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Research Paper

Random assembly of SUR subunits in K_{ATP} channel complexes

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Sulfonylurea receptors (SURs) associate with Kir6.x subunits to form tetradimeric K_{ATP} channel complexes. SUR1 and SUR2 confer differential channel sensitivities to nucleotides and pharmacological agents, and are expressed in specific, but overlapping, tissues. This raises the question of whether these different SUR subtypes can assemble in the same channel complex and generate channels with hybrid properties. To test this, we engineered dimeric constructs of wild type or N160D mutant Kir6.2 fused to SUR1 or SUR2A. Dimeric fusions formed functional, ATP-sensitive, channels. Coexpression of weakly rectifying SUR1-Kir6.2 (WTF-1) with strongly rectifying SUR1-Kir6.2[N160D] (NDF-1) in COSm6 cells results in mixed subunit complexes that exhibit unique rectification properties. Coexpression of NDF-1 and SUR2A-Kir6.2 (WTF-2) results in similar complex rectification, reflecting the presence of SUR1- and SUR2A-containing dimers in the same channel. The data demonstrate clearly that SUR1 and SUR2A subunits associate randomly, and suggest that heteromeric channels will occur in native tissues.

Introduction

The ATP-sensitive potassium channel is formed from two distinct subunits: Kir6.x, members of the inward rectifier family of potassium channels, and SURx, sulfonylurea receptors that belong to the ATP-binding cassette superfamily,[1] The vertebrate genome encodes two Kir6 genes (Kir6.1 and Kir6.2) and two SUR genes (SUR1 and SUR2A/B). The Kir6.2-SUR1 channel complex forms a tetradimeric structure consisting of four Kir6.2 subunits, which make up the potassium selective pore, and four SUR1 subunits, which serve regulatory functions.[2,3] Although it has not been systematically examined, it is likely that K_{ATP} channels of other subunit combinations (e.g., SUR2A and Kir6.2; SUR2B and Kir6.1) also form tetradimeric protein complexes.

Sulfonylurea receptors confer variable sensitivity to both pharmacological agents, such as potassium channel blockers and openers,[4,5] and physiological regulators of channel function, such as MgADP.[6] Different tissue expression of SUR genes gives rise, in part,

to the physiological diversity of K_{ATP} channel function. SUR1 is highly expressed in pancreatic beta cells,[7] SUR2A in cardiac and skeletal muscle,[5] and SUR2B in vascular tissue although its expression is fairly ubiquitous.[8] Consistent with the notion that tissue specific expression determines the properties of the native channel, mouse knockouts of SUR1 show a disruption in insulin secretion,[9] whereas knockouts of SUR2 show a disruption of glucose uptake in skeletal muscle,[10] vasospasm, high blood pressure and sudden death.[11]

A number of studies suggest that the molecular architecture of the K_{ATP} channel can be more complex. Splice variants of SUR1 and SUR2 have been found that form channels with unique functional properties.[12,13] In addition, the two pore forming subunit isoforms (Kir6.2 and Kir6.1) have been shown to coassemble, at least in cell lines when both are expressed.[14,15] SUR1 and SUR2 can also be present together, in tissues such as the heart and brain,[16] and transgenic overexpression of either SUR1 or SUR2A in the mouse heart leads to suppression of sarcolemmal K_{ATP} current,[17] suggesting that either subunit can interfere with normal channel expression in this tissue.

Despite apparent overlap of SUR subunit expression in tissues, it remains unclear whether a single K_{ATP} channel can contain more than one SUR isotype. Giblin et al.[18] examined the assembly of SUR isotypes by testing the coimmunoprecipitation of K_{ATP} complexes and pharmacological properties of K_{ATP} currents from cells expressing both SUR1 and SUR2A. They concluded that SUR1 and SUR2A are unable to form mixed channel complexes when stably coexpressed with Kir6.2 in HEK293 cells. In the present study, we used an alternative approach to examine whether SUR isotypes can coassemble in the same K_{ATP} channel. By coexpressing dimeric fusions of wild type Kir6.2 or Kir6.2[N160D] (that confers strong inward rectification) with SUR1 or SUR2A, the formation of mixed channel complexes can be observed as channels with intermediate rectification properties characteristic of combinations of WT or N160D mutant Kir6.2 subunits. The experiments demonstrate clearly that SUR1 and SUR2A can readily assemble into the same channel complex.

Results and Discussion

Fusion constructs form functional ATP sensitive channels. The half maximal ATP inhibitory concentration (K_i) of Kir6.2 coexpressed with either SUR1 or SUR2A is about 10 μM.[6] We engineered fusion constructs consisting of SUR1 or SUR2A covalently linked to the N-terminus of Kir6.2 by a hexa-glycine linker.[3] Fusion channels are also inhibited by ATP, but show decreased sensitivity compared to unfused wild-type constructs (Fig. 1). Estimated K_i

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values for SUR1-Kir6.2 (WTF-1) and SUR1-Kir6.2[N160D] (NDF-1) fusion constructs (Fig. 1B) were similar to those reported previously.[2,3] Interestingly, SUR2A-Kir6.2 fusion (WTF-2) is much less sensitive to ATP inhibition than its SUR1 counterpart. Nevertheless, these data show that these fusion proteins produce functional K$_{ATP}$ channels.

Complex rectification from WTF-1 and NDF-1 mixtures. The substitution of the negatively charged aspartate for asparagine at position 160 in the M2 helix is sufficient to confer strong inward rectification on the normally weak rectifying Kir6.2, whether expressed together with SUR1, or as a SUR1-Kir6.2 fusion.[2] Figure 2A shows currents through WTF-1, NDF-1 and WTF-2 fusion channels. In each case, relative conductance-voltage (Grel-V) relations obtained in the presence of spermine are well fit by a single Boltzmann distribution (Fig. 2B). While NDF-1 fusion channels rectify strongly, with steep voltage-dependence, both WTF-1 and WTF-2 fusion channels are relatively insensitive to spermine block with very similar voltage-dependencies (Fig. 2C).

When expressed together (Fig. 3), WTF-1 and NDF-1 readily coassemble and form a population of heteromeric channels that exhibit complex voltage-dependent rectification[2]. The relative conductance-voltage relationship (Grel-V) is well fit (Fig. 3B) with the sum of five Boltzmann functions:[2]

$$G_{rel} = \sum_{i=0}^{4} A_i \cdot \{1 + \exp[(F/RT) \cdot z_i \cdot (V - V_i)]\}^{-1} \quad \text{Eqn. 1}$$

where A_i, V_i and z_i are the amplitude, voltage of half-maximal inhibition, and effective valency, respectively, of the ith component. Assuming random subunit association, the total amplitude (A_{tot}) reflects the individual contributions of each of the possible combinations of wild type and mutant subunits determined by the binomial probability distribution,

$$A_{tot} = \sum_{i=0}^{4} P^i \cdot (1-P)^{4-i} \quad \text{Eqn. 2}$$

where P, the probability that WTF-1 assembles in the channel complex, is determined by its relative expression level. We fixed the values of $V_{1/2}$ and z for the homomeric populations (i = 0 NDF-1 tetramers and i = 4 WTF-1 tetramers) using the values determined from experiments where dimeric constructs were expressed alone (Fig. 2C). The same parameters of the mixed species were not fixed, but constrained such that $V_0 < V_1 < V_2 < V_3 < V_4$ and $z_0 > z_1 > z_2 > z_3 > z_4$.[2] Figure 3C plots the intermediate values of $V_{1/2}$ and z for the heteromultimers containing one, two or three NDF-1 subunits within the tetramer (n = 3).

Heteromeric channels from mixing WTF-2 and NDF-1 fusions. In order to test whether or not SUR1 and SUR2A can coassemble within the same channel, we measured the rectification properties of currents generated by coexpression of SUR1-containing (NDF1) and
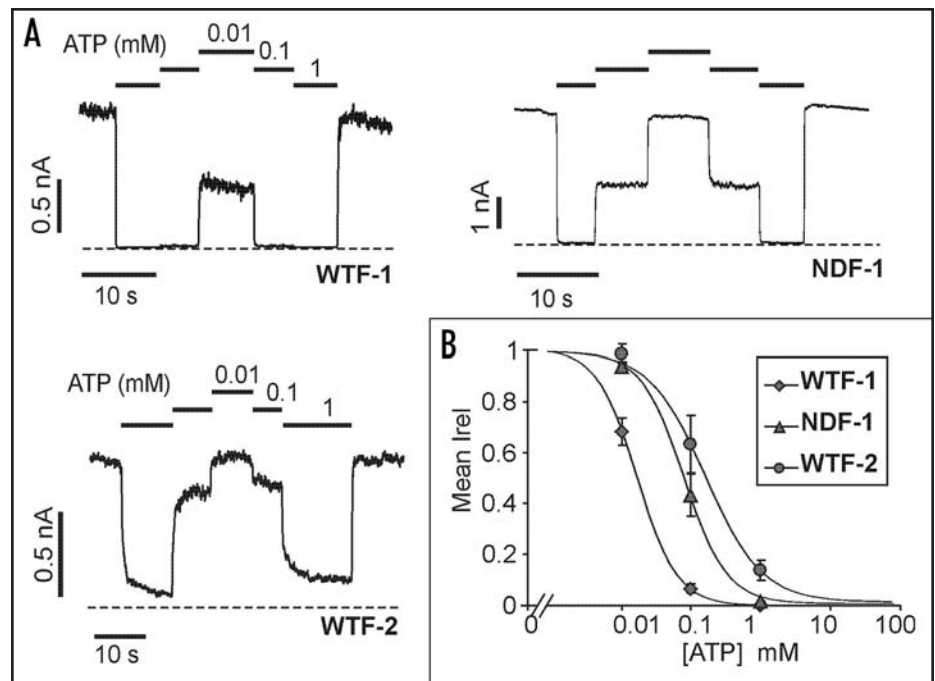

Figure 1. SURx-Kir6.2 fusion proteins generate ATP-sensitive K channels. (A) Representative inside-out membrane patch recordings from COSm6 cells expressing SUR1-Kir6.2 (WTF-1), SUR2A-Kir6.2 (WTF-2), and SUR1-Kir6.2[N160D] (NDF-1) fusions, in absence and presence of ATP, as indicated. (B) Relative current—[ATP] relationships from experiments as in (A). Relationships were fit by Hill equation, with the following estimated K$_i$ values (μM): 18 ± 2 for WTF-1 (n = 12, ± SEM), 90 ± 10 for NDF-1 (n = 5) and 220 ± 70 for WTF-2 (n = 6).

SUR2A-containing (WTF-2) fusion constructs. Because expression of WTF-2 was less efficient than expression of SUR1-containing fusions, we coexpressed WTF-2 and NDF-1 at a cDNA ratio of 10:1 in order to obtain similar expression levels of each construct.

The resulting currents again showed complex voltage-dependent rectification (Fig. 4A) in the presence of spermine, suggesting that WTF-1 and NDF-2 coassemble to form heteromeric channels. To confirm this, we fitted Grel-V relations by the sum of five Boltzmann distributions with additional weighting. Since rectification is a property of the channel pore, it can be assumed that the rectification properties of all mixed channel combinations will be the same regardless of the SUR subtype. Thus, we fixed the values of V_i and z_i using the values obtained from mixtures of WTF-1 and NDF-1 fusions determined above (Fig. 3C). Only two remaining parameters were varied for the fit: P, the fraction of NDF-1 in the mixture (1-P = fraction of WTF-2), and T, the tendency of unlike subunits to associate relative to the tendency of like subunits to associate. The following expression for a five-component binomial distribution was then used to calculate the Grel-V relation:

$$Grel = P^4 \cdot G_1 + 4T^2P^3(1-P) \cdot G_2 + (4T^2+2T^4)P^2(1-P)^2 \cdot G_3 + 4T^2P(1-P)^3 \cdot G_4 + (1-P)^4 \cdot G_5 \quad \text{Eqn. 3}$$

where G_i is the conductance of the ith component. The additional weighting parameter, xT^n, is assigned to each component that has unlike SUR subunits associating, to take into account the tendency (T) of unlike subunits to associate, relative to the tendency of like subunits to associate. n is the number of unlike subunit contacts in a given channel combination, and x is the number of permutations
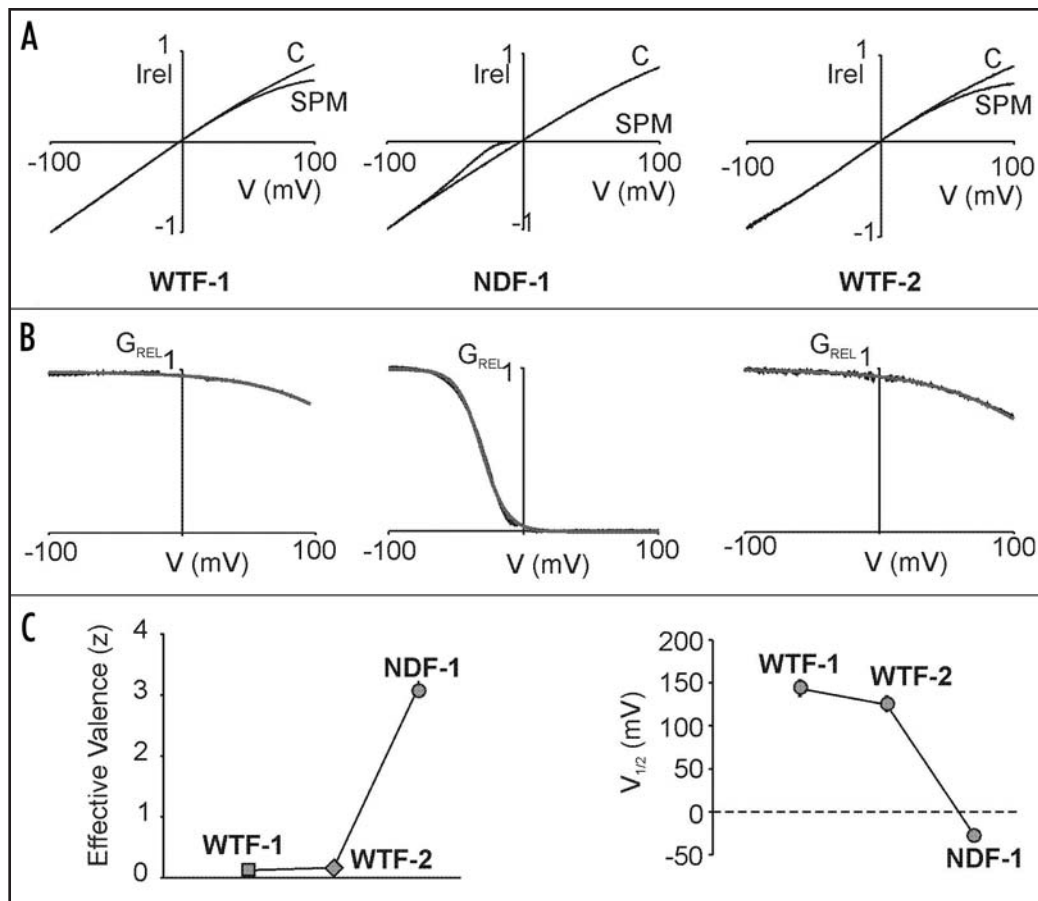
Figure 2. Spermine-induced rectification in fusion channels. (A) Currents in response to ramp from -100 mV to +100 mV (1 second) in absence and presence of 100 μM spermine for WTF-1, NDF-1 and WTF-2 homomeric channels. In all patches, leak current was determined by recording in 10 mM ATP and 1 mM spermine, and subtracted from raw currents. (B) Relative conductance-voltage relationships of the data above, fitted with single Boltmann functions. (C) Average estimates of half-maximal inhibition ($V_{1/2}$) and effective valence (z) in 100 μM spermine (± SEM): 144 ± 9 mV and 0.70 ± 0.07 for WT-SUR1 (n = 3), -28 ± 1 mV and 3.2 ± 0.2 for N160D SUR1 (n = 3) and 130 ± 10 mV and 0.57 ± 0.07 for WT-SUR2A (n = 4).

of each channel combination. When T = 1 (i.e., if the tendency to associate with like or unlike SURs is the same), the Grel-V relationship is as predicted by Equations 1 and 2.

Figure 4 shows the results of a typical experiment. Rectification clearly contains multiple phases (Fig. 4A), and the Grel-V relationship (Fig. 4B) cannot be predicted by a T value of zero (i.e., if unlike SUR subunits cannot associate), nor by T values >> 1 (i.e., if unlike SUR subunits preferentially associate). The relationship is actually best fit by a T-value very close to one (Fig. 4B). Over all data sets with mixed NDF-1/WTF-2 co-expression, (n = 9), the average T value was 0.77 ± 0.07 (mean ± SEM, range 0.55–1.03), and the average P value was 0.52 ± 0.05 (range 0.36–0.83, reflecting similar levels of expression of each fusion construct).

Implications of these results. The analysis demonstrates clearly that fusion subunits containing SUR1 and SUR2A coexpressed in COSm6 cells can combine and form heteromeric channels and that the association is close to random, i.e., there is little or no bias towards like subunits associating in the same complex. It is important to note that this finding is at odds with conclusions from an earlier study[18] in which SUR1 and SUR2A in stably transfected HEK293 cells were not coimmunoprecipitated and no population of pharmacologically unique channels was detected. However, interpretation

of such results to imply failure of SUR1 and SUR2A to associate in heteromeric channels requires first that the entire octameric channel complex be immunoprecipitated by anti-Kir6.2 antibodies, and second that a channel consisting of different SUR subtypes would exhibit unique properties. Unlike the intermediate rectification properties found in wild-type Kir6.2 and Kir6.2[N160D] mixed channel complexes used in the present study as a reporter for coassembly, there is no evidence to suggest that mixed SUR channels should have detectably novel pharmacological properties.

Yoshida et al. have demonstrated that Kir6.1, Kir6.2 and SUR2B subunits can form a heteromultimeric channel complex in human coronary artery endothelial cells by coimmunoprecipitation.[19] Intuitively, if the Kir subunits can form mixed complexes, it may be even more likely that the SUR subunits, which are not known to directly interact with one another, are promiscuous in their assembly. Our average value of T (0.77), very close to one (random association), argues that SUR1 and SUR2A accessory subunits form mixed channels almost as readily as homomeric ones. Much is still unknown about the regulation of K_{ATP} channels in mammalian tissue and our results indicate that one may expect heteromeric channels to exist in tissues that express multiple SUR subtypes, consistent with the functional consequences of transgenic expression of two different SUR subtypes in the heart.[17]
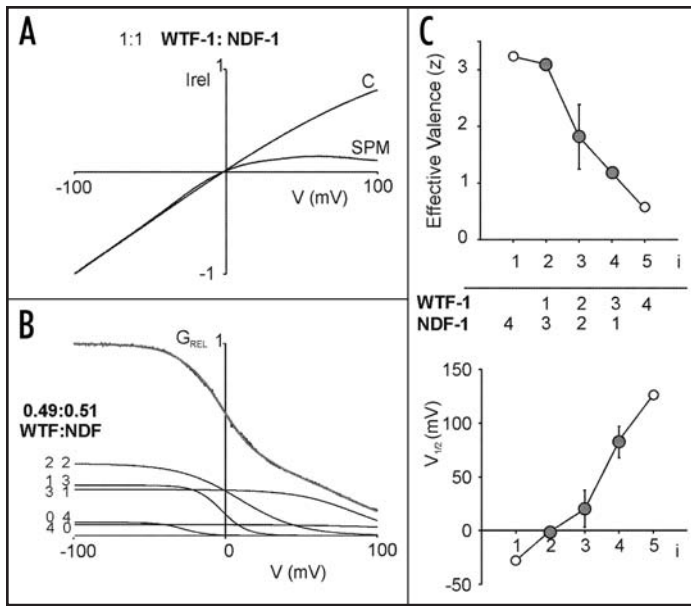
Figure 3. Intermediate rectification in mixed WTF-1 and NDF-1 channels. (A) Currents in response to ramp from -100 mV to +100 mV (1 second) in absence and presence of 100 μM spermine for a patch expressing WTF-1 and NDF-1, expressed at 1:1 cDNA ratios. (B) Relative conductance-voltage relationship from above. Five components of rectification distributed according to a binomial distribution of subunit expression fitted the data, with p (probability of WTF-1 incorporation) = 0.49. Each component, appropriately scaled is shown on the graph (see text). (C) Mean values for half-maximal inhibition (V$_{1/2}$) and effective valence (z) for homomeric channels (from Fig. 2, white circles) and for intermediate rectifying heteromeric channels (from experiments as in A, n = 3, grey circles). These components were constrained such that $V_1 < V_2 < V_3 < V_4 < V_5$ and $z_1 > z_2 > z_3 > z_4 > z_5$. Average values of V_i and z_i at 100 μM spermine (n = 3, SE): V_2 = -1 ± 2 mV, z_2 = 3.10 ± 0.08, V_3 = 20 ± 17 mV, z_3 = 1.8 ± 0.6, V_4 = 82 ± 14 mV and z_4 = 1.20 ± 0.08.

Methods

Construction of dimeric fusion proteins. The construction of the SUR1-Kir6.2 (WTF-1) or SUR1-Kir6.2[N160D] (NDF-1) cDNA constructs have been described previously.[3] Briefly, SUR1 was joined in a head-to-tail fashion to the NH$_3$-terminus of either wild type Kir6.2 or the Kir6.2[N160D] mutant via a hexa-glycine (Gly$_6$) linker. SUR2A-Kir6.2 dimeric cDNA constructs were generated in a similar fashion and all cDNAs were subcloned into the same mammalian expression vector (pECE).

Expression of K$_{ATP}$ channels in COSm6 cells. COSm6 cells were plated at an approximate density of 2.5 x 10^5 cells per well (30 mm six-well dishes) and cultured in Dulbecco's Modified Eagle Medium plus 10 mM glucose and fetal calf serum (10%). After 24 hours, DNA constructs were transfected into the cells using FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). Typically, the following amount of DNA was used for each construct: 0.4 μg Kir6.2, 0.6 μg SUR1, 0.6 μg SUR2A and 1 μg of the fusion constructs (SUR1-Kir6.2WT, SUR2A-Kir6.2WT, SUR1-Kir6.2[N160D], SUR2A-Kir6.2[N160D]). When coexpressing SUR1-Kir6.2[N160D] plus SUR2A-Kir6.2WT, a 1:10 ratio was used (0.1 μg to 1 μg, respectively). The DNA of interest was added to 0.3 μg GFP, as a marker of transfection, and 3 μl FuGENE6. After incubation for 24 hours, the cells were
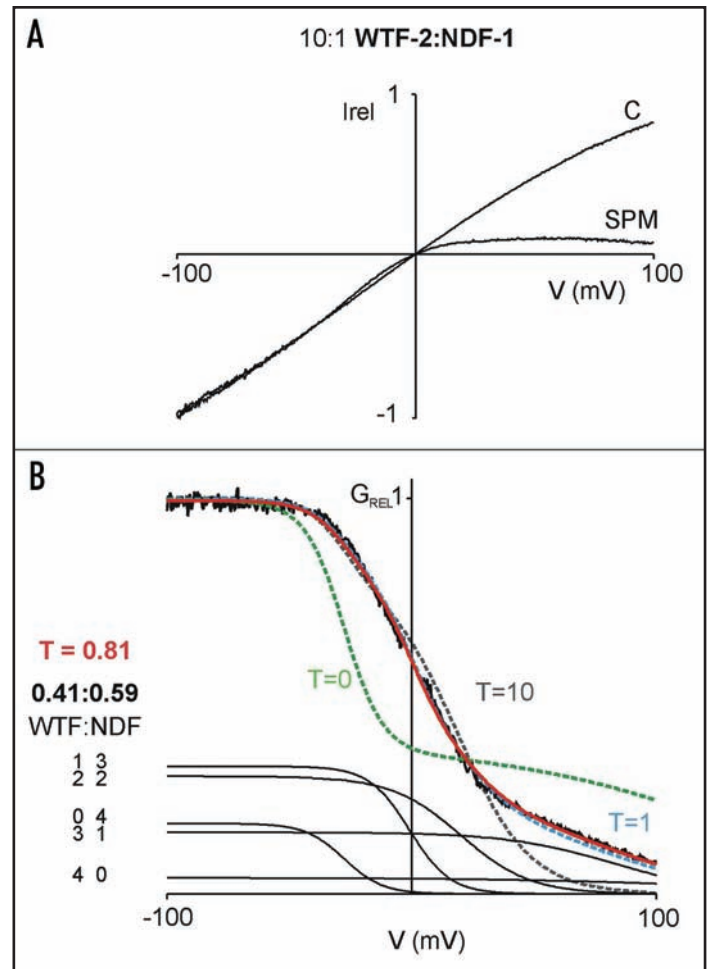

Figure 4. SUR1 and SUR2A randomly associate. (A) Representative currents in response to ramp from -100 mV to +100 mV (1 second) in absence and presence of 100 μM spermine for coexpressed WTF-2 and NDF-1, expressed at 10:1 cDNA ratios. (B) Relative conductance-voltage relationship from above. All components of the Boltzmann fit were constrained with the average V$_{1/2}$ and z values from the pure SUR1-fusion analyses (Fig. 3C). Only the relative ratio of N160D subunit expression, P, and the relative tendency for unlike subunits to associate, T, were allowed to vary. Best-fit values (red) were p = 0.59 and T = 0.81. Predictions for T = 0 (green), 1 (blue) and 10 (grey) are also shown (see text).

plated in sterile glass coverslips and incubated overnight prior to patch-clamp experiments.

Electrophysiology. Inside-out membrane patches were voltage-clamped at room temperature in an oil-gate chamber with four channels that run into the same end-pool where the ground electrode is placed (Lederer and Nichols, 1989). The chamber is mounted on the stage of an inverted microscope (Nikon, Garden City, NY). The solution level is sensed by a float connected to a tension transducer that is used to control the outflow from the end-pool. COSm6 cells (2–3 days after transfection) that fluoresced from GFP expression were chosen for patch-clamping. Membrane patches were voltage-clamped using a CV-4 headstage, an Axopatch 1-D amplifier, a Digidata 1322A digitizer board (all from Axon Instruments, Union City, CA), and a MP-225 micromanipulator (Sutter Instrument Company, Novato, CA). Bath and pipette solutions, with K-ATP added to the bath solution where indicated,

contained, in mM: 150 KCl, 10 HEPES and 1 EGTA (pH 7.4). For the spermine sensitivity experiments, the following solution was used, in mM: 150 KCl, 2 phosphate and 1 EGTA (pH 7.4). Data were analyzed using the pClamp 8.2 software suite (Axon Instruments, Union City, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA).

Note Added in Proof

The above work was originally presented at the Biophysical Society Annual Meeting in March, 2007. Chan et al. have since reported the use of a similar approach to demonstrate random assembly of SUR1 and SUR2A subunits in functional KATP channels [Chan KW, Wheeler A, Csanády L. <http://www.ncbi.nlm.nih.gov/pubmed/18079561?ordinalpos=1&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum> (2008) Sulfonylurea receptors type 1 and 2A randomly assemble to form heteromeric KATP channels of mixed subunit composition. Gen Physiol 2008;131:43-58.]. Their findings and conclusions are strongly in agreement with our own.

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