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The tolbutamide site of SUR1 and a mechanism for its functional coupling to K_{ATP} channel closure

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Abstract Micromolar concentrations of tolbutamide will inhibit $(SUR1/K_{IR}6.2)_4$ channels in pancreatic β-cells, but not $(SUR2A/K_{IR}6.2)_4$ channels in cardiomyocytes. Inhibition does not require Mg^{2+} or nucleotides and is enhanced by intracellular nucleotides. Using chimeras between SUR1 and SUR2A, we show that transmembrane domains 12–17 (TMD12-17) are required for high-affinity tolbutamide inhibition of K_{ATP} channels. Deletions demonstrate involvement of the cytoplasmic N-terminus of $K_{IR}6.2$ in coupling sulfonylurea-binding with SUR1 to the stabilization of an interburst closed configuration of the channel. The increased efficacy of tolbutamide by nucleotides results from an impairment of their stimulatory action on SUR1 which unmasks their inhibitory effects. The mechanism of inhibition of β-cell K_{ATP} channels by sulfonylureas during treatment of non-insulin-dependent diabetes mellitus thus involves two components, drug-binding and conformational changes within SUR1 which are coupled to the pore subunit through its N-terminus and the disruption of nucleotide-dependent stimulatory effects of the regulatory subunit on the pore. These findings uncover a molecular basis for an inhibitory influence of SUR1, an ATP-binding cassette (ABC) protein, on $K_{IR}6.2$, a ion channel subunit.

Key words: ATP-sensitive potassium inward rectifier; Gating; ATP-binding cassette; Transport ATPase; SUR2A; $K_{IR}6.0$

1. Introduction

The inhibition of K_{ATP} channels in pancreatic β-cells by micromolar concentrations of tolbutamide or nanomolar concentrations of glibenclamide [1] has been the pharmacological basis for treatment of non-insulin-dependent diabetes mellitus with these first and second generation sulfonylureas for almost 60 years [2]. The high-affinity sulfonylurea receptor SUR1 is an ATP-binding cassette (ABC) protein [3] that associates with the inward rectifier, $K_{IR}6.2$ [4], to form the octameric, $(SUR1/K_{IR}6.2)_4$ β-cell K_{ATP} channel [5,6]. These channels provide a unique example of functional coupling between an ABC or transport ATPase protein and a K_{IR} pore forming subunit [7] and are the paradigm for other $(SURx/K_{IR}6.0)_4$ K_{ATP} channels [8].

Sensitivity to micromolar concentrations of tolbutamide, poor reversibility of high-affinity inhibition by nanomolar concentrations of glibenclamide and enhancement of the sulfonylurea block by intracellular MgADP are hallmarks of β-cell [9] but not cardiac K_{ATP} channels [10]. Reconstitution of sarcolemmal K_{ATP} channels by co-expression of $K_{IR}6.2$ and SUR2A, a low-affinity glibenclamide receptor, demonstrated that the differential response to sulfonylureas was specified by the SUR isoform [11]. These data and the fact that glibenclamide consists of the tolbutamide moiety plus the non-sulfonylurea meglitinide group known to inhibit K_{ATP} channels [12,13] imply that SUR1 contains a high-affinity tolbutamide-binding site missing in SUR2A. Sulfonylureas do not block the open pore of a K_{ATP} channel or affect its unitary conductance or intraburst kinetics. Sulfonylureas do shorten the mean burst duration and lengthen the intervals between bursts by placing channels in long-lived interburst closed states [14]. The structural determinants involved in coupling sulfonylurea-binding with closure of the $(K_{IR}6.2)_4$ pore are unknown. How intracellular nucleotides increase the efficacy of sulfonylurea inhibition of β-cell channels is unknown [15]. Two reports suggest that sulfonylureas can alter the ADP stimulation of $SUR1/K_{IR}6.2$, but not $SUR2A/K_{IR}6.2$ channels [16,17] and reduce the stability of 8-azido ATP-binding to SUR1 [18]. These effects are dependent on Mg^{2+} and require intact nucleotide-binding folds (NBFs), suggesting that sulfonylurea-binding can uncouple Mg-nucleotide di- and triphosphate-dependent stimulatory effects of SUR1 on the $K_{IR}6.2$ gate. As these stimulatory effects antagonize nucleotide inhibition on $K_{IR}6.2$, uncoupling unmasks the inhibitory action of nucleotides prompting K_{ATP} channel closure.

Using matched chimeras of human SUR1 and SUR2A, we show that the transmembrane domains 12–17 (TMD12-17) segment of SUR1 confers the high-affinity component of tolbutamide inhibition to chimeric $SUR/K_{IR}6.2$ channels. High-affinity tolbutamide attenuation of channel activity does not require the addition of Mg^{2+} and nucleotides. Progressive truncations of the N-terminus of $K_{IR}6.2$ impair inhibition by tolbutamide or glibenclamide, implying that the N-terminus is involved in the allosteric coupling of conformational changes in SUR1 to the gate of the pore, consistent with the importance of this cytoplasmic domain for limiting channel bursting [19]. A relationship between the degree of nucleotide stimulation and tolbutamide inhibition demonstrates that sulfonylureas impair the stimulatory action of intracellular nucleotides unmasking their inhibitory action. Our results suggest a two component mechanism for the high-affinity effects of sulfonylureas: binding to the TMD12-17 segment of SUR1 causes conformational changes that stabilize an interburst closed configuration via a link that involves the N-terminus of $K_{IR}6.2$. The same conformational changes are proposed to impair the stimulatory interactions of intracellular nucleotides with the NBFs of SUR1 that balance the inhibitory effects of nucleotides on $K_{IR}6.2$.

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Structures and Function and 43rd Biophysical Society Meetings [20].

2. Materials and methods

The human SUR1, SUR2A and $K_{IR}6.2$ cDNAs have been described previously [21], as have the $\Delta NK_{IR}6.2$ and $K_{IR}6.2\Delta C$ constructs [19,22] and the matched SUR chimeras [23]. Two additional chimeras, XVII and XVIII, were generated as follows using the constructs shown in Fig. 2A. Chim XVII was constructed by replacing a *Sal*I-*Not*I fragment from Chim XIII, containing the N-terminus of SUR1 with the *Sal*I-*Not*I fragment from Chim IV which contains the N-terminal half of SUR2 and TMD12-17 from SUR1. Chim XVIII was constructed using overlapping PCR primers to engineer a *Hin*dIII site into SUR2A to match one in SUR1. This changed SUR2 residues R1197 and M1198 in the intermediate vector to K and L, respectively. A *Hin*dIII-*Hin*dIII fragment from SUR1, containing residues 1020–1226, was then used to replace the corresponding fragment in the intermediate vector to generate Chim XVIII. The resulting plasmids were sequenced in both directions to verify that no mutations were introduced. The transfection and cultivation of COSm6 cells for electrophysiological experiments and the recording of currents using the patch-clamp technique were done as described previously [24]. All recording was done using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA), at −40 mV holding and 23–24°C unless otherwise noted. Pipettes were filled with the K^+-rich external solution containing (mM): KCl 145, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, pH 7.4 (KOH), unless otherwise noted. The Mg^{2+}-free internal solution contained (mM) KCl 140, EDTA 5, HEPES 5, KOH 10, pH 7.2 (KOH), while the Mg^{2+}-containing 'intracellular' solution had the following composition (mM): KCl 140, $MgCl_2$ 1, EGTA 5, HEPES 5, KOH 10, pH 7.2 (KOH). The free Mg^{2+} concentration in the Mg^{2+}-containing internal solution supplemented with nucleotides was kept at a quasi-cytosolic level of ~0.7 mM by adding $MgCl_2$ to account for the Mg^{2+}-binding to nucleotides [25]. Nucleotides and other compounds were from Sigma (St. Louis, MO, USA). Bathing solutions were applied using a programmable rapid solution changer (RSC-200, Biologic, Claix, France). The relative NPo, used as a measure of channel activity in the presence of a test compound, was estimated and the single channel kinetics and nucleotide-sensitivity were analyzed as described earlier [24]. A conventional single pseudo-Hill equation or a two component pseudo-Hill equation were fit to the tolbutamide dose-response data. The latter included the fraction of the maximal NPo (L) remaining when all of the high-affinity inhibitory sites are occupied [17] and the concentrations IC_{50h} and IC_{50l} at which inhibition is half maximal at the high and low-affinity sites with corresponding h_h and h_l slope factors, respectively. Non-linear curve fitting was done using the Marquardt-Levenberg algorithm. Averaged data are expressed as means ± S.E.M. for $n \geq 5$ with error bars equal to the 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.

3. Results

3.1. SUR1 specifies high-affinity tolbutamide inhibition, which is enhanced by stimulatory nucleotides

Fig. 1A shows that inhibition of $SUR1/K_{IR}6.2$ channels by tolbutamide, in the absence of nucleotides, can be described by a two site model where the IC_{50h} and IC_{50l} values differ by approximately three orders of magnitude, 1.9 ± 0.2 μM ($h_h = 1.04 \pm 0.08$) versus $IC_{50L} = 1399 \pm 137.3$ μM ($h_l = 1.13 \pm 0.1$). Inhibition of the $SUR2A/K_{IR}6.2$ channels can be described by a single site model with an IC_{50} value, 1057.4 ± 52.1 μM ($h_l = 0.95 \pm 0.04$), similar to the low-affinity site in the $SUR1/K_{IR}6.2$ channels, in agreement with our earlier estimate [24]. Comparable results were found for channels reconstituted from rat SUR1 or SUR2A plus mouse $K_{IR}6.2$ in *Xenopus* oocytes [17]. The results are consistent with the idea that the high sensitivity of the β-cell K_{ATP} channel to sulfo-

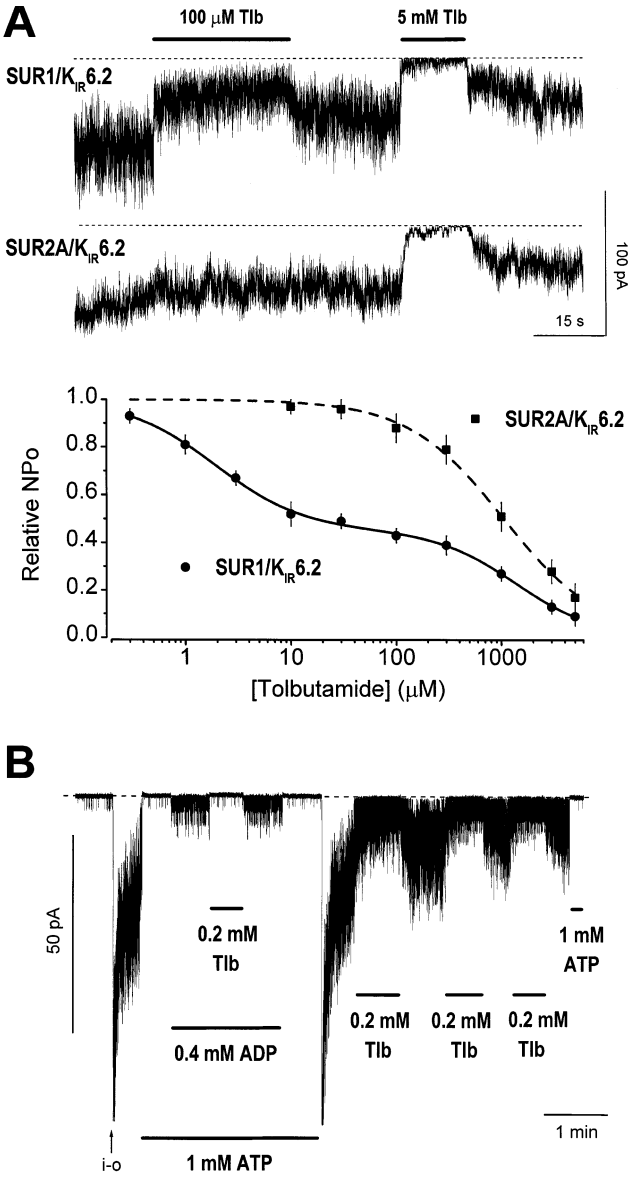

Fig. 1. SUR1 specifies the high-affinity tolbutamide inhibition of K_{ATP} channels which is enhanced by Mg-nucleotides. A: A comparison of the currents and tolbutamide dose-response curves from $SUR1/K_{IR}6.2$ versus $SUR2A/K_{IR}6.2$ K_{ATP} channels indicates that the β-cell channel can be described by two components versus a single component for the cardiac channel. The solid curve through the $SUR1/K_{IR}6.2$ data is the best fit to a two component pseudo-Hill equation giving $IC_{50h} = 1.9 \pm 0.2$ μM, $h_h = 1.04 \pm 0.08$, $IC_{50l} = 1399 \pm 137.3$ μM, $h_l = 1.13 \pm 0.1$ and $L = 0.451 \pm 0.015$. The dashed line through the $SUR2A/K_{IR}6.2$ data is the best fit to a single component pseudo-Hill equation giving $IC_{50} = 1057.4 \pm 52.1$ μM and $h = 0.95 \pm 0.04$. These experiments were done in the absence of nucleotides in the Mg^{2+}-containing internal solution described in Section 2. B: The addition of ADP to the ATP-containing internal solution at ~0.7 mM free Mg^{2+} stimulates $SUR1/K_{IR}6.2$ channels and markedly increases the degree of high-affinity tolbutamide inhibition. Here and in the following figures, the arrow and i-o indicate isolation of the inside-out patch, a downward deflection of the current trace corresponds to inwardly directed current, the horizontal dotted lines show the level of current when all K_{ATP} channels are closed and the thick solid lines indicate superfusion with a test compound at the concentration shown. Tlb = tolbutamide. The higher ratio of the mean current to macrocurrent noise for $SUR2A/K_{IR}6.2$ channels is consistent with the cardiac channels having a higher Po_{max} [23].

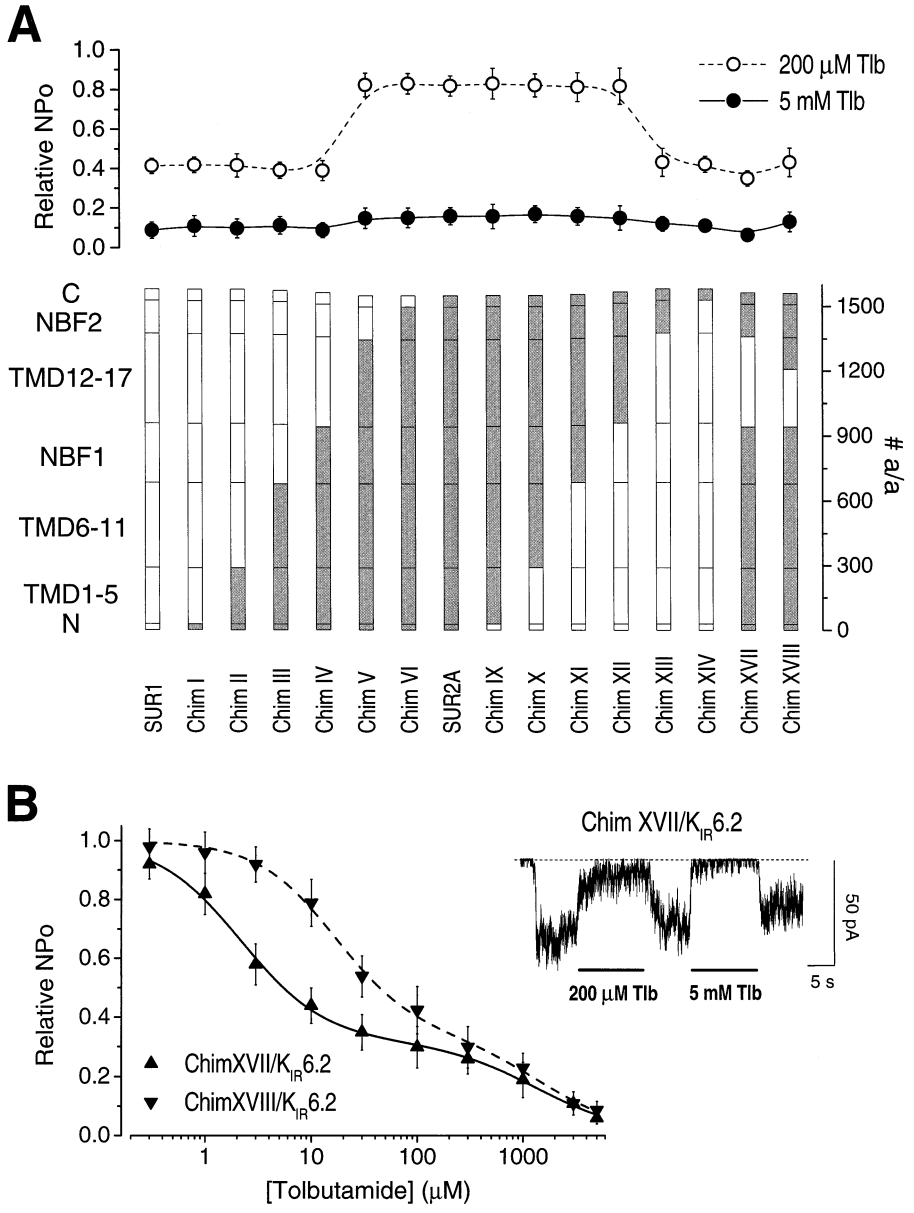

Fig. 2. Delineation of the SUR1 segment required for high-affinity tolbutamide inhibition of K_{ATP} channels. A: The top panel shows inhibition of channels assembled from $K_{IR}6.2$ and SUR1, SUR2A or the chimeric SURs illustrated. Chimeras I–VI and IX–XIV were described in a previous report [23]. The topological features of SUR are given on the left, the corresponding amino acids on the right axis. Segments from SUR1 and SUR2A are shown in white and gray, respectively. The presence of TMD12-17 from SUR1 is sufficient to confer a high-affinity interaction with tolbutamide. B: The inset gives a representative example of inhibition of the Chim XVII/$K_{IR}6.2$ channel. These channels contain TMD1-5 which specifies the higher Po_{max} of the cardiac channel [23], indicated by the greater ratio of the mean current to macrocurrent noise (compare with Fig. 1A). The dose-response curves for the Chim XVII/$K_{IR}6.2$ and Chim XVIII/$K_{IR}6.2$ channels show that TMD12-17 from SUR1 specifies a high-affinity interaction with tolbutamide ($IC_{50h} = 2.2 \pm 0.3$ μM, $h_h = 1.11 \pm 0.11$, $IC_{50l} = 1374.1 \pm 393.2$ μM, $h_l = 0.97 \pm 0.21$, compare with the values for SUR1 in Fig. 1). Reducing the size of the swapped segment reduces the apparent affinity of the chimeric channel for tolbutamide ($IC_{50h} = 16.7 \pm 4.3$ μM, $h_h = 1.18 \pm 0.19$, $IC_{50l} = 1504.6 \pm 914.5$ μM and $h_l = 1.01 \pm 0.37$) without significantly affecting the apparent efficacy ($L = 0.32 \pm 0.029$ versus 0.355 ± 0.092 for Chim XVII versus XVIII, respectively). The ionic conditions and holding potential are as in Fig. 1.

nylureas is determined by a high-affinity sulfonylurea-binding site in SUR1 [3] missing in SUR2A [11,17,24]. Low-affinity inhibition of the SUR1/$K_{IR}6.2$ channels is attributed to an interaction of sulfonylureas with $K_{IR}6.2$ [17]. The data are not sufficient to conclude that low-affinity binding of tolbutamide to SUR2A [26] makes no contribution and that inhibition is entirely due to binding to $K_{IR}6.2$. High-affinity inhibition of SUR1/$K_{IR}6.2$ channels, specified by sulfonylurea-binding to SUR1, is saturated at ~40% of the maximal Po in the absence of nucleotides (Po_{max}) by ~100 μM tolbutamide. A substantial channel activity remains at several mM tolbutamide. Two-hundred μM tolbutamide was used to saturate the high-affinity tolbutamide-binding site in all further experiments.

Fig. 1B illustrates the enhancing effect of a quasi-intracellular mixture of ADP and ATP at ~0.7 mM free Mg^{2+} on the inhibition of reconstituted human β-cell K_{ATP} channels by 200 μM tolbutamide. Using these simulated intracellular con-

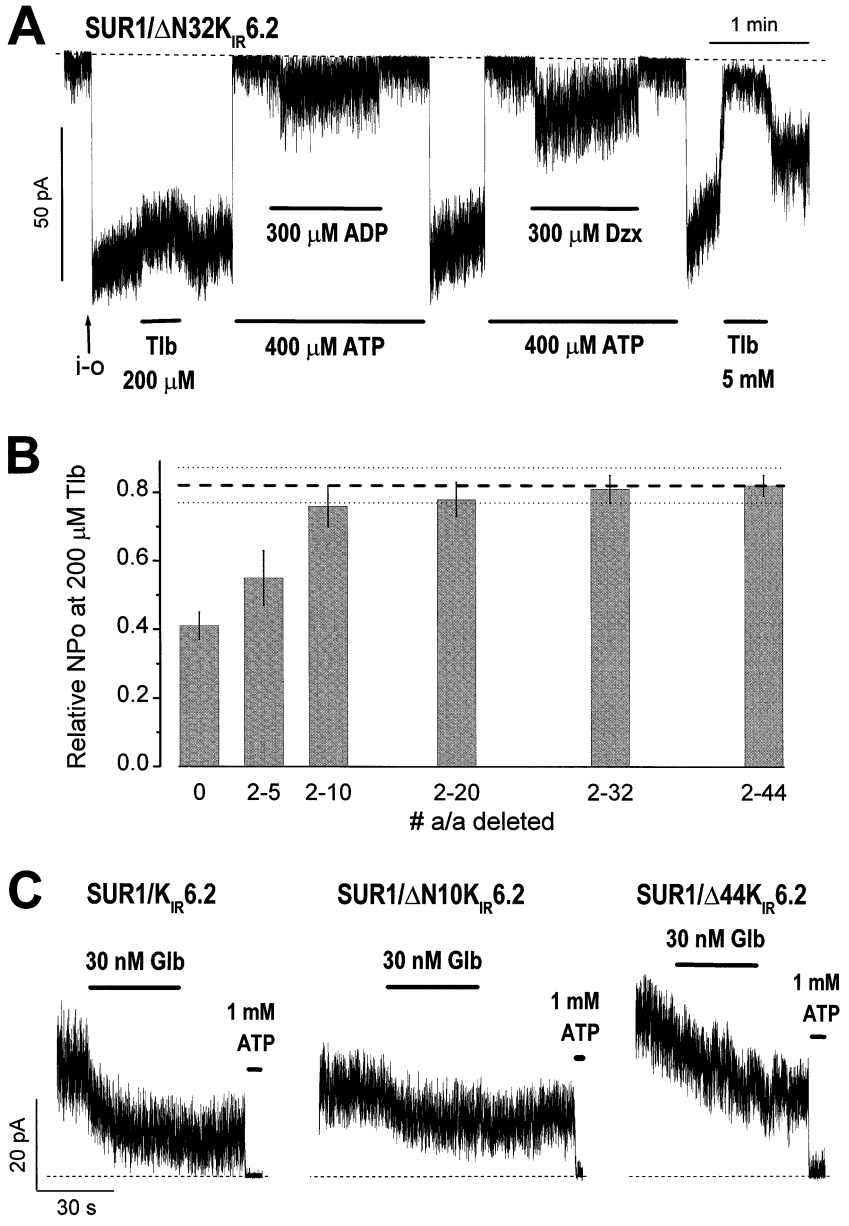

Fig. 3. Deletion of the N-terminus of $K_{IR}6.2$ impairs high-affinity sulfonylurea inhibition. A: Deletion of 32 amino acids, ΔN32, from the N-terminus of $K_{IR}6.2$ eliminates high-affinity inhibition of β-cell K_{ATP} channels, but not low-affinity tolbutamide inhibition, or stimulation by diazoxide and nucleotides. Mg-nucleotides also stimulated all chimeric channels. B: Progressive truncation of the N-terminus of $K_{IR}6.2$ results in a gradual impairment of high-affinity inhibition to the level of SUR2A/$K_{IR}6.2$ channels marked here by the thick dashed and dotted lines (mean ± S.D.). C: N-terminal deletion progressively eliminates the inhibitory action of glibenclamide. The holding potential was 0 mV. The quasi-physiological external pipette solution contained (mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, pH 7.4 (NaOH). The single channel kinetics, ATP- and tolbutamide-sensitivity of the SUR1/ΔN44$K_{IR}6.2$ channels using similar experimental conditions were reported previously [7,19]. Free [Mg^{2+}] in the internal solution was ~0.7 mM.

ditions [27], ADP stimulates channel activity pre-inhibited by 1 mM ATP, while 200 μM tolbutamide reduces channel activity ~10-fold compared with ~2.5-fold inhibition in the absence of nucleotides. Tolbutamide (200 μM) did not affect the unitary conductance or intraburst kinetics of SUR1/$K_{IR}6.2$ channels (see [19] for values obtained under similar conditions). Note that comparable tolbutamide concentrations will nearly eliminate the K_{ATP} conductance of whole β-cells and that others have described the 'enhanced' inhibition of β-cell K_{ATP} channels when MgADP [9] or other nucleotide di- or triphosphates are present at the cytoplasmic face of isolated patches [15].

3.2. High-affinity inhibition by tolbutamide requires the TMD12-17 segment of SUR1

Matched SUR chimeras [23] were used to delineate the region of SUR1 which confers high-affinity tolbutamide inhibition by comparing the effect of 200 μM tolbutamide on chimeric channels (Fig. 2A). The results indicate that the TMD12-17 segment of SUR1 is critical for high-affinity tolbutamide inhibition. Ashfield et al. [28] reported a similar result using unmatched chimeras. Chim XVII and XVIII show that TMD12-17 is sufficient to confer high-affinity inhibition when swapped into SUR2A. Comparison of the tolbutamide dose-response curves for the Chim XVII/$K_{IR}6.2$ and

Chim XVIII/K_{IR}6.2 channels (Fig. 2B) show that the smaller TMD12-15 segment reduces the affinity for tolbutamide without affecting the efficacy of high-affinity inhibition (1-L), consistent with a tolbutamide-binding site within TMD12-17. Our previous studies [23] have shown that the TMD1-5 segment, not the TMD12-17 segment, specifies the β-cell versus sarcolemmal channel isoform differences in Po_{max}. Differences in Po_{max} are determined by the occupancy of interburst closed state(s), suggested to be the key state(s) that bind nucleotides and sulfonylureas that inhibit K_{ATP} channels [19,29,30]. This result and the observation that the intraburst kinetics of the Chim XVII/K_{IR}6.2 and Chim XVIII/K_{IR}6.2 channels are similar to those of wild-type channels [23] argue that changes in tolbutamide-sensitivity of the chimeric channels can not solely be due to modification of the single channel kinetics by swapping TMD12-17 segments. The conservative conclusion is that a part of the high-affinity sulfonylurea-binding site is formed by the TMD12-17 segment of SUR1.

3.3. The N-terminus of $K_{IR}6.2$ is involved in coupling sulfonylurea-binding to SUR1 with closure of the K_{ATP} channel pore

We have shown previously that truncation of the N-terminus of K_{IR}6.2 increases the Po_{max} of β-cell K_{ATP} channels by reducing the occupancy of the interburst closed state [19]. Truncation moderately increases the apparent $IC_{50(ATP)}$ [19,31], but millimolar concentrations of ATP will reduce the Po to nearly undetectable levels. The effects of N-terminal deletions have not been observed for homomeric K_{IR}6.2ΔC channels [31] whose steady-state ATP-sensitivity is lower than that of SUR1/ΔNK_{IR}6.2ΔC channels (see [22] versus [19] for a statistically representative side by side comparison of K_{IR}6.2ΔC versus SUR1/ΔNK_{IR}6.2ΔC channels). The results indicate that deletion of as many as 44 amino acids from the N-terminus [19] does not affect closure of the pore or remove the low-affinity nucleotide site from K_{IR}6.2, but does disrupt functional coupling between SUR and K_{IR}6.2 [7]. The results shown in Fig. 3 support this hypothesis. As illustrated in Fig. 3A, SUR1/Δ$N32K_{IR}$6.2 channels are inhibited by 200 μM tolbutamide as poorly as the SUR2A/K_{IR}6.2 channels (relative NPo values of 0.84 ± 0.03 versus 0.82 ± 0.05, respectively). At the same time, the SUR1/Δ$N32K_{IR}$6.2 channels were stimulated by ADP or the potassium channel opener diazoxide (300 μM), in the presence of ATP and Mg^{2+}. Tolbutamide at a high concentration (5 mM) inhibited SUR1/Δ$N32K_{IR}$6.2, SUR1/Δ$N32K_{IR}$6.2ΔC35 and SUR2A/K_{IR}6.2 channels equivalently in the absence of Mg-nucleotides (relative NPo values of 0.16 ± 0.05, 0.18 ± 0.07 and 0.17 ± 0.06, respectively). These values are consistent with the IC_{50} for tolbutamide inhibition reported for K_{IR}6.2ΔC26 channels [17], arguing that the deleted segments of the N- and C-termini of K_{IR}6.2 are not part of the low-affinity tolbutamide-binding site. Fig. 3B summarizes the data for a series of deletions. Truncation of as few as five amino acids was sufficient to compromise high-affinity tolbutamide inhibition. Truncation of 10 or 20 amino acids gave ∼85–90% of the maximum attenuation of the inhibition. Analysis of the tolbutamide dose-response of the SUR1/Δ$N10K_{IR}$6.2 channels showed a significantly increased L value (0.88 ± 0.028 versus 0.451 ± 0.015 for the wild-type channel shown in Fig. 2) with no significant changes in the parameters related to tolbutamide-binding (IC_{50h} = 1.7 μM at h_h = 1.36 and IC_{50l} = 1479.4 μM at h_l = 0.97). As shown in Fig. 3C, high-affinity inhibition by glibenclamide was also impaired by N-terminal deletion.

As reported for other SUR1/ΔNK_{IR}6.2 channels [19], the $IC_{50(ATP)}$ and Po_{max} values for the SUR1/Δ$N10K_{IR}$6.2 channels were slightly increased, with about a 3-fold increase in the steady-state $IC_{50(ATP)}$ (20.1 ± 2.8 μM, mean ± S.D. for n = 4 versus 5.9 ± 0.5 μM for wild-type channels) and approximately a 10% increase in Po_{max} (by a direct estimate from three different single channel records, see also a prediction from macrocurrent analysis [31]). The decreased apparent ATP-sensitivity of the SUR1/ΔNK_{IR}6.2 channels could result from a decreased occupancy of interburst closed state(s) that bind ATP [19]. Although a similar mechanism has been suggested for the insensitivity of SUR1/$K_{IR}6.2_{C166S}$ channels to 100 μM tolbutamide [30], it is unlikely that a decreased occupancy of the closed state(s) can explain the decreased fraction of the high-affinity component of tolbutamide inhibition seen for the SUR1/Δ$N10K_{IR}$6.2 channels. Reducing the Po of SUR1/ΔNK_{IR}6.2 channels to the Po_{max} of wild-type channels by ∼5–10 μM ATP in Mg^{2+}-free conditions or by spontaneous run-down did not increase the fraction of the high-affinity component of tolbutamide inhibition (L ∼ 0.85). Similarly, increasing the Po_{max} of K_{ATP} channels with an intact K_{IR}6.2 did not affect the fraction of the high-affinity component. Chim XVII (Fig. 2), like Chim II, contains the TMD1-5 segment of SUR2A, which specifies higher Po_{max} values, and has a Po_{max} value comparable to those of the SUR1/Δ$N10K_{IR}$6.2 channels, which are greater than the Po_{max} values of SUR1/K_{IR}6.2 channels [23]. Irrespective of the lower occupancy of the interburst closed state, the two chimeric channels were inhibited by tolbutamide as efficiently as wild-type channels (a somewhat lower L was observed for Chim XVII/K_{IR}6.2 channels, as shown in Fig. 2B). The latter results are not complicated by the presence of nucleotides, by the possibility of altered interactions between subunits in run-down channels or by possible effects of the K_{IR}6.2 modification. The observation is consistent with the idea that coupling of tolbutamide-binding with SUR1 to closure of the gate is not a purely kinetic phenomenon and suggests a specific role for the N-terminus in transduction of an inhibitory signal from SUR1 to the pore.

3.4. Sulfonylurea-binding to SUR1 impairs the stimulatory action of intracellular nucleotides

Fig. 4A shows that the degree of inhibition by 200 μM tolbutamide is always higher in the presence of Mg^{2+} and nucleotides (either ATP, ADP or both at variable ratios with a quasi-cytosolic concentration of Mg^{2+}, ∼0.7 mM). Inhibition of SUR1/K_{IR}6.2 channels by glibenclamide (100 nM) is also enhanced by MgADP [17]. To test whether saturation of the sulfonylurea-binding site on SUR1 could abolish the stimulatory action of either, or both, ATP or ADP, we compared the effects of tolbutamide under stimulatory and inhibitory conditions (presence of Mg-nucleotides versus presence of nucleotides without Mg^{2+} plus 5 mM EDTA, respectively). Each paired set of internal solutions was tested on a membrane fragment selected for a high density of channels and fast diffusional access (comparable with that shown in Fig. 4A) to increase the significance of current measurements at millimolar concentrations of nucleotide(s) and to minimize errors due to variable 'fading' of Mg-nucleotide stimulation of

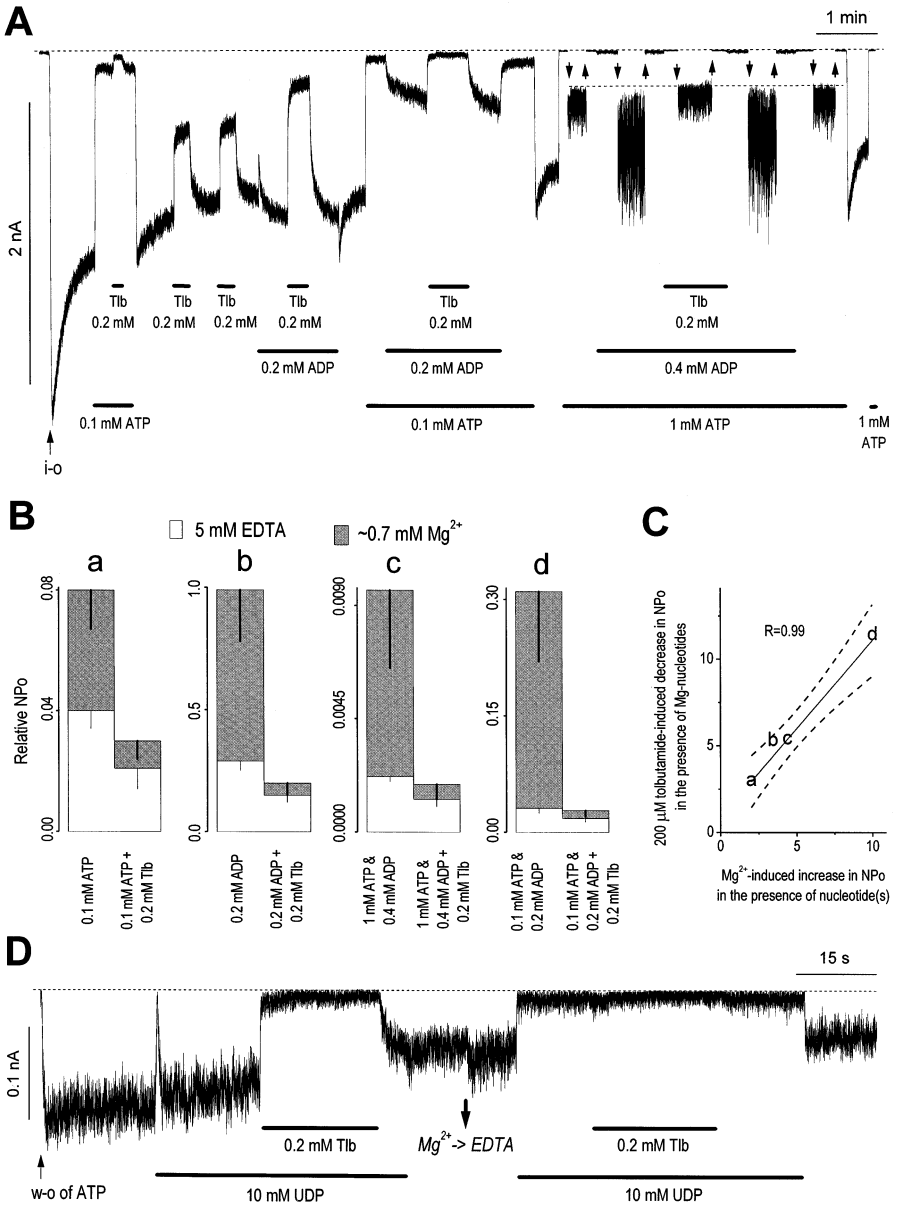

Fig. 4. Tolbutamide-binding uncouples the Mg-nucleotide-dependent stimulatory action of SUR1 on $K_{IR}6.2$. A: The degree of high-affinity inhibition of SUR1/$K_{IR}6.2$ channel currents is greater in the presence of either MgATP, MgADP or both nucleotides as a result of their stimulatory action on SUR1 [27,46]. In a representative conventional inside-out patch containing $\sim 10^3$ K_{ATP} channels, with fast diffusional access, 200 μM tolbutamide inhibited the current 2.85, 5.32, 11.09 and 5.05-fold versus 1.95 ± 0.02-fold (mean ± S.D., two applications) in the presence of 0.1 mM ATP, 0.2 mM ADP, 0.1 mM ATP plus 0.2 mM ADP, 1 mM ATP plus 0.4 mM ADP versus no nucleotides, respectively. The measurements were done in Mg^{2+}-containing internal solution. Interestingly, fast diffusional access to these channels allows for resolution of a rapid current transient upon application of ADP, which may be the result of more rapid inhibition than stimulation. B: The enhancement of high-affinity tolbutamide inhibition of SUR1/$K_{IR}6.2$ channels by MgATP and/or MgADP is the result of tolbutamide uncoupling the stimulatory action of Mg-nucleotides, thus accentuating their inhibitory action. Currents were recorded as described above. Note that the scales of relative NPo values change. The relative NPo values after tolbutamide inhibition in Mg^{2+}-containing and Mg^{2+}-free conditions were not significantly different. C: A plot of the tolbutamide-induced decrease in NPo in the presence of Mg-nucleotide(s) versus the Mg-nucleotide-stimulated increase in NPo shows a correlation. The alphabetical symbols correspond to the individual experiments in (B). The solid line shows a linear regression ($R \sim 1$), the dashed lines give the 95% confidence limits. D: Tolbutamide inhibition of MgUDP-stimulated SUR1/$K_{IR}6.2$ channel currents. MgUDP was applied before there was substantial run-down of SUR1/$K_{IR}6.2$ channels. The strong stimulatory action of MgUDP seen at this high, non-physiological concentration may be the result of a markedly reduced inhibitory action of UDP (estimated $IC_{50(UDP)} > 1$ mM, data not shown), rather than a unique stimulatory action (as was suggested originally for cardiac K_{ATP} channels [29]). Like MgADP, 10 mM MgUDP produced a diffusion-limited offset of the current followed by a rise to nearly the level of current seen after removal of 1 mM ATP (in Mg^{2+}) used to refresh the channels (upward arrow). Removing Mg^{2+} by perfusion with 5 mM EDTA (downward arrow) caused a minor increase in activity which was inhibited by application of 10 mM UDP.

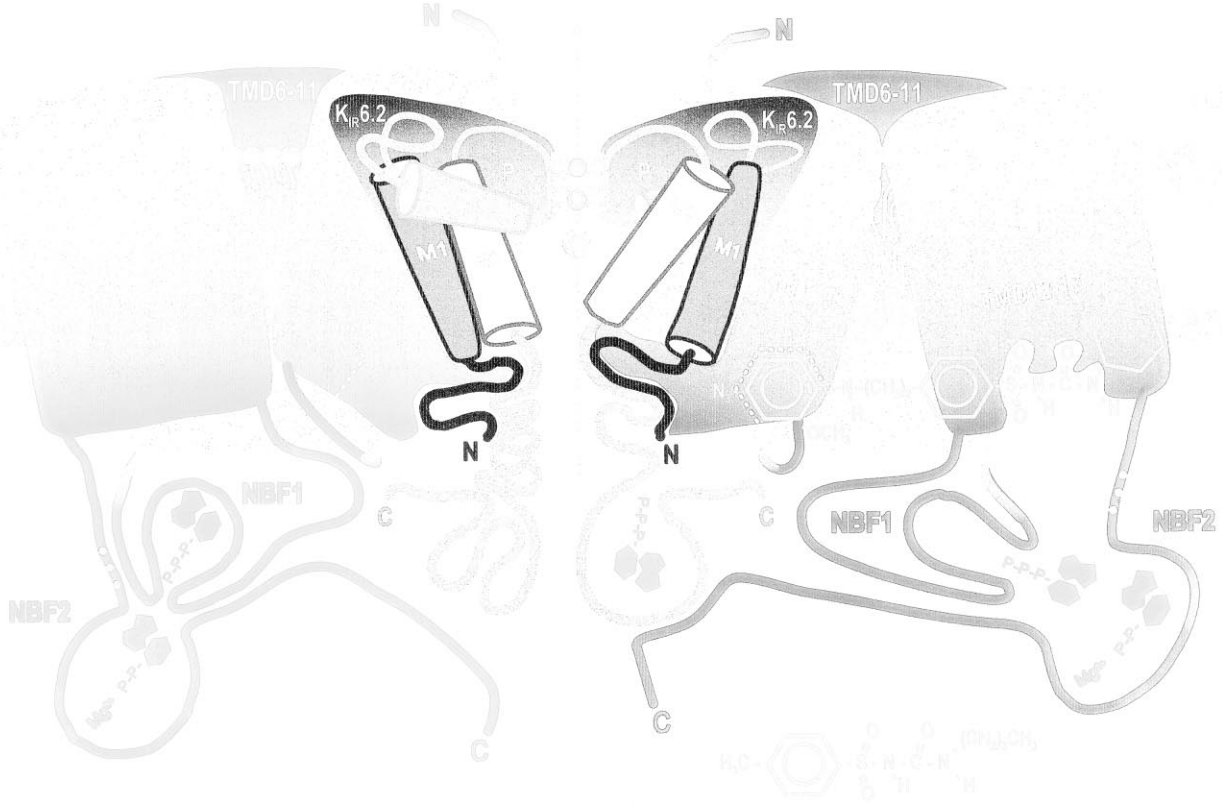

Fig. 5. The proposed interactions involved in modulation of nucleotide-dependent gating of K_{ATP} channels by sulfonylureas. Two pairs of SUR/K_{IR}6.2 subunits, a cardiac pair on the left and a β-cell pair on the right, are illustrated around a central pore. The SURs surround K_{IR}6.2 consistent with absence of data, suggesting that they contribute to the permeation pathway. The triplet of dots indicates the location of ER retention signals, which ensure that only completely assembled channels reach the plasma membrane [47]. Our chimeric channel data were used to position the TMD domains based on the finding that TMD1-5 specifies the isoform differences in kinetics [23], which presumably requires its closer proximity to the K_{IR}6.2 gate. The C-terminus of SUR is placed near the C-terminal cytoplasmic segment of K_{IR}6.2 based on the specification of the isoform differences in $IC_{50(ATP)}$ by the last 42 amino acids of SUR. The K_{IR} pore is modeled on the bacterial KcsA channel [40] without implying an exact packing of M1 and M2, which may be different in K_{IR}s [48]. The K^+ selectivity filter is formed by the backbone carbonyl groups (=O) of the -GFG- motif. Two dehydrated K^+ ions (smaller spheres) occupy the filter, a larger hydrated K^+ ion is stabilized by pore helix dipoles in the central cavity [49]. The M2 internal helices line the aqueous canal within the transmembrane electrical field. 'Gated access' [39] to the pore is controlled by translation rotational movements of 'rigid bodies' composed of the α-helical M2 and possibly the adjacent segment of the C-terminus [41]. A periodicity in cysteine accessibility to Cd^{2+} in these segments [50] is consistent with the model. Movement of the internal helices is indicated by their different positions in the two halves of the pore, the left pair is illustrated in an open state with the M2 helix orientation allowing for 'gated access' to the pore. No inhibitory nucleotide is shown in the proposed low-affinity C-terminal ATP-binding site of K_{IR}6.2 [51]. Both NBFs on SUR are occupied and are drawn to indicate cooperative interactions of Mg^{2+}-independent high-affinity ATP-binding on NBF1 and low-affinity MgADP-binding on NBF2 [18]. The right pair illustrates a closed state induced by glibenclamide. The glibenclamide-binding site consists of a benzamido (meglitinide)-binding site (dotted benzene ring) on TMD1-5 and a sulfonylurea (tolbutamide)-binding site on TMD12-17, consistent with earlier observations [3] and the data presented here and in [28]. The higher affinity of the second generation sulfonylureas, like glibenclamide, for SUR presumably arises from binding at both sites. A molecule of tolbutamide displaced by azido-iodoglibenclamide is shown. Cooperative interactions between NBF1 and NBF2 are disrupted by the conformational change (shown here as a change in orientation of TMD1-5 and 12-17) induced by sulfonylurea-binding, consistent with the observations of Ueda et al. [18]. This conformational switch repositions the M2 helix to form an interburst closed state. This requires an intact N-terminus on K_{IR}6.2. Thus, high-affinity sulfonylurea inhibition of $(SUR1/K_{IR}6.2)_4$ channels in vivo is determined by an integration of the inhibitory signals from occupied sulfonylurea-binding site(s) and from ATP inhibitory site(s), both of which stabilize a long-lived closed state of the $(K_{IR}6.2)_4$ pore.

K_{ATP} channels following patch excision [32]. A summary of the results from a number of such experiments (Fig. 4B) illustrates three points. (1) Nucleotides alone do not affect the action of tolbutamide. The ~2-fold inhibition by 200 μM tolbutamide in the absence of Mg^{2+} was similar to the degree of inhibition seen in nucleotide-free conditions irrespective of Mg^{2+} (an example of tolbutamide inhibition in the absence of nucleotides with Mg^{2+} present is illustrated in Fig. 4A). (2) Stimulatory Mg-nucleotides do not affect the final level of inhibition, irrespective of the nucleotide composition tested, 200 μM tolbutamide decreased the NPo to nearly the same level in the presence or absence of Mg^{2+}. As a result, (3) the degree of tolbutamide inhibition in the presence of Mg-nucleotide(s) correlates with the degree of NPo increase stimulated by Mg-nucleotides (Fig. 4C), arguing that saturation of the sulfonylurea-binding site eliminates the stimulatory but not the inhibitory action of Mg-nucleotides. This conclusion is consistent with the observation that 100 μM tolbutamide will 'inhibit the channel activating effect of MgGDP in a noncompetitive manner' [33]. Fig. 4D illustrates a similar result for SUR1/K_{IR}6.2 channels stimulated maximally by 10 mM MgUDP. Two-hundred μM tolbutamide eliminates stimulated K_{ATP} channel activity when applied prior to significant rundown of the current. In agreement with previous studies, we

saw no stimulation of SUR1/K_{IR}6.2 channels by nucleotide monophosphates, which weakly inhibited ($IC_{50} > 1$ mM, see also [31]). We conclude that the increased efficacy of sulfonylureas for K_{ATP} channels in β-cells is due to elimination of the stimulatory action of intracellular nucleotide di- and triphosphates, thus accentuating their inhibitory action.

4. Discussion

Initial insight into how sulfonylureas work came with the identification of K_{ATP} channels in β-cells [34] and the finding that their activity was inhibited by tolbutamide and by glibenclamide [1]. Several studies reported on the differential sensitivity of β-cell versus cardiac K_{ATP} channels to sulfonylureas ([9] versus [10]) and noted the enhancing effects of Mg-nucleotides for their action on β-cell K_{ATP} channels [9,15]. In this study, we used tolbutamide, a reversible inhibitor, rather than glibenclamide. The latter is nearly irreversible and is pharmacologically more complex since it contains both a sulfonylurea moiety and the benzamido moiety of meglitinide, a compound that inhibits both β-cell and SUR2A/K_{IR}6.2 channels [17,35] and may thus bind to SURs at a separate site [12,13,17,36]. Glibenclamide photolabelling of the TMD1-5 segment of SUR1 [3] is consistent with a complex-binding site in which the sulfonylurea moiety binds to TMD12-17 and the benzamido group is in close proximity to the TMD1-5 segment (see Fig. 5). The simultaneous binding of second generation sulfonylureas at two sites would account for their lower K_D values [26].

Comparison of dose-response curves shows that tolbutamide reduces the Po of β-cell K_{ATP} channels through high- and low-affinity interactions, while low-affinity interactions account for inhibition of sarcolemmal channels (Fig. 1 and [17]). The high-affinity component requires SUR1 while the low-affinity component appears to be a direct effect on K_{IR}6.2 [17]. It is not clear whether direct binding to K_{IR}6.2 can account completely for the inhibition of SUR2A/K_{IR}6.2 channels. Dorschner et al. [26] have estimated a $K_D \sim 0.3$ mM for binding of tolbutamide to SUR2A and simulation of inhibition through two low-affinity binding sites versus a single site on K_{IR}6.2 suggests that either model can account for the available data. A binding site has not been localized on K_{IR}6.2, but 5 mM tolbutamide inhibits channels missing the first 44 and last 35 amino acids, indicating that they are not critical. Similarly, the specificity of the low-affinity site has not been established. Meglitinide will inhibit homomeric K_{IR}6.2ΔC channels with an $IC_{50} \sim 10^3$ times greater than for heteromeric channels [17], although the IC_{50} values for inhibition of the heteromeric channels are consistent with the affinities of meglitinide for SUR1 and SUR2A [26]. It is worth noting that inhibition of channel activity by supra-pharmacological concentrations of sulfonylureas does not mean that SUR/K_{IR} type K_{ATP} channels are present and should be used with caution to infer interactions between SURs and K_{IR}s.

We used chimeras between SUR1 and SUR2A to determine the region of SUR1 important for the high-affinity inhibitory effects of tolbutamide. Inclusion of the TMD12-17 segment of SUR1 was sufficient to confer high-affinity inhibition, while swapping a shorter TMD12-15 segment into SUR2A increased the IC_{50h} ~8-fold without decreasing the apparent efficacy of inhibition. Substitution of SUR1 TMD12-17 into SUR2A did not affect the occupancy of the interburst closed state that is critical for interaction with inhibitory nucleotides [19], implying that TMD12-17 specifies tolbutamide-binding rather than coupling binding at a separate site, common to SUR1 and SUR2, to K_{IR}6.2. The speculation [17] that SUR2A binds sulfonylureas as well as SUR1 (see for example [37,38]) without closing the channel is eliminated by the finding that [^{3}H]P1075 is displaced from SUR2 by tolbutamide or glibenclamide with estimated K_D values of ~0.3 mM or ~0.3 μM, respectively, values markedly higher than those estimated for tolbutamide or glibenclamide displacement of [^{3}H]glibenclamide from SUR1 [26]. Furthermore, unpublished data of Schwanstecher and colleagues suggest an essential role for TMD12-17 from SUR1 for high-affinity [^{3}H]glibenclamide-binding.

Our observations are consistent with tolbutamide-binding with a micromolar affinity to TMD12-17 of SUR1, inducing a conformational change with at least two consequences. (1) The change is coupled directly to K_{IR}6.2 through its N-terminus, decreasing the Po by ~60%. Deletions of 5–44 amino acids from the N-terminus disrupt this high-affinity inhibition, but do not affect the kinetics, ATP and low-affinity tolbutamide inhibition of homomeric K_{IR}6.2ΔC channels. This direct coupling does not require nucleotides or intact NBFs and cannot be attributed to kinetic effects, although a reduced occupancy of the long-lived closed state has been suggested to account for the insensitivity of SUR1/K_{IR}6.2$_{C166S}$ channels to tolbutamide [30]. Unlike the N-terminal deletions, this mutation dramatically increases the Po_{max} and $IC_{50(ATP)}$ of homomeric K_{IR}6.2ΔC26 channels and appears to modify 'gated access' to the pore [39], based on its role in gating of KcsA channels [40,41]. Tolbutamide inhibition of SUR1/ΔNK_{IR}6.2 channels partially pre-inhibited with ATP and of chimeric channels displaying an increased Po_{max} demonstrates that a normal occupancy of the interburst closed state does not guarantee normal coupling of sulfonylurea-binding to stabilization of the K_{IR}6.2 pore in a long-lived closed conformation. We suggest that the N-terminus couples the sulfonylurea-induced conformational changes in SUR1 to the gate of K_{IR}6.2 through an interaction with a C-terminal segment adjacent to the M2-helix. This is consistent with recent evidence for a cytoplasmic bundle whose rearrangement might affect the positioning of the pore lining helices of bacterial channels during gating [41,42]. (2) The conformation changes disrupt stimulation of SUR1/K_{IR}6.2 channel activity by Mg-nucleotides. At variable concentrations of ATP and/or ADP, 200 μM tolbutamide decreases SUR1/K_{IR}6.2 channel activity to nearly the same level in the presence or absence of Mg^{2+}. The final NPo values depend on the nucleotide concentration and are close to what is expected from adding the inhibitory effects of nucleotides to the ~2-fold decrease in Po seen in the absence of Mg-nucleotides described above. This two-part mechanism, consisting of the action of tolbutamide plus the effect of inhibitory nucleotides, predicts that the 'apparent' IC_{50} for sulfonylureas will inversely correlate with the nucleotide concentration. This would explain the variable IC_{50} values for sulfonylurea inhibition of K_{ATP} channels seen in whole β-cell experiments versus estimates from excised patches (see, for example, [43]) and the variation in apparent IC_{50} values for sulfonylurea inhibition of SUR1/K_{IR}6.2 channels in inside-out patches with the nucleotide concentration. Whether Mg-nucleotides affect the K_D for sulfonylurea-binding is ques-

tionable, but at least two reports suggest that Mg-nucleotides inhibit sulfonylurea-binding, i.e. attenuating rather than accentuating the effects of these compounds [38,44]. We have been unable to demonstrate a systematic effect of nucleotides on the binding of [^{125}I]iodoglibenclamide to recombinant SUR1 (Bryan, unpublished data). Our finding that the degree of tolbutamide inhibition in the presence of 0.1 mM MgATP+0.2 mM MgADP is higher than in the presence of 1 mM MgATP+0.4 mM MgADP suggests that enhancement cannot be explained solely by a nucleotide-dependent increase in the affinity of SUR1 for sulfonylureas.

How a tolbutamide-induced conformational change in SUR1 disrupts the stimulation of SUR1/K_{IR}6.2 channels by Mg-nucleotides is unclear. Allosteric effects that impair nucleotide-binding at the NBFs and disruption of the coupling between SUR1 and K_{IR}6.2 are two possibilities. Although disruption of coupling cannot be ruled out, allosteric effects are consistent with biochemical data indicating that glibenclamide affects the stability of 8-azido ATP-binding on NBF1 of SUR1 [18]. Ueda et al. [18] propose that K_{ATP} channels are open when ATP is bound to NBF1 and MgADP occupies NBF2 while binding of glibenclamide releases ATP from NBF1, thus closing channels. The result is consistent with tolbutamide-induced conformational changes being transmitted through the N-terminus of K_{IR}6.2 to the pore in the absence of nucleotides, but are at variance with our observation that SUR1/K_{IR}6.2 channels can be reversibly, and repeatedly, inhibited by tolbutamide in nucleotide-free solution (see Figs. 1B and 4A). One way to reconcile these observations is to propose (see Fig. 5) that both ATP-binding to NBF1 and MgADP-binding to NBF2 (resulting from hydrolysis of MgATP) are required for Mg-nucleotide stimulation of K_{ATP} channels. In this case, loss of ATP from NBF1 would eliminate stimulation by Mg-nucleotides, consistent with the requirement that the NBFs are intact for stimulation to occur [27,45].

We propose a two component mechanism for high-affinity tolbutamide inhibition of SUR1/K_{IR}6.2 channels that explains the selective inhibition of pancreatic β-cell versus sarcolemmal K_{ATP} channels (Fig. 5). Sulfonylurea-binding to TMD12-17 induces a conformational change in SUR1 irrespective of whether Mg^{2+} and/or nucleotides are present. This conformational switch uses the N-terminus of K_{IR}6.2 to stabilize a long-lived closed state in which gated access of potassium ions to the pore is denied and also disrupts the Mg-nucleotide-induced stimulatory action of SUR1 on K_{IR}6.2. The stimulatory signal does not require the N-terminus and normally acts to antagonize the inhibitory action of intracellular nucleotides on K_{IR}6.2. Impairment of this signal reveals the inhibitory action of nucleotides. Nucleotide-independent stabilization of the closed state reduces the Po by ~60%, while disrupting stimulation by Mg-nucleotides causes a further reduction whose extent depends upon the intracellular nucleotide concentration. This model accounts for the increased efficacy of sulfonylureas on SUR1/K_{IR}6.2 channels in vivo.

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