monomers. ○, α; ●, β; ▲, mutant β (mutβ). D: $^{86}Rb^+$ efflux from COS1 cells transfected with pCMV vector alone (1 μg; □), pCMVα plus pCMVmutβ (1 μg each; ▲), and pCMVα plus pCMVβ (1 μg each; ●), in the presence of metabolic inhibitors. Since the data points are tightly clustered, the symbols have been offset ±1 or 2 min for clarity. E: $^{86}Rb^+$ efflux from COS1 cells reconstituted from mutant $\beta\alpha_2$ polypeptides. The β•mutα•α polypeptide has an amino acid residue serine-132 instead of glycine in the H5 region of the first α subunit of the $\beta\alpha_2$ polypeptide. The β•α•mutα polypeptide has the same substitution in the corresponding region of the second α subunit of the polypeptide. Each value shows % $^{86}Rb^+$ efflux at 40 min from COS1 cells transfected with pCMV$\beta\alpha_2$ alone (1 μg), pCMV$\beta\alpha_2$ plus pCMVβ (1 μg each), pCMVβ•mutα•α alone (1 μg), pCMVβ•mutα•α plus pCMVβ (1 μg each), pCMVβ•α•mutα alone (1 μg), pCMVβ•α•mutα plus pCMVβ (1 μg each), or pCMV vector alone (1 μg), in the presence of metabolic inhibitors. Each value is the averages of 3-5 independent experiments. The error bars indicate SEM.

ical studies should clarify the direct interaction between the two subunits.

Acknowledgements: We thank K. Sakurai, A. Tamamoto, T. Yoneda, and K. Watanabe for their technical assistance. This work was supported by Scientific Research Grants and Grant-in-Aid for Scientific Research on Priority Areas of 'Channel-Transporter Correlation' from the Ministry of Education, Science, Sports and Culture and Scientific Research Grants from the Ministry of Health and Welfare, Japan; by Uehara Memorial Foundation; by a grant for studies on pathophysiology and complications of diabetes from Tsumura; by a grant from Yamanouchi Foundation for Research on Metabolic Disorders; and by a grant from the Juvenile Diabetes Foundation International.

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