Sulphonylureas, such as tolbutamide and glibenclamide, are widely used to treat non-insulin-dependent diabetes mellitus. These drugs stimulate insulin release by closing ATP-sensitive K⁺ channels (K<sub>ATP</sub> channels) in the plasma membrane of pancreatic β-cells (Ashcroft & Ashcroft, 1992). This leads to β-cell depolarization, activation of voltage-gated Ca<sup>2+</sup> channels, Ca<sup>2+</sup> entry and thereby to exocytosis of the insulin-containing secretory granules (Ashcroft & Rosman, 1989; Ashcroft & Ashcroft, 1990).

There is evidence that the sensitivity of the K<sub>ATP</sub> channel to sulphonylureas can be modulated by cytosolic nucleotides such as MgADP and MgGDP (Zündler, Lins, Ohno-Shosaku, Trube & Panten, 1988; Schwanstecher, Diedel & Panten, 1992, 1994). In pancreatic β-cells, for example, single-channel recordings have shown that intracellular ADP appears to increase the sensitivity of the K<sub>ATP</sub> channel to tolbutamide. This effect is dependent on the presence of intracellular Mg<sup>2+</sup>, but the mechanism by which MgADP modulates the tolbutamide sensitivity is unknown.

The interaction of nucleotides with the tolbutamide block of cloned ATP-sensitive K⁺ channel currents expressed in <i>Xenopus</i> oocytes: a reinterpretation

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1. We have examined the mechanism by which nucleotides modulate the tolbutamide block of the β-cell ATP-sensitive K⁺ channel (K<sub>ATP</sub> channel), using wild-type and mutant K<sub>ATP</sub> channels heterologously expressed in <i>Xenopus</i> oocytes. This channel is composed of sulphonylurea receptor (SUR1) and pore-forming (Kir6.2) subunits.

2. The dose–response relation for tolbutamide block of wild-type K<sub>ATP</sub> currents in the absence of nucleotide showed both a high-affinity (K<sub>i</sub> = 2·0 μM) and a low-affinity (K<sub>i</sub> = 1·8 mM) site.

3. The dose–response relation for tolbutamide block of Kir6.2ΔC36 (a truncated form of Kir6.2 which is expressed independently of SUR1) was best fitted with a single, low-affinity site (K<sub>i</sub> = 1·7 mM). This indicates that the high-affinity site resides on SUR1, whereas the low-affinity site is located on Kir6.2.

4. ADP (100 μM) had a dual effect on wild-type K<sub>ATP</sub> currents: the nucleotide enhanced the current in the presence of Mg<sup>2+</sup>, but was inhibitory in the absence of Mg<sup>2+</sup>. Kir6.2ΔC36 currents were blocked by 100 μM ADP in the presence of Mg<sup>2+</sup>.

5. For wild-type K<sub>ATP</sub> currents, the blocking effect of 0·5 mM tolbutamide appeared greater in the presence of 100 μM Mg ADP (84 ± 2 %) than in its absence (59 ± 4 %). When SUR1 was mutated to abolish Mg ADP activation of K<sub>ATP</sub> currents (K719A or K1384M), there was no difference in the extent of tolbutamide inhibition in the presence or absence of Mg ADP.

6. The K<sub>i</sub> for tolbutamide interaction with either the high- or low-affinity site was unaffected by 100 μM Mg ADP, for both wild-type and K719A–K1384M currents.

7. MgGDP (100 μM) enhanced wild-type K<sub>ATP</sub> currents and was without effect on K719A–K1384M currents. It did not affect the K<sub>i</sub> for tolbutamide block at either the high- or low-affinity site.

8. Our results indicate that interaction of tolbutamide with the high-affinity site (on SUR1) abolishes the stimulatory action of Mg ADP. This unmasks the inhibitory effect of ADP and leads to an apparent increase in channel inhibition. Under physiological conditions, abolition of Mg ADP activation is likely to constitute the principal mechanism by which tolbutamide inhibits the K<sub>ATP</sub> channel.

Keywords: Potassium channel, Nucleotide, Magnesium

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(−100 μM) stimulate activity. The stimulatory effect requires the presence of MgATP while the inhibitory effect is independent of the cation. The molecular identity of the β-cell KATP channel has recently been resolved. It is a complex of (at least) two different proteins, both of which are required for the formation of a functional KATP channel (Inagaki et al. 1995; Sakura, Ammålä, Smith, Gribble & Ashcroft, 1995). One of these proteins is an inwardly rectifying K+ channel subunit (Kir6.2) and it is likely that four of these subunits come together to form the channel pore (Clement et al. 1997). The other subunit is a sulphonylurea receptor (SUR1; Aguilar-Bryan et al. 1995), which acts as a regulator of channel activity (Ammålä, Moorhouse & Ashcroft, 1996c; Inagaki et al. 1996). Both subunits are required for functional KATP channels since expression of Kir6.2 alone does not result in measurable currents. Truncation of the last thirty-six residues of Kir6.2 (Kir6.2Δ36), however, enables functional expression of the protein in the absence of SUR1 (Tucker, Gribble, Zhao, Trapp & Ashcroft, 1997). Studies of Kir6.2Δ36 expressed in the absence and presence of SUR1 have shown that the site at which ATP and ADP mediate channel inhibition lies on Kir6.2, while SUR1 confers sensitivity to the sulphonylureas, diazoxide and the stimulatory effects of MgADP (Tucker et al. 1997).

SUR1 is a member of the ATP-binding cassette (ABC) transporter superfamily (Higgins, 1992), and its predicted membrane topology consists of thirteen to seventeen transmembrane domains with two large intracellular loops which contain consensus sequences for nucleotide binding. Each nucleotide binding domain (NBD) contains a highly conserved Walker A (WA) and Walker B (WB) motif (Walker, Saraste, Runswick & Gay, 1982). Mutations in either of these domains abolish the ability of MgADP to stimulate channel activity (Nichols et al. 1996; Gribble, Tucker & Ashcroft, 1997b). In other ATPases and ABC transporters a lysine residue in the WA motif is essential for ATP hydrolysis (Azzaria, Schurr & Gros, 1989; Saraste, Sibbald & Wittinghofer, 1990; Tian, Yan, Jiang, Kishi, Nakazawa & Tsai, 1990; Higgins, 1992; Ko & Pedersen, 1995; Koronakis, Hughes, Milislav & Koronakis, 1995). Mutation of this residue in either of the NBDs of SUR1 abolishes the putative potassium ATPase denoted in other ABC transporters, whereas the low-affinity site lies on SUR1, whereas the low-affinity site is located on Kir6.2. Previous studies have assumed that tolbutamide interacts with the KATP channel at a single site (Zünkl et al. 1988; Schwanstecher et al. 1992, 1994). If a single-site, rather than a two-site, model is used, the K_i for tolbutamide inhibition appears to shift in the presence of MgADP. This finding led to the earlier idea that the nucleotide alters the affinity of the β-cell KATP channel for sulphonylureas. We now show that this idea is incorrect, because the K_i for tolbutamide interaction with the high-affinity site is unaffected by MgADP. Instead, our results suggest that the interaction of tolbutamide with SUR1 abolishes the stimulatory action of MgADP. This unmasks the inhibitory effect of MgADP and leads to an apparent increase in channel inhibition. Under physiological conditions, reduction of MgADP activation is likely to constitute the principal mechanism by which tolbutamide inhibits the KATP channel.

**METHODS**

**Molecular biology**

Site-directed mutagenesis was carried out by subcloning the appropriate fragments into the pALTER vector (Pronge, Madison, WI, USA). The lysine residues at position 719 or 1384 of SUR1 were replaced by an alanine or methionine, respectively (K719A, K1384M). The C-terminus deletion of Kir6.2 (Kir6.2Δ36) was created by introduction of a stop codon at residue 355. Synthesis of mRNA encoding Kir6.2Δ36 or wild-type mouse Kir6.2 (Genbank D50581), and of wild-type and mutant rat SUR1 (Genbank L40624) was carried out as previously described (Gribble et al. 1997b). All SUR1 mutations were coexpressed with wild-type Kir6.2 and the resulting currents are referred to by the SUR1 mutation only (thus, wild-type Kir6.2 plus SUR1–K719A currents are referred to as K719A currents). The term wild-type KATP current refers to wild-type Kir6.2 coexpressed with wild-type SUR1.

**Electrophysiology**

**Oocyte collection.** Female *Xenopus laevis* were anaesthetized with MS-222 (2 g l⁻¹) added to the water. One ovary was removed via a mid-line laparotomy and the animal allowed to recover. Once the wound had completely healed, the second ovary was anaesthetized with MS-222 and manually defolliculated. They were then injected with ~2 ng each of mRNAs encoding wild-type Kir6.2 and either wild-type or mutant SUR1, or injected with Kir6.2Δ36 alone (Gribble, Ashfield, Ammålä & Ashcroft, 1997a). Control oocytes were injected with water. The final injection volume was ~50 nl per oocyte in all cases. Isolated oocytes were maintained in modified
Barth’s solution containing (mM): 88 NaCl, 1 KCl, 1·7 MgSO_4·7H_2O, 0·47 Ca(NO_3)_2, 0·41 CaCl_2, 24 NaHCO_3, 10 Hepes (pH 7·4 with NaOH), supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 5 mM pyruvate. Currents were studied 1–4 days after injection.

**Giant patch recordings.** Macropatches were recorded from giant excised inside-out patches (Hilgemann, Nicoll & Phillipson, 1991) at 20–24 °C. Patch electrodes were pulled from thick-walled borosilicate glass (GC150; Clark Electromedical Instruments, Pangbourne, UK) and had resistances of 200–400 kΩ when filled with pipette solution. Currents were not leak corrected because the leak current (that remained in the presence of 1 μM ATP) was < 1 % of the total currents. To control for run-down, the control solution before and after application of the test compound. Most data were obtained by alternating test solutions with control (drug- and nucleotide-free) solutions. To control for run-down, the control solution was used as the means of that obtained in the solution before and after application of the test compound. Most currents were not leak-corrected because the leak current (that remaining in the presence of 1 mM ATP) was < 1 % of the total current. However, as the amplitude of Kir2.2A3C36 currents was smaller, we corrected the dose–response curves for leak by subtraction of the mean conductance measured in patches excised from control oocytes.

The conductance (G) is plotted as a fraction of that obtained in the control solution (G_c). In each case, the dose–response curve was fitted with the following equation:

\[ \frac{G}{G_c} = A B x y, \]  

where A is a factor describing the activation of the fractional conductance by nucleotide; B is a factor describing the block of the fractional conductance by nucleotide (both A and B have a value of one in the absence of nucleotide diphosphate); x is a term describing the high-affinity site and y is a term describing the low-affinity site.

\[ x = \frac{1 - L}{1 + ([Tolb]/K_B)^{h_B}} \]  

and

\[ y = \frac{1}{1 + ([Tolb]/K_A)^{h_A}} \]

where [Tolb] is the tolbutamide concentration, K_A and K_B are the tolbutamide concentrations at which inhibition is half-maximal at the high- and low-affinity sites, respectively, and L is the fractional conductance remaining when all of the high-affinity inhibitory sites are occupied. When only a single binding site is present, eqn (1) reduces to:

\[ \frac{G}{G_c} = A B y. \]  

In most cases, 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). In the case of the K_i, the statistics were carried out on log-transformed data and are presented as the geometric mean (with 1 s.e.m. range). Statistical significance was tested using Student’s unpaired t test or ANOVA, as appropriate. P values of < 0·05 were taken to indicate that the data were significantly different.

**RESULTS**

Tolbutamide block of wild-type channels

The inhibitory effect of tolbutamide on wild-type K_{ATP} currents is shown in Fig. 1A. As previously reported for native K_{ATP} currents, the drug inhibited wild-type Kir2.2–SUR1 currents in a voltage-independent fashion. Tolbutamide (0·5 mM) blocked the slope conductance by 59±1 ± 4·3 % (n = 11; Fig. 3). The extent of this inhibition was considerably less than that found for wild-type K_{ATP} currents when the drug was applied to the extracellular surface of the membrane of intact oocytes; under these conditions, 0·5 mM tolbutamide blocked the current at −100 mV by 96 % (Gribble et al. 1997). A similar difference has been observed for native β-cell K_{ATP} channels, where it was attributed to the presence of MgADP at the inner surface of the membrane in intact cells (Zünkler et al. 1988). Figure 1B shows that when tolbutamide was applied in the presence of intracellular MgADP the extent of inhibition of the K_{ATP} current was enhanced. Tolbutamide (0·5 mM) blocked the wild-type K_{ATP} conductance by 84 ± 2 % (n = 7) in the presence of 100 μM MgADP, compared with 59 % in the absence of the nucleotide diphosphate (Fig. 3). Furthermore, whereas 100 μM MgADP caused a marked activation of K_{ATP} currents when applied by itself (Fig. 1C), the nucleotide diphosphate was actually inhibitory when applied in the presence of tolbutamide (Fig. 1D). Similar findings with native K_{ATP} channels have been used to argue that MgADP potentiates the tolbutamide block by making the channel more sensitive to tolbutamide (Zünkler et al. 1988, 1992, 1994). However, since MgADP has both stimulatory and inhibitory effects on K_{ATP} channel activity (Bokvist et al. 1991; Gribble et al. 1997b; Tucker et al. 1997), an alternative interpretation is that tolbutamide abolishes the potentiatory effect of MgADP, so unmasking its inhibitory effect. This idea is supported by the fact that the extent of block by 100 μM MgADP in the presence of tolbutamide was not significantly different from the inhibitory effect of 100 μM ADP in Mg^{2+}-free solution (Fig. 4), a condition in which the stimulatory action of MgADP is abolished.

**Tolbutamide inhibition of mutant channel currents**

To further investigate the possibility that tolbutamide abolishes the potentiatory effect of MgADP, we studied the
interaction between MgADP and tolbutamide on mutant KATP channels in which the stimulatory effect of MgADP was selectively abolished. Mutation of the W4 lysine residues in either NBD1 (K719A) or NBD2 (K1384M), or both (K719A–K1384M) prevented the MgADP activation of KATP currents and unmasked the inhibitory effect of MgADP (Figs 2C and 4; Gribble et al. 1997b). In the absence of nucleotides, the extent of block of K719A–K1384M, K719A and K1384M currents by 0·5 mM tolbutamide was similar to that of wild-type channels (Fig. 3). This suggests that the W4 lysines are not required either for tolbutamide binding or for the transduction of tolbutamide binding into inhibition of the KATP channel. Addition of 100 μM MgADP to the cytosolic side of the membrane blocked

Figure 1. Effects of MgADP and tolbutamide on wild-type KATP currents
Macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from −110 to +100 mV (holding potential, 0 mV). The oocytes were coinjected with mRNAs encoding wild-type Kir6.2 and wild-type SUR1. MgADP (100 μM) and tolbutamide (0·5 mM) were added to the internal (bath) solution as indicated.

Figure 2. Effects of MgADP and tolbutamide on K719A–K1384M currents
Macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from −110 to +100 mV (holding potential, 0 mV). The oocytes were coinjected with mRNAs encoding wild-type Kir6.2 and K719A–K1384M SUR1. MgADP (100 μM) and tolbutamide (0·5 mM) were added to the internal (bath) solution as indicated.
K719A–K1384M currents by 67% (Fig. 2C). This contrasts with its stimulatory action on wild-type K\textsubscript{ATP} channels (Fig. 4; Gribble \textit{et al.} 1997b). Furthermore, MgADP did not enhance tolbutamide inhibition of K719A–K1384M currents (Figs 2 and 3). Similar results were observed for K719A and K1384M currents (Figs 3 and 4), which confirms that the W\textsubscript{A} lysine at each of the NBDs of SUR1 is needed both for the stimulatory effect of MgADP itself (Gribble \textit{et al.} 1997b), and for the ability of MgADP to enhance tolbutamide block.

**Mutation of the W\textsubscript{B} aspartate residues in either NBD1 (D853N) or NBD2 (D1505N) of SUR1 abolished the stimulatory effects of MgADP on K\textsubscript{ATP} currents (Fig. 4) and prevented the MgADP-dependent enhancement of tolbutamide block (Fig. 3).** These mutations are predicted to prevent the interaction of the Mg\textsuperscript{2+} ion of MgADP with the Tolbutamide block of K\textsubscript{ATP} channels.

**Figure 3. Mean effects of tolbutamide on wild-type and mutant K\textsubscript{ATP} channels**

Mean macroscopic conductance of wild-type or mutant K\textsubscript{ATP} channels recorded in response to 0.5 mM tolbutamide with or without 100 \textmu M MgADP. Conductance is expressed as a percentage of its amplitude in the absence of tolbutamide (\(G_{Tol}/G_{Tc}\)). The dashed line indicates the conductance of wild-type channels in the presence of 0.5 mM tolbutamide (ADP free) solution. The number of patches is indicated in parentheses. Oocytes were injected with wild-type Kir6.2 and mutant SUR1 as indicated.

**Figure 4. Mean effects of MgADP on wild-type and mutant K\textsubscript{ATP} channels**

Mean macroscopic conductance of wild-type or mutant K\textsubscript{ATP} channels recorded in response to 100 \textmu M MgADP, 100 \textmu M MgADP in 0.5 mM tolbutamide, and 100 \textmu M ADP in the absence of Mg\textsuperscript{2+}. All data are expressed as a percentage of the conductance in the absence of MgADP (\(G_{ADP}/G_{Tc}\)). The dashed line indicates the conductance level in the absence of MgADP. The number of patches is indicated in parentheses. Oocytes were injected with wild-type Kir6.2 and mutant SUR1, as indicated.
NBDs of SUR1. The results indicate that the W8 aspartates at both NBDs of SUR1 are needed not only for the activatory effect of MgADP but also for the ability of the nucleotide to enhance tolbutamide block.

Effects of nucleotides on the dose–response relation for tolbutamide block

The relation between tolbutamide concentration and the amplitude of the wild-type K\textsubscript{ATP} conductance is given in Fig. 5. It is clear that the dose–response curve cannot readily be fitted by a single-site model. Instead, the data indicate that tolbutamide blocks at both a high-affinity and a low-affinity site and that occupation of the high-affinity site produces a maximum of 57% inhibition. When the dose–response curve was fitted to a two-site model (eqn (1)), the best fit was obtained with a mean $K_1$ of 2.0 μM (1.2–3.5 μM) and a Hill coefficient of 1.0 ± 0.1 for the high-affinity site and a $K_2$ of 1.8 mM (1.4–2.3 mM) and a Hill coefficient of 1.3 ± 0.2 for the low-affinity site ($n = 8$). The Hill coefficients were close to unity for both sites, which suggests that only a single tolbutamide molecule need interact with either site to cause channel inhibition.

Figure 5. Dose–response curve for tolbutamide inhibition of wild-type and mutant K\textsubscript{ATP} channels

Relationship between tolbutamide concentration and the macroscopic K\textsubscript{ATP} conductance, expressed as a fraction of its amplitude in the absence of the drug ($G/G_c$).

- (continuous line), wild-type K\textsubscript{ATP} currents ($n = 8$).
- (dashed line), K719A–K1384M currents ($n = 6$). The lines are the best fit of the data to eqn (1). Wild-type Kir6.2–SUR1 channel currents: $K_1 = 2.0$ μM, $h_1 = 1.0$, $K_2 = 1.8$ mM, $h_2 = 1.3$, $L = 0.43$. K719A–K1384M channel currents: $K_1 = 1.6$ μM, $h_1 = 0.7$, $K_2 = 2.1$ mM, $h_2 = 1.4$, $L = 0.40$.

Figure 6. Effects of MgADP and MgGDP on the relation between K\textsubscript{ATP} conductance and tolbutamide concentration

A, relation between tolbutamide concentration and the macroscopic K\textsubscript{ATP} conductance, expressed as a fraction of its amplitude in control solution ($G/G_c$) lacking both drug and nucleotide. • wild-type currents, no nucleotides ($n = 8$). ■, wild-type currents, 100 μM MgADP ($n = 8$). □, K719A–K1384M currents, 100 μM MgADP ($n = 6$). The lines are the best fit of the data to eqn (1). In the presence of 100 μM MgADP: $K_1 = 6.6$ μM, $h_1 = 1.1$, $K_2 = 4.3$ mM, $h_2 = 1.0$, $L = 0.13$, $A = 4.3$, $B = 0.33$, for wild-type channel currents; and $K_1 = 3.1$ μM, $h_1 = 1.2$, $K_2 = 5.5$ mM, $h_2 = 1$, $L = 0.44$, $A = 1.0$, $B = 0.33$, for K719A–K1384M currents. B, mean relationship between tolbutamide concentration and the macroscopic K\textsubscript{ATP} conductance, expressed as a fraction of its amplitude in control solution lacking both drug and nucleotide. • wild-type currents, no nucleotides ($n = 8$). ▲, wild-type currents, 100 μM MgGDP ($n = 6$). △, K719A–K1384M currents, 100 μM MgGDP ($n = 6$). The lines are the best fit of the data to eqn (1). In the presence of 100 μM MgGDP: $K_1 = 6.0$ μM, $h_1 = 1.0$, $K_2 = 2.0$ mM, $h_2 = 1.0$, $L = 0.39$, $A = 1.9$, $B = 0.9$, for wild-type channel currents; and $K_1 = 3.8$ μM, $h_1 = 1.0$, $K_2 = 2.8$ mM, $h_2 = 1.3$, $L = 0.56$, $A = 1.0$, $B = 0.9$, for K719A–K1384M currents.
We next examined the dose–response curve for tolbutamide block of K719A–K1384M currents. In the absence of nucleotides, the dose–inhibition curve was not significantly different from that of the wild-type channel and could be well fitted with the same parameters (Fig. 5). The best fit to the $W_A$ mutant channel currents was obtained with a $K_i$ of 1.6 ± 0.1 (1.1–2.4 μM) and a Hill coefficient of 0.7 ± 0.1 for the high-affinity site and a $K_i$ of 2.1 mM (1.8–2.5 mM) and a Hill coefficient of 1.4 ± 0.2 for the low-affinity site ($n = 6$). This provides further support for the idea that mutation of the $W_A$ lysines does not influence the tolbutamide sensitivity of the $K_{ATP}$ channel.

We next examined the effects of intracellular MgADP on the efficacy of tolbutamide block (Fig. 6A). In the absence of the drug, MgADP caused a marked activation of wild-type $K_{ATP}$ currents (155 ± 13 %, $n = 5$). The dose–response curve for tolbutamide block was also altered. In particular, greater inhibition was observed at high tolbutamide concentrations in the presence of MgADP. The $K_i$ and Hill coefficient for the high-affinity site were 6.6 μM (4.9–8.9 μM) and 1.1 ± 0.1, respectively ($n = 5$) in the presence of 100 μM MgADP. These values are not significantly different (t test) from those obtained in the absence of MgADP, which suggests that MgADP does not markedly alter the sensitivity of the wild-type $K_{ATP}$ channel to tolbutamide. The small size of the currents at high tolbutamide concentrations in the presence of MgADP precludes an accurate analysis of the $K_i$ of the low-affinity site in this solution.

Unlike wild-type $K_{ATP}$ currents, K719A–K1384M channel currents were blocked by 100 μM MgADP (by 63.4 ± 2.8%, $n = 17$). Tolbutamide caused a further block at all concentrations without affecting the shape of the dose–response curve (Fig. 6A). There was no detectable difference in the $K_i$ of the high-affinity site measured in the presence or absence of MgADP. The best fit to the mutant channel currents in 100 μM MgADP was obtained with a $K_i$ of 3.1 μM (2.1–4.7 μM, $n = 5$), which is not significantly different (ANOVA) from that of wild-type currents, or K719A–K1384M currents in the absence of ADP. The Hill coefficient for the high-affinity site was 1.2 ± 0.1 ($n = 5$).

The most likely explanation for our results is that binding of tolbutamide to the high-affinity site produces a concentration-dependent inhibition of the stimulatory action of MgADP, and so unmasks the inhibitory effect of the nucleotide. This would account for the fact that MgADP has no effect on the tolbutamide block of K719A–K1384M currents, which are not stimulated by the nucleotide. It is also consistent with the finding that at high tolbutamide concentrations the dose–response curves for wild-type and mutant channels converge (Fig. 6A), suggesting that the stimulatory effect on MgADP on wild-type $K_{ATP}$ channels is abolished. If this idea is correct, we would expect that nucleotides which...
potentiate activity, but which have minimal blocking effect, such as MgGDP, should not enhance the tolbutamide block. We therefore next explored this possibility.

In contrast to MgADP, 100 μM MgGDP had little effect on K719A—K1384M currents, which were 90 ± 3 % (n = 6) of their amplitude in control solution. This indicates that the guanine nucleotide is much less effective at the inhibitory nucleotide-binding site (Fig. 6B), in agreement with the fact that 100 μM MgGDP causes little or no block of Kir6.2ΔC26 currents (Trapp, Tucker & Ashcroft, 1997). MgGDP was, however, a potent activator of wild-type channels, enhancing the macroscopic conductance by 173 ± 15 % (n = 6). This confirms that MgGDP is able to act at the stimulatory nucleotide-binding site in wild-type, but not K719A—K1384M, channels. Tolbutamide produced a concentration-dependent inhibition of wild-type currents in the presence of MgGDP, but even at high drug concentrations the block never exceeded that found in the absence of the nucleotide. In the presence of 100 μM MgGDP, the Ki values were 6.0 μM (4.7—7.6 μM) and 2.0 mM (1.6—2.5 mM), and the Hill coefficients were 1.0 ± 0.1 and 1.0 ± 0.2, for the high- and low-affinity sites, respectively (n = 6). These values are not significantly different from those obtained in the absence of MgGDP (by t test). The best fit to the mutant channel currents was obtained with a Ki of 3.8 μM (1.9—7.6 μM) and a Hill coefficient of 1.0 ± 0.2 for the high-affinity site and a Ki of 2.8 mM (2.2—3.5 mM) and a Hill coefficient of 1.3 ± 0.1 for the low-affinity site (n = 6). The convergence of the dose—response curves for wild-type and mutant channels at high tolbutamide concentrations (Fig. 6B) suggests that activation of wild-type currents by MgGDP is abolished by the drug.

The low-affinity site may reside on Kir6.2

We next explored whether the low-affinity site lies on the sulphonylurea receptor or on Kir6.2 by studying the effect of tolbutamide on the Kir subunit in the absence of SUR1. Expression of Kir6.2 alone does not result in measurable KATP currents (Sakura et al. 1995; Inagaki et al. 1995). We therefore examined the effect of tolbutamide on a mutant form of Kir6.2 (Kir6.2ΔC36) in which the last thirty-six C-terminal amino acids had been deleted: expression of this protein produces large currents in the absence of SUR1 (Tucker et al. 1997). Mean Kir6.2ΔC36 currents upon patch excision were 1.0 ± 0.2 nA (n = 6) at −100 mV. Figure 7A shows that these currents were blocked by high concentrations of tolbutamide. The dose—response curve for tolbutamide inhibition (Fig. 7B) was well fitted by a single-site model with a Ki of 1.7 mM (1.7—1.8 mM, n = 6) and a Hill coefficient of 1.2 ± 0.1 (n = 6). These values are not significantly different from those obtained for the low-affinity site of wild-type KATP (Kir6.2—SUR1) currents (Ki = 1.8 mM, k = 1.0). These data confirm that the high-affinity binding site for sulphonylureas resides on SUR1 (Aguilar-Bryan et al. 1995). They also suggest that the low-affinity site resides on Kir6.2.

We tested whether MgADP interacted with the low-affinity site for tolbutamide directly, by measuring the dose—response curve for tolbutamide inhibition of Kir6.2ΔC36 currents in the presence of 100 μM MgADP (Fig. 7B). The mean Ki was 1.9 mM (1.7—2.1 mM, n = 4) and the mean Hill coefficient was 1.5 ± 0.2 (n = 4), values which are not significantly different (t test) from those found in the absence of MgADP. Thus, it appears that MgADP at this concentration does not affect the low-affinity site.

**DISCUSSION**

Our results indicate that tolbutamide interacts with two sites to inhibit wild-type KATP currents, which have Ki values of ~2 μM and ~2 mM. The former value is in good agreement with previous reports for both native β-cell (7 μM; Pienten et al. 1989) and cloned (4 μM; Sakurai et al. 1995) KATP channels in whole-cell recordings. The presence of two sites which mediate sulphonylurea inhibition of KATP channel currents is consistent with the results of studies of the binding of [3H]glibenclamide to β-cell membranes, which have also shown both high- and low-affinity sulphonylurea receptors (Ashcroft & Ashcroft, 1992). The Ki for tolbutamide displacement of [3H]glibenclamide binding to the high-affinity binding site was 15—25 μM, close to that observed for high-affinity inhibition of KATP currents in the present study. This suggests that tolbutamide binds to the high-affinity sulphonylurea receptor to mediate high-affinity inhibition of KATP currents. No Ki value for tolbutamide displacement of [3H]glibenclamide binding to the low-affinity binding site of β-cell membranes has been reported. However, [3H]glibenclamide binds approximately 1000 times less strongly to the low-affinity binding site of HIT T15 insulinoma cell membranes (Niki, Kelly, Ashcroft & Ashcroft, 1988; Aguilar-Bryan, Nelson, Vu, Humphrey & Boyd, 1990). Since the Ki for low-affinity tolbutamide inhibition of the cloned KATP channels obtained in this study was also 1000 times greater than that for high-affinity inhibition, this may suggest that the low-affinity inhibition we observed is mediated by tolbutamide binding to the low-affinity site observed in sulphonylurea-binding studies. Furthermore, in at least one of the binding studies, the number of high- and low-affinity binding sites was the same (~546 vs. 575 fmol (mg protein)−1; Niki et al. 1989), in agreement with the 1:1 stoichiometry observed for Kir6.2 and SUR1 (Clement et al. 1997; Inagaki, Goni & Seno, 1997).

Interaction of tolbutamide with the high-affinity site blocks the macroscopic KATP conductance by about 50%. It is difficult to explain why this block is not complete. It cannot result from a partial reduction of the single-channel current amplitude since single-channel recordings have shown that tolbutamide has no effect on the single-channel conductance but affects only the channel open probability (Trube, Rosman & Ohno-Shosaku, 1986; Gillis, Gee, Hammoumi, McDaniel, Falk & Misler, 1989). It also cannot be explained by postulating that only 50% of KATP channels are
associated with a sulphonylurea receptor, because wild-type Kir6.2 does not form functional channels in the absence of SUR1. One possibility is that the high-affinity binding site for tolbutamide can exist in two states, one of which induces $K_{\text{ATP}}$ channel inhibition and one which has no effect on channel activity, and that there is an approximately equal probability of being in either state. (Our results do not allow us to distinguish whether channels that are unaffected by the drug simply do not bind tolbutamide, or whether they fail to translate the effect of tolbutamide binding into closure of the $K_{\text{ATP}}$ channel pore.)

**Identity of the two sulphonylurea-binding sites**

There is good evidence that the high-affinity site which mediates tolbutamide inhibition of $K_{\text{ATP}}$ channel currents resides on SUR1. First, the $K_i$ for tolbutamide displacement of $[^{3}H]$glibenclamide binding to SUR1 in the absence of Kir6.2 was 59 μM (Ammlak et al. 1996b), a value which is closest to that of the high-affinity site which regulates $K_{\text{ATP}}$ currents. Second, in the absence of SUR1, Kir6.2ΔC36 currents do not show high-affinity inhibition by tolbutamide.

The low-affinity site is present when Kir6.2ΔC36 currents are expressed independently of SUR1. It must therefore reside on Kir6.2, or on a separate protein, endogenously expressed in *Xenopus* oocytes, which regulates Kir6.2 activity. Although we cannot exclude the latter possibility, we favour the idea that Kir6.2 is itself sensitive to sulphonylureas. This is because Kir6.2ΔC36 currents were expressed at high levels in oocytes and were completely blocked by 10 μM tolbutamide; thus, an endogenous oocyte protein would also have to be expressed at a similar high density.

**Effects of MgADP on tolbutamide inhibition of $K_{\text{ATP}}$ channels**

The dose–inhibition curve for tolbutamide in the presence of nucleotide diphosphates is best fitted with a two-site model, as it is under control conditions. Neither MgADP nor MgGDP markedly altered the $K_i$ for tolbutamide inhibition at either the high- or low-affinity site. This suggests that nucleotide diphosphates do not measurably alter the affinity of the interaction between the drug and the $K_{\text{ATP}}$ channel, in contrast to previous suggestions (Zünkler et al. 1988; Schwantescher et al. 1992, 1994).

It is well established that ADP has both potentiatory and inhibitory effects on the $K_{\text{ATP}}$ channel (Bovkivist et al. 1991; Gribble et al. 1997b; Tucker et al. 1997). The greater inhibition of $K_{\text{ATP}}$ channel currents by tolbutamide in the presence of MgADP is most easily explained by assuming that the drug reduces the stimulatory action of MgADP and thereby unmasks the inhibitory action of the nucleotide diphosphate. Several findings support this idea. First, we observed no significant difference in the $K_i$ of either the high- or low-affinity sites in the presence and absence of MgADP. Second, the tolbutamide block of K719A–K1384M currents, which are not potentiated by MgADP, was not altered by MgADP. Third, tolbutamide inhibition of Kir6.2ΔC36 currents, which are also not potentiated by MgADP, was unaffected by the nucleotide. Fourth, MgGDP, at a concentration which produces activation but not inhibition of $K_{\text{ATP}}$ currents, did not support an enhanced block by tolbutamide. Since Mg$^{2+}$ is required for the stimulatory effects of MgADP and MgGDP (Bovkivist et al. 1991; Gribble et al. 1997b), our results may also explain why nucleotide diphosphates only influence tolbutamide inhibition of $K_{\text{ATP}}$ currents in the presence of Mg$^{2+}$; in the absence of Mg$^{2+}$, the effects of ADP would resemble those found for K719A–K1384M currents.

**Mechanism of tolbutamide block**

The mechanism by which tolbutamide inhibits the MgADP potentiation of channel activity is not clear. Nucleotide diphosphates mediate their stimulatory effects by interaction with the NBDs of SUR1 (Nichols et al. 1996; Gribble et al. 1997b). One possibility, therefore, is that binding of tolbutamide to SUR1 prevents the interaction of MgADP with either or both NBDs. Another idea is that tolbutamide disrupts the mechanism by which the interaction of MgADP with the NBDs of SUR1 is translated into $K_{\text{ATP}}$ channel activation. We emphasize that this does not necessarily mean that tolbutamide interacts directly with the NBDs of SUR1—it may simply mediate its effects allosterically by binding to a site elsewhere on SUR1.

Mutation of either the $W_A$ lysine or the $W_B$ aspartate in a single NBD of SUR1 is sufficient to abolish the stimulatory effect of MgADP (Nichols et al. 1996; Gribble et al. 1997b). We cannot tell whether tolbutamide prevents the stimulatory action of MgADP at NBD1 or NBD2, or both, because the enhancement of channel activity by MgADP is abolished if either one of the NBDs is mutated.

**Reinterpretation of previous studies**

Previous studies have fitted tolbutamide dose–inhibition curves with a single-site model in both the presence and absence of intracellular nucleotide diphosphates (Zünkler et al. 1988; Schwantescher et al. 1992, 1994). Our data show that this is inappropriate. In the absence of nucleotide diphosphates, tolbutamide clearly interacts with two sites to inhibit the wild-type $K_{\text{ATP}}$ channel, one of which has a high affinity and the other a low affinity for the drug. This is easily discerned in our studies because the high expression levels of cloned $K_{\text{ATP}}$ channels in *Xenopus* oocytes allows a more accurate determination of the dose–response relation, but it is not always so readily apparent in β-cells and the data have traditionally been fitted with a single-site model. However, careful re-examination of the tolbutamide dose–response curve in β-cells (e.g. Schwantescher et al. 1994) confirms that they may also be better fitted with a two-site model, suggesting that tolbutamide also inhibits the native β-cell $K_{\text{ATP}}$ channel by interaction with both a high- and a low-affinity site.

The model used to fit the tolbutamide dose–response curve has functional implications. If a single-site, rather than a
two-site model is used, the $K_i$ for tolbutamide inhibition appears to shift in the presence of MgADP (Zinkler et al. 1988; Schwanstecher et al. 1992, 1994). For example, an apparent shift in $K_i$ from 55 to 14 $\mu$M, in the absence and presence of 100 $\mu$M MgADP, respectively, was observed when a single-binding site model was used to fit the tolbutamide dose–response curve (Zinkler et al. 1988). This led to the idea that the nucleotide alters the affinity of the $\beta$-cell $K_{ATP}$ channel for sulphonylureas. It is clear from our studies that this is not the case. Rather, tolbutamide not only has a direct inhibitory action but also appears to have the additional effect of reducing the stimulatory action of MgADP.

**Physiological significance**

In the normal $\beta$-cell, glucose metabolism causes $K_{ATP}$ channel closure. Recent studies argue that this is principally mediated by the fall in intracellular MgADP, rather than the concomitant rise in ATP (Nichols et al. 1996; Gribble et al. 1997). At therapeutic concentrations (1–10 $\mu$M), our results indicate that the primary mechanism by which tolbutamide blocks the $K_{ATP}$ channels is by reducing the potentiationary effects of MgADP. Thus, both glucose and tolbutamide mediate their inhibitory effects on the $K_{ATP}$ channel by modulating the stimulatory action of MgADP: the former directly alters the MgADP concentration and the latter prevents its effect on channel activity.


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