

Involvement of the N-terminus of Kir6.2 in coupling to the sulphonylurea receptor

Frank Reimann, Stephen J. Tucker, Peter Proks and Frances M. Ashcroft

University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK

(Received 27 November 1998; accepted after revision 12 April 1999)

1. ATP-sensitive potassium (K_{ATP}) channels are composed of pore-forming Kir6.2 and regulatory SUR subunits. ATP inhibits the channel by interacting with Kir6.2, while sulphonylureas block channel activity by interaction with a high-affinity site on SUR1 and a low-affinity site on Kir6.2. MgADP and diazoxide interact with SUR1 to promote channel activity.
2. We examined the effect of N-terminal deletions of Kir6.2 on the channel open probability, ATP sensitivity and sulphonylurea sensitivity by recording macroscopic currents in membrane patches excised from *Xenopus* oocytes expressing wild-type or mutant Kir6.2/SUR1.
3. A 14 amino acid N-terminal deletion ($\Delta N14$) did not affect the gating, ATP sensitivity or tolbutamide block of a truncated isoform of Kir6.2, Kir6.2 $\Delta C26$, expressed in the absence of SUR1. Thus, the N-terminal deletion does not alter the intrinsic properties of Kir6.2.
4. When Kir6.2 $\Delta N14$ was coexpressed with SUR1, the resulting K_{ATP} channels had a higher open probability ($P_o = 0.7$) and a lower ATP sensitivity ($K_i = 196 \mu M$) than wild-type (Kir6.2/SUR1) channels ($P_o = 0.32$, $K_i = 28 \mu M$). High-affinity tolbutamide block was also abolished.
5. Truncation of five or nine amino acids from the N-terminus of Kir6.2 also enhanced the open probability, and reduced both the ATP sensitivity and the fraction of high-affinity tolbutamide block, although to a lesser extent than for the $\Delta N14$ deletion. Site-directed mutagenesis suggests that hydrophobic residues in Kir6.2 may be involved in this effect.
6. The reduced ATP sensitivity of Kir6.2 $\Delta N14$ may be explained by the increased P_o . However, when the P_o was decreased (by ATP), tolbutamide was unable to block Kir6.2 $\Delta N14$ /SUR1-K719A,K1385M currents, despite the fact that the drug inhibited Kir6.2-C166S/SUR1-K719A,K1385M currents (which in the absence of ATP have a P_o of > 0.8 and are not blocked by tolbutamide). Thus the N-terminus of Kir6.2 may be involved in coupling sulphonylurea binding to SUR1 to closure of the Kir6.2 pore.

The ATP-sensitive potassium channel (K_{ATP}) serves to couple the metabolic status of the cell to its electrical activity (Ashcroft & Rorsman, 1989). One of the defining characteristics of this channel is that its open probability is decreased by intracellular ATP and enhanced by Mg²⁺ nucleotides, like MgADP. K_{ATP} channels are also blocked by sulphonylurea drugs, such as glibenclamide and tolbutamide, and activated by K⁺ channel openers, such as diazoxide (Zünckler *et al.* 1988b).

The β -cell K_{ATP} channel is a tightly associated heterooctameric complex of an inwardly rectifying potassium channel (Kir6.2) and a regulatory subunit, the sulphonylurea receptor (SUR1) (Inagaki *et al.* 1995; Sakura *et al.* 1995; Aguilar-Bryan *et al.* 1995; Clement *et al.* 1997; Inagaki *et al.* 1997; Shyng & Nichols, 1997). Truncation of 26 amino acids from the C-terminus of Kir6.2 permits functional expression of this subunit in the absence of

SUR1. Studies of this truncated subunit (Kir6.2 $\Delta C26$) have clearly demonstrated that Kir6.2 forms the pore of the channel and that it is inhibited by ATP, whereas SUR1 confers the stimulatory effects of MgADP and the K⁺ channel opener diazoxide, as well as the characteristic high-affinity block by sulphonylureas (Tucker *et al.* 1997; Babenko *et al.* 1998). Kir6.2 $\Delta C26$, in the absence of SUR1, can also be inhibited by high concentrations of sulphonylureas but this subunit is at least three orders of magnitude less sensitive than the high-affinity sulphonylurea binding site on SUR1 (Gribble *et al.* 1997b). SUR1 is able to 'enhance' or 'sensitize' the direct block of Kir6.2 by ATP, although the precise mechanism by which this occurs is as yet unresolved (Tucker *et al.* 1997; Trapp *et al.* 1998; Koster *et al.* 1999). Thus SUR1 plays a key role in determining the ATP sensitivity of Kir6.2, as well as transducing the effects of high-affinity sulphonylurea binding into the closure of the

pore formed by Kir6.2. However, as yet there is little information about the precise regions involved in the functional coupling of SUR1 to Kir6.2.

A role for the intracellular N-domain of Kir6.2 in controlling the ATP sensitivity of the K_{ATP} channel has recently been reported. Mutations within this domain have been shown to have profound effects on the ATP sensitivity of the channel (Tucker *et al.* 1998; Proks *et al.* 1999), whilst truncation of the N-terminus reduces the ATP sensitivity indirectly, by altering the open probability of the channel in the presence of SUR1 (Koster *et al.* 1999; Babenko *et al.* 1999). In a previous study (Tucker *et al.* 1997), we showed that deletion of 14 amino acids from the N-terminus of Kir6.2 (Kir6.2 Δ N14) was not sufficient to permit functional expression of this subunit in the absence of SUR1. In the present work, we demonstrate that functional expression of this N14 deletion can be achieved by additionally truncating the C-terminus (Kir6.2 Δ C26- Δ N14). By expression of the Kir6.2 Δ C26- Δ N14 deletion in the absence of SUR1, we demonstrate that deletion of the N-terminus has no effect on the intrinsic ATP sensitivity or gating kinetics of the Kir6.2 Δ C26 subunit. In contrast, coexpression of this subunit with SUR1 produces K_{ATP} channels with a reduced ATP sensitivity, an effect that probably results from the increased open probability of this channel.

We also find that whilst Kir6.2 Δ N14/SUR1 channels retain the ability to be stimulated by MgADP and by the K^+ channel opener diazoxide they exhibit no high-affinity sulphonylurea block. Indeed, the Kir6.2 Δ N14 deletion not only removes the ability of SUR1 to produce high-affinity sulphonylurea block of channel activity but also that of the related sulphonylurea receptor SUR2A. Smaller deletions of the N-terminus produce a graded decrease in high-affinity block. This effect is not simply the consequence of the altered channel kinetics. Therefore, in addition to a role for the N-terminus of Kir6.2 in the control of ATP sensitivity by SUR1 we propose a role for this domain in the functional coupling of sulphonylurea sensitivity between SUR1 and Kir6.2.

METHODS

Molecular biology

Mouse Kir6.2 (GenBank D50581; Inagaki *et al.* 1995; Sakura *et al.* 1995), rat SUR1 (GenBank L40624; Aguilar-Bryan *et al.* 1995; provided by Dr G. Bell, University of Chicago) and rat SUR2A (GenBank D83598; Inagaki *et al.* 1996; provided by Dr S. Seino, Chiba University School of Medicine) were used in this study.

A 26 or 36 amino acid C-terminal deletion of mouse Kir6.2 (Kir6.2 Δ C) was made by introduction of a stop codon at the appropriate residue (Tucker *et al.* 1997). No differences were apparent in currents encoded by Kir6.2 Δ C26 and Kir6.2 Δ C36 except that Kir6.2 Δ C36 expressed larger currents (Tucker *et al.* 1997). N-terminal deletions were made by using the polymerase chain reaction to replace the indicated residue with an initiator methionine residue. In this paper, we use the term Δ N5 to refer to

the deletion of residues 2–5, Δ N9 to refer to the deletion of residues 2–9, and Δ N14 to refer to the deletion of residues 2–14. Site-directed mutagenesis of Kir6.2 Δ C26, Kir6.2 and SUR1 was carried out by subcloning the appropriate fragments into the pALTER vector (Promega, Madison, WI, USA). We use the abbreviation SUR1-KAKM to refer to mutation of the Walker A lysines in both NBD1 (K719A) and NBD2 (K1385M) of SUR1. Synthesis of capped mRNA was carried out using the mMessage mMachine *in vitro* transcription kit (Ambion, Austin, TX, USA). Amino acids are indicated by the single-letter code.

Electrophysiology

Oocyte collection. Female *Xenopus laevis* were anaesthetized with MS222 (2 g l⁻¹ added to the water; Sigma). One ovary was removed via a mini-laparotomy, the incision sutured and the animal allowed to recover. Once the wound had completely healed, the second ovary was removed in a similar operation and the animal was then killed by decapitation whilst under anaesthesia. Immature stage V–VI *Xenopus* oocytes were manually defolliculated and injected with 2 ng of mRNA encoding wild-type or mutant forms of Kir6.2 Δ C26, or co-injected with ~0.1 ng Kir6.2 (wild-type or mutant) and ~2 ng of SUR. The final injection volume was ~50 nl per oocyte. Control oocytes were injected with water. Isolated oocytes were maintained in Barth's solution and studied 1–4 days after injection (Gribble *et al.* 1997a).

Electrophysiology. Patch pipettes were pulled from thick-walled glass and had resistances of 250–500 k Ω when filled with pipette solution. Macroscopic currents were recorded from giant excised inside-out patches at a holding potential of 0 mV and at 20–24 °C (Gribble *et al.* 1997a). Currents were evoked by repetitive 3 s voltage ramps from –110 to +100 mV and recorded using an EPC7 patch-clamp amplifier (List Electronic). They were filtered at 5 kHz and stored on digital audiotape. Currents were subsequently filtered at 0.5 kHz, digitized at 1 kHz using a Digidata 1200 interface and analysed using pCLAMP software (Axon Instruments). Single-channel currents were recorded from small inside-out patches, filtered at 1 kHz and sampled at 3 kHz.

The pipette (external) solution contained (mM): 140 KCl, 1.2 MgCl₂, 2.6 CaCl₂, 10 Hepes (pH 7.4 with KOH). The intracellular (bath) solution contained (mM): 107 KCl, 2 MgCl₂, 1 CaCl₂, 10 EGTA, 10 Hepes (pH 7.2 with KOH; final K^+ ~140 mM) and nucleotides as indicated. Tolbutamide was made up as a 0.1 M stock solution in 0.15 M KOH and diazoxide as a 0.068 M stock solution in 0.1 M KOH. Solutions containing nucleotides were made up fresh each day. The pH of all solutions was checked and readjusted, if required, after drug and nucleotide addition. Rapid exchange of solutions was achieved by positioning the patch in the mouth of one of a series of adjacent inflow pipes placed in the bath.

Data analysis

The slope conductance was measured by fitting a straight line to the current–voltage relationship between –20 and –100 mV: the average of five consecutive ramps was calculated in each solution. ATP dose–response relationships were measured by alternating the control solution with a test ATP concentration. The conductance (G) was then expressed as a fraction of the mean of the value obtained in the control solution before and after exposure to ATP (G_c). Dose–response curves were fitted to the Hill equation:

$$G/G_c = 1/(1 + ([ATP]/K_1)^h), \quad (1)$$

where [ATP] is the ATP concentration, K_1 is the concentration at which inhibition is half-maximal and h is the slope factor (Hill coefficient).

Tolbutamide dose–response curves were fitted to the following equation (Gribble *et al.* 1997a):

$$G/G_c = xy, \quad (2)$$

where x is a term describing the high-affinity site and y a term describing the low-affinity site.

$$x = L + \frac{(1 - L)}{(1 + ([\text{Tolb}]/K_{11})^{h_1})}, \quad (3)$$

$$y = 1/(1 + ([\text{Tolb}]/K_{12})^{h_2}), \quad (4)$$

where $[\text{Tolb}]$ is the tolbutamide concentration; K_{11} and K_{12} are the tolbutamide concentrations at which inhibition is half-maximal at the high- and low-affinity sites, respectively; h_1 , h_2 are the Hill coefficients (slope factors) for the high- and low-affinity sites, respectively; and L is the fractional conductance remaining when all of the high-affinity inhibitory sites are occupied.

Single-channel currents were analysed using a combination of pCLAMP and in-house software written by Dr P. A. Smith (Oxford University). Single-channel current amplitudes were calculated from an all-points amplitude histogram. Channel activity (NP_o) was measured as the mean patch current divided by the single-channel current amplitude, for segments of the current records of ~ 1 min duration. Open probability (P_o) was calculated from NP_o/N , where N is the number of channels in the patch and was estimated from the maximum number of superimposed events.

Data are given as means ± 1 s.e.m., and the symbols in the figures indicate the mean and the vertical bars indicate 1 s.e.m. (where this is larger than the symbol).

RESULTS

ATP sensitivity

Figure 1A shows that deletion of the first 14 amino acids from the N-terminus did not affect the ATP sensitivity of Kir6.2 Δ C26. Half-maximal inhibition (K_i) of Kir6.2 Δ C26- Δ N14 was produced by $109 \pm 8 \mu\text{M}$ ATP ($n = 7$), while a K_i of $106 \pm 4 \mu\text{M}$ ($n = 7$) was found for Kir6.2 Δ C26. Coexpression of SUR1 with Kir6.2 enhances the channel ATP sensitivity, by a mechanism that is not yet clear (Tucker *et al.* 1998). Figure 1B shows that this effect was impaired when the N-terminus of Kir6.2 is truncated: the K_i for ATP block of Kir6.2 Δ N14/SUR1 currents was $196 \pm 17 \mu\text{M}$ ($n = 10$) compared with $28 \pm 5 \mu\text{M}$ ($n = 5$) for Kir6.2/SUR1 currents. This suggests that the N-terminus of Kir6.2 may be involved in functionally coupling this subunit to SUR1.

In addition to enhancing the ability of ATP to inhibit Kir6.2, SUR1 confers sensitivity to the stimulatory effects of MgADP and diazoxide, and to the inhibitory effects of sulphonylureas (Ashcroft & Gribble, 1998). Therefore, we examined whether the N-terminus of Kir6.2 is also involved in transducing the interaction of these agents with SUR1 into changes in the gating of Kir6.2.

Effects of K⁺ channel activators

As shown in Fig. 2, both diazoxide and MgADP were able to increase Kir6.2 Δ N14/SUR1 currents blocked by $100 \mu\text{M}$

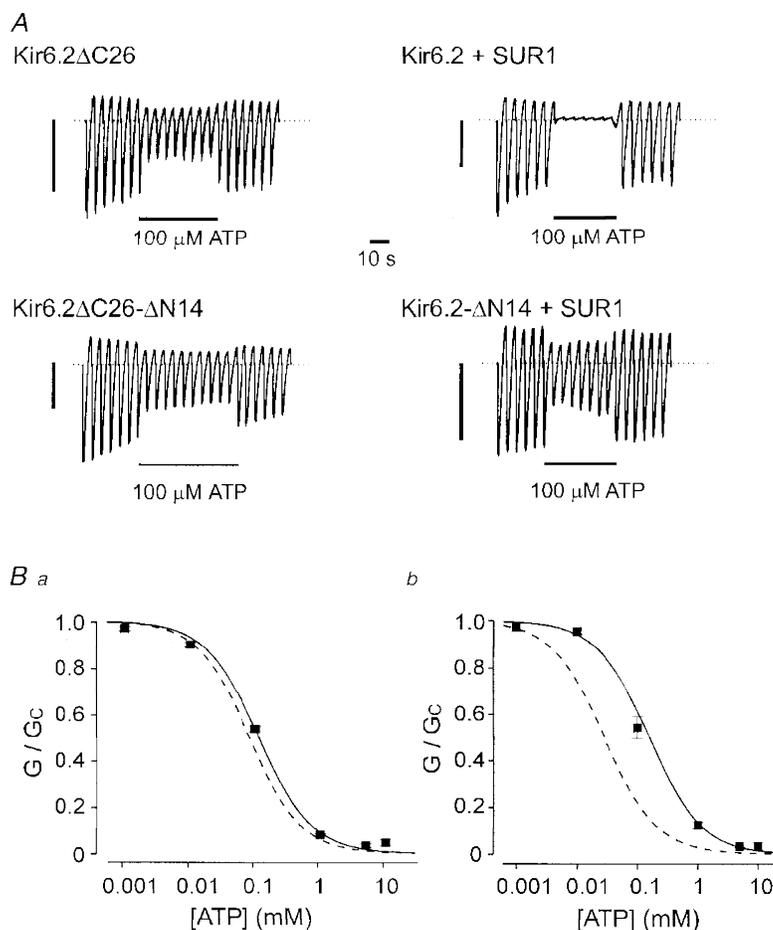
Figure 1. Effects of ATP

A, macroscopic currents recorded from inside-out patches excised from oocytes injected with mRNA encoding Kir6.2 Δ C26, Kir6.2 Δ C26- Δ N14, Kir6.2/SUR1, Kir6.2 Δ N14/SUR1.

Currents were elicited in response to a series of voltage ramps from -110 to $+100$ mV.

ATP ($100 \mu\text{M}$) was added as indicated by the bars. The current scale bars are 0.5 nA for Kir6.2 Δ C26 and 2 nA for the other currents.

B, mean ATP dose–response relationships for Kir6.2 Δ N14- Δ C26 currents (■, $n = 7$) and Kir6.2 Δ C26 (dashed line) (a); and for Kir6.2 Δ N14 (■, $n = 10$) or Kir6.2 (dashed line) coexpressed with SUR1 (b). The slope conductance (G) is expressed as a fraction of the mean of that obtained in control solution (G_c) before and after exposure to ATP. The continuous lines are the best fit of the data to the Hill equation using the mean values for K_i and h given in Table 1. The dashed lines are taken from Tucker *et al.* (1997) (a) and Gribble *et al.* (1997a) (b).



ATP, as is found for the wild-type K_{ATP} channel (Kir6.2/SUR1). Diazoxide increased both currents to approximately the same amplitude but, possibly because ATP blocks Kir6.2 Δ N14/SUR1 currents less potently, the relative increase in current was smaller than for Kir6.2/SUR1. Likewise, MgADP did not enhance Kir6.2 Δ N14/SUR1 currents to the same extent as wild-type currents.

Sulphonylurea block

Tolbutamide interacts with a low-affinity site on Kir6.2 and a high-affinity site on SUR1 (Gribble *et al.* 1997*b*). Truncation of the N-terminus did not alter the low-affinity

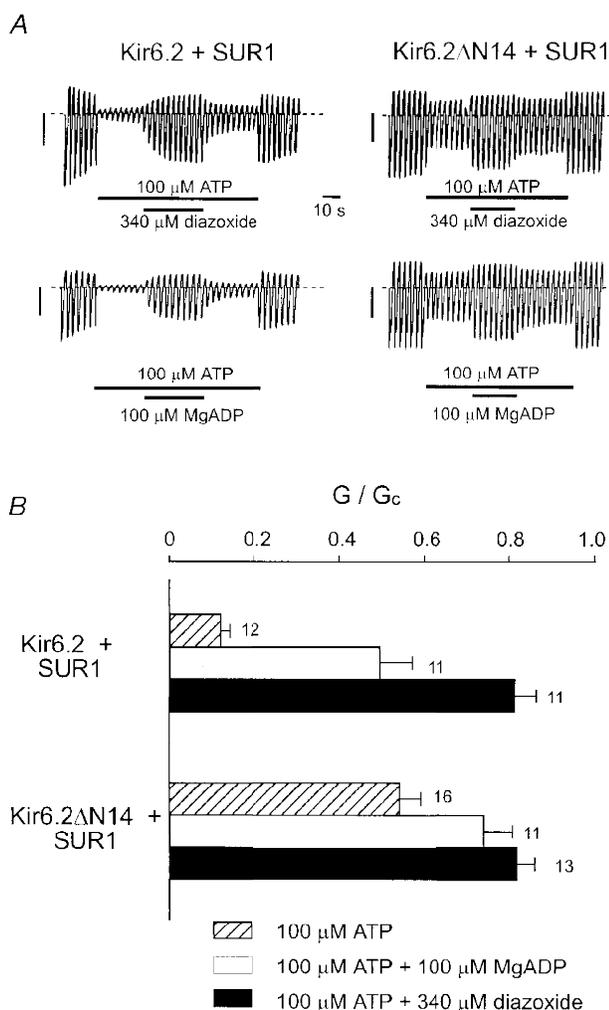


Figure 2. Effects of activators

A, macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from -110 to $+100$ mV from oocytes co-injected with SUR1 plus either Kir6.2 (left) or Kir6.2 Δ N14 (right). ATP (0.1 mM), diazoxide (340 μ M) or MgADP (100 μ M) were added to the intracellular solution as indicated by the bars. The current scale bars are 2 nA in each case. *B*, mean slope conductance (G) recorded in the presence of the additions indicated, expressed as a percentage of that (G_c) in control solution (no additions), for Kir6.2/SUR1 and Kir6.2 Δ N14/SUR1. The number of patches is given alongside each bar.

block of Kir6.2 (Fig. 3). When expressed in the absence of SUR1, the K_i for tolbutamide inhibition was 1.7 mM for Kir6.2 Δ C36 (Gribble *et al.* 1997*b*) and 2.0 ± 0.1 mM ($n = 5$) for Kir6.2 Δ C26- Δ N14. This indicates that the first 14 amino acids of the N-terminus do not form part of the low-affinity site for tolbutamide on Kir6.2.

Truncation of the N-terminus profoundly affects the ability of SUR1 to cause high-affinity inhibition of Kir6.2. As previously reported (Gribble *et al.* 1997*b*), when SUR1 is coexpressed with Kir6.2 the dose-response curve for tolbutamide block is best fitted with two binding sites (Fig. 4). In contrast to the wild-type K_{ATP} channel, only a single low-affinity site was observed for Kir6.2 Δ N14/SUR1 currents. The K_i of this low-affinity site (2.6 ± 0.2 mM, $n = 5$) was similar to that of wild-type K_{ATP} channels (2.4 ± 0.1 mM, $n = 6$) and of Kir6.2 Δ C36 (Gribble *et al.* 1997*b*), indicating that it resides on Kir6.2. The K_i of the high-affinity site measured for Kir6.2/SUR1 was 1.4 ± 0.4 μ M ($n = 6$), a value similar to that previously reported (Gribble *et al.* 1997*b*).

We next examined whether the N-terminus of Kir6.2 is also concerned in coupling this subunit to SUR2A, a sulphonylurea receptor that shares 68% sequence identity with SUR1 (Inagaki *et al.* 1996). Although Kir6.2/SUR2A currents do not show high-affinity inhibition by tolbutamide, they are blocked by glibenclamide with a potency similar to that of SUR1 (Gribble *et al.* 1998*b*). Figure 5 shows that neither Kir6.2 Δ N14/SUR1 nor Kir6.2 Δ N14/SUR2A currents were blocked by glibenclamide at a concentration (100 nM) sufficient to cause maximal high-affinity inhibition of wild-type K_{ATP} channels. Thus an intact N-terminus of Kir6.2 is required for the inhibitory effect of both tolbutamide and glibenclamide, and SUR1 and SUR2A transduce the effects of sulphonylureas by a mechanism that involves common elements.

Mutations that affect coupling

To localize further residues within the N-terminus of Kir6.2 involved in coupling to SUR1, we made a number of additional truncations and mutations within this region. Deletion of residues 2–5 (Δ N5) or 2–9 (Δ N9) caused a progressive reduction in the amount of high-affinity tolbutamide inhibition (Fig. 6). The maximal inhibition produced at the high-affinity site was 54% for wild-type K_{ATP} channels: this decreased to 38%, 25% and was finally completely abolished when the N-terminus of Kir6.2 was truncated by 5, 9 and 14 residues, respectively (Table 1). There was also a small shift in the K_i for the high-affinity site, but the Hill coefficient remained unchanged (Table 1).

Figure 7 shows the extent of block produced by 100 μ M tolbutamide for wild-type K_{ATP} currents, for the N-terminal truncations and for a number of mutations in the N-terminus of Kir6.2. No single mutation was sufficient to decrease tolbutamide block to that observed for Kir6.2 Δ N14/SUR1: rather, the results suggest the involvement of a number of residues. Neutralization of the

Figure 3. Tolbutamide inhibition of Kir6.2 currents

Relationship between tolbutamide concentration and the macroscopic K_{ATP} conductance, expressed as a fraction of its amplitude in the absence of the drug. \circ , Kir6.2 Δ C36 currents ($n = 6$); \blacktriangledown , Kir6.2 Δ C26- Δ N14 currents ($n = 5$). The line is the best fit of the data obtained for Kir6.2 Δ C26- Δ N14 to eqn (4): $K_1 = 2$ mM, $h = 0.9$. The data for Kir6.2 Δ C36 are the same as those in Gribble *et al.* (1997b).

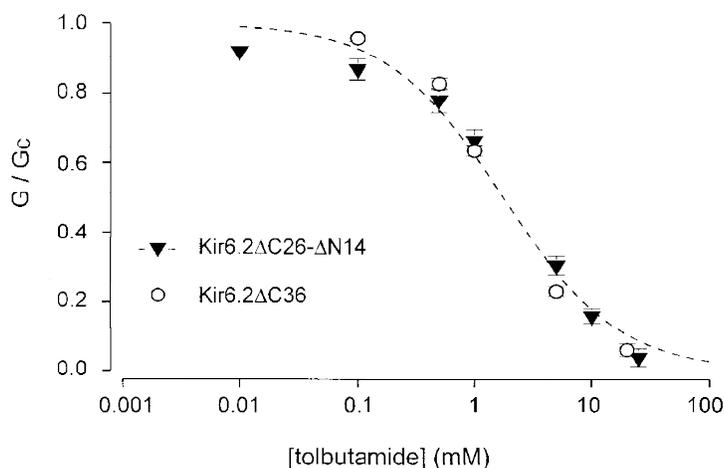


Figure 4. Tolbutamide block of Kir6.2/SUR1 and Kir6.2 Δ N14/SUR1 currents

A, macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from -110 to $+100$ mV from oocytes co-injected with SUR1 plus either Kir6.2 (left) or with Kir6.2 Δ N14 (right). Tolbutamide (0.1 mM) was added to the intracellular solution as indicated by the bar. *B*, relationship between tolbutamide concentration and the macroscopic K_{ATP} conductance, expressed as a fraction of its amplitude in the absence of the drug. Kir6.2/SUR1 currents (\circ , $n = 6$) were fitted with eqn (1) and Kir6.2 Δ N14/SUR1 currents (\blacktriangledown , $n = 5$) were fitted with eqn (4) using the parameters given in Table 1.

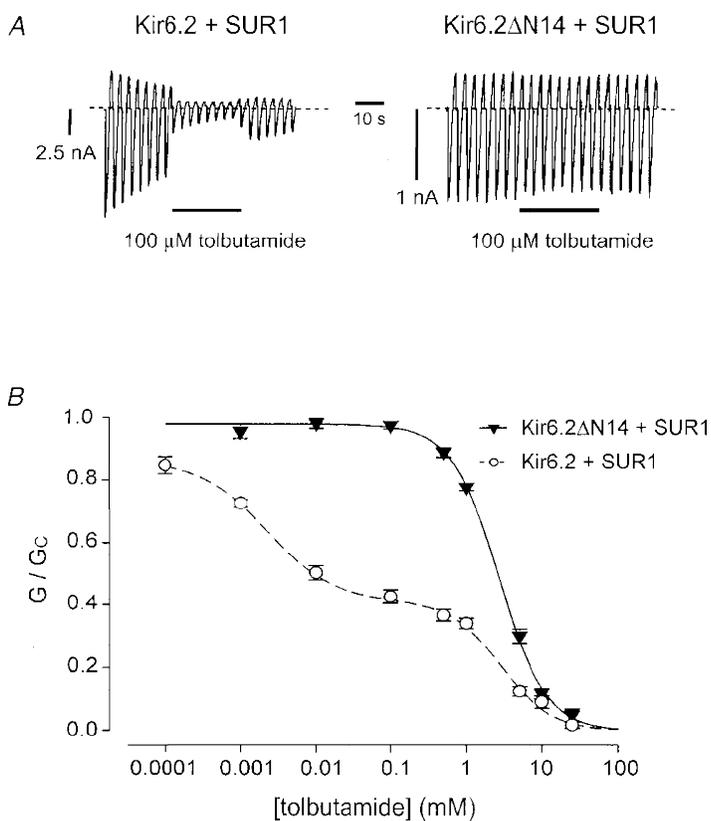


Figure 5. Effects of glibenclamide

Mean slope conductance recorded in the presence of 100 nM glibenclamide, expressed as a percentage of that in the absence of the drug. The dotted line indicates the level of activity in the absence of glibenclamide. Currents were recorded from oocytes expressing Kir6.2 (\blacksquare) or Kir6.2 Δ N14 (\boxtimes), with SUR1 or SUR2A, as indicated. The number of patches is given above each bar.

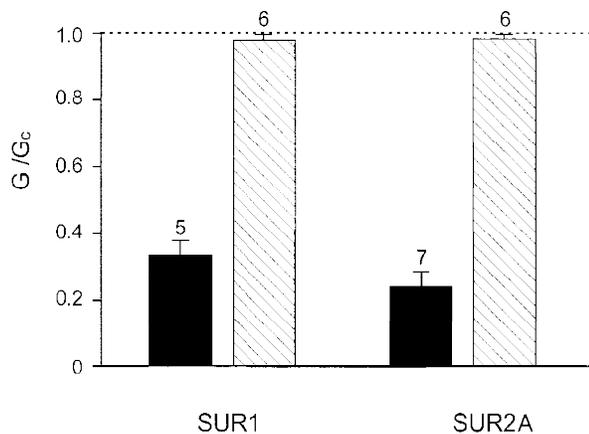


Table 1. Tolbutamide block of K_{ATP} channels

Clone*	K_{i1} (μM)	h_1	K_{i2} (mM)	h_2	L	n
Kir6.2	1.42 ± 0.35	0.92 ± 0.06	2.40 ± 0.1	1.20 ± 0.1	0.46 ± 0.03	6
Kir6.2 $\Delta\text{N}5$	6.99 ± 2.02	1.01 ± 0.03	2.19 ± 0.33	1.45 ± 0.14	0.62 ± 0.03	6
Kir6.2 $\Delta\text{N}9$	22.52 ± 5.56	0.99 ± 0.01	2.24 ± 0.35	1.37 ± 0.09	0.75 ± 0.02	4
Kir6.2 $\Delta\text{N}14$	—	—	2.56 ± 0.17	1.34 ± 0.05	—	5

* All coexpressed with SUR1. Data were fitted with a two-site model for tolbutamide inhibition (eqn (2)). K_{i1} , tolbutamide concentration producing half-maximal block; h , Hill coefficient; L , maximum level of block produced by the high-affinity site.

Table 2. ATP block of K_{ATP} channels

Clone	K_i (μM)	h_1	n	P_o	n
Without SUR1					
Kir6.2 $\Delta\text{C}36$	106 ± 4	1.2 ± 0.1	7	0.11 ± 0.03	4
Kir6.2 $\Delta\text{C}26$ - $\Delta\text{N}14$	109 ± 8	0.9 ± 0.1	7	0.10 ± 0.02	4
With SUR1					
Kir6.2	28 ± 5	1.0 ± 0.01	5	0.32 ± 0.10	4
Kir6.2 $\Delta\text{N}5$	50 ± 10	1.0 ± 0.1	5	0.44 ± 0.12	4
Kir6.2 $\Delta\text{N}9$	96 ± 26	1.0 ± 0.1	6	0.61 ± 0.08	4
Kir6.2 $\Delta\text{N}14$	196 ± 17	1.2 ± 0.1	10	0.70 ± 0.09	4

K_i , ATP concentration producing half-maximal block; h , Hill coefficient; P_o , open probability.

charged residues K5, E10 and E11 did not significantly alter the extent of tolbutamide block, suggesting that these amino acids do not interact with SUR1. Neutralization of the arginine at position 4, however, reduced the extent of block to that found for the $\Delta\text{N}5$ truncation: thus R4 may be involved in functional interaction with SUR1. Hydrophobic interactions may also be important, since L2A, and the combination of V13N and L14N, also reduced tolbutamide inhibition. Furthermore, tolbutamide block was abolished when residues 7, 8, 13 and 14 were simultaneously mutated to asparagine. However, since multiple mutations may destroy the local structure of the protein, the precise involvement of these hydrophobic residues is hard to assess.

Single-channel currents

One mechanism by which truncation of the N-terminus may alter the channel ATP and tolbutamide sensitivity in the presence of SUR1 is by impairing the ability of the channel to close (Shyng *et al.* 1997a; Tucker *et al.* 1998). No difference was observed between the single-channel kinetics of Kir6.2 $\Delta\text{C}26$ and Kir6.2 $\Delta\text{C}26$ - $\Delta\text{N}14$ (Fig. 8, Table 2), indicating that the intrinsic gating of the channel is unaffected by removal of residues 2–14. The ability of SUR1 to enhance the open probability of Kir6.2 (Proks & Ashcroft, 1997), however, was much greater when the N-terminus of Kir6.2 was truncated. As Fig. 8 and Table 2 also show, when Kir6.2 containing the $\Delta\text{N}5$ or $\Delta\text{N}14$ truncation was

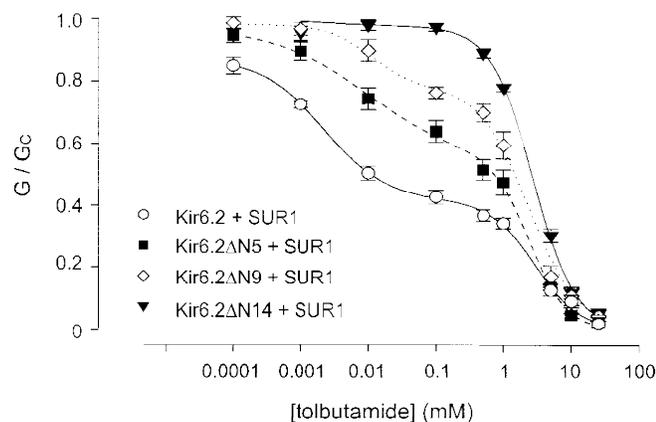


Figure 6. Effect of tolbutamide on N-terminal deletions of different length

Relationship between tolbutamide concentration and the macroscopic K_{ATP} conductance, expressed as a fraction of its amplitude in the absence of the drug. Kir6.2/SUR1 (\circ , $n = 6$), Kir6.2 $\Delta\text{N}5$ /SUR1 (\blacksquare , $n = 6$) and Kir6.2 $\Delta\text{N}9$ /SUR1 (\diamond , $n = 4$) currents were fitted with eqn (1). Kir6.2 $\Delta\text{N}14$ /SUR1 currents (\blacktriangledown , $n = 5$) were fitted with eqn (4). The parameters used to fit the data are given in Table 1.

coexpressed with SUR1, the open probability was greatly increased: the mean P_o was 0.32 for the wild-type K_{ATP} channel, but rose to 0.44 for Kir6.2 Δ N5/SUR1, 0.61 for Kir6.2 Δ N9/SUR1 and 0.70 for Kir6.2 Δ N14/SUR1. There was also a clear correlation between the increase in P_o and that in the K_i for ATP inhibition (Table 2). Thus it seems likely that the reduced ATP sensitivity is a consequence of the altered gating kinetics.

How do N-terminal deletions alter tolbutamide sensitivity?

We next explored whether the reduced sulphonylurea block of Kir6.2 Δ N14/SUR1 is also merely a consequence of the enhanced channel open probability. One way to test this idea would be to decrease P_o by some independent means and then examine whether tolbutamide inhibition is affected. If the reduced sulphonylurea sensitivity of Kir6.2 Δ N14/SUR1 results only from its altered kinetics, we would expect tolbutamide to block the channel when the P_o was reduced prior to drug application: conversely, we predict that tolbutamide block would be independent of P_o if deletion of the N-terminus of Kir6.2 impairs the mechanism by which sulphonylurea binding to SUR1 is translated into pore closure.

The simplest way to reduce the P_o of the K_{ATP} channel is to apply ATP. Figure 9Aa shows that tolbutamide blocked Kir6.2 Δ N14/SUR1 currents when applied in the presence of 100 μ M ATP. This result requires careful interpretation, however, because of the interaction between Mg-nucleotides and sulphonylureas (Züinkler *et al.* 1988b; Gribble *et al.* 1997b). Like MgADP, MgATP interacts with the K_{ATP} channel at two sites, one that induces channel inhibition (on Kir6.2) and one that causes channel activation (on SUR1) (Gribble *et al.* 1998a). The overall action of MgATP is therefore a balance between these two opposing effects. It has been previously shown that tolbutamide abolishes the stimulatory action of MgADP, whilst leaving the inhibitory effect of the nucleotide on Kir6.2 intact (Gribble *et al.* 1997b). As a consequence, the extent of sulphonylurea block is greater when MgADP is present, because it results from the combined actions of MgADP and tolbutamide. Mutation of the Walker A lysines of SUR1 prevents MgATP binding (Ueda *et al.* 1997) and Mg-nucleotide stimulation of channel activity (Gribble *et al.* 1997b; Shyng *et al.* 1997b; Trapp *et al.* 1998). Figure 9Ab shows that the ability of tolbutamide to block the channel in the presence of MgATP was also abolished when both Walker A lysines were mutated (SUR1-K719A,K1385M, abbreviated SUR1-KAKM). This suggests that tolbutamide simply interferes with the stimulatory action of MgATP on Kir6.2 Δ N14/SUR1 currents but has no direct blocking effect itself.

When SUR1-KAKM is coexpressed with full-length Kir6.2, rather than Kir6.2 Δ N14, tolbutamide causes channel inhibition in the absence of ATP (Gribble *et al.* 1997b). To confirm that this is also the case in the presence of ATP, we used a mutant form of Kir6.2 (Kir6.2-R50E) which is

blocked by ATP to a similar extent as Kir6.2 Δ C26- Δ N14 (Proks *et al.* 1999). This is because both the wild-type channel and Kir6.2/SUR1-KAKM are highly sensitive to ATP and the stimulatory effect of MgATP is not observed at low ATP concentrations (Gribble *et al.* 1998a).

As Fig. 9Ac shows, when Kir6.2-R50E and SUR1 were coexpressed, the resulting channel was not inhibited by 100 μ M ATP: indeed in some patches, as shown here, the channel was actually activated. This may be attributed to the stimulatory effect of MgATP mediated by the nucleotide binding domains of SUR1, because ATP was an effective inhibitor of the channel when the Walker A lysines in both NBDs were mutated (Fig. 9Ad). Addition of tolbutamide in the presence of ATP produced a much more profound block

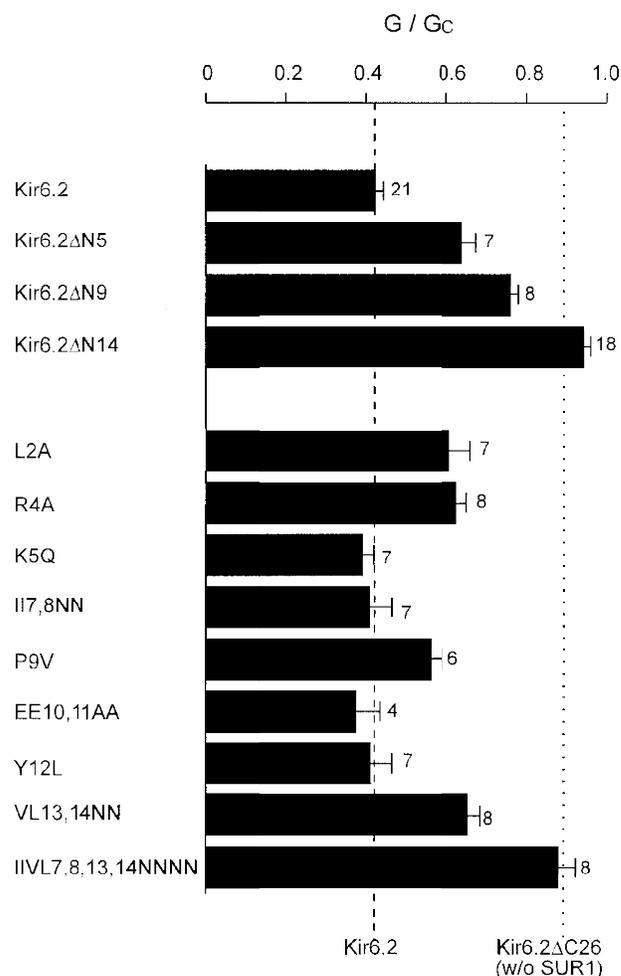


Figure 7. Effect of tolbutamide on wild-type and mutant K_{ATP} currents

Mean current amplitude recorded in the presence of 100 μ M tolbutamide (G), expressed as a fraction of that in the absence of the drug (G_c). Currents were recorded from oocytes expressing SUR1 and either wild-type or mutant Kir6.2 Δ C26, as indicated. The number of patches is given alongside each bar. The mean values obtained for Kir6.2 Δ C26 and Kir6.2/SUR1 channels are indicated by the dotted and dashed lines, respectively.

of Kir6.2-R50E/SUR1 than was observed for Kir6.2 Δ N14/SUR1. Moreover, the drug remained able to block the channel when the stimulatory effect of MgATP was abolished by coexpression of Kir6.2-R50E with SUR1-KAKM (Fig. 9Ad), in marked contrast to the lack of tolbutamide effect on Kir6.2 Δ N14/SUR1-KAKM (Fig. 9Ab).

Taken together these results suggest that the ability of tolbutamide to uncouple the potentiatory effect of MgATP is not impaired by deletion of the N-terminus of Kir6.2. They further argue that the loss of tolbutamide block (most evident in the absence of nucleotide) involves a functional uncoupling of Kir6.2 Δ N14 from SUR1, rather than being simply a secondary consequence of the enhanced P_o .

To confirm this hypothesis, we used a mutant form of Kir6.2 that shows a markedly enhanced open probability. Mutation of cysteine 166 to serine (C166S) increases the P_o to 0.8, and concomitantly reduces the ATP sensitivity and abolishes inhibition by 0.1 mM tolbutamide (Trapp *et al.* 1998). Figure 10A demonstrates that when the P_o of Kir6.2-C166S/SUR1-KAKM was decreased to 50% by addition of ATP, 100 μ M tolbutamide caused significant inhibition of the current. In contrast, Kir6.2 Δ N14/SUR1-KAKM currents were unaffected when the P_o was reduced. Mean data for the effect of tolbutamide at different ATP concentrations are given in Fig. 10B, and show that as the ATP concentration was increased, tolbutamide block of Kir6.2-C166S/SUR1-KAKM was enhanced, while the drug

continued to remain ineffective on Kir6.2 Δ N14/SUR1-KAKM. Indeed, there was a reciprocal correlation between the extent of ATP block and that of tolbutamide inhibition in the presence of ATP for Kir6.2-C166S/SUR1-KAKM, but not for Kir6.2 Δ N14/SUR1-KAKM (Fig. 10C). These data provide additional support for the idea that the lack of tolbutamide sensitivity of Kir6.2 Δ N14/SUR1 is not the result of the enhanced P_o .

DISCUSSION

Our results show that partial deletion of the N-terminus of Kir6.2 has no effect on the intrinsic properties of Kir6.2, but influences K_{ATP} channel behaviour when Kir6.2 is coexpressed with SUR1. In particular, the open probability is enhanced, the sensitivity to the inhibitory effects of ATP and sulphonylureas is decreased and there is a reduction in the stimulatory action of diazoxide and MgADP. These data argue that the first 14 amino acids of Kir6.2 are involved in functional coupling to SUR1.

Gating

The open probability of Kir6.2 Δ C26 is not affected by N-terminal deletions of up to 14 amino acids, indicating that these residues are not involved in the intrinsic gating of the Kir6.2 subunit. When wild-type Kir6.2 is coexpressed with SUR1, however, the open probability (P_o) of the K_{ATP} channel is enhanced – from a P_o of \sim 0.1 to \sim 0.3 (Proks & Ashcroft, 1997; Table 2). The effect of SUR1 is magnified if

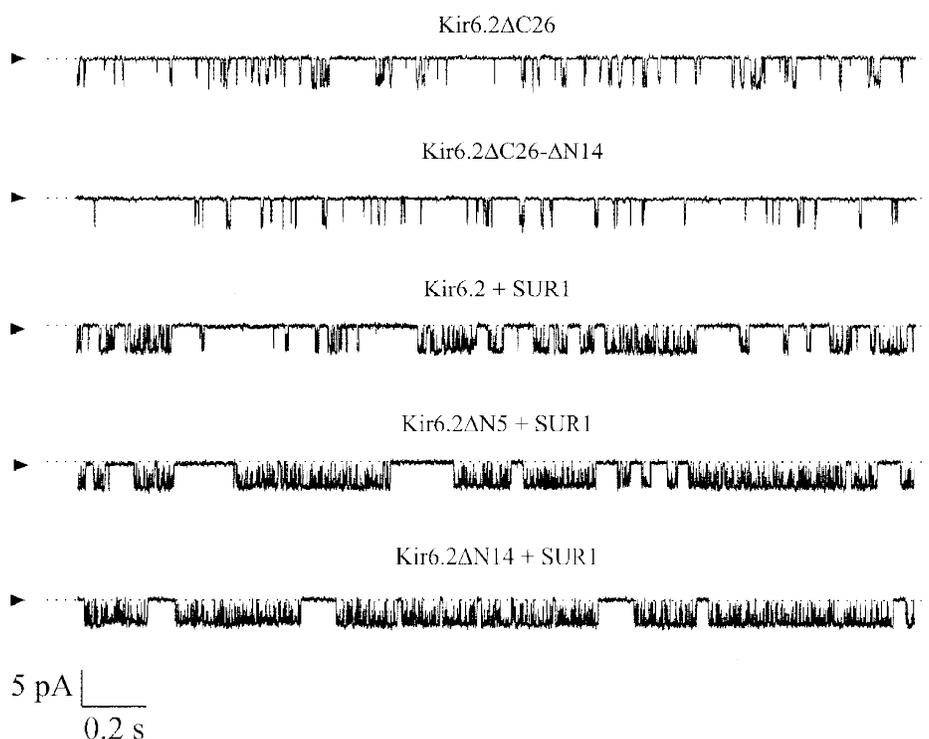


Figure 8. Single-channel currents recorded from wild-type and mutant K_{ATP} channels. Single-channel currents recorded at -60 mV from inside-out patches excised from oocytes injected with mRNA(s) indicated. The arrowheads indicate the zero current level.

between 5 and 14 residues are deleted from the N-terminus of Kir6.2, the P_o increasing in parallel with the length of the deletion. These data argue that the N-terminus of Kir6.2 may exert a negative effect on the ability of SUR1 to promote K_{ATP} channel activity. They also demonstrate that the ability of SUR1 to enhance the P_o (Proks & Ashcroft, 1997) cannot involve the first 14 residues of Kir6.2. Finally, our results raise the possibility that SUR1 may physically interact with the N-terminus of Kir6.2 to influence channel gating.

ATP sensitivity

There is accumulating evidence that ATP interacts with Kir6.2 to mediate K_{ATP} channel inhibition. This derives from the observations that a C-terminally truncated form of

Kir6.2 (Kir6.2 Δ C), expressed in the absence of SUR1, is blocked by ATP (Tucker *et al.* 1997), that mutations within Kir6.2 affect the channel ATP sensitivity (Shyng *et al.* 1997*a*; Tucker *et al.* 1997, 1998; Drain *et al.* 1998), and that Kir6.2 can be photo-affinity labelled with 8-azido-[³²P]ATP (Tanabe *et al.* 1999). The results presented here demonstrate that the first 14 residues of Kir6.2 are not involved in ATP binding since deletion of these residues was without effect on ATP inhibition.

A marked decrease in ATP sensitivity was observed when Kir6.2 Δ N14 was coexpressed with SUR1. A similar finding has been reported for N-terminal truncations of 10–30 residues by others (Koster *et al.* 1999). Although we cannot exclude a direct involvement of the N-terminus of Kir6.2 in

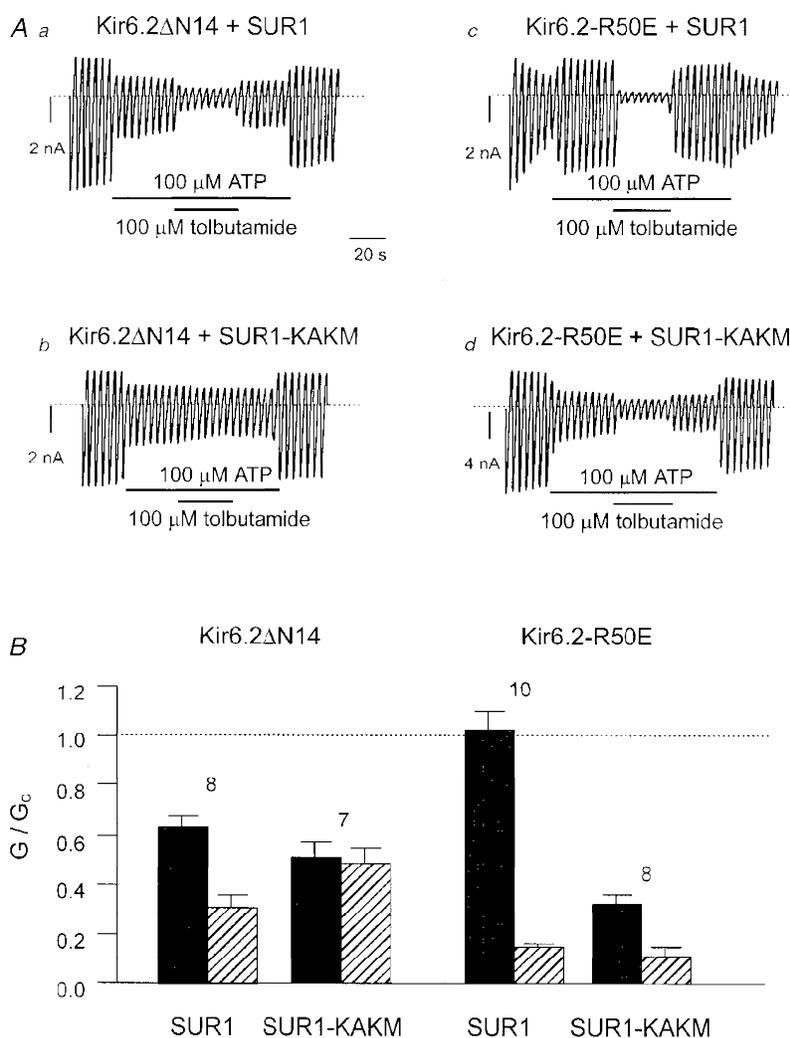


Figure 9. Tolbutamide block of mutant K_{ATP} channels

Aa–d, macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from -110 to $+100$ mV from oocytes with the indicated mRNAs. Tolbutamide (0.1 mM) or ATP (0.1 mM) were added to the intracellular solution as indicated by the bars. *B*, mean slope conductance recorded in the presence of 100 μ M ATP (■) or in 100 μ M ATP + 100 μ M tolbutamide (▨), expressed as a fraction of that in control solution (lacking drugs and nucleotide). Currents were recorded from oocytes expressing Kir6.2 Δ N14, or Kir6.2-R50E, together with either SUR1 or SUR1-KAKM, as indicated. The number of patches is given above each bar. The current level in control solution is indicated by the dotted line.

the mechanism by which SUR1 enhances the ATP sensitivity of the K_{ATP} channel (Tucker *et al.* 1997), we favour the idea that the decrease in ATP sensitivity of Kir6.2 Δ N14/SUR1 is a secondary consequence of the reduced channel open probability. It has been shown that mutations which increase

P_o also increase the K_i for ATP inhibition (Shyng *et al.* 1997a; Trapp *et al.* 1998). Our results demonstrate that there is a reciprocal increase in P_o and a decrease in ATP sensitivity for N-terminal truncations of different length. Thus the reduced efficacy of ATP may be an indirect result

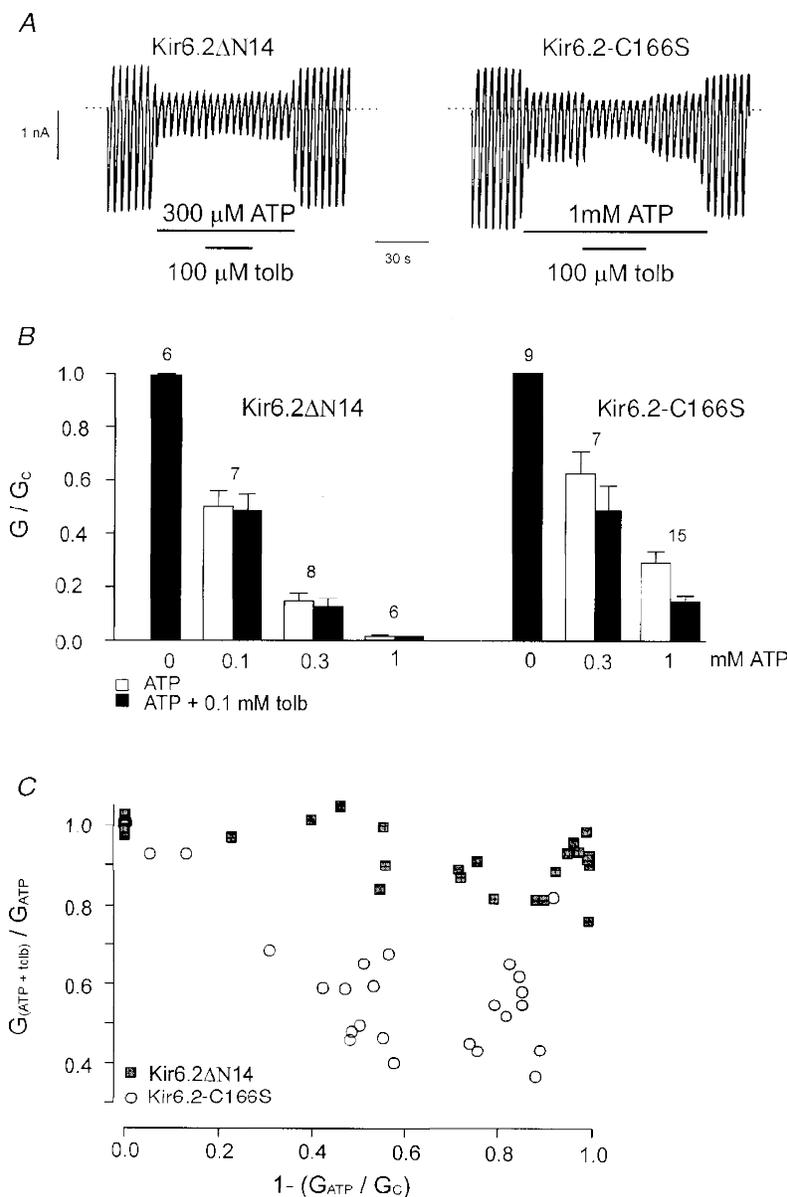


Figure 10. Tolbutamide block of mutant K_{ATP} channels

A, macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from -110 to $+100$ mV from oocytes expressing SUR1-KAKM together with either Kir6.2 Δ N14 (Δ N14) or Kir6.2-C166S. Tolbutamide (tolb; 0.1 mM) or ATP were added to the intracellular solution as indicated by the bars. *B*, mean current amplitude recorded in the presence of various concentrations of ATP (\square), or various concentrations of ATP + 0.1 mM tolbutamide (\blacksquare), expressed as a fraction of that in control solution (lacking drugs and nucleotide). Currents were recorded from oocytes expressing Kir6.2 Δ N14, or Kir6.2-C166S, together with SUR1-KAKM. The number of patches is given above each bar. *C*, relationship between the fractional block by tolbutamide in the presence of ATP, measured in the same patch. \blacksquare , Kir6.2 Δ N14/SUR1-KAKM; \circ , Kir6.2-C166S/SUR1-KAKM. Data were obtained at different ATP concentrations. The conductance in the presence of ATP is expressed as a fraction of its value in ATP-free solution ($1 - (G_{ATP}/G_c)$). The conductance in the presence of ATP and 100 μ M tolbutamide is expressed as a fraction of its value in ATP-containing solution ($G_{(ATP+tolb)}/G_{ATP}$).

of the altered gating kinetics. A similar conclusion was reached by Koster *et al.* (1999).

Nucleotide activation

Our results argue that deletion of the N-terminus of Kir6.2 does not alter the ability of MgADP or MgATP/diazoxide to enhance channel activity. Although the percentage increase in channel activity is less for Kir6.2ΔN14/SUR1 than for wild-type channels, the final current amplitude in the presence of the nucleotide is similar (or even enhanced). Since the channel has an upper limit of activity ($P_o \approx 0.9$), the smaller increase in current observed for Kir6.2ΔN14/SUR1 is probably an inevitable consequence of the enhanced basal P_o .

Tolbutamide modulation of MgADP sensitivity

The ability of tolbutamide to abolish the stimulatory effects of MgATP was unaffected by deletion of the first 14 residues of Kir6.2. This result indicates that tolbutamide binding to SUR1 is unaffected by the deletion of the N-terminus of Kir6.2. It also indicates that the distal N-terminus is not required for this action of tolbutamide.

Sulphonylureas might interfere with the potentiatory effects of Mg-nucleotides either by reducing nucleotide binding (to the NBDs of SUR1) or by preventing the translation of binding into channel activation. Because glibenclamide is able to block the co-operative binding of MgATP and MgADP to SUR1 (Ueda *et al.* 1999), the former explanation seems more likely. The fact that removal of the N-terminus of Kir6.2 does not affect the ability of tolbutamide to inhibit MgADP activation is consistent with this idea.

Tolbutamide sensitivity

Removal of the first 14 residues of Kir6.2 did not alter the low-affinity block by tolbutamide, indicating that these amino acids do not contribute to the low-affinity binding site on Kir6.2. When Kir6.2ΔN14 was coexpressed with either SUR1 or SUR2A, however, high-affinity inhibition by sulphonylureas was completely abolished. Indeed, truncation of the N-terminus of Kir6.2 by as few as five amino acids greatly decreased the amount of block. There is good evidence that the high-affinity sulphonylurea binding site lies on SUR (Aguilar-Bryan *et al.* 1995; Tucker *et al.* 1997). It therefore seems unlikely that N-terminal truncations of Kir6.2 influence the affinity of the sulphonylurea binding site on SUR. This idea is supported by the fact that tolbutamide is still able to abolish the stimulatory effects of Mg-nucleotides.

We have considered two possible mechanisms by which N-terminal deletion of Kir6.2 might affect the efficacy of sulphonylurea inhibition. First, it may be a secondary consequence of the greater open probability of Kir6.2ΔN14/SUR1 channels; second, it may result because the N-terminus is involved in transducing sulphonylurea binding to SUR1 into closure of the pore of Kir6.2. It is undeniable that a reduction of the channel open probability will concomitantly decrease sulphonylurea sensitivity: as previously described, Kir6.2-C166S/SUR1 channels have

an open probability ~4-fold greater than wild-type and are unaffected by 100 μM tolbutamide, a concentration specific for the high-affinity site on SUR1 (Trapp *et al.* 1998). Thus the enhanced P_o of Kir6.2ΔN14/SUR1 may be expected to reduce sulphonylurea inhibition. Our results show, however, that this effect masks a more interesting phenomenon. When the P_o is lowered by other means, tolbutamide is still unable to block Kir6.2ΔN14/SUR1 currents, despite causing marked inhibition of Kir6.2-C166S/SUR1 channels. We therefore conclude that the N-terminus of Kir6.2 is involved in coupling sulphonylurea binding to SUR1 into closure of the Kir6.2 pore. Whether this results from a direct physical interaction of the N-terminus of Kir6.2 with SUR1, or whether it involves an allosteric interaction between the two subunits, will require biochemical investigation.

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Acknowledgements

We thank Yamanouchi Research Institute International, The Wellcome Trust and the British Diabetic Association for support. F.R. is a Yamanouchi Research Institute Fellow. S.J.T. is a Wellcome Trust Research Fellow.

Corresponding author

F. M. Ashcroft: University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK.

Email: frances.ashcroft@physiol.ox.ac.uk