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

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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 β-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 β-cell stimulus–secretion coupling is that when plasma glucose levels rise, glucose uptake and metabolism by the pancreatic β-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 β-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 β-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 β-cell and inhibiting insulin release (Dunne et al., 1993). Diazoxide is sometimes used to treat PHHI.

The regulation of the β-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 β-cell K-ATP channel (Dunne et al., 1993). In β-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 β-cell K-ATP channel is a complex of two proteins (Inagaki et al., 1995; Sakura et al., 1995): Kir6.2 and

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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 (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 conserved 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 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 co-injected 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 ± 15-fold (n = 10). The mean current amplitudes at –100 mV following patch excision were: –3.9 ± 0.8 nA (n = 12) for wild-type, –2.2 ± 0.8 nA (n = 12) for K719A, –5.0 ± 1.9 nA (n = 11) for K1384M and –2.6 ± 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 β-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 ± 0.4 μM (n = 10) for wild-type, 13.6 ± 0.2 μM (n = 8) for K719A, 15.7 ± 0.2 μM (n = 6) for K1384M and 16.5 ± 0.3 μM (n = 5) for K719A/K1384M. There was no significant difference between the \(K_i\) obtained for...
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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). The lines are the best fit to the data of the but that Conductance was measured between –20 and –100 mV and is the wild-type currents in the presence of intracellular Mg2+. Test solutions were alternated with control solutions and the slope conductance (G) is expressed as a fraction of the mean (Gc) 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 Kc 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 Wα lysines, being 1.03 ± 0.06 (n = 10) for currents formed from Kir6.2 and wild-type SUR1, 1.35 ± 0.13 (n = 6) for K1384M and 0.99 ± 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 β-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 β-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 ATP in the absence of Mg2+ (Figure 2C). Since all Wα mutant channels showed ‘refreshment’ in the presence of Mg2+ (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 Mg2+, but that α-β-methylene Mg-ADP (a non-hydrolysable ADP analogue), or ADP in the absence of Mg2+, were inhibitory. This confirms earlier studies of native K-ATP channels (Larsson et al., 1993). All of the Wα 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 the ‘refreshment’ of channel activity takes place at the NBDs of SUR1.

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., 1993). All of the Wα 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 Wα 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 Wα mutant K-ATP currents.
Mutation of the W4 lysines did not significantly alter recorded in control solution lacking nucleotides on current sensitivity of the wild-type channel (Dunne et al., 1991). The reduced ATP inhibition of wild-type and mutant channels. ADP plus diazoxide were even larger than those in the presence of 100 μM Mg-ATP, 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 W4 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 μM 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 μM ADP plus diazoxide were even larger than those in the presence of 10 μM ATP plus diazoxide. The mean currents evoked by 340 μM diazoxide were 194 ± 22% (n = 5) with 100 μM ADP compared with 131 ± 7% (n = 6) with 10 μM 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.

The activity of native β-cell K-ATP channels is inhibited by Mg2+ ions, with a K_i of 5.4 mM (Ashcroft and Kakei, 1988). Mutation of the W4 lysines did not significantly alter the sensitivity of the cloned channel to Mg2+ ions. Magnesium (1.4 mM, total) blocked the conductance (measured between -20 and -100 mV) by 15.3 ± 1.6% (n = 8) in wild-type channels, by 17.4 ± 0.6% (n = 5) in K719A channels, 18.2 ± 1.0% (n = 4) in K1384M channels and by 12.9 ± 2.3% (n = 6) in K719A/K1384M channels (n.s. by t-test between wild-type and mutant channels).

Effects of diazoxide

The drug diazoxide is a potent potentiator of K-ATP channel activity (Dunne et al., 1993; Kozlowski, 1994). In β-cells, this effect requires the presence of hydrolysable ATP at the cytoplasmic side of the membrane (Kozlowski et al., 1989; Larsson et al., 1993). Figure 6A shows that, in the presence of 100 μM 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 μM, 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 W4 lysines of both NBDs at low ATP concentrations supports this idea.

The 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
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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 µM 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 WA 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 β-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 WA motifs of the NBDs of SUR1 subunit of the channel constitute an essential part of the latter site.

Mutation of the WA 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 Mg²⁺ 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 WA mutant channels were slightly more sensitive to ATP than wild-type channels. First, Mg-ADP may be formed from hydrolysis 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 a₃ and the F₁/F₀ 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 the WA motifs of the SUR1 subunit. There are at least two possible explanations for our finding that all of the WA mutant channels were slightly more sensitive to ATP than wild-type channels. First, Mg-ADP may be formed from hydrolysis by the WA 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 WA 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 Mg²⁺ (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

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**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 µM) and diazoxide (340 µM) 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 µM Mg-ADP (hatched bars) or 100 µM Mg-ADP plus 340 µM 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.
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 W₁ lysines completely prevents Mg-ADP binding to the stimulatory site and that neither the wild-type nor the mutant channel is blocked by α-β-methylene Mg-ADP as strongly as by ADP. Alternatively, the W₁ 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 W₁ mutations are not expected to affect nucleotide binding. How then might mutation of the W₁ 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 α-β-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 Mg²⁺, a cation which is required for nucleotide hydrolysis. Secondly, the non-hydrolysable analogue α-β-methylene Mg-ADP did not cause channel activation. Thirdly, Mg-ADP is actually inhibitory when the W₁ lysines are mutated. Since mutation of the W₁ 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 W₁ 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 W₁α or W₁β 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 W₁ 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 W₁ 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 W₁ 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 W₁ lysine in a single NBD is sufficient to prevent Mg-ADP activation of the K-ATP channel. However, the fact that the W₁ 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 W₁ 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 µM 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 W₁ 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 Mg²⁺, 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 β-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 β-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 Duoupin et al. (1995). Kir6.2 refers to BIRR (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 β-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 cocrafted 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, Bokvist, K., A¨ mma ¨ la¨ , C., Ashcroft, F.M., Berggren, P.O., Larsson, O. and mdr-1 Xenopus (pH 7.4), supplemented with 100 U/ml penicillin, 100 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 MΩ. 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 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 MgCl2, 1.8 CaCl2, 5 HEPES (pH 7.4) supplemented with 100 U/ml penicillin, 100 μg/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 MΩ. 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 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 MgCl2, 1.8 CaCl2, 5 HEPES (pH 7.4) 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 (1000x) 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 ± 0.1 μA in control solution and 0.2 ± 0.1 μA in the presence of 3 mM azide.

Macroscopic currents were recorded from giant excised inside-out patches (Helgemann et al., 1991), at 20–24°C using 200–400 kΩ electrodes. The pipette solution contained (mM): 140 KCl, 1.2 MgCl2, 2.6 CaCl2, 10 HEPES (pH 7.4 with KOH) and the internal (bath) solution contained (mM): 110 KCl, 1.44 MgCl2, 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 CaCl2, 10 EDTA, 10 HEPES (pH 7.2 with KOH). Rapid switching 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 Electronic, Darmstadt, Germany), filtered at 0.2 kHz and sampled at 0.5 kHz.

Data analysis

All data are given as mean ± 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:

\[
G_i = \frac{1}{1 + ([ATP]/K_i)^n}
\]

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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References


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