

# The essential role of the Walker A motifs of SUR1 in K-ATP channel activation by Mg-ADP and diazoxide

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**The ATP-sensitive K-channel (K-ATP channel) plays a key role in insulin secretion from pancreatic  $\beta$ -cells. It is closed by glucose metabolism, which stimulates insulin secretion, and opened by the drug diazoxide, which inhibits insulin release. Metabolic regulation is mediated by changes in ATP and Mg-ADP, which inhibit and potentiate channel activity, respectively. The  $\beta$ -cell K-ATP channel consists of a pore-forming subunit, Kir6.2, and a regulatory subunit, SUR1. We have mutated (independently or together) two lysine residues in the Walker A ( $W_A$ ) motifs of the first (K719A) and second (K1384M) nucleotide-binding domains (NBDs) of SUR1. These mutations are expected to inhibit nucleotide hydrolysis. Our results indicate that the  $W_A$  lysine of NBD1 (but not NBD2) is essential for activation of K-ATP currents by diazoxide. The potentiatory effects of Mg-ADP required the presence of the  $W_A$  lysines in both NBDs. Mutant currents were slightly more sensitive to ATP than wild-type currents. Metabolic inhibition led to activation of wild-type and K1384M currents, but not K719A or K719A/K1384M currents, suggesting that there may be a factor in addition to ATP and ADP which regulates K-ATP channel activity.**

**Keywords:** ATP-sensitive K-channel/diazoxide/ADP/Kir6.2/nucleotide/SUR1

## Introduction

ATP-sensitive potassium channels (K-ATP channels) are inhibited by an increase in the intracellular ATP concentration. They thereby couple cell metabolism to electrical activity and play important roles in the physiology and pathophysiology of many tissues (Ashcroft and Ashcroft, 1990). In pancreatic  $\beta$ -cells, for example, the K-ATP channel regulates insulin secretion in response both to glucose—the primary physiological stimulus—and to clinically important drugs (reviewed by Ashcroft and Rorsman, 1989). Furthermore, mutations in this channel cause persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), a disease associated with unregulated insulin secretion (Thomas *et al.*, 1995). The consensus view of  $\beta$ -cell stimulus–secretion coupling is that when plasma glucose levels rise, glucose uptake and metabolism by the pancreatic  $\beta$ -cell is increased. The resulting elevation of intracellular ATP, and concomitant lowering of intracellular Mg-ADP,

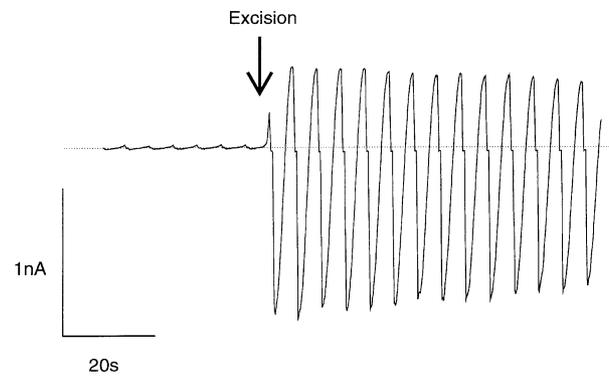
result in the closure of K-ATP channels in the  $\beta$ -cell plasma membrane, because ATP inhibits, whereas Mg-ADP activates, channel activity. The closure of K-ATP channels produces a membrane depolarization which activates voltage-dependent  $Ca^{2+}$  channels, increases  $Ca^{2+}$  influx into the  $\beta$ -cell and triggers insulin release. K-ATP channels are also regulated by two important classes of drug: the sulfonylureas and the K-channel openers. Sulfonylureas, widely used in the treatment of non-insulin-dependent diabetes mellitus, inhibit K-ATP channel activity and thereby stimulate insulin release (Ashcroft and Ashcroft, 1992). In contrast, the K-channel opener, diazoxide, activates K-ATP channels thereby hyperpolarizing the  $\beta$ -cell and inhibiting insulin release (Dunne *et al.*, 1993). Diazoxide is sometimes used to treat PHHI.

The regulation of the  $\beta$ -cell K-ATP channel by adenine nucleotides is extremely complex. In addition to its well-known inhibitory effect, Mg-ATP enhances channel activity as evidenced by the fact that when Mg-ATP is removed, K-ATP channel activity is greater than that recorded in the control solution prior to application of the nucleotide (Ohno-Shosaku *et al.*, 1987). This ‘refreshment’ of channel activity is not observed in the absence of  $Mg^{2+}$ , nor is it supported by non-hydrolysable ATP analogues, indicating that Mg-ATP hydrolysis is required. ADP also has both stimulatory and inhibitory actions. In the absence of  $Mg^{2+}$ , ADP blocks channel activity. When  $Mg^{2+}$  is present, however, high concentrations of ADP are inhibitory whereas low concentrations potentiate channel activity (Dunne and Petersen, 1986; Kakei *et al.*, 1986; Hopkins *et al.*, 1992). This suggests that Mg-ADP both activates and inhibits the channel and that the inhibitory effect dominates in Mg-free solutions or at high Mg-ADP concentrations (Bokvist *et al.*, 1991; Hopkins *et al.*, 1992). There is evidence that nucleotides also modulate the response of the K-ATP channel to drugs. Diazoxide, for example, antagonizes the inhibitory effects of Mg-ATP on the  $\beta$ -cell K-ATP channel (Dunne *et al.*, 1993). In  $\beta$ -cells, this drug has no effect, or is even inhibitory, in the absence of internal  $Mg^{2+}$  or when ATP is replaced by non-hydrolysable ATP analogues (Dunne, 1989; Kozlowski *et al.*, 1989). This result has been used to support the idea that the action of diazoxide requires protein phosphorylation. However, diazoxide is also effective in the presence of hydrolysable ADP (Larsson *et al.*, 1993), suggesting that it is more likely that the effect of the drug requires nucleotide hydrolysis rather than phosphorylation. Studies of single K-ATP channel currents have shown that nucleotides, and drugs such as diazoxide and sulfonylureas, do not alter the single-channel current amplitude but mediate their effects by modulating the channel open probability (Ashcroft and Rorsman, 1989).

The  $\beta$ -cell K-ATP channel is a complex of two proteins (Inagaki *et al.*, 1995; Sakura *et al.*, 1995): Kir6.2 and

the sulfonylurea receptor (SUR1). One of these proteins (Kir6.2) is an inwardly rectifying K-channel (Kir channel) subunit and it is believed that four Kir6.2 subunits come together to form the channel pore. The other protein, SUR1, is a member of the ATP-binding cassette (ABC) transporter family (Higgins, 1992; Aguilar-Bryan *et al.*, 1995) and acts as a regulator of channel activity conferring sensitivity to sulfonylureas and diazoxide (Åmmälä *et al.*, 1996; Inagaki *et al.*, 1996). Both Kir6.2 and SUR1 subunits are required to form a functional K-ATP channel and, unlike many other inward rectifier channels, Kir6.2 does not form functional channels in the absence of the sulfonylurea receptor. SUR1 has two groups of putative transmembrane domains (9 + 4), each of which is followed by a large cytoplasmic domain which contains a consensus sequence for nucleotide binding (Aguilar-Bryan *et al.*, 1995). Each nucleotide binding domain (NBD) contains a highly conserved Walker A ( $W_A$ ) and Walker B ( $W_B$ ) motif (Walker *et al.*, 1982). Studies of many ATPases and ABC transporters have shown that these motifs catalyse ATP hydrolysis. An aspartate in the  $W_B$  motif co-ordinates the  $Mg^{2+}$  ion of Mg-ATP and is required for nucleotide binding, while a lysine in the  $W_A$  motif interacts with the  $\gamma$  and  $\beta$  phosphate groups of ATP and is essential for ATP hydrolysis (Azzaria *et al.*, 1989; Saraste *et al.*, 1990; Tian *et al.*, 1990; Higgins, 1992; Carson *et al.*, 1995; Ko and Pedersen, 1995).

The presence of the NBDs in SUR1 raises the possibility that these may constitute one of the sites at which nucleotides regulate K-ATP channel activity. In support of this idea, recent studies have indicated that the second NBD (NBD2) plays an important role in the modulation of K-ATP channel activity by Mg-ADP (Nichols *et al.*, 1996). For example, mutation of the  $W_B$  aspartate (D1505) in NBD2 removes the ability of Mg-ADP to stimulate channel activity. This mutation is predicted to decrease Mg-ADP binding. As indicated above, however, there is also evidence that nucleotide hydrolysis is required for the effects of both Mg-ADP and diazoxide on K-ATP channel activity (Dunne *et al.*, 1993; Larsson *et al.*, 1993). We have therefore examined the effects of mutating the critical lysine in the  $W_A$  motifs of either NBD1 (K719A) or NBD2 (K1384M), or both (K719A/K1384M), of SUR1, on K-ATP currents heterologously expressed in *Xenopus* oocytes. These mutations are predicted to abolish or severely impair nucleotide hydrolysis without significantly affecting nucleotide binding (Azzaria *et al.*, 1989; Saraste *et al.*, 1990; Tian *et al.*, 1990; Higgins, 1992; Carson *et al.*, 1995; Ko and Pedersen, 1995). Our results indicate that the  $W_A$  lysine of NBD1 (but not NBD2) is essential for channel activation by diazoxide. This suggests that a conformational change induced by nucleotide binding or hydrolysis at NBD1 is involved in diazoxide action. The stimulation of channel activity by Mg-ADP may involve nucleotide hydrolysis since none of the  $W_A$  mutant channels were potentiated by Mg-ADP and, in wild-type channels, neither non-hydrolysable ADP analogues nor ADP in the absence of  $Mg^{2+}$  were effective. Alternatively, Mg-ADP binding (but not its hydrolysis) may induce a conformational change in SUR1 leading to channel opening, which is prevented by the  $W_A$  mutations. Mutant currents were slightly more sensitive to ATP than wild-type currents demonstrating that the  $W_A$  lysines are not



**Fig. 1.** Macroscopic K-ATP currents in inside-out membrane patches. Macroscopic currents recorded from a giant inside-out patch excised from an oocyte coinjected with Kir6.2 and SUR1. The holding potential was 0 mV and the voltage was successively ramped from  $-110$  mV to  $+100$  mV over a 4 s period. The patch was excised at the arrow into ATP-free solution.

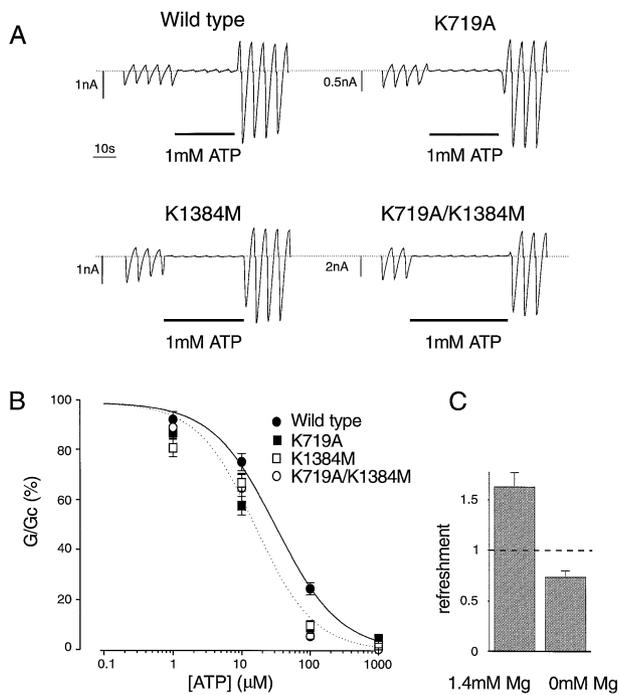
involved in nucleotide inhibition of channel activity and suggesting that Mg-ATP hydrolysis may partially relieve the inhibitory action of ATP on wild-type currents. We also found that metabolic inhibition led to activation of wild-type and K1384M currents, but not K719A or K719A/K1384M currents. This argues that there may be a factor in addition to ATP and ADP that regulates channel activity. These studies clarify the mechanism of action of nucleotides on K-ATP channels and demonstrate that the  $W_A$  lysines of SUR1 play an essential role in the channel activation by Mg-ADP and diazoxide.

## Results

### Effects of ATP

We examined the effects of ATP on wild-type and  $W_A$  mutant K-ATP channels, using inside-out giant patches excised from oocytes coinjected with Kir6.2 and either wild-type or mutant SUR1. The patch conductance was very low in the cell-attached configuration but increased rapidly following excision of the patch into nucleotide-free solution (Figure 1). This may be attributed to relief of the blocking effect of cytoplasmic ATP (Gribble *et al.*, 1997). For wild-type channels, the mean increase in current at  $-100$  mV was  $65 \pm 15$ -fold ( $n = 10$ ). The mean current amplitudes at  $-100$  mV following patch excision were:  $-3.9 \pm 0.8$  nA ( $n = 12$ ) for wild-type,  $-2.2 \pm 0.8$  nA ( $n = 12$ ) for K719A,  $-5.0 \pm 1.9$  nA ( $n = 11$ ) for K1384M and  $-2.6 \pm 0.9$  nA ( $n = 8$ ) for K719A/K1384M. These values are not significantly different (n.s., ANOVA), indicating that expression of the  $W_A$  mutant channels is not compromised by the mutation.

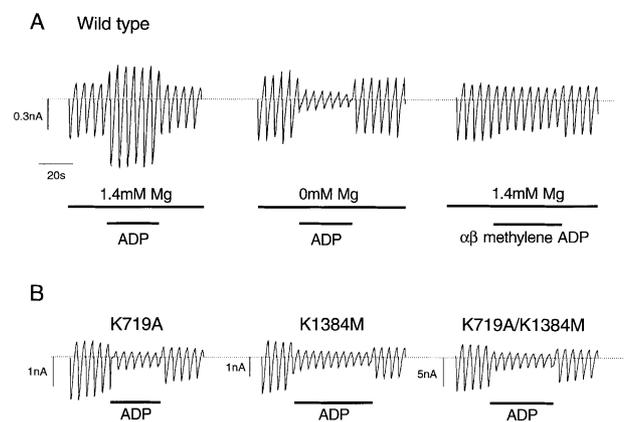
Native  $\beta$ -cell K-ATP channels are blocked by ATP in the absence of  $Mg^{2+}$  or by non-hydrolysable ATP analogues, suggesting that channel inhibition does not involve ATP hydrolysis (Ashcroft and Rorsman, 1989). In agreement with this idea, all of the  $W_A$  mutant channels were blocked by application of ATP to the intracellular side of the membrane (Figure 2). Indeed, they were all slightly more ATP sensitive ( $P < 0.005$ , ANOVA) than the wild-type channel: the mean  $K_i$  was  $34.5 \pm 0.4$   $\mu$ M ( $n = 10$ ) for wild-type,  $13.6 \pm 0.2$   $\mu$ M ( $n = 8$ ) for K719A,  $15.7 \pm 0.2$   $\mu$ M ( $n = 6$ ) for K1384M and  $16.5 \pm 0.3$   $\mu$ M ( $n = 5$ ) for K719A/K1384M. There was no significant difference between the  $K_i$  obtained for



**Fig. 2.** Effects of ATP on wild-type and mutant K-ATP channels. (A) Macroscopic currents recorded from four different inside-out patches in response to a series of voltage ramps from  $-110$  to  $+100$  mV (holding potential,  $0$  mV). Oocytes were coinjected with mRNAs encoding Kir6.2 and either wild-type SUR1, K719A-SUR1, K1384M-SUR1 or the double SUR1 mutant K719A/K1384M.  $1$  mM Mg-ATP was added to the internal solution as indicated by the bar. (B) Mean ATP dose-response relationships for wild-type K-ATP channels (●,  $n = 10$ ) and the mutant K-ATP channels K719A (■,  $n = 8$ ), K1384M (□,  $n = 6$ ) or K719A/K1384M (○,  $n = 5$ ). Test solutions were alternated with control solutions and the slope conductance ( $G$ ) is expressed as a fraction of the mean ( $G_c$ ) of that obtained in control solution before and after exposure to ATP. Conductance was measured between  $-20$  and  $-100$  mV and is the mean of five voltage ramps. The lines are the best fit to the data of the Hill equation (equation 1) using the mean values for  $K_i$  and  $n$  given in the text. Solid line fit to wild-type currents; dotted line fit to K719A/K1384M currents. (C) Mean amplitude of wild-type K-ATP currents recorded after exposure to  $1$  mM ATP, expressed as a fraction of the current amplitude before exposure to ATP, in Mg-free ( $n = 5$  patches) or Mg-containing solution ( $n = 12$  patches). The dashed line indicates the control current level (before ATP).

the mutant channels (n.s., ANOVA). The Hill coefficients were unaffected by mutation of the W<sub>A</sub> lysines, being  $1.03 \pm 0.06$  ( $n = 10$ ) for currents formed from Kir6.2 and wild-type SUR1,  $0.99 \pm 0.10$  ( $n = 8$ ) for K719A,  $1.35 \pm 0.13$  ( $n = 6$ ) for K1384M and  $0.99 \pm 0.14$  ( $n = 5$ ) for K719A/K1384M (n.s., ANOVA). These data demonstrate that neither the Walker A lysine of NBD1 nor that of NBD2 is essential for ATP-induced inhibition of the  $\beta$ -cell K-ATP channel.

In excised patches, K-ATP channel activity declines with time. This run-down may be reversed by the addition of Mg-ATP to the intracellular solution (Ohno-Shosaku *et al.*, 1987). Since ATP also blocks K-ATP currents, ‘refreshment’ of channel activity is only observed following ATP removal (Figure 2A). Studies on native  $\beta$ -cells have suggested that ‘refreshment’ requires ATP hydrolysis, since it is not supported by non-hydrolysable ATP analogues (Ohno-Shosaku *et al.*, 1987). In agreement with this idea, wild-type K-ATP currents were not refreshed by



**Fig. 3.** Effects of ADP on wild-type and mutant K-ATP channels. (A) Macroscopic wild-type K-ATP currents recorded from the same inside-out patch in response to a series of voltage ramps from  $-110$  mV to  $+100$  mV (holding potential,  $0$  mV). Nucleotides were applied as indicated by the bars. Left, effect of  $100$   $\mu$ M ADP in the presence of  $1.4$  mM Mg<sup>2+</sup>; centre, effect of  $100$   $\mu$ M ADP in the absence of Mg<sup>2+</sup>; right, effect of  $100$   $\mu$ M  $\alpha$ - $\beta$ -methylene ADP in the presence of  $1.4$  mM Mg<sup>2+</sup>. (B) Macroscopic currents recorded from mutant K-ATP channels in inside-out patches in response to a series of voltage ramps from  $-110$  to  $+100$  mV (holding potential,  $0$  mV).  $1.4$  mM Mg<sup>2+</sup> was present throughout and ADP ( $100$   $\mu$ M) was applied as indicated by the bar.

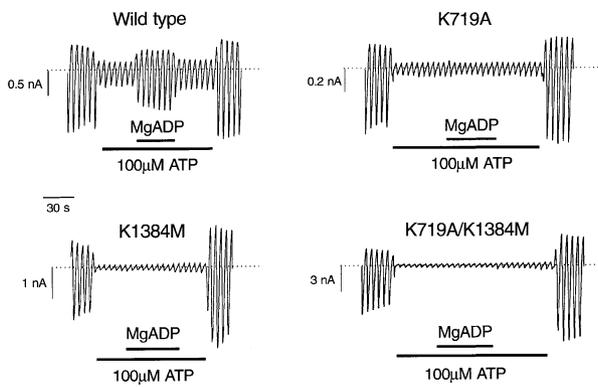
ATP in the absence of Mg<sup>2+</sup> (Figure 2C). Since all W<sub>A</sub> mutant channels showed ‘refreshment’ in the presence of Mg<sup>2+</sup> (Figure 2A), it is unlikely that the ATP hydrolysis required for ‘refreshment’ of channel activity takes place at the NBDs of SUR1.

#### Effects of Mg-ADP

Next, we investigated the effects of ADP on K-ATP currents. Figure 3A and C shows that ADP potentiated wild-type currents in the presence of intracellular Mg<sup>2+</sup> but that  $\alpha$ - $\beta$ -methylene Mg-ADP (a non-hydrolysable ADP analogue), or ADP in the absence of Mg<sup>2+</sup>, were inhibitory. This confirms earlier studies of native K-ATP channels (Larsson *et al.*, 1993). All of the W<sub>A</sub> mutant channel currents were inhibited rather than activated by Mg-ADP (Figures 3B and 5), indicating that the effects of Mg-ADP are mediated by interaction of the nucleotide diphosphate with the NBDs of SUR1 and that the Walker A lysine residues play a critical role in this interaction. Furthermore, both NBDs are required for channel activation: interaction of Mg-ADP with a single NBD is not sufficient because neither K719A nor K1384M currents were enhanced by Mg-ADP. In this context, it is of interest that Hopkins *et al.* (1992) required the presence of two MgADP binding sites in order to model the stimulatory effects of the nucleotide on K-ATP channel activity.

It is well established that Mg-ADP can, at least partially, reverse the inhibitory effects of ATP on the K-ATP channel (Dunne and Petersen, 1986; Kakei *et al.*, 1986). This was not the case for the W<sub>A</sub> mutant channels (Figures 4 and 5), which argues that interaction of Mg-ADP with both NBDs is needed to relieve channel inhibition by ATP. It also indicates that the potentiatory effect of Mg-ADP is mediated by the same mechanism in both the presence and absence of ATP.

It is noteworthy that, in contrast to wild-type channels, Mg-ADP inhibits the W<sub>A</sub> mutant K-ATP currents



**Fig. 4.** Effects of ADP on ATP-inhibition of wild-type and mutant K-ATP channels. Macroscopic currents recorded from four different inside-out patches in response to a series of voltage ramps from  $-110$  mV to  $+100$  mV (holding potential,  $0$  mV). Oocytes were coinjected with mRNAs encoding Kir6.2 and either wild-type SUR1, K719A-SUR1, K1384M-SUR1 or the double SUR1 mutant K719A/K1384M;  $100 \mu\text{M}$  MgATP and  $100 \mu\text{M}$  MgADP were added to the internal solutions as indicated by the bars.

(Figure 5). The extent of this inhibition was comparable with that observed for wild-type channels when ADP was applied in the absence of  $\text{Mg}^{2+}$ . The inhibitory effects of  $\alpha$ - $\beta$ -methylene Mg-ADP, or ADP in the absence of  $\text{Mg}^{2+}$ , were also similar for wild-type and mutant channels. These data support the idea that Mg-ADP potentiates and inhibits K-ATP channel activity by separate mechanisms and that the inhibitory effect is unmasked when activation is abolished (Bokvist *et al.*, 1991). The reduced ATP sensitivity of the wild-type channel (Figure 2B) may thus result from a simultaneous activation by Mg-ADP (formed from hydrolysis of Mg-ATP) which does not occur in  $W_A$  mutant channels. The fact that the percentage block of the  $W_A$  mutant currents by Mg-ADP is smaller in the presence of ATP than in its absence, also suggests that ATP and ADP may compete for the same inhibitory site.

#### Effects of AMP

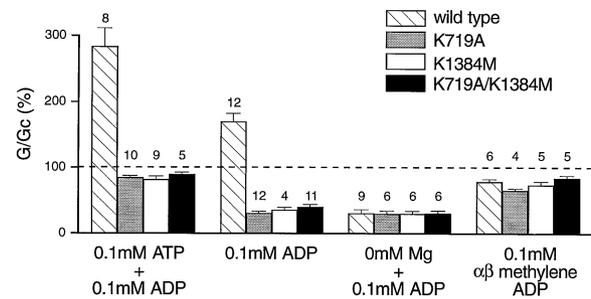
Mutation of the  $W_A$  lysines did not alter the effects of AMP on the cloned channel. AMP ( $100 \mu\text{M}$ ) blocked wild-type currents by  $3.9 \pm 5.7\%$  ( $n = 6$ ) and K719A/K1384M currents by  $12.8 \pm 2.0\%$  ( $n = 6$ ) (n.s. by *t*-test between wild-type and mutant channels).

#### Effects of $\text{Mg}^{2+}$

The activity of native  $\beta$ -cell K-ATP channels is inhibited by  $\text{Mg}^{2+}$  ions, with a  $K_i$  of  $5.4$  mM (Ashcroft and Kakei, 1988). Mutation of the  $W_A$  lysines did not significantly alter the sensitivity of the cloned channel to  $\text{Mg}^{2+}$  ions. Magnesium ( $1.4$  mM, total) blocked the conductance (measured between  $-20$  and  $-100$  mV) by  $15.3 \pm 1.6\%$  ( $n = 8$ ) in wild-type channels, by  $17.4 \pm 0.6\%$  ( $n = 5$ ) in K719A channels,  $18.2 \pm 1.0\%$  ( $n = 4$ ) in K1384M channels and by  $12.9 \pm 2.3\%$  ( $n = 6$ ) in K719A/K1384M channels (n.s., ANOVA).

#### Effects of diazoxide

The drug diazoxide is a potent potentiator of K-ATP channel activity (Dunne *et al.*, 1993; Kozłowski, 1994). In  $\beta$ -cells, this effect requires the presence of hydrolysable ATP at the cytoplasmic side of the membrane (Kozłowski



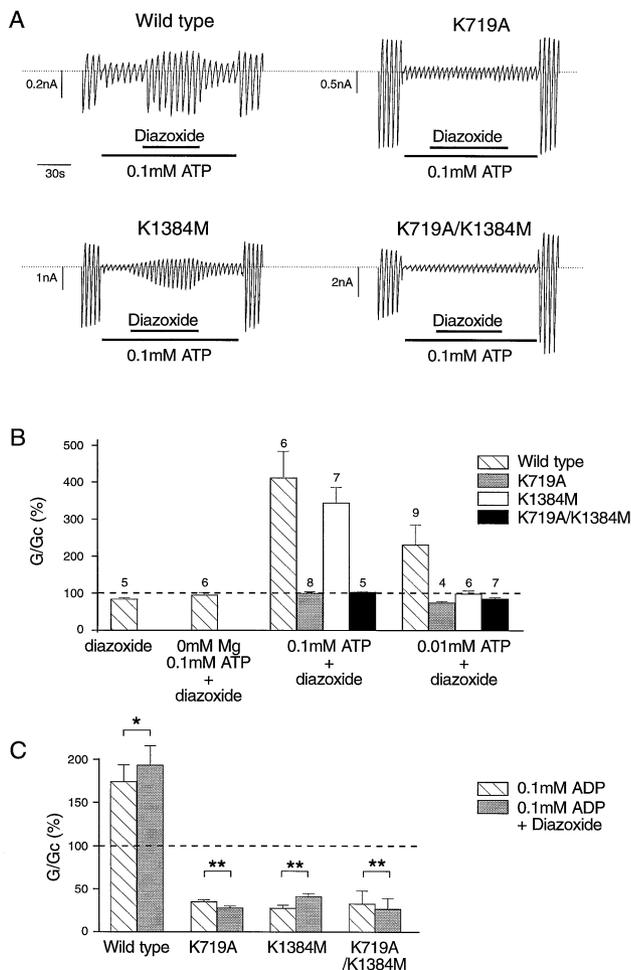
**Fig. 5.** Mean amplitude of wild-type or mutant K-ATP currents recorded in intracellular solution with the additions indicated, expressed as a percentage of the current amplitude in the absence of ADP. The dashed line indicates the control (ADP-free) current level. The number of patches is indicated above each bar. Unless indicated, all solutions contain  $1.4$  mM  $\text{Mg}^{2+}$ .

*et al.*, 1989; Larsson *et al.*, 1993). Figure 6A shows that, in the presence of  $100 \mu\text{M}$  Mg-ATP, diazoxide activated both wild-type and K1384M currents but did not affect K719A or K719A/K1384M currents. When Mg-ATP was reduced to  $10 \mu\text{M}$ , wild-type currents were enhanced by diazoxide, K719A and K719A/K1384M were inhibited and K1384M was unaffected (Figure 6B). In the absence of ATP, or in Mg-free ATP solution, wild-type currents were also blocked by diazoxide (Figure 6B). Diazoxide acted more slowly on K1384M than the wild-type channel (Figure 6A), suggesting that NBD2 may enhance diazoxide activation or that the NBDs interact. The requirement for the  $W_A$  lysines of both NBDs at low ATP concentrations supports this idea.

The effects of diazoxide in the presence of the nucleotide diphosphate Mg-ADP are summarized in Figure 6C. In the presence of  $100 \mu\text{M}$  Mg-ADP, diazoxide slightly potentiated wild-type and K1384M currents and inhibited K719A and K719A/K1384M currents. There are several possible explanations for the fact that Mg-ADP appears less efficient at supporting the stimulatory action of diazoxide than Mg-ATP. First, the level of channel activity is already high in the presence of Mg-ADP, whereas it is low in the presence of Mg-ATP: the relative extent of channel activation may therefore be limited by the fact that the channel open probability cannot exceed one. Indeed, wild-type currents in the presence of  $100 \mu\text{M}$  ADP plus diazoxide were even larger than those in the presence of  $10 \mu\text{M}$  ATP plus diazoxide. The mean currents evoked by  $340 \mu\text{M}$  diazoxide were  $194 \pm 22\%$  ( $n = 5$ ) with  $100 \mu\text{M}$  ADP compared with  $131 \pm 7\%$  ( $n = 6$ ) with  $10 \mu\text{M}$  ATP, when expressed as a percentage of the current recorded in control solution lacking nucleotides and diazoxide. Secondly, if nucleotide hydrolysis is required for diazoxide action, this hydrolysis may occur more slowly with MgADP than with MgATP. A third possibility, which cannot be completely excluded, is that our ADP solution contains a small quantity of ATP either as a contaminant or formed from ADP by the action by enzymes present in the patch membrane.

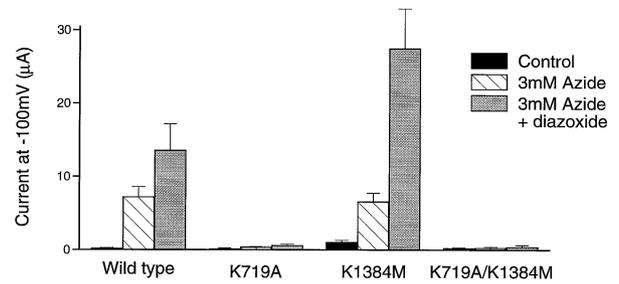
#### Effects of metabolic inhibition

We examined the effects of metabolic inhibition and of diazoxide on whole-cell currents recorded from intact oocytes (Figure 7). Under resting conditions, small currents, similar to those observed in control oocytes, were



**Fig. 6.** Effects of diazoxide on wild-type and mutant K-ATP channels. (A) Macroscopic currents recorded from wild-type, or mutant, K-ATP channels in inside-out patches in response to a series of voltage ramps from  $-110$  mV to  $+100$  mV (holding potential,  $0$  mV). Mg-ATP ( $100$   $\mu$ M) and diazoxide ( $340$   $\mu$ M) were applied as indicated by the bar. (B) Mean amplitude of wild-type or mutant K-ATP currents recorded in intracellular solution with the additions indicated, expressed as a percentage of the current amplitude in the absence of diazoxide. The dashed line indicates the control (diazoxide-free) current level. The number of patches is indicated above each bar. (C) Mean amplitude of wild-type or mutant K-ATP currents recorded in the presence of  $100$   $\mu$ M Mg-ADP (hatched bars) or  $100$   $\mu$ M Mg-ADP plus  $340$   $\mu$ M diazoxide (grey bars), expressed as a percentage of the current amplitude in the absence of either. The number of patches was five in each case. The dashed line indicates the control current level. \* $P < 0.05$ ; \*\* $P < 0.01$  using paired  $t$ -test.

recorded from oocytes expressing either wild-type or mutant K-ATP channels. Addition of Na-azide ( $3$  mM), which lowers cellular ATP levels by inhibition of cytochrome  $a_3$  and the  $F_1/F_0$  ATPase (Vasilyeva *et al.*, 1982), produced an increase in whole-cell currents in oocytes coinjected with wild-type SUR1 and Kir6.2, as previously described (Gribble *et al.*, 1997). Steady-state activation of K-ATP currents was achieved within  $15$  min. Metabolic inhibition increased, and diazoxide further potentiated both wild-type and K1384M whole-cell currents but was without effect in oocytes expressing K719A or K719A/K1384M. Similar results were found using another metabolic inhibitor, FCCP, which acts by uncoupling the mitochondrial electron transport chain from



**Fig. 7.** Effects of metabolic inhibition on wild-type and mutant whole-cell K-ATP currents. Mean whole-cell currents recorded at  $-100$  mV (holding potential,  $-10$  mV) before exposure to  $3$  mM azide,  $10$  min afterwards, and in the presence of azide plus  $340$   $\mu$ M diazoxide. The number of oocytes was: wild type ( $n = 13$ ), K719A ( $n = 8$ ), K1384M ( $n = 12$ ) and K719A/K1384M ( $n = 5$ ).

ATP synthesis (data not shown). In excised patches, azide induced a small ( $<10\%$ ) block of K-ATP currents which was not significantly different in wild-type and mutant channels (S.Trapp, personal communication). These data therefore argue that the  $W_A$  lysine in NBD1, but not NBD2, is essential for metabolic regulation of K-ATP channel activity.

## Discussion

Our results are consistent with the idea that nucleotides interact with the  $\beta$ -cell K-ATP channel at three sites: an inhibitory site which binds free nucleotides, a site involved in Mg-ATP 'refreshment' of channel activity and a site which mediates channel activation by Mg-ADP and diazoxide. They also show that the  $W_A$  motifs of the NBDs of the SUR1 subunit of the channel constitute an essential part of the latter site.

Mutation of the  $W_A$  lysine in either NBD1 or NBD2 did not prevent channel inhibition by ATP. This mutation is predicted to abolish ATP hydrolysis without affecting nucleotide binding and we therefore conclude that ATP hydrolysis by the NBDs of SUR1 is not required for channel inhibition by ATP. This is in agreement with numerous earlier studies which have shown that native K-ATP channels are strongly blocked by ATP in the absence of  $Mg^{2+}$  and by non-hydrolysable ATP analogues (Ashcroft and Rorsman, 1989). Our results do not allow us to decide whether or not the site at which ATP binds to inhibit K-ATP channel activity is located on either of the NBDs of SUR1. There are at least two possible explanations for our finding that all of the  $W_A$  mutant channels were slightly more sensitive to ATP than wild-type channels. First, Mg-ADP may be formed from hydrolysis of Mg-ATP (either by the  $W_A$  motifs themselves or by other ATPases present in the patch) and partially relieve the inhibitory effects of ATP in wild-type, but not mutant, channels. Secondly, Mg-ATP hydrolysis is predicted to occur at the  $W_A$  motifs of SUR1. It is possible that this hydrolysis reaction may itself result in enhanced channel activity in wild-type channels. The greater ATP sensitivity of the mutant channels is in agreement with the enhanced ATP sensitivity reported for native K-ATP channels in the absence of  $Mg^{2+}$  (Ashcroft and Kakei, 1989).

The prevailing view in the literature is that Mg-ADP activates K-ATP channels by binding to some regulatory site on the channel or an associated control protein and

producing a conformational change which enhances the channel open probability (Ashcroft and Rorsman, 1989; Bokvist *et al.*, 1991). This suggests two explanations for our results. One possibility is that mutation of the  $W_A$  lysines completely prevents Mg-ADP binding to the stimulatory site and that neither the wild-type nor the mutant channel is blocked by  $\alpha$ - $\beta$ -methylene Mg-ADP as strongly as by ADP. Alternatively, the  $W_A$  mutations do not alter Mg-ADP binding but instead prevent transduction of the conformational change which leads to channel activation. Although we cannot exclude the former possibility, we favour the latter because the  $W_A$  mutations are not expected to affect nucleotide binding. How then might mutation of the  $W_A$  lysine affect the ability of bound Mg-ADP to cause channel opening? The simplest explanation is that the mutation causes a structural change which prevents a conformational change induced by Mg-ADP binding: both alanine and methionine are uncharged and have smaller side-chains than lysine. In this case, the failure of  $\alpha$ - $\beta$ -methylene Mg-ADP to potentiate wild-type currents is explained by a lower binding affinity and/or a reduced ability to cause a conformational change.

Another possibility, however, is that the conformational change which facilitates channel opening requires hydrolysis of Mg-ADP at the NBDs of SUR1. Several pieces of evidence support this idea. First, Mg-ADP was inhibitory in the absence of  $Mg^{2+}$ , a cation which is required for nucleotide hydrolysis. Secondly, the non-hydrolysable analogue  $\alpha$ - $\beta$ -methylene Mg-ADP did not cause channel activation. Thirdly, Mg-ADP is actually inhibitory when the  $W_A$  lysines are mutated. Since mutation of the  $W_A$  lysines abolishes or markedly decreases ATP hydrolysis in all ABC transporters examined (Azzaria *et al.*, 1989; Carson *et al.*, 1995; Ko and Pedersen, 1995; Koronakis *et al.*, 1995), a similar effect may be expected for K719A, K1384M and K719A/K1384M. It therefore seems possible that, in addition to ATP hydrolysis, Mg-ADP hydrolysis is impaired by the  $W_A$  mutations. Although this idea has not been previously suggested in the literature, it is one which is consistent with our data. Finally, the function of the other ABC transporters is impaired by mutations in the conserved  $W_A$  or  $W_B$  motifs which reduce ATP hydrolysis but do not abolish ATP binding or Mg-ATP-induced conformational changes (Koronakis *et al.*, 1995). A second possibility, therefore, is that MgADP hydrolysis at the NBDs might produce some conformational change in SUR1 which enhances K-ATP channel opening. If this idea is correct, then hydrolysis at both NBDs must be required to sustain channel activation because mutation of only one NBD removed the ability of Mg-ADP to activate the current. Furthermore, since Mg-AMP was without effect on either wild-type or native (Kakei *et al.*, 1986) channels, the process of hydrolysis itself, rather than the reaction product (AMP), must induce the conformational change. An analogy would be the myosin head where it is the hydrolysis of ATP which induces a conformational change of the molecule.

Although our data argue that the  $W_A$  lysine residues are required for Mg-ADP binding to produce a conformational change which leads to channel activation, they do not allow us to conclude whether or not this conformational change requires nucleotide diphosphate hydrolysis. This

will require measurement of the capacity of the NBDs of SUR1 to hydrolyse ATP and ADP.

The mechanism by which Mg-ATP refreshes channel activity must be different from that which mediates the potentiatory effects of Mg-ADP, since the  $W_A$  lysine mutants are unaffected by Mg-ADP but show normal refreshment of channel activity with Mg-ATP. Perhaps this is not surprising since the effects of Mg-ATP persist for some time after its removal whereas Mg-ADP effects are immediately reversed when the nucleotide is removed. The persistence of refreshment after Mg-ATP removal suggests that it may involve protein phosphorylation. Whether this phosphorylation occurs on the Kir6.2 subunit or on SUR1 remains to be determined.

Our results also shed light on the molecular mechanism of diazoxide action. It has previously been argued that diazoxide action involves protein phosphorylation, either of the K-ATP channel or of a regulatory protein: indeed, diazoxide has even been suggested to act by stimulating a protein kinase (Dunne *et al.*, 1993; Kozlowski, 1994). Our data suggest that this is not the case. Rather, they demonstrate that the interaction of MgATP (or Mg-ADP) with the  $W_A$  lysine of NBD1 is needed for channel activation by diazoxide, and that while NBD2 is not essential, it speeds the rate of channel activation by the drug. This suggests that the NBDs of SUR1 may interact with each other, as has been suggested for other ABC transporters (Higgins, 1992); such interaction may also account for the fact that mutation of the  $W_A$  lysine in a single NBD is sufficient to prevent Mg-ADP activation of the K-ATP channel. However, the fact that the  $W_A$  lysine of NBD1, but not NBD2, is absolutely required for the effects of metabolic inhibition and diazoxide indicates that the two NBDs are not functionally equivalent. We speculate that nucleotide binding, or hydrolysis, at NBD1 potentiates or prolongs K-ATP channel opening and that diazoxide may stabilize this active state. Our data are reminiscent of the differential action of the NBDs of CFTR on the intrinsic chloride channel activity of that protein, where it has been suggested that nucleotide hydrolysis at NBD1 initiates a burst of activity whereas hydrolysis at NBD2 terminates the burst (Carson *et al.*, 1995).

Our results suggest that the  $W_A$  lysine of NBD1, but not NBD2, is required for coupling metabolic inhibition to channel activation in intact oocytes, because whole-cell K1384M currents, but not K719A currents, were activated by exposure to azide. Since both K719A and K1384M channels have similar ATP sensitivities and neither are upregulated by 100  $\mu$ M Mg-ADP, this result also argues that, in addition to ATP and ADP, there may be a previously unrecognized intracellular substance that regulates channel activity. Nichols *et al.* (1996) reported that mutation of the  $W_B$  aspartate 1505 in NBD2 to alanine, which is expected to decrease nucleotide binding, removed the ability of metabolic inhibition to activate the cloned K-ATP channel. Thus we speculate that hydrolysis at NBD1, and binding of Mg-ADP at NBD2, facilitates the ability of an additional coupling factor to enhance K-ATP channel activity. Although the identity of the coupling factor remains unknown, we can exclude  $Mg^{2+}$ , reduced pyridine nucleotides and oleoyl co-A, all of which have been postulated to serve as metabolic coupling

factors, because all of these had similar effects on wild-type and mutant channels (above and our unpublished observations). We point out that our data do not allow us to distinguish between a coupling factor whose concentration changes with metabolism and a coupling factor which is present at a constant level in the cell, but which requires the interaction of MgADP with NBD1 for its action. The oocyte is, of course, not a  $\beta$ -cell and may exhibit different metabolic pathways: it therefore remains to be established if the coupling factor also contributes to regulation of K-ATP channel activity in  $\beta$ -cells.

In conclusion, our results provide new insight into the way in which SUR1 regulates K-ATP channel activity. It is becoming clear that, like SUR1, many other ABC transporters regulate ion channels and that this regulation is of clinical relevance, as, for example, in cystic fibrosis, diabetes and PHHI (Higgins, 1995). It seems possible that at least some of the mechanisms by which SUR1 and Kir6.2 interact may be generally applicable to other ABC transporter-channel interactions.

## Materials and methods

### Nomenclature

In this paper we use the nomenclature of Duopnik *et al.* (1995). Kir6.2 refers to BIR1 (GenBank accession No. D50581). Kir6.2 was cloned from a mouse insulinoma cDNA library (Sakura *et al.*, 1995) and SUR1 (Aguilar-Bryan *et al.*, 1995; GenBank accession No. L40624) was cloned from rat insulinoma cells.

### Molecular biology

Mouse Kir6.2 and wild-type or mutant rat SUR1 were cloned into the vector pBF (which provides 5' and 3' untranslated regions of the *Xenopus*  $\beta$ -globin genes; B.Fakler) for mRNA synthesis. Capped mRNA was synthesized by *in vitro* transcription from linearized cDNA, as described previously (Tucker *et al.*, 1996). Site-directed mutagenesis of SUR1 was carried out by subcloning the appropriate fragments into the pALTER vector (Promega).

### Electrophysiology

*Xenopus* oocytes were defolliculated and coinjected with ~5 ng each of mRNAs encoding Kir6.2 and either wild-type or mutant SUR1, as indicated. 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 (in mM): 88 NaCl, 1 KCl, 1.7 MgSO<sub>4</sub>, 0.47 Ca(NO<sub>3</sub>)<sub>2</sub>, 0.41 CaCl<sub>2</sub>, 2.4 NaHCO<sub>3</sub>, 10 HEPES (pH 7.4), supplemented with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 5 mM pyruvate. Currents were studied 1–4 days after injection.

Whole-cell currents were measured using a 2-electrode voltage-clamp (Geneclamp 500, Axon Instruments, Foster City, USA). Voltages were applied and currents recorded using a microcomputer with an Axolab interface and pClamp software (Axon Instruments). Currents were filtered at 1 kHz and digitized at 4 kHz. Current and voltage electrodes were filled with 3 M KCl and had resistances of 0.5–2 M $\Omega$ . Transmembrane potential was measured differentially between the intracellular electrode and a second bath electrode positioned close to the oocyte on the downstream side, in order to minimize series resistance errors. The bath electrodes consisted of Ag/AgCl<sub>2</sub> pellets connected to the bath by agar bridges filled with 3 M KCl. Oocytes were perfused continuously with a solution containing (mM): 90 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 5 HEPES (pH 7.4 with KOH), plus drugs as indicated, at 18–24°C. Diazoxide was prepared from stock solution (68 mM) in water as required. Na-azide was prepared as a stock solution (1000 $\times$ ) in water. Whole-cell currents were measured 280–295 ms after the start of the voltage pulse. Control oocytes were injected with water and had mean currents of 0.3  $\pm$  0.1  $\mu$ A in control solution and 0.2  $\pm$  0.1  $\mu$ A in the presence of 3 mM azide.

Macroscopic currents were recorded from giant excised inside-out patches (Hilgemann *et al.*, 1991), at 20–24°C using 200–400 k $\Omega$  electrodes. The pipette solution contained (mM): 140 KCl, 1.2 MgCl<sub>2</sub>, 2.6 CaCl<sub>2</sub>, 10 HEPES (pH 7.4 with KOH) and the internal (bath) solution contained (mM): 110 KCl, 1.44 MgCl<sub>2</sub>, 30 KOH, 10 EGTA, 10 HEPES

(pH 7.2 with KOH) and nucleotides as indicated. The Mg-free solution contained (in mM): 110 KCl, 30 KOH, 2.6 CaCl<sub>2</sub>, 10 EDTA, 10 HEPES (pH 7.2 with KOH). 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. Currents were recorded with an EPC7 amplifier (List Elektronik, Darmstadt, Germany), filtered at 0.2 kHz and sampled at 0.5 kHz.

### Data analysis

All data are given as mean  $\pm$  SEM. The symbols in the figures indicate the mean and the vertical bars one SEM (where this is larger than the symbol). ATP dose-response relationships were fitted to the Hill equation:

$$\frac{G}{G_c} = \frac{1}{1 + ([ATP]/K_i)^n} \quad \text{equation 1}$$

where [ATP] is the ATP concentration,  $K_i$  is the ATP concentration at which inhibition is half maximal and  $n$  is the slope factor (Hill coefficient).

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