# Sulfonylurea receptors set the maximal open probability, ATP sensitivity and plasma membrane density of $K_{ATP}$ channels

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**Abstract $K_{ATP}$ channels are heteromultimers of SUR and $K_{IR}6.2$. C-terminal truncation of $K_{IR}6.2$ allows surface expression of the pore. $K_{IR}6.2\Delta C35$ channels display ~7-fold lower maximal open probability, ~35-fold reduced ATP sensitivity, reduced mean open time, a markedly increased transition rate from a burst into a long-lived closed state, and have no counterpart in vivo. SUR1 and SUR2A restore wild-type bursting, ATP sensitivity and increase channel density in the plasma membrane. The high $IC_{50(ATP)}$ of ~4 mM for $K_{IR}6.2\Delta C_{K185Q}$ channels results from the additive effects of SUR removal and $K_{IR}6.2$ modification. The results demonstrate allosteric interaction(s) are essential for normal intrinsic activity, ATP inhibition, and trafficking of $K_{ATP}$ channels.**



*Key words:* Allosterism; ATP-sensitive potassium channel; SUR1; SUR2A; Trafficking

## 1. Introduction

ATP-sensitive $K^+$ channels link changes in metabolism to membrane excitability in a variety of cells. These channels are heteromultimers of a $K^+$ inward rectifier, $K_{IR}6.x$, and a sulfonylurea receptor, SURx, an ATP binding cassette protein [1–4] assembled with an octameric stoichiometry, $(SURx/K_{IR}6.x)_4$ [2]. Both subunits are required to assemble native channels, for example $K_{IR}6.2$ and SUR1 form the $K_{ATP}$ channels from pancreatic β-cells [5] while $K_{IR}6.2$ and SUR2A comprise the cardiac sarcolemmal channels [6,7]. Recent reports [2,8,9] have shown that $K_{IR}6.2$ forms the pore of the channel while SUR serves a regulatory role conferring sensitivity to sulfonylureas, potassium channel openers and activation by MgADP, the proposed physiologic regulator [10]. The failure of mutations in the NBFs of SUR to affect the nucleotide inhibition of $K_{ATP}$ channels and the observation that expression of $K_{IR}6.2$ in the absence of SUR, either as C-terminally truncated proteins [9] missing an endoplasmic reticulum (ER) retention signal [11] or by strong over-expression [12], can generate an ATP-sensitive $K^+$ conductance has led to the idea that SUR may not play an important role in the ATP inhibition of channel activity [9]. On the other hand, the ATP sensitivity of the homomeric channels is markedly reduced, being 20–40-fold lower for $K_{IR}6.2\Delta C35$ vs. $SUR1/K_{IR}6.2$ channels [9,13,14], indicating that SUR modifies the apparent sensitivity to inhibitory ATP. A comparison of the reported ATP sensitivity of recombinant $SUR2A/K_{IR}6.2$ channels [6] with the $IC_{50}$ of homomeric $K_{IR}6.2\Delta C$ channels, ~0.1 mM [9], furthered speculation that SUR might not have a critical role in channel inhibition by ATP. There is ample reason to question this view: more recent work [7] comparing the steady-state ATP inhibition of native human cardiac and recombinant human $SUR2A/K_{IR}6.2$ channels has shown that the reports of the apparent $IC_{50(ATP)}$ ~0.1 mM for native and recombinant cardiac $K_{ATP}$ overestimated this value by at least a factor of four for a variety of reasons. This implies, as we show here, that SUR2A, like SUR1, will markedly increase the sensitivity of $K_{IR}6.2$ channels to ATP. Similarly, while mutations in $K_{IR}6.2\Delta C$ subunits can clearly attenuate ATP inhibition by poorly understood mechanisms, larger effects observed in the homomeric channels, as shown in this report, are the result of the additive effects of the $K_{IR}6.2$ mutation and the removal of SUR. The data imply that allosteric interactions between the receptor and $K_{IR}$ determine spontaneous bursting and the apparent affinity of $K_{ATP}$ channels for ATP.

Are homomeric $K_{IR}6.2$ channels physiologically relevant? This question is difficult to resolve for all pathological conditions, but there are two cases where their presence should be detectable, in the β-cells of patients with persistent hyperinsulinemic hypoglycemia of infancy (PHHI) and in SUR1 knockout mice. In both cases there is a lack of detectable ATP-inhibited $K^+$ currents [15,16] consistent with the idea that $K_{IR}6.2$ is retained in the ER in the absence of a functional SUR. We have used single channel recording to compare human $K_{IR}6.2\Delta C35$ channels with and without SURs and find that the $P_{omax}$, the density in the plasma membrane, the mean open time, the mean burst duration, and the apparent sensitivity to inhibitory ATP for the homomeric $K_{IR}$s are significantly lower than those of heteromeric channels. The currents observed in cells expressing homomeric $K_{IR}6.2$ channels have no known counterpart in vivo. The results indicate that the functions of SUR and $K_{IR}6.2$ are tightly integrated and raise the question of whether surface expression of $K_{IR}6.2$ alone is deleterious to cell function.

This work was presented originally at the 42nd annual meeting of the Biophysical Society [14].

## 2. Materials and methods

### 2.1. Molecular biology

The last 35 amino acids of human $K_{IR}6.2$ were deleted using PCR primers designed to introduce a termination codon at the desired position. The K185Q mutation was introduced into $K_{IR}6.2$ or $K_{IR}6.2\Delta C35$ using overlapping PCR primers. These were combined with appropriate flanking forward and reverse primers including unique restriction enzyme sites. Two amplifications were carried out, the first set of reactions matched the forward and reverse flanking primers with the appropriate overlapping primer. The resulting PCR products were purified by agarose gel electrophoresis, mixed and used as the template for the second PCR reaction with the flanking

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primers. The product of the second reaction was cut with an appropriate pair of restriction enzymes and used to replace the corresponding wild-type fragment. All the PCR products and restriction sites were sequenced to verify the constructions.

### 2.2. Transfection and cultivation of cells for electrophysiological experiments

Approximately $1.8\times10^5$ COSm6 cells per 35 mm dish were transfected with 1 μg of SUR plasmid and/or 1 μg of the wild-type or modified $K_{IR}6.2$ plasmid, and 0.5 μg of a GFP (pGreen Lantern-1, Life Technologies, Baltimore, MD) plasmid. Transfections were done using FuGene6 following the manufacturer's directions (Boehringer Mannheim, Indianapolis, IN). Transfected cells were cultured overnight, trypsinized and replated on glass cover slips. Cells expressing GFP as a marker were selected for analysis.

### 2.3. Patch-clamp recording and single-channel kinetics analysis

The currents through reconstituted $K_{ATP}$ channels were recorded in inside-out configurations of the patch-clamp technique at 23–24°C, as described previously [7,17]. Pipettes filled with the quasi-physiological external solution containing (mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, pH 7.4 (NaOH), or the $K^+$-rich external solution containing: KCl 145, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, pH 7.4 (KOH) had resistance of 4–8 MΩ. Smaller pipettes were used in some experiments to record single channel currents. The internal, nominally $Mg^{2+}$-free, solution containing: KCl 140, EDTA 5, HEPES 5, KOH 10, pH 7.2 (KOH) was used as control bath solutions. ATP disodium salt, glibenclamide, and other compounds were from Sigma (St. Louis, MO). Bathing solutions were applied using a programmable RSC-200 rapid solution changer (Biologic Inc, Claix, France), and quasi-steady-state ATP inhibition was estimated as described previously [7]. Under experimental conditions used we observed stable sensitivity to inhibitory ATP ($>30$ min long recordings with the repetitively applied dose-response protocol were performed for majority of patches). For single channel kinetics analysis, we only accepted patch current records with no superimposed openings where the cumulative $P_o$ increased linearly for more than 30 s in a continuous recording activated immediately after patch isolation. In the case of heteromeric channels displaying $P_o>0.5$, these criteria were sufficient to be confident that we were analyzing a single channel without significant run-down. In the case of homomeric channels that display low activity, we applied an additional statistical test to evaluate the probability of our observing $n$ number of openings, without superimposed events, if we had $N$ (two or more) independent equivalent channels with mean open time, $t_O$, that was significantly smaller than the mean closed time, $t_C$. This probability $P\approx\pi^n$, where $\pi\approx[1+(t_O(N-1)/(t_CN))]^{-1}$ [18], was $<10^{-10}$ for all of the records accepted for kinetic analysis; thus the probability of overestimating $P_o$ was negligible. Dwell time distributions were constructed and the burst analysis at different burst criteria was performed as described previously [7,19–21].

### 2.4. Expression of data

Averaged data were expressed as means ± S.E.M. for $n\geq5$ with error bars equal to S.E.M. unless otherwise noted. Significance was evaluated using the Student's $t$-test; differences with values of $P<0.05$ were considered to be significant.

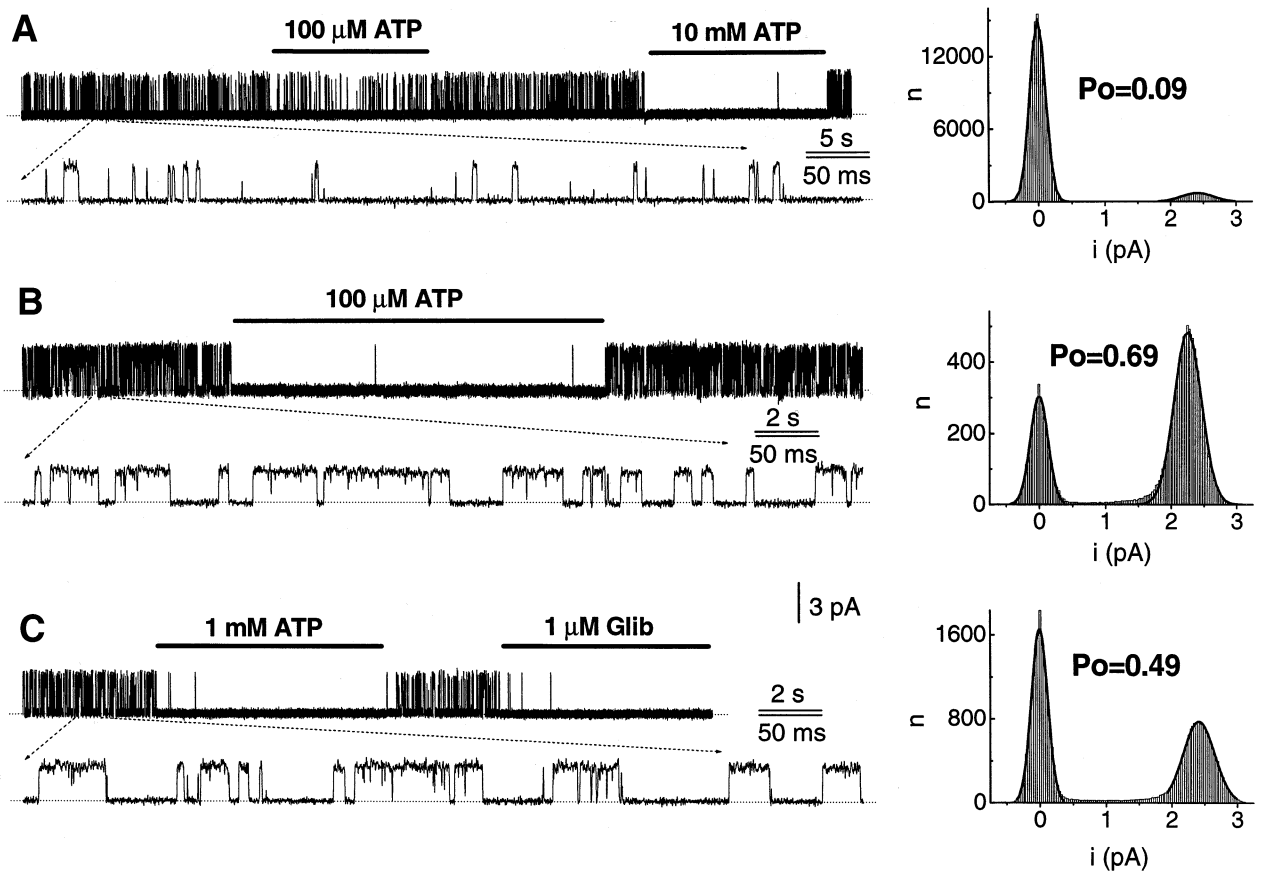
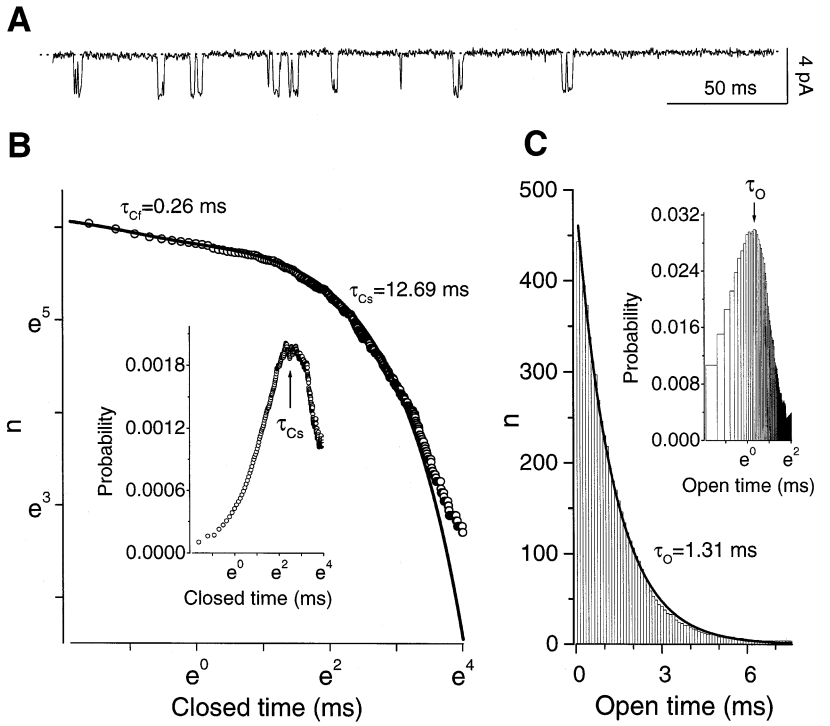



Fig. 1. Homomeric $K_{IR}6.2\Delta C35$ channels display low activity and low ATP sensitivity that are restored to those of $K_{ATP}$ channels by co-assembly with SUR1. A, B, and C: Currents through single $K_{IR}6.2\Delta C35$, SUR1/$K_{IR}6.2$, and SUR1/$K_{IR}6.2\Delta C35$ channels, respectively, along with their corresponding all-points current amplitude histograms (shown on the right) and maximal $P_o$ values. Inside-out patches were isolated in the control internal solution with the quasi-physiological external solution in the pipette and held at 0 mV. The maximal $P_o$ value was estimated from a double-Gaussian fit to an all-points current amplitude histogram constructed from a continuous segment of record activated immediately after patch excision (a ~2 s long fragment of the segment is shown at an expanded time scale). Here and in the following figures, upward deflection of the current trace corresponds to outward current, and the horizontal dotted lines show the level of current when all $K_{ATP}$ channels are closed. Glib, glibenclamide.

### 3. Results and discussion

Fig. 1A shows a representative record of current through a single human $K_{IR}6.2\Delta C35$ channel. The maximal open probability ($P_{omax}$) of the $K_{IR}6.2\Delta C$ channels, immediately after isolation of an inside-out patch into a nucleotide-free solution, is markedly lower than that of the human SUR1/$K_{IR}6.2$ $K_{ATP}$ channel (Fig. 1B), $0.09 \pm 0.01$ vs. $0.69 \pm 0.01$ ($n = 4$ for both channels, with the last value close to that estimated indirectly using macro-current noise analysis [8]). Co-assembly with SUR1, which was verified by determining the sensitivity of the channels to glibenclamide (Fig. 1C), nearly restores the normal $P_{omax}$ ($0.49 \pm 0.08$; $n = 3$), bursting pattern, and sensitivity to inhibitory ATP (see also Fig. 4). Kinetic analysis of inward currents through single $K_{IR}6.2\Delta C35$ channels (Fig. 2A) shows that the reduced $P_{omax}$ of the homomeric channels is the result of a marked increase in the percentage of long-lived closures. The mean lifetime of the short-lived gaps is not significantly affected ($\tau_{Cf} = 0.25 \pm 0.03$ ms in Fig. 2B vs. $0.22 \pm 0.02$ ms for the wild-type channel at $-40$ mV); however, the mean open time of the pore is reduced $\sim$2.2-fold in the absence of SUR ($\tau_O = 1.38 \pm 0.09$ ms in Fig. 2C vs. $3.01 \pm 0.21$ ms for the wild-type channel) indicating SUR affects the ATP-independent intraburst kinetics [22] which are dependent on the $K^+$ driving force [23,24]. Analysis of spontaneous bursting, a prominent feature of $K_{ATP}$ channels, shows a large reduction in the mean number of openings in the burst suggesting the homomeric channels have a higher rate of transition out of a burst (Table in Fig. 2D). Similar 'flickery' currents, with $\tau_O \sim 1$ ms, have been observed in multi-channel patches from HEK293 cells over-expressing full-length $K_{IR}6.2$ [12]. The single channel kinetic analysis shows a clearly distinct mode of gating in the homomeric vs. heteromeric $K_{ATP}$ channels. On average the mean burst duration for either β-cell or cardiac $K_{ATP}$ channels [7,19,21] is 10–100 times longer than observed here for the homomeric channels.




D

| Burst criterion (ms) | Mean N of openings in the burst | Mean burst duration (ms) |
|---|---|---|
| 1.0 | **1.3** | **2.0** |
| 2.0 | **1.5** | **2.5** |
| 3.0 | **1.6** | **3.0** |
| 4.0 | **1.7** | **3.7** |
| 5.0 | **1.9** | **4.8** |
| 10.0 | **2.9** | **11.1** |

Fig. 2. Single channel kinetics of homomeric $K_{IR}6.2\Delta C$ channels. A: A record of inward current through a single $K_{IR}6.2\Delta C35$ channel with the $K^+$-rich external pipette solution at $-40$ mV. B: Closed time distribution fit with a sum of two exponent where $\tau_{Cf}$ and $\tau_{Cs}$ define the fast and slow components respectively. The inset shows a plot of the relative total time the channel spent in a closed state vs. the duration of the state. C: Open time histogram fit with a single-exponential function with time constant $\tau_O$, and a plot of the relative total time the channel spent in an open state vs. the duration of the state (inset). D: Results of burst analysis applying variable burst criteria.

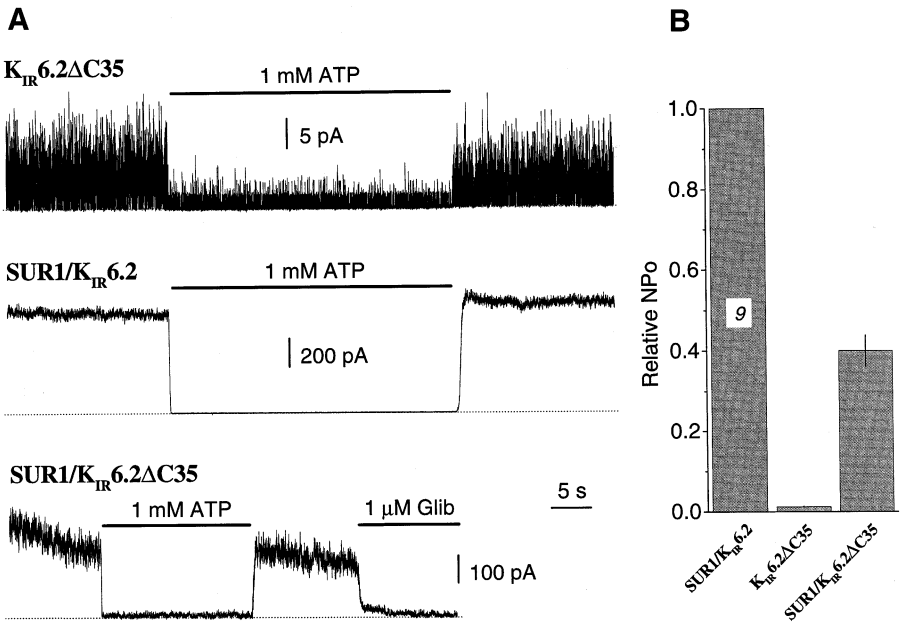



Fig. 3. SUR1 increases the density of $K_{IR}6.2\Delta C35$ pores in the plasma membrane. A: Currents in inside-out patches from COSm6 cells transfected with $K_{IR}6.2\Delta C35$ without SUR1, $K_{IR}6.2$ with SUR1, or $K_{IR}6.2\Delta C35$ with SUR1, recorded under the same experimental conditions as in Fig. 1A–C. In these experiments the ratio of SUR1 to $K_{IR}$ plasmids was 4:1. B: The relative $NP_o$ values for each type of channel. Twenty-seven patches from nine triplets of cells from three independent transfections using three different combinations of plasmids were compared.

The results demonstrate that co-assembly with SUR increases the stability of the open state ~2.2-fold and markedly reduces the rate of transition to an interburst closed state. The restoration of wild-type kinetics by SUR indicates there are allosteric interactions between the regulatory subunit and $K_{IR}6.2$ that convert the poorly operational $K_{IR}$ into the normal high $P_{omax}$ $K_{ATP}$ channel.

The estimated $P_{omax}$ values for homomeric, heteromeric SUR1/$K_{IR}6.2\Delta C$ and wild-type channels were used to examine whether co-expression with SUR1 would affect the density of channels in the plasma membrane. $NP_o$ values were estimated from ATP-inhibited currents (Fig. 3A) in inside-out patches from identically transfected cells using conditions designed to avoid outward currents through other channels [7]. The currents in patches from cells co-expressing SUR1 and $K_{IR}6.2\Delta C35$ were equally inhibited by ATP and glibenclamide showing that the observed currents were through heteromeric channels. As shown in Fig. 3B, the relative $NP_o$ values, normalized to the wild-type value, were $0.012 \pm 0.001$ and $0.4 \pm 0.039$ for the $K_{IR}6.2\Delta C35$ and SUR1/$K_{IR}6.2\Delta C35$ channels respectively. Thus co-expression with SUR1 increases the relative $N$ of $K_{IR}6.2\Delta C35$ based channels ~7-fold to ~60% of the wild-type value. Together the reduced intrinsic activity and the reduced density of the homomeric channels account for the relatively low level of $K_{IR}6.2\Delta C$ channel macro-currents we observe and reported by others [9]. The increased channel density suggests SUR either facilitates transit to the plasma membrane and/or slows their recycling to an interior compartment thus increasing their steady-state density. The data are consistent with our initial observations [2] that co-expression of SUR1 with $K_{IR}6.x$ caused altered glycosylation of the receptor and demonstrated that SUR1 trafficked poorly to the cell surface in the absence of $K_{IR}6.x$. The lack of $K^+$ currents similar to homomeric $K_{IR}6.2$ channels in PHHI β-cells and in β-cell from SUR1 null mice [15,16] implies that $K_{IR}6.2$ trafficks poorly to the cell surface in the absence of a functional SUR, in agreement with the recent discovery of an ER retention signal on the C-terminus of $K_{IR}6.2$ [11].

Fig. 4A provides examples of records showing the ATP inhibition of $K_{IR}6.2\Delta C$, wild-type, and SUR1/kir$6.2_{K185Q}$ channels. Fig. 4B shows that homomeric $K_{IR}6.2\Delta C35$ channels are inhibited by ATP with an $IC_{50}$ of $221 \pm 12.1$ μM while

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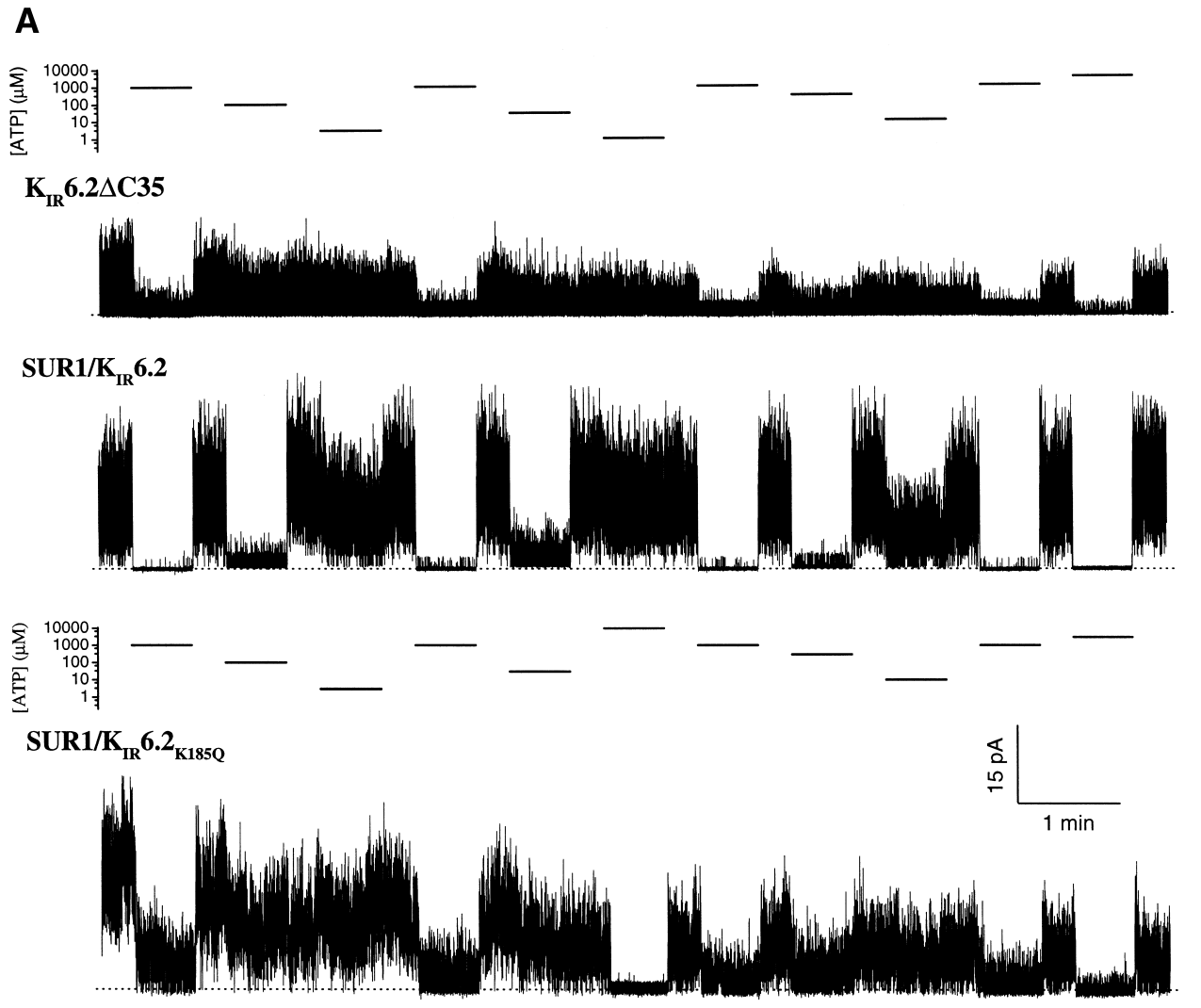
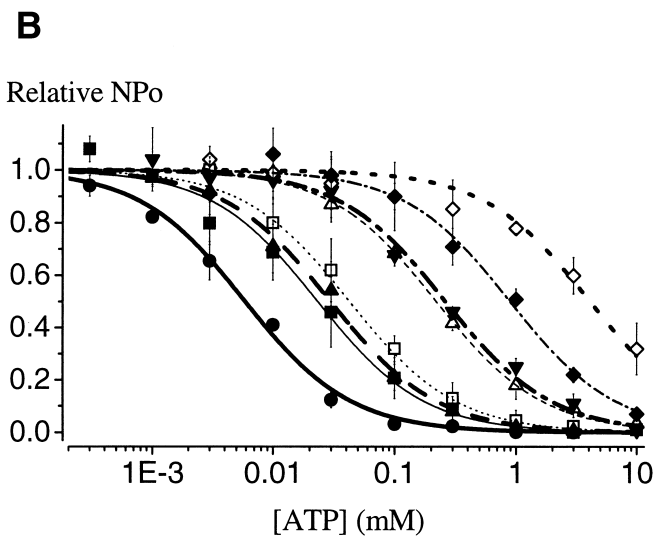
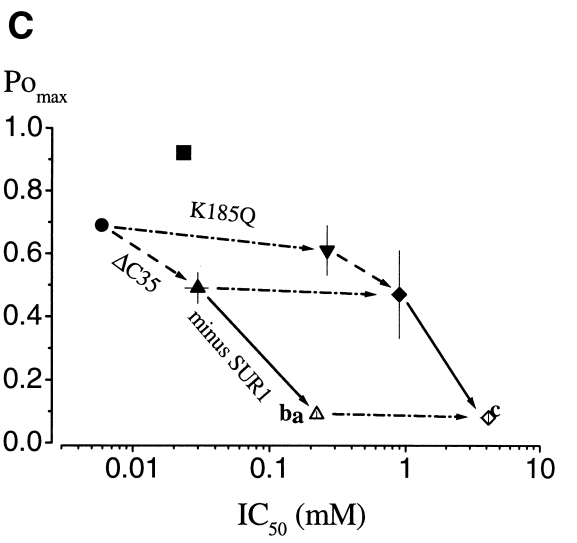
Fig. 4. Effects of SURs and the K185Q mutation on ATP-inhibition of $K_{ATP}$ channels. A: Representative examples of the ATP dose responses for different channels taken as described previously [7] using the experimental conditions as in Fig. 1A. B: Semi-logarithmic plots of normalized channel activity vs. [ATP] for all channels examined. At least five different patches were examined for each channel type (error bars = S.E.M.) The corresponding apparent $IC_{50(ATP)} \pm$error (in μM) with pseudo-Hill coefficients were estimated from fits of a conventional pseudo-Hill function to the averaged relative $NP_o$ obtained from measurements of quasi-steady-state $NP_o$ at different [ATP] for each channel type as marked: solid circle and thick solid line for SUR1/$K_{IR}6.2$, $5.9 \pm 0.5$; solid square and thin solid line for SUR2A/$K_{IR}6.2$, $23.4 \pm 2.6$; open up triangle and thin dashed line for $K_{IR}6.2\Delta C35$, $221.1 \pm 12.1$; open square and thin dotted line for SUR2A/$K_{IR}6.2\Delta C35$, $42.7 \pm 4.5$; solid up triangle and thick dashed line for SUR1/$K_{IR}6.2\Delta C35$, $29.8 \pm 1.9$; solid down triangle and thick dashed line for SUR1/$K_{IR}6.2K_{185Q}$, $262.7 \pm 21.9$; open diamonds and thick dotted line for $K_{IR}6.2\Delta C35_{K185Q}$, $4123.8 \pm 570.3$; solid diamonds and thin dash-dotted line for SUR1/$K_{IR}6.2\Delta C35_{K185Q}$, $897.2 \pm 113.1$. The pseudo-Hill coefficients for all of these channels were not significantly different from 1. C: A semi-logarithmic plot of the $IC_{50(ATP)}$ vs. maximal $P_o$ for a number of different mutants summarizing the points discussed in the text. The open and solid symbols (consistent with their designation in B) give the values for the homomeric and heteromeric channels respectively. The different line styles correspond to different modifications indicated. The alphabetical symbols mark similar channels characterized by others: a: $K_{IR}6.2$ channel [12]; b: $K_{IR}6.2\Delta C26$ and c: $K_{IR}6.2\Delta C26_{K185Q}$ [25].


A
[ATP] (μM)
10000
1000
100
10
1
K_IR6.2ΔC35
SUR1/K_IR6.2
SUR1/K_IR6.2_K185Q
15 pA
1 min


B
Relative NPo
1.0
0.8
0.6
0.4
0.2
0.0
1E-3
0.01
0.1
1
10
[ATP] (mM)


C
Po_max
1.0
0.8
0.6
0.4
0.2
0.0
K185Q
ΔC35
minus SUR1
b
a
c
0.01
0.1
1
10
IC_50 (mM)

the heteromeric channels assembled with $K_{IR}6.2$ or $K_{IR}6.2\Delta C$ are all significantly more sensitive (e.g. the $IC_{50(ATP)} = 5.9 \pm 0.5$ μM for the SUR1/$K_{IR}6.2$ channel). This includes the SUR2A/$K_{IR}6.2$ channels ($23.4 \pm 2.6$ μM; see also [7]) and SUR2A/$K_{IR}6.2\Delta C35$ channels ($42.7 \pm 4.5$ μM). This result eliminates the previous speculation that the SUR2 receptor does not have a role in determining the ATP sensitivity of the cardiac channel [9]. Note that while the K185Q mutation in the $K_{IR}6.2\Delta C$ channels increases their $IC_{50(ATP)}$ to $>4$ mM (see also [9]), co-assembly with SUR1 increases the apparent ATP sensitivity giving an $IC_{50(ATP)}$ for the SUR1/$K_{IR}6.2_{K185Q}$ channels which is close to that of the unmutated homomeric channels ($262.7 \pm 21.9$ vs. $221.1 \pm 12.1$ μM). Thus modification of $K_{IR}6.2$ and SUR removal make additive contributions to yield the markedly reduce ATP sensitivity of the mutant homomeric channel. Fig. 4C summarizes our data in the form of a map of maximal $P_o$ vs. $IC_{50(ATP)}$. In all of the channels tested, and in similar channels reported elsewhere, the removal of SUR1 has two major effects, reducing the maximal $P_o$ and increasing the $IC_{50(ATP)}$. The higher $IC_{50}$ values observed in the mutant homomeric channels are the result of the additive effects of not having SUR present and of the mutations. Note that the correlation of an increase in the $P_{omax}$ with a decrease in $IC_{50(ATP)}$ observed when $K_{IR}6.2$ is assembled with an SUR is the opposite of the trend reported first for the N160D substitution [8] and seen for other mutations of $K_{IR}6.2$ that alter gating [13,25,26]. Since $K_{IR}6.2$ in the absence of SUR spends more time in a long-lived closed state which others have argued binds inhibitory ATP [13,26], and SUR increases the $P_{omax}$ mainly by shortening the interburst interval, this negative correlation between the $P_{omax}$ and $IC_{50(ATP)}$ implies SUR either increases the affinity of an ATP binding site on $K_{IR}6.2$ in the interburst closed state and/or improves the linkage between the binding site and the gate thus amplifying the effect of ATP binding. An alternative to this allosteric mechanism is that SUR and $K_{IR}6.2$ co-assemble a shared ATP binding site and that this shared site has a slightly higher affinity for ATP in the β-cell vs. the cardiac $K_{ATP}$ channel. Biochemical measurements of ATP binding will be required to distinguish these possibilities.

In summary, SUR is required for efficient trafficking of $K_{IR}6.2$ to the plasma membrane and determination of the maximal $P_o$ and apparent sensitivity of physiologically relevant $K_{ATP}$ channels. We are aware of no description of $K^+$ currents that resemble those recorded from cells expressing $K_{IR}6.2\Delta C$, or over-expressing $K_{IR}6.2$ subunits. It appears that cells have evolved a mechanism(s) that couples trafficking with channel assembly whereby incompletely assembled channels to do reach the cell surface. This mechanism(s) appears to insure that $K_{IR}6.2$ subunits are not expressed in the plasma membrane without SUR, and if they are, that the resulting homomeric channels will be poorly operational. The reason for this is unclear, but we have observed greater death of cells transfected with $K_{IR}6.2\Delta C$ vs heteromeric wild-type channels, presumably the result of the increased energy consumption associated with unregulated $K^+$ efflux.

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