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Topical Review

The β-Cell K_{ATP} Channel

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Introduction

Insulin secretion from the pancreatic β-cell is regulated by changes in blood glucose concentration and an integrated network of hormonal, neural and paracrine signals (Ashcroft et al., 1994; Ashcroft & Ashcroft, 1992*a;* Brunicardi, Shavelle & Andersen, 1995; Smith & Bloom, 1995; D'Alessio, 1997; Prentki, Tornheim & Corkey, 1997). Of critical importance for initiation of secretion is the intracellular concentration of calcium ions (Wollheim, 1981; Hellman et al., 1992*b;* Berggren & Larsson, 1994). In the unstimulated β-cell $[Ca^{2+}]_i$ is maintained at a low level by the action of the plasma membrane and endoplasmic reticulum Ca-ATPases (Váradi, Molnár & Ashcroft, 1995). In response to an increase in blood glucose concentration to a stimulatory level the β-cell plasma membrane depolarizes to a potential at which voltage-dependent Ca-channels open. The resultant influx of calcium leads to an elevation of $[Ca^{2+}]_i$ which initiates exocytosis. Studies at the single-cell level have shown that the Ca signal is oscillatory as a result of a complex interplay between movement across the plasma membrane and uptake and release from the endoplasmic reticulum (Hellman et al., 1992*a*). Other agents capable of modifying the β-cell membrane potential have corresponding effects on insulin release. Of particular importance are the sulfonylureas such as tolbutamide (Loubatières, 1955) which are used to treat type II diabetes and which depolarize the β-cell and hence stimulate insulin secretion (Ashcroft et al., 1993). A related sulfonamide, diazoxide (Loubatières et al., 1966), on the other hand, which hyperpolarizes the β-cell and thereby inhibits insulin secretion, is used to treat the hyperinsulinemia arising from excessive insulin secretion (Dunne, Aynsley-Green & Lindley, 1997*a*). The story of the discovery of these compounds has been reviewed (Loubatières, 1969; Henquin, 1992; Ashcroft & Ashcroft, 1992*b*). Hormones and neurotransmitters modulate the primary response to glucose through a variety of second messenger pathways (Berggren et al., 1992; Strubbe & Steffens, 1993; Howell, Jones & Persaud, 1994; Brunicardi et al., 1995; Smith & Bloom, 1995; D'Allesio, 1997). Activation of muscarinic cholinergic receptors, for example, stimulates phospholipase C and potentiates glucose-induced insulin release through liberation of IP_3 (Biden, 1984) and diacyglycerol (Weng, Davies & Ashcroft, 1993). Epinephrine, however, leads to inhibition of insulin release via α2-adrenergic receptors (Morgan, 1987; Hirose et al., 1993).

Early studies into the biochemical basis for control of β-cell membrane potential and hence insulin secretion established that the effect of glucose is mediated by intracellular metabolism of the sugar (Ashcroft, 1980). It was further found that the changes in membrane potential elicited by glucose and by sulfonylureas reflect changes in K-permeability (Dean & Matthews, 1968; Henquin, 1980*a,b;* Henquin & Meissner, 1982). Patch-clamp studies identified the channel responsible as an ATP-sensitive K-channel (K_{ATP} channel) (Cook & Hales, 1984; Ashcroft, Ashcroft & Harrison, 1986), a type of inwardly rectifying K-channel first described in cardiac muscle cells (Noma, 1983). Cloning of the K_{ATP} channel subunits (Aguilar-Bryan et al., 1995; Inagaki et al., 1995*a;* Sakura et al., 1995) has led in the last few

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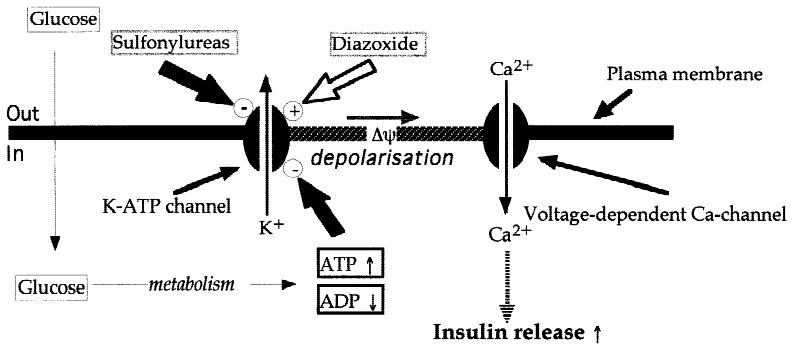

Fig. 1. Role of the K_{ATP} channel in control of insulin secretion. Closure of β-cell K_{ATP} channels occurs in response to an increase in glucose concentration via an increase in intracellular [ATP]/[ADP]. The ensuing depolarization leads to opening of voltage-dependent Ca-channels and an influx of calcium ions which triggers secretion. Sulfonylureas initiate secretion by directly binding to the K_{ATP} channel leading to channel closure. Diazoxide inhibits secretion by binding to and opening K_{ATP} channels.

years to a rapid growth in knowledge of the molecular details of the channel. In this review I shall summarize current views on the structure and function of the β-cell K_{ATP} channel.

The K_{ATP} Channel Plays a Central Role in β-Cell Function

The resting β-cell membrane potential of approximately −70 mV is determined by the activity of K_{ATP} channels (Ashcroft & Ashcroft, 1989). When blood glucose increases over the physiological range there is a corresponding increase in the rate of glucose utilization. Since glucose entry into the β-cell is rapid and non-rate limiting (Hellman, Sehlin & Taljedal, 1971) the metabolic response to an increase in glucose concentration is determined by D-glucose ATP-phosphotransferase which in the β-cell is the high K_m isoform, glucokinase (Ashcroft & Randle, 1970). Metabolic control analysis has demonstrated that the flux control coefficient for glucokinase is close to unity (Wang & Iynedjian, 1997) confirming earlier studies indicating that this enzyme may constitute the 'glucoreceptor' (Ashcroft, 1980). Consistent with this concept, altered expression of β-cell glucokinase either as a result of mutation in man (Froguel et al., 1992) or using gene knock-out technology in mice (Terauchi et al., 1995; Sakura et al., 1998) can lead to disordered insulin secretion. The β-cell has little capacity for glycogen synthesis, for pentose phosphate pathway flux or for fatty acid synthesis; the major metabolic fate of glucose is therefore glycolysis and oxidation via the Krebs cycle (Ashcroft, Hedeskov & Randle, 1971; Ashcroft et al., 1972). Unusually, the β-cell does not show a Pasteur effect i.e., an increase in [ATP]/[ADP] ratio does not cause feedback inhibition of glycolytic flux (Ashcroft, Weerasinghe & Randle, 1973; Hellman et al., 1975). Hence in the β-cell an increased rate of glucose metabolism leads to an increase in cytosolic [ATP]/[ADP]. This results in closure of K_{ATP} channels (Rorsman & Trube, 1985). The resulting depolarization leads to insulin release as described above. Measurements of β-cell [ATP]/[ADP] ratio have demonstrated that the increase occurs with sufficient rapidity to account for the subsequent changes in K_{ATP} channel activity and intracellular [Ca] (Detimary, Gilon & Henquin, 1998).

In contrast to the indirect closure of K_{ATP} channels by glucose, sulfonylureas elicit closure of K_{ATP} channels by direct interaction with the channel (Sturgess et al., 1985; Trube et al., 1986; Dunne, Illot & Petersen, 1987; Ashcroft et al., 1993). Diazoxide also interacts directly with the channel but leads to channel opening and thereby acts to inhibit insulin release (Trube, 1986; Dunne et al., 1987; Sturgess et al., 1998).

Thus, as summarized in Fig. 1, both the major physiological modulator of insulin secretion, glucose, and the main pharmacological agents used to alter insulin secretion rates clinically operate through K_{ATP} channels which are therefore central to β-cell function. The properties and function of the β-cell K_{ATP} channel have been extensively reviewed (Bryan & Aguilar-Bryan, 1997; Aguilar-Bryan et al., 1998; Ashcroft & Gribble, 1998; Babenko, Aguilar-Bryan & Bryan, 1998; Tucker & Ashcroft, 1998; Aguilar-Bryan & Bryan, 1999; Ashcroft & Gribble, 1999; Dunne et al., 1999; Miki, Nagashima & Seino, 1999; Schwanstecher et al., 1999; Seino, 1999).

K_{ATP} Channels Display a Characteristic Burst Activity

K_{ATP} channels are weakly inwardly rectifying K-selective channels (Ashcroft & Rorsman, 1989; Ashcroft & Ashcroft, 1990). Patch-clamp studies show that the β-cell K_{ATP} channel has a characteristic pattern of activity in which the channels rapidly flicker between the open state and a short closed state. The bursts are separated by long closed states. ATP increases the time spent in the long closed state by prolonging the interburst interval and reducing the burst duration without affecting the intraburst kinetics or the unitary conductance. This effect of ATP does not require the presence of Mg^{2+} ions. Sulfonylureas also shorten the mean burst duration and lengthen the interburst interval by stabilizing the long closed state (Gillis et al., 1989). ADP, in the presence of

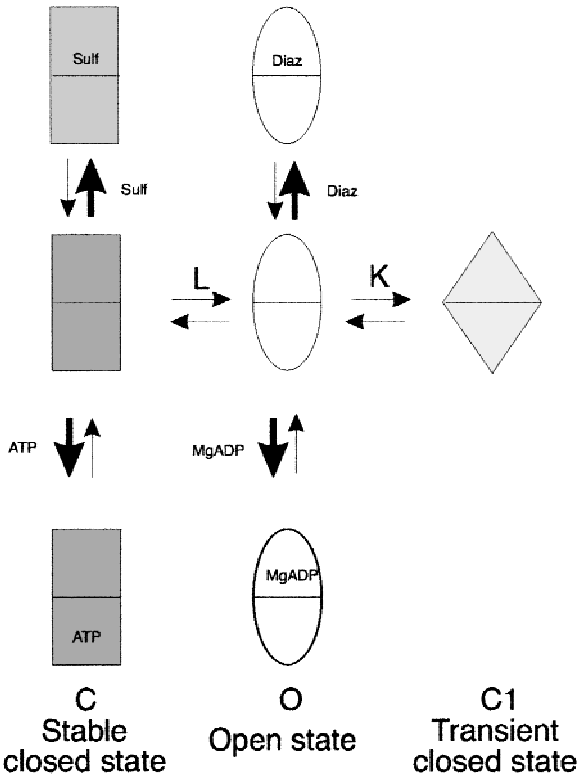

Fig. 2. Allosteric model for K_{ATP} channel function. The figure shows a minimal allosteric model for the β-cell K_{ATP} channel. In the absence of ligands the K_{ATP} channel can exist in two states, a stable long closed state (*C*) and an open state (*O*) which displays spontaneous rapid transitions to a short closed state (C1). Occupancy of the two states is described by the equilibrium constant L which determines the open probability of the channel. The intraburst kinetics are determined by the constant, K, for the transition to the short closed state. ATP and sulfonylureas bind preferentially to *C* and thereby reduce the time spent in the open state. MgADP and diazoxide bind preferentially to *O* and thereby activate the channels. By shifting the equilibrium between the two conformational states a ligand increases the fraction of the channel protein in the state with the higher affinity for that ligand while decreasing the apparent (macroscopic) affinity for the other ligand.

Mg ions, increases the open probability by shortening the interburst period and prolonging the burst duration, again without significant effect on intraburst kinetics or unitary channel conductance (Nichols et al., 1996). Channel openers such as diazoxide have a similar effect to MgADP (Kozlowski, Hales & Ashford, 1989; Larsson et al., 1993).

A minimal model to encompass these properties, using the allosteric theory of ligand-protein interaction (Monod, Wyman & Changeux, 1965), is shown in Fig. 2. In the absence of ligands the K_{ATP} channel can exist in two states, a stable closed state (*C*) and an open state (*O*) which displays spontaneous stochastic rapid transitions to a transient closed state (C_1) (Kakei & Noma, 1984). Occupancy of the two states is described by the equilibrium constant L. ATP and sulfonylureas bind preferentially to C and thereby reduce the time spent in the open state. MgADP and diazoxide bind preferentially to the open state and thereby activate the channels. By shifting the equilibrium between the two conformational states a ligand increases the fraction of the channel protein in the state with the higher affinity for that ligand while decreasing the apparent (macroscopic) affinity for the other ligand. The general equation for the effect of a ligand *X* on the apparent value, L_X, of the equilibrium constant, *L*, for the two states *C* and *O* each with *n* binding sites is given by:

$$L_x = L\left[\frac{(1+[X]/K_O)^n}{(1+[X]/K_C)^n}\right]$$

where K_O and K_C represent the intrinsic dissociation constants for the binding of *X* to the *O* and *C* states, respectively.

It is important to note that a mutation that alters the value of *L* will necessarily affect the apparent affinity for ligands. Therefore such a shift in apparent affinity does not imply that the mutation has affected the binding of the ligand. The model of Fig. 2 can serve as a basis for discussion of K_{ATP} channel structure-function relationships; however it should be noted that detailed analysis of channel kinetics has led to models incorporating additional closed states (Gillis et al., 1989; Alekseev, Brady & Terzic, 1998; Trapp et al., 1998*a*). The challenge is to elucidate at the molecular level the inter- and intrasubunit interactions determining channel structure and function, the nature of the ligand binding sites, and the structural basis for the conformational transitions between different open and closed states.

The K_{ATP} Channel Contains Two Subunits

Since the binding of sulfonylureas to intact β-cells or to β-cell membrane preparations paralleled their effects on K_{ATP} channel activity (Zunkler et al., 1988; Panten et al., 1989) it was clear that the 'sulfonylurea receptor, SUR' was either the K_{ATP} channel itself or a closely associated protein. Cloning of the β-cell SUR (Aguilar-Bryan et al., 1995) resolved this issue. The cloned protein when expressed in mammalian cells showed high affinity glibenclamide-binding activity but did not give rise to a K-channel. Since the K_{ATP} channel shows weak inward rectification it was predicted that the missing channel subunit might belong to the inward rectifier family. Homology screening of β-cell cDNA libraries with probes for an inwardly rectifying K-channel led to the cloning of a new member of the family which, for reasons related to its primary sequence in comparison with other inward rectifiers, was denoted Kir6.2 (Sakura et al., 1995; In-

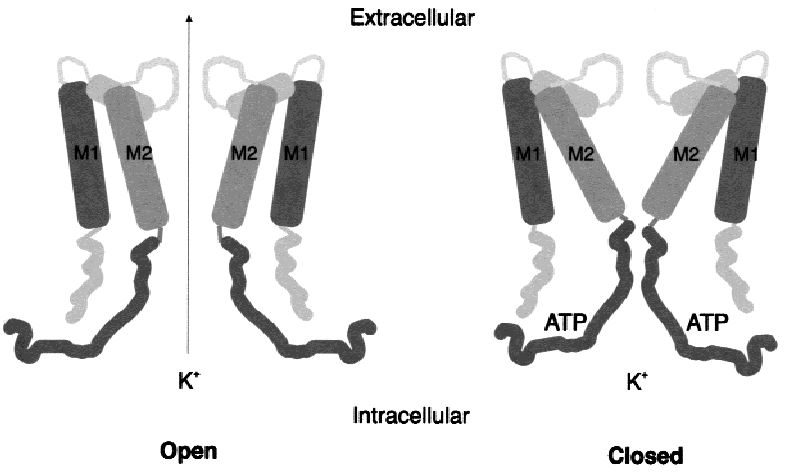

Fig. 3. Open and closed states of Kir6.2. In Kir6.2 the M2 helix is believed to form the permeability pathway and, by analogy with KcsA, the four M2 helices are likely to be arranged in an inverted tepee fashion converging at the cytoplasmic face. Relative movement of M1 and M2 forms the gate that can open and close the channel. The P loop, positioned near the external face, acts as a selectivity filter composed of the backbone carbonyl oxygen atoms of the GYG sequence. Below the selectivity filter lies a space some 10 Å in diameter connected via a water-filled tunnel lined with hydrophobic residues to the intracellular milieu.

agaki et al., 1995*a*). When cells were cotransfected with β-cell SUR and Kir6.2 K_{ATP} channel activity was obtained which in all key respects reproduces the behavior of the native K_{ATP} channel. Reconstitution of active K_{ATP} channels by co-expression of β-cell SUR and Kir6.2 has been achieved in mammalian cell lines (Inagaki et al., 1995*a;* Sakura et al., 1995), *Xenopus* oocytes (Gribble et al., 1997*a*), and in insect cells using a baculovirus system (Mikhailov et al., 1998). Surprisingly, and in contrast to other members of the IR family, Kir6.2 alone did not normally give rise to active channels in mammalian cells; the reason for this is discussed below.

A protein related to the β-cell SUR was subsequently cloned and denoted SUR2 (Inagaki et al., 1996; Isomoto et al., 1996), with the original β-cell isoform being now referred to as SUR1. At least three splice variants of SUR2 (SUR2A, SUR2B, SUR2C) exist differing in exon usage at the C-terminus. Channels formed by co-expression of Kir6.2 with SUR1 or SUR2 show different responses to sulfonylureas. Thus tolbutamide blocks SUR1/Kir6.2, but not SUR2/Kir6.2, with high affinity (Ashcroft & Gribble, 1999). A second gene in the Kir6.x family has also been identified (Inagaki et al., 1995*b*); Kir6.1, which shows 70% amino acid sequence identity to Kir6.2 and wide tissue distribution. Available evidence, reviewed in (Seino, 1999), suggests that SUR1/Kir6.2 constitutes the β-cell and neuronal K_{ATP} channel; SUR2A/Kir6.2 forms the heart and skeletal muscle K_{ATP} channel; and both SUR2B/Kir6.2 and SUR2B/Kir6.1 may serve as smooth muscle K_{ATP} channels.

In *Drosophila*, however, a novel sulfonylurea receptor (DSUR) has been discovered which possesses intrinsic sulfonylurea and ATP sensitive K-channel activity (Nasonkin et al., 1999). The DSUR sequence is highly related to the vertebrate SUR family but contains 1.7 kb of a unique sequence that shows no homology to any known gene. Only a single DSUR gene is present in the *Drosophila* genome. Study of DSUR is likely to lead to novel insights into K_{ATP} channel function.

Kir6.2 Forms the Channel Pore

Human Kir6.2 is a protein of 390 amino acids with an Mr of 43.5 kDa. Hydropathy plots predict that, like other members of the IR family (Doupnik, Davidson & Lester, 1995), Kir6.2 has two transmembrane (TM) domains, M1 and M2. These segments flank a sequence similar to the P (pore) or H5 loop first identified in the voltage-gated K-channel family and shown to function as part of the K-selectivity filter. Both termini of the protein are intracellular. The recent determination of the structure of the pore in a related protein, the KcsA channel of *Streptomyces lividans* (Doyle et al., 1998), has provided insight into the probable architecture of Kir6.2. The KcsA channel is tetrameric with four identical subunits arranged around the central pore. The M2 helix forms the permeability pathway and the four M2 helices are arranged in an inverted tepee fashion converging at the cytoplasmic face to form a gate that can open and close. The P loop is positioned near the external face to act as a selectivity filter composed of the backbone carbonyl oxygen atoms of the GYG sequence. Below the selectivity filter lies a vestibule some 10 Å in diameter connected via a water-filled tunnel lined with hydrophobic residues to the intracellular milieu.

Despite the overall similar domain structure of Kir channels and KcsA, however, there are, as discussed by (Reimann & Ashcroft, 1999) some significant points of difference and the amino acid sequence of KcsA is more homologous to the voltage-gated K-channel than to the Kir family. Mutagenesis studies of Kir2.1, for example, point to a different organization of transmembrane domains compared with KcsA (Minor et al., 1999). The KcsA structure may be best regarded as a useful guide rather than a definitive description of Kir6.2 structure. A depiction of Kir6.2 based on KcsA is shown in Fig. 3.

The Regulatory Subunit, SUR1, is an ABC Protein

Human SUR1 is a protein of 1582 amino acids and an Mr of approximately 176 kDa. The protein belongs to the ATP-binding cassette (ABC) family which includes CFTR, P-glycoprotein, and multidrug-resistance associated protein (MRP). SUR1 contains multiple putative transmembrane domains and two potential nucleotide-binding domains (NBD1 and NBD2). The NBDs each contain a Walker A (-GXXGXGKS- where X is any residue) and Walker B (-YYYYD- where Y is a hydrophobic residue) motifs (Walker et al., 1982). The segment linking the Walker A and B motifs contains a conserved motif (-LSGGQ-in NBD1 and -FSQGQ- in NBD2). Hydropathy plots and comparison with the most closely similar ABC proteins, the MRP family, suggest that SUR1 has an extracellular N-terminus followed by a set (TMD0) of five TM helices connected by a short intracellular loop to a second set (TMD1) of six TM regions. Between TMD1 and a third set (TMD2) of TM segments occurs the large intracellular domain, Mr ~40 kDa, containing NBD1. A second large C-terminal intracellular domain, Mr 38 kDa, after TMD1 contains NBD2.

There is direct evidence from glycosylation and protease protection studies for the above topology (Raab-Graham et al., 1999). Deglycosylation using peptide-N-glycosidase F and site-directed mutagenesis established that N10, near the amino terminus, is on the extracellular side of the membrane. Fusion proteins containing 1–5 hydrophobic segments of the TMD0 region fused to the reporter prolactin were expressed in vitro and subjected to a protease protection assay that indicated the accessibility of the prolactin epitope. The results confirmed that the TMD0 region is comprised of 5 transmembrane segments.

SUR1 is differentially glycosylated (Aguilar-Bryan & Bryan, 1999). Mature SUR1, either in or in transit to the plasma membrane, has an Mr of 150–170 kDa and is complex glycosylated with sialic acid residues. An immature form of Mr 140 kDa constitutes a core glycosylated protein with mannose-containing glycosyl groups. Two N-glycosylation sites are present at residues 10 and 1050 and mutation of these residues eliminates glycosylation but does not prevent formation of active K_{ATP} channels when co-expressed with Kir6.2. The predicted topology of Kir6.2 and SUR1 is shown in Fig. 4.

The K_{ATP} Channel is an Octamer

The KcsA channel is a homotetramer and several studies indicate that both Kir and Kv channels are also tetrameric (MacKinnon, 1991; Glowatzki et al., 1995; Yang, Jan & Jan, 1995). There is good evidence that the K_{ATP} channel is a heterooctamer containing four molecules each of Kir6.2 and SUR1. Firstly a digitonin stable complex of Kir6.2 and SUR1 had an estimated Mr of 950 kDa by sucrose gradient centrifugation consistent with the expected molecular mass of a $(SUR1/Kir6.2)_4$ heterooctamer (Clement et al., 1997). Expression of fusion constructs SUR1 ~ Kir6.2 and SUR1 ~ $(Kir6.2)_2$ further supported this model (Clement et al., 1997; Inagaki, Gonoi & Seino, 1997, Shyng, & Nichols, 1997). SUR1 ~ Kir6.2 gave rise to active K_{ATP} channels and also produced a 950 kDa complex on sucrose gradient analysis. Thus a 1:1 stoichiometry is sufficient for K_{ATP} channel formation. That it is also necessary was shown using the triple fusion protein SUR1 ~ $(Kir6.2)_2$ which did not lead to expression of K_{ATP} channels when expressed alone but did so when rescued by co-expression of monomeric SUR1. To confirm the 4:4 stoichiometry advantage was taken of the fact that the weakly rectifying channels formed by co-expression of Kir6.2 and SUR1 can be converted to strongly rectifying channels by mutation of D160 in M2 of Kir6.2 (Shyng, Ferrigni & Nichols, 1997*a*). When a mixture of SUR1 ~ $(Kir6.2)_2$ and SUR1 ~ $(Kir6.2_{N160D})_2$ was rescued by monomeric SUR1 three classes of channels were expressed including a channel with intermediate rectification properties. The formation of the latter species implies that two triple fusion proteins, one containing wild-type Kir6.2 and the other $Kir6.2_{N160D}$, can interact to form the pore, as predicted from a tetrameric architecture for the pore-forming unit.

The suggested subunit structure of the K_{ATP} channel is indicated in Fig. 5.

Nucleotides Bind to Kir6.2

Since SUR1 possesses two putative NBDs whereas Kir6.2 does not have an obvious nucleotide-binding sequence, it was originally envisaged that effects of nucleotides on K_{ATP} channel function were mediated by SUR1. However it was subsequently found that active K-channels can be formed by expression of C-terminally deleted Kir6.2 (Kir6.2ΔC) in the absence of SUR1; these truncated isoforms of Kir6.2 were sensitive to inhibition by ATP but were not stimulated by MgADP (Tucker et al., 1997). Wild-type Kir6.2 has also been shown to exhibit functional ATP-inhibitable K-channels independently of SUR1 (John et al., 1998; Mikhailov et al., 1998). The sensitivity of Kir6.2 to ATP is lower (Ki ~ 100 μM) when expressed in the absence of SUR1 than when the two proteins are co-expressed (Ki ~ 10 μM). In addition mutations in Kir6.2 have been found that markedly decrease the sensitivity of Kir6.2ΔC currents to inhibition by ATP (Tucker et al., 1997; Drain, Li & Wang, 1998; Tucker et al., 1998; Koster et al., 1999*b*). These data suggest that Kir6.2 contains a functionally important nucleotide-binding site. Direct evidence for this has been obtained by photoaffinity labeling of Kir6.2

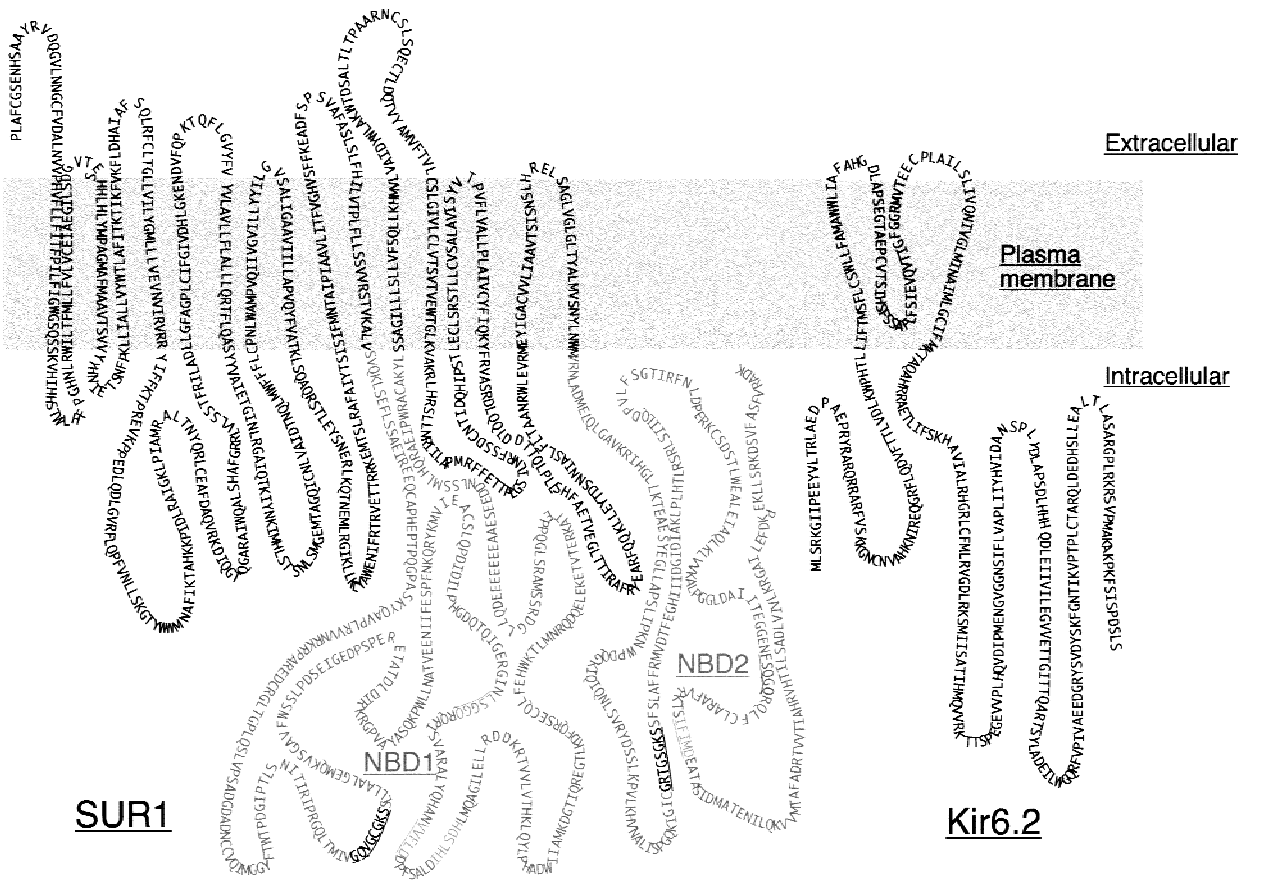

Fig. 4. Predicted topology of K_{ATP} channel subunits. The structures of human SUR1 and Kir6.2 are given with their putative membrane topology indicated. The positions of the Walker A, Walker B and linking sequences in the two NBDS of SUR1 are shown underlined.

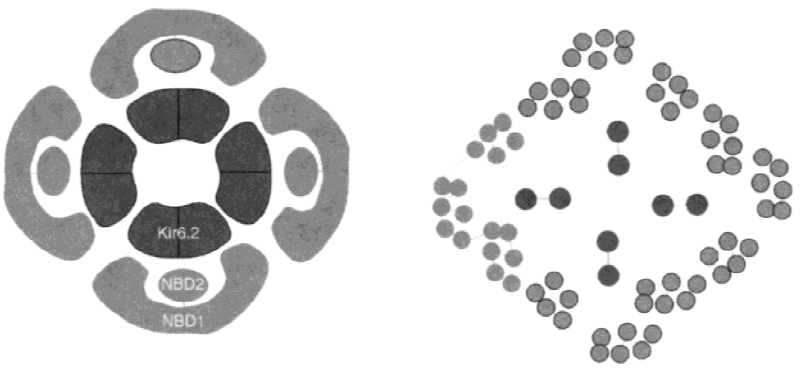

Fig. 5. Subunit structure of K_{ATP} channels. The K_{ATP} channel is an octamer. Four Kir6.2 subunits form the pore and one SUR1 subunit is associated with each Kir6.2. On the left is depicted a view of the K_{ATP} channel from the cytosol showing the N- and C-terminal domains of Kir6.2 and the large cytosolic NBDs of SUR1. On the right is a view from the outside showing the two TM domains of each Kir6.2 and the 17 TM domains of each SUR1.

by [γ-^{32}P]-8-azido-ATP (Tanabe et al., 1999). Thus it is now clear that nucleotides interact with both SUR1 and Kir6.2. SUR1 mediates the stimulatory effects of MgADP on K_{ATP} channel activity whereas inhibition by ATP is mediated by binding to Kir6.2. The functional effect of ATP binding to Kir6.2 is however modulated by SUR1.

The molecular determinants of K_{ATP} channel inhibition by ATP have been studied using Kir6.2ΔC (Tucker et al., 1998). The effect is highly specific for the adenine moiety of ATP. GTP, UTP, ITP, CTP and UTP are all ineffective. ADP (Ki ~ 260 μM) is almost as potent as ATP (Ki ~ 115 μM) but AMP is markedly less effective (Ki ~ 9 mM). The regions of Kir6.2 responsible for inhibition by ATP were studied by mutational analysis of the N- and C-terminal regions of Kir6.2 expressed in *Xenopus* oocytes (Tucker et al., 1998). Five mutations (R50G, C166S, I167M, K185Q, T171A) were found that

significantly reduced the potency of ATP, with several other mutations producing smaller changes. The effective mutations were situated in two distinct regions, one at the N-terminus just before M1 and the other in the C-terminus just after M2. The mutations R50G and K185Q lowered the sensitivity to ATP without affecting the single-channel kinetics. These mutations may therefore modify the affinity of the binding site for ATP or the mechanism by which ATP binding is transduced into channel closure. Direct evidence that these mutation do impair ATP binding was obtained by showing that photoaffinity labeling of Kir6.2 by [γ-^{32}P]-8-azido-ATP was decreased by more than 50 per cent (Tanabe et al., 1999). Since effects on the binding site may be either direct or allosteric it is not possible from these data to conclude whether either of these mutations resides in the actual ATP binding site (but see below). The decrease in ATP sensitivity found with C166S, I167M and T171A was associated with a change in the channel gating consisting of a marked decrease in the frequency of the long closed state. If ATP binds preferentially to this state then a mutation that decreased the probability of a channel being in the long closed state would result in decreased ATP sensitivity without any necessary change in ATP binding or transduction. A detailed mutational analysis of C166 (Trapp et al., 1998*a*) indicated that this residues may play a role in the intrinsic gating of the channel and confirmed that the apparent decrease in sensitivity to ATP observed in C166 mutants is a consequence of an alteration in the probability of transition from the open to the long closed state. A similar correlation between channel open probability and sensitivity to ATP has been reported in a study of mutations of N160 (Shyng et al., 1997*a*).

The role of K185 has been examined in detail (Reimann et al., 1999*a*). Substitution of a negative charge (glutamate) for the positively charged lysine markedly decreased the channel ATP sensitivity. However substitution of arginine, another positively charged residue, had little effect. Intermediate effects were obtained with neutral amino acids. Thus ATP inhibition requires a positively charged amino acid residue at position 185. Since inhibition by ADP was also reduced in K185Q or K185A, the terminal phosphate of ATP is unlikely to interact with K185. There was no correlation of the ability of the amino acid at position 185 to form hydrogen bonds and its effect on inhibition by ATP. Nor did neutralization of K185 or substitution of a negative charge affect the selectivity to purine nucleotide triphosphates. These data suggest that the side-chain of K185 does not interact directly with ATP. Instead it is suggested that in the wild-type channel the side-chain of K185 is directed towards a hydrophobic pocket where it interacts with a negatively charged residue and thereby stabilizes the tertiary structure of Kir6.2. Any mutation that disrupts this interaction leads to allosteric effects on the ATP binding site that result is decreased sensitivity to ATP. This model accounts for several aspects of the effects of substitution at K185. Thus substitution of glycine has little effect on ATP inhibition since the small size of glycine means that it does not approach the negative charge and allows water and ions to enter the pocket and stabilize the charge. However bulkier amino acids would approach more closely the negative residue and prevent entry of water and ions leading to increased charge destabilization. Hydrophobic residues would even more effectively exclude water. The major effect of substitution of a negative charge is explicable as a severe disruption of the hydrophobic pocket by charge repulsion.

The effects of mutation of R50 have also received detailed attention by mutational analysis (Proks et al., 1999). Replacement of the positively charged arginine by a negatively charged glutamate had only a minor effect on sensitivity to ATP. In general ATP sensitivity was not correlated with the ability of the amino acid side chain to form chemical interactions but rather with the size of the residue; the smaller the side chain the less was the inhibition by ATP. Mutation of the adjoining I49 residue to glycine also markedly reduced sensitivity to ATP without affecting the single channel kinetics whereas mutation of E51 to glycine had no effect. The data suggest that R50 does not interact directly with ATP but that residues 49 and 50 are critical for maintenance of the ATP binding site. The introduction of small residues at these positions has a marked allosteric effect on the ATP-binding site which leads to loss of sensitivity to ATP.

When a mutation at the N-terminus (R50S) was combined with one at the C-terminus (E179Q), the single channel conductance and the channel kinetics were not different from wild-type Kir6.2ΔC (Proks et al., 1999). However the sensitivity to ATP was further reduced compared to either mutation alone. This suggests that the N- and C-termini of Kir6.2 interact cooperatively to affect channel inhibition by ATP. Direct evidence for such interaction is discussed below.

The effects of the R50G and K185Q mutations have also been studied in COS cells expressing heteromeric SUR1/Kir6.2 channels (Babenko, Gonzalez & Bryan, 1999*a*). Comparison of wild-type, SUR1/Kir6.2$_{K185Q}$ and SUR1/Kir6.2$_{R50Q/K185Q}$ channels showed that the K185Q mutation reduced the K_i for ATP some 40-fold while the double mutation R50Q/K185Q had a K_i for ATP some 400-fold lower than wild-type. In agreement with results in *Xenopus* oocytes (Tucker et al., 1998), SUR1/Kir6.2$_{K185Q}$ had unaltered channel kinetics. However the double mutant also had increased open probability suggesting that the R50Q mutation leads to reduced occupancy of the long closed state. The reason for this discrepancy with the conclusions from the *Xeno-*

pus expression system (Proks et al., 1999) is unclear. Truncation of up to 44 amino acid residues from the N-terminus also increased open probability and decreased sensitivity to ATP (Babenko et al., 1999*a;* Koster et al., 1999*b;* Reimann et al., 1999*b*). However removal of up to 36 amino acids from the C-terminus (without loss of functional channels on co-expression with SUR1) did not affect the ATP sensitivity of co-expressed channels (Koster et al., 1999*b*). It may be concluded that the two cytoplasmic domains of Kir6.2 participate in ATP-inhibitory gating via different mechanisms; the N-terminal deletions stabilize the ATP-independent open state, whereas the $Kir6.2_{K185Q}$ mutation alters the stability of ATP binding.

Another chimeric and point mutagenesis study on Kir6.2 expressed in *Xenopus* oocytes identified distinct functional domains of the C-terminal segment of Kir6.2 that play an important role in inhibition by ATP (Drain et al., 1998). The results suggested that one C-terminal domain is associated with inhibitory ATP binding and another with gate closure.

Agents reacting with SH-groups produce an irreversible inhibition of K_{ATP} channel activity (Lee et al., 1994). The cysteine residue involved in this effect has been shown to reside on Kir6.2 and has been located to C42 within the N-terminal cytosolic domain (Trapp, Trucker & Ashcroft, 1998*b*). Since ATP protects against the effect of sulfhydryl modifying agents, it appeared possible that C42 may form part of the ATP-binding site. However, mutagenesis of C42 to valine or alanine did not affect the ATP sensitivity of Kir6.2ΔC. Thus ATP is unlikely to interact directly with this residue. Instead it has been suggested that C42 becomes inaccessible to sulfhydryl modifying agents when the channel is closed by ATP (Trapp et al., 1998*b*).

Thus, none of these studies have yet defined the actual ATP-binding site. However it has been suggested that the sequence -F_{333}GNTIK_{338}- which resembles the F-X_4-K motif found in ion-motive ATPases may be a candidate (Drain et al., 1998).

Nucleotides Bind to SUR1

SUR1 has the highly conserved Walker A and B motifs and the SGGQ ABC signature in each putative NBD. The binding of nucleotides to SUR1 was studied by introducing point mutations into NBD1 and NBD2 and characterizing the ability of [^{32}P]-azido ATP to photolabel wild-type and mutant SUR1 (Ueda, Inagaki & Seino, 1997). SUR1 was labelled with [^{32}P]-azido ATP in the absence or presence of Mg^{2+}. NBD1 mutations impaired photolabeling but NBD2 mutations did not. Preincubation with MgADP antagonized photolabeling by [^{32}P]-azido ATP and this antagonism was reduced by mutation of NBD2. It was concluded that SUR1 binds ATP at NBD1 and MgADP at NBD2. MgADP bound at NBD2 was assumed to facilitate binding of MgADP at NBD1 and thus prevent interaction with [^{32}P]-azido ATP, i.e., there is cooperative action between NBD1 and NBD2. This interaction was further studied using a protocol in which [^{32}P]-azido ATP was first incubated with membrane proteins and free ligand removed before exposure to MgADP or MgATP (Ueda et al., 1999*a*). Using this procedure it was found that MgATP and MgADP, but not the Mg salt of ATPγS, stabilize the binding of prebound [^{32}P]-azido ATP to SUR1. Mutations in the Walker A or B motifs of NBD2 of SUR1 abolished this stabilizing effect of MgADP. These results suggest that SUR1 binds [^{32}P]-azido ATP strongly at NBD1 and that MgADP, either by direct binding to NBD2 or by hydrolysis of bound MgATP at NBD2, stabilizes prebound [^{32}P]-azido ATP binding at NBD1. Further evidence for this view has been provided by studies in which tryptic fragments of SUR1 containing NBD1 or NBD2 were immunoprecipitated by antibodies specific for NBD1 or NBD2 after photoaffinity labeling with [^{32}P]azidoATP. A 35 kDa fragment was labeled with [α-^{32}P]azidoATP even in the absence of Mg^{2+} and was immunoprecipitated with an anti-NBD1 antibody. A 65 kDa fragment which was immunoprecipitated by anti-NBD2 antibody was labeled with [α-^{32}P]azidoATP only in the presence of Mg^{2+} and required higher concentrations of the azido-ATP. The data indicate that NBD1 binds azido-ATP strongly in the absence of Mg^{2+} whereas NBD2 binds azidoATP only weakly in a Mg^{2+}-dependent manner (Matsuo et al., 2000). It was further found that the 65 kDa fragment was not labeled by [γ-^{32}P]azidoATP whereas the 35 kDa fragment was. This was interpreted to imply that NBD2 but not NBD1 possesses significant ATPase activity.

Analysis of the dose dependence of ATP inhibition indicates that although four molecules of ATP bind per channel molecule, channel closure results from binding a single ATP (Ashcroft & Gribble, 1998).

Sulfonylureas Bind with High Affinity to SUR1

Studies on the binding of radioactive sulfonylureas to intact β-cells and β-cell membranes established that [^{3}H]-glibenclamide bound to both high affinity (Kd ~ 1 nM) and low affinity (Kd ~ 1 μM) sites. Unfortunately it has not so far proved possible to detect directly binding of tolbutamide or meglitinide, presumably because of rapid dissociation of these drugs during the separation of bound from unbound during the assay procedure. Instead affinities for sulfonylureas other than glibenclamide have had to be estimated by measurement of competition with [^{3}H]-glibenclamide binding. The rank order of potency corresponds closely to the ability of the drugs to close K_{ATP} channels and stimulate insulin se-

cretion. However the absolute values for the potencies of sulfonylureas and meglitinide to inhibit the activity of native or recombinant β-cell K_{ATP} channels is 3- to 6.4-fold higher than their binding affinities for SUR1 (Dörschner et al., 1999). [^{3}H]-glibenclamide also binds to cells expressing Kir6.2/SUR2B channels but with affinity some 400-fold lower than for SUR1-Kir6.2. This affinity is too weak to permit detection of [^{3}H]-glibenclamide binding to membranes containing SUR2 isoforms. However the high affinity binding of the K-channel opener P-1075 to membranes containing SUR2 can be displaced by [^{3}H]-glibenclamide and has been used to measure the affinity of SUR2 for sulfonylureas and meglitinide. All drugs inhibited the activity of transiently expressed SUR2B-Kir6.2 channels with EC_{50} values significantly lower than the K_d values for binding to SUR2B (Dörschner et al., 1999). Uniquely, meglitinide has a binding affinity for SUR2B similar to that for SUR1. The leftward shift of potencies versus affinities has been suggested to indicate that binding of sulfonylureas to any one of the four binding sites per channel is sufficient to cause channel closure (Dörschner et al., 1999). This conclusion has been challenged, however (Russ et al., 1999). Homologous competition studies of [^{3}H]-glibenclamide binding to whole cells expressing SUR2B gave a K_d value of 32 nM whereas heterologous competition binding experiments with [^{3}H]-P1075 gave values some 30 to 45 times higher. Moreover coexpression of Kir6.1 with SUR2B reduced the K_d for [^{3}H]-glibenclamide binding to 6 nM. When this value is compared with the IC_{50} for Kir6.1-SUR2B channel inhibition by glibenclamide (43 nM) it is apparent that the channel inhibition curve was shifted to the right of the binding curve by a factor of ~7 (*cf.* the leftward shift reported by Dörschner et al. (1999)). Since this is close to the value expected for binding of 4 molecules of glibenclamide to 4 identical independent sites (shift to the right of the binding curve by a factor of $(2^{1/4} - 1)^{-1} = 5.3$) it was concluded, in direct opposition to (Dörschner et al., 1999), that occupancy of all 4 binding sites per channel is necessary for channel closure by sulfonylureas (Russ et al., 1999). The discrepancy may be related to the different experimental conditions used by the two groups and possible factors are discussed in detail by Russ et al. (1999).

Early studies on native K_{ATP} channels showed that the binding of [^{3}H]-glibenclamide to β-cell membranes is markedly affected by nucleotides (Ashcroft & Ashcroft, 1992*b*). Both MgATP and MgADP displace [^{3}H]-glibenclamide binding to β-cell membranes (Niki, Nicks & Ashcroft, 1990). MgATP increased the apparent Km for [^{3}H]-glibenclamide (Schwanstecher et al., 1991); however the apparent K_d was not the linear function of MgATP concentration expected for competitive inhibition indicating that MgATP does not compete for the sulfonylurea binding site (Schwanstecher et al., 1992*b*). Although MgADP also inhibited [^{3}H]-glibenclamide binding the possibility that the effect is mediated by formation of MgATP by transphosphorylation reactions in the microsomal membrane used can not be discounted (Schwanstecher et al., 1991; Schwanstecher et al., 1992*b*). These data suggest that nucleotide binding to the NBDs of SUR1 modifies the glibenclamide-binding site. To what extent this effect of nucleotides involves hydrolysis has not been established although early studies suggested that this may be the case since non-hydrolyzable ATP analogues do not reproduce the effect of MgATP, and ATP is ineffective in the absence of Mg ions (Schwanstecher et al., 1992*b*). It has been suggested that phosphorylation of SUR1 is involved since a study of the kinetics of reversal of MgATP-induced inhibition of [^{3}H]-glibenclamide binding to β-cell membranes showed that reversal was slower than the rate of association of glibenclamide with the receptor (Schwanstecher et al., 1991) consistent with its depending on rate-limiting protein dephosphorylation. Moreover inhibition of [^{3}H]-glibenclamide binding by MgATP was augmented in the presence of NaF, a nonspecific protein phosphatase inhibitor (Ashcroft et al., 1993). The finding that fluorescein derivatives which have been shown to bind to nucleotide-binding sites in diverse proteins are also able to displace [^{3}H]-glibenclamide from β-cell membranes was proposed to be consistent with the idea that binding at the NBD(s) influences binding at the sulfonylurea site (De Weille, Müller & Lazdunski, 1992). However kinetic analysis of this interaction suggested otherwise since the data indicated that fluorescein derivatives and glibenclamide may bind to a common site (Schwanstecher et al., 1995). More recently, clear evidence for communication between the NBDs and the glibenclamide-binding site has been provided by the demonstration that glibenclamide causes release of pre-bound 8-azido-[α-^{32}P]ATP from SUR1 in the presence of MgADP or MgATP in a concentration-dependent manner (Ueda et al., 1999*a*).

Channels formed by co-expression of Kir6.2 with SUR1 exhibit different responses to sulfonylureas to those formed from Kir6.2 plus SUR2A (Gribble et al., 1998*b*) as also observed for native K_{ATP} channels from β-cell and heart. Tolbutamide blocks Kir6.2-SUR1 with high affinity but not Kir6.2-SUR2A. Glibenclamide blocks both types of channels but its effect is much more readily reversible with Kir6.2-SUR2A. These observations allowed construction of chimeras between SUR1 and SUR2A to identify the regions of SUR1 that confer high affinity tolbutamide block. Using co-expression of SUR chimeras with Kir6.2 in *Xenopus* oocytes (Ashfield et al., 1999) it was found that high-affinity tolbutamide inhibition of macroscopic K_{ATP} channel currents could be conferred on SUR2A by replacing M14–M16 with the

corresponding sequences from SUR1. Conversely, when M13–M16 of SUR1 were replaced with the corresponding SUR2A sequence tolbutamide inhibition of K_{ATP} channel currents was abolished, as was also binding of [^{3}H]-glibenclamide to COS cell membranes expressing the chimera. The mutation of a single serine residue within this region (S1237Y) in the intracellular loop between M15 and M16 was itself sufficient to abolish high-affinity tolbutamide inhibition and glibenclamide binding. In contrast to tolbutamide, meglitinide blocked Kir6.2-$SUR1_{S1238Y}$ currents to a similar extent. This is consistent with the view that meglitinide, which corresponds to the nonsulfonylurea moiety of glibenclamide (see below), binds to a different site to tolbutamide. The ability of SUR1 to confer activation by diazoxide or by MgADP was not impaired in Kir6.2-$SUR1_{S1238Y}$. These data indicated that the binding site for tolbutamide resides in the C-terminal set of TM helices (TMD2). The conclusion that TMD2 of SUR1 confers high-affinity tolbutamide inhibition was subsequently confirmed by a similar study using chimeras of human SUR1 and SUR2A (Babenko, Gonzalez & Bryan, 1999*b*).

In addition to a sulfonylurea moiety glibenclamide possesses a benzamido group. The differential effects of tolbutamide and meglitinide which represent sulfonylurea and benzamido functionality respectively on Kir6.2/SUR1 and Kir6.2/SUR2A channels expressed in *Xenopus* oocytes (Gribble et al., 1998*b*) suggest that SUR1 may possess separate high-affinity binding sites for sulfonylurea and benzamido groups. The high potency and relative irreversibility of glibenclamide binding can be explained by its binding to both sites.

An alternative approach has been taken to locating the glibenclamide binding site (Mikhailov & Ashcroft, 2000). SUR1 was divided into two halves at P1064 which is located in the putative extracellular loop between M12 and M13 of TMD2. Both half-molecules were shown to be inserted into the plasma membrane when expressed as green fluorescent protein fusion proteins in Sf9 insect cells using a baculovirus system previously shown to permit expression of active K_{ATP} channels (Mikhailov et al., 1998). However glibenclamide binding activity could not be detected in cells expressing either the N-terminal or the C-terminal moiety alone. When both half-molecules were co-expressed, however, significant glibenclamide binding activity was obtained. These data indicate that the two halves of SUR1 interact and that the glibenclamide binding site requires residues from both halves of the molecule. It was further shown that NBD2 plays no significant role in forming the glibenclamide binding site since co-expression of the N-terminal half-molecule with a truncated C-terminal moiety lacking NBD2 also resulted in appearance of glibenclamide binding. Consistent with these results, a novel SUR isoform isolated from a hypothalamic cDNA library and lacking NBD2 retained sensitivity to sulfonylureas (Sakura et al., 1999).

These data suggest that inhibition by glibenclamide requires interaction between TMD2 domains of the C-terminal half of SUR1 (the tolbutamide binding site) with cytosolic and/or TM domains in the N-terminal half-molecule (the meglitinide (benzamido) binding site). Photolabeling studies (Aguilar-Bryan et al., 1995) suggest that the N-terminal domain is likely to reside in TMD0.

Glibenclamide Binds with Low Affinity to Kir6.2

It is clear that SUR1 confers on the β-cell K_{ATP} channel sensitivity to high affinity inhibition by sulfonylureas. However in β-cell membranes and intact β-cells there is additional low-affinity glibenclamide-binding activity (Niki et al., 1989; Nelson, Aguilar-Bryan & Bryan, 1992; Schwanstecher et al., 1994*a*). Studies on Kir6.2ΔC found effects of high concentrations of sulfonylureas on channel activity in the absence of SUR1 (Gribble, Tucker & Ashcroft, 1997*c;* Gribble et al., 1998*b*) suggesting that the low affinity site is on Kir6.2. Direct evidence for this has been provided by studies on the binding of [^{3}H]-glibenclamide to COS cells expressing Kir6.2 (Gros et al., 1999). A single class of low affinity (Ki ~ 1.6 μM) sites was found with pharmacological specificity similar to those observed for low affinity binding to MIN6 β-cells. In particular, in contrast to the high affinity sites, tolbutamide, gliclazide, glipizide and glibornuride were unable to displace [^{3}H]-glibenclamide—only gliquidone was effective (Ki ~ 20 μM). The clinical relevance of the low affinity site is doubtful in view of the high concentration of glibenclamide necessary to exert an effect.

Diazoxide and Other Channel Openers Bind to SUR1

Potassium channel openers e.g., diazoxide, pinacidil, cromakalim, are a structurally varied group of drugs which open K_{ATP} channels in diverse tissues and thereby cause hyperpolarization and reduction of electrical activity. Early studies established that diazoxide and other KCOs were effective in opening β-cell K_{ATP} channels only if MgADP or MgATP were present at the inside face of the membrane (Dunne, 1990; Dunne, Aspinall & Peterson, 1990; Jaggar et al., 1993). Although direct binding of diazoxide could not be measured in these studies, it was also found that in the presence of MgATP, but not in its absence, diazoxide (Niki & Ashcroft, 1991; Schwanstecher et al., 1991) and pinacidil (Schwanstecher et al., 1992*a*) are able to displace [^{3}H]-glibenclamide from β-cell membranes. The presence of

MgATP was also shown to be essential for binding of [^{3}H]-P1075 to membranes from various tissues (Löffler-Walz & Quast, 1998; Quast et al., 1993). The requirement for Mg^{2+} and the inability of nonhydrolyzable ATP analogues to support the effect of diazoxide on the β-cell K_{ATP} channel were interpreted as suggesting an involvement of protein phosphorylation (Schwanstecher et al., 1991; Kozlowski & Ashford, 1992; Dunne et al., 1999). However the ability of MgADP and hydrolyzable ADP analogues to permit channel opening by diazoxide led to the alternative suggestion that the effects of diazoxide require nucleotide hydrolysis (Larsson et al., 1993). The availability of cloned K_{ATP} channel subunits has permitted these interactions to be explored in detail.

It has been established that the tissue specific differences in the response of K_{ATP} channels to KCOs are conferred by the SUR subunit. Thus Kir6.2/SUR1 currents are activated by diazoxide but not by pinacidil (Gribble et al., 1997*a*), Kir6.2/SUR2A currents are activated strongly by pinacidil but only weakly by diazoxide (Inagaki et al., 1996) whereas Kir6.2/SUR2B currents are activated by both drugs (Schwanstecher et al., 1998). However the situation appears to be more complex since it has been shown that Kir6.2/SUR2A currents in excised patches become as sensitive to diazoxide as Kir6.2/SUR1 in the presence of MgADP (but not ADP in the absence of Mg ions) at concentrations above 100 μM (D'hahan et al., 1999*b*). Moreover in heart cells treated with oligomycin to block mitochondrial ATP generation diazoxide was able to activate K_{ATP} currents if cytosolic ADP was increased by a creatinine kinase inhibitor. Electrophysiological studies with mutated forms of SUR1 co-expressed with Kir6.2 in *Xenopus* oocytes showed that the Walker A lysine of NBD1 (but not NBD2) was essential for activation of K_{ATP} currents by diazoxide, whereas the potentiatory effects of Mg-ADP required the presence of the Walker A lysines in both NBDs (Gribble, Tucker & Ashcroft, 1997*b*). However the conclusion that NBD2 is not important for the action of KCOs has been questioned by subsequent studies which have confirmed that NBD1 is involved but have in addition shown that NBD2 also plays a role in response to KCOs (D'hahan et al., 1999*b;* Schwanstecher et al., 1998; Shyng et al., 1997*b*). Mutations in the linker region and Walker A motif of NBD2, including G1479D, G1485D, G1485R, Q1486H and D1506A, all abolished stimulation by diazoxide of Kir6.2/SUR1 currents in transfected COSm6 cells (Shyng et al., 1997*b*). Analogous mutations in NBD1, including G827D, G827R, and Q834H, were still stimulated by diazoxide but with altered channel kinetics. Channel opening required the presence of MgADP or MgATP and a model was proposed in which MgADP and diazoxide stabilizes a channel conformation which is desensitized to ATP inhibition; mutations at the NBDs were suggested to alter the channel response to diazoxide and MgADP by altering nucleotide hydrolysis rates or coupling of hydrolysis to channel activation. The importance of NBD2 for activation by diazoxide and by MgADP is also supported by the finding that the current obtained when a splice variant of SUR1 lacking NBD2 is co-expressed with Kir6.2 in *Xenopus* oocytes is insensitive to stimulation by these agents (Sakura et al., 1999).

Using a heterologous displacement assay, binding of diazoxide to SUR1 was shown to require Mg and ATP or a hydrolyzable ATP analogue (Schwanstecher et al., 1998). ADP was not effective if the ATP concentration was kept low by the hexokinase reaction suggesting that nucleoside diphosphates may act via enzymatic nucleoside triphosphate formation. It was suggested that KCO binding requires a conformational change induced by hydrolysis of ATP in both NBFs. However the available evidence discussed above, although as yet indirect, is that NBD1 has little or no ATPase activity (Matsuo et al., 2000).

The regions of sulfonylurea receptors involved in binding KCOs have been explored by constructing chimeras between SUR1 and SUR2A (D'hahan et al., 1999*a*) or SUR2B (Uhde et al., 1999). The characteristic sensitivity to cromakalim of SUR2A was found to be conferred by the third group of transmembrane helices, TMD2, which may contain, therefore, at least part of the binding site (D'hahan et al., 1999*a*). Diazoxide sensitivity, however, could not be linked to a single domain. It had been anticipated that the C-terminal region of SUR would be critical for diazoxide action since this is the region of difference between SUR2A (diazoxide insensitive) and SUR2B (diazoxide sensitive). However chimeras of SUR1 with the C-terminus of SUR2A retained significant sensitivity to diazoxide. Moreover, despite their functional differences, SUR1 and SUR2A have comparable affinities for diazoxide (Schwanstecher et al., 1998). Hence it was suggested that the diazoxide binding site may be located within conserved regions but that the isoform variability in response may involve changes in coupling of binding to channel opening (D'hahan et al., 1999*a*). Two distinct regions of TMD2, part of the putative cytosolic loop between M13 and M14 (T1059-L1087; KCO1) and M16 and M17 (R1218-N1320; KCO2), were found to be essential for high affinity [^{3}H]P1075 binding to SUR2B (Uhde et al., 1999). Substitution of the SUR2B sequence by the SUR1 sequence resulted in complete loss of [^{3}H]P1075 binding whereas combined transfer of these domains into SUR1 induced a 6,200-fold increase in [^{3}H]P1075 affinity. The changes in binding affinity were paralleled by changes in K_{ATP} channel response. The high affinity for glibenclamide of SUR1 was not reduced in this chimera indicating that high affinities for both sulfonylureas and KCOs can co-exist in the same molecule. Both domains there-

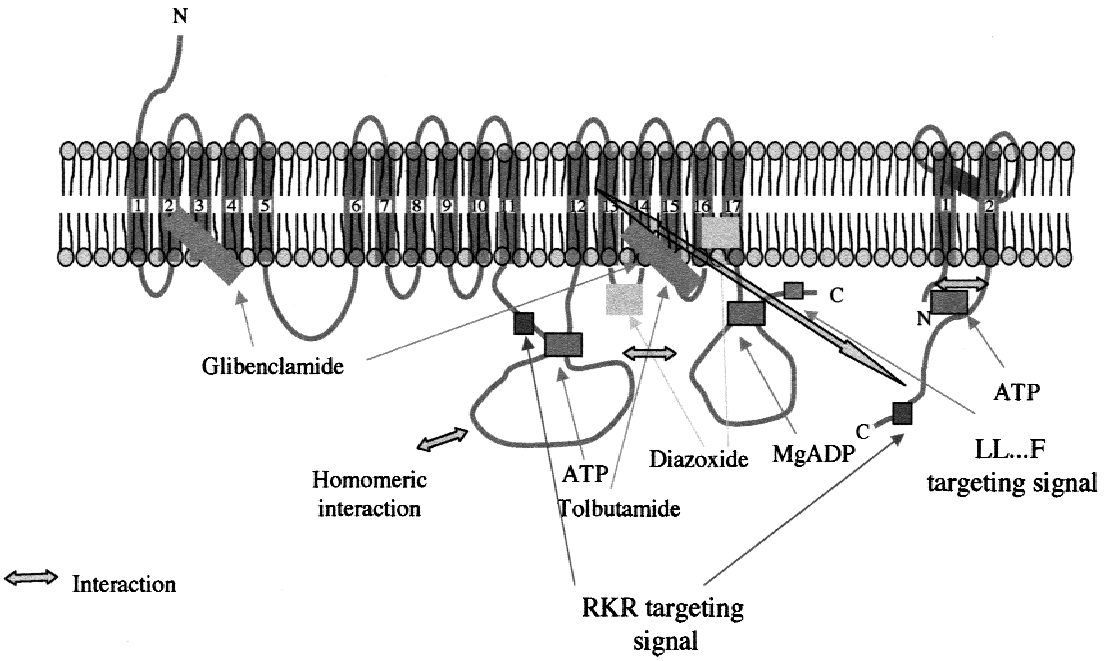

Fig. 6. Functionally important regions of the β-cell K_{ATP} channel. The figure depicts sites in the β-cell K_{ATP} channel so far identified as important for binding ligands, for homo- and heteromeric interactions, and for targeting to the plasma membrane.

fore may interact to form the KCO binding site. However full KCO affinity required further domains. Identification of KCO1 in a putative intracellular region suggests that KCOs may have to cross the plasma membrane to exert their effects as previously argued for sulfonylureas (Schwanstecher et al., 1994*b*). In agreement with Ashfield et al. (1999) M14 and M15, the region between KCO1 and KCO2, conferred high affinity sulfonylurea binding (Uhde et al., 1999). Thus TM helices M14–M17 within TMD2 of SURs are of key importance for drug-induced K_{ATP} channel responses. The core region of this domain forms the sulfonylurea binding domain and the flanking regions make up at least part of the KCO receptor site.

Summary of Binding Sites on K_{ATP} Channel Subunits

The regions of the β-cell KATP channel that have so far been implicated in ligand binding are summarized in Fig. 6.

The studies described above establish that Kir6.2 contains a site at which ATP binds in a Mg-independent manner to cause channel closure. The binding site has not been defined. However cooperative interactions between the N- and C-terminal cytosolic regions appear to be important. The efficacy with which ATP binds and leads to channel closure is modified by the presence of SUR1. The ATP sensitivity is also markedly affected by PIP_2 which may interact with Kir6.2 and Kir6.2 also contains binding sites for fatty acyl-CoAs and for imidazolines (see below). A low affinity binding site for sulfonylureas also exists on Kir6.2.

SUR1 contains two nucleotide binding sites in NBD1 and NBD2. Consideration of the binding data described above (Ueda et al., 1997; Matsuo et al., 2000) suggests the following model (Ueda et al., 1999*b*). When [ATP]/[ADP] is relatively low, NBD1 binds ATP and NBD1 binds MgADP. In this conformation of SUR1 its interactions with Kir6.2 result in the affinity of Kir6.2 for ATP being low and K_{ATP} channels are open. When [ATP]/[ADP] is increased, the decrease in MgADP leads to release of bound MgADP from NBD2 and a consequential release of ATP from NBD1. A resulting conformational change in SUR1 leads to an increase in affinity of Kir6.2 for ATP and the K_{ATP} channels close.

SUR1 contains the binding sites for sulfonylureas and KCOs. M14 and M15 of TMD2 are of importance for sulfonylurea binding but residues nearer the N-terminus are also required. Two regions, the cytosolic loop between M13 and M14 (T1059-L1087 (KCO1) and M16 and M17 R1218-N1320 (KCO2) form at least part of the KCO binding site.

Imidazolines Interact with Kir6.2

A number of drugs containing an imidazoline moiety, such as phentolamine and efaroxan, are potent stimula-

tors of insulin secretion (Jonas, Plant & Henquin, 1992; Brown et al., 1993). The effect has been shown to involve closure of K_{ATP} channels (Chan et al., 1991; Rustenbeck et al., 1999). Phentolamine was found to interact directly with Kir6.2 since the drug produces a voltage-independent reduction in channel activity of Kir6.2ΔC in the absence of SUR1 (Proks & Ashcroft, 1997). The single-channel conductance was unaffected. Although the ATP molecule also contains an imidazoline group, the site at which phentolamine blocks is not identical to the ATP-inhibitory site, because phentolamine block of the ATP-insensitive mutant (K185Q) was normal.

Fatty Acyl CoA Modulates K_{ATP} Channels

Patch clamp studies have demonstrated that both saturated and unsaturated long chain acyl-CoA esters induce a rapid, slowly reversible opening of β-cell K_{ATP} channels (Larsson et al., 1996). This effect could be physiologically important since elevated circulating levels of free fatty acids such as occur in diabetes and obesity lead to an increase in β-cell levels of long chain acyl-CoA which, by opposing the effects of glucose on closure of K_{ATP} channels, could diminish the β-cell secretory response to glucose. The stimulation by long chain acyl-CoA involved increased occupancy of a prolonged open state without change in single channel unitary conductance (Bränström et al., 1997). The effect was additive with that of MgADP; moreover after brief treatment of the patch with trypsin channel opening induced by long chain acyl-CoA was unaffected whereas that induced by MgADP was abolished. This indicates that long chain acyl-CoA and MgADP do not bind to the same site. The site of action of long chain acyl-CoA has been shown to be Kir6.2 since long chain acyl-CoA was found to increase Kir6.2ΔC currents in *Xenopus* oocytes in the absence of SUR1 and the effect was not augmented by co-expression with SUR1 (Bränström et al., 1998; Gribble et al., 1998*a*).

It is not clear whether acyl CoAs interact directly with Kir6.2 or whether they influence channel activity by some other means such as an interaction with the lipid membrane. Specific interactions of acyl-CoAs with proteins are known to occur e.g., transcription factors. Binding studies are required to resolve this point.

PIP_2 is a Potent Activator of K_{ATP} Channels

Phosphatidylinositol bisphosphate (PIP_2) has been shown to be a potent activator of K_{ATP} channels in β-cells, cardiac myocytes, skeletal muscle cells, and SUR/Kir6.2 channels stably expressed in a mammalian cell line (Fan & Makielski, 1997; Baukrowitz et al., 1998; Shyng & Nichols, 1998; Koster, Sha & Nichols, 1999*a;* Ribalet, 1999; Shyng & Nichols, 1999). The effect of PIP_2 involves a dramatic decrease in ATP sensitivity of K_{ATP} channels—application of 5 μM PIP_2 to the intracellular side of the membrane rapidly increases the $K_{0.5}$ from around 10 μM to 3 mM (Shyng & Nichols, 1998). PIP_2 also abolishes high affinity tolbutamide sensitivity by stabilizing the open state of the channel and thereby driving the channel away from closed state(s) that are preferentially affected by high affinity tolbutamide binding (Koster et al., 1999*a*). The effectiveness was proportional to the number of negative charges on the head group of the anionic phospholipid and was antagonized by screening negative charges with polyvalent cations (Fan & Makielski, 1997). Treatment with phospholipases that reduced the charge on the lipids also reduced or eliminated the effect. In intact cells receptor-mediated activation of phospholipase C resulted in inhibition of K_{ATP}-mediated currents (Baukrowitz et al., 1998). These results suggest that intact phospholipids with negative charges are the critical requirement for activation of K_{ATP} channels rather than the usual cell signaling pathway through phospholipids that require cleavage. It is possible, therefore, that the gradual run-down of K_{ATP} channels seen in isolated patches ("washout") may result from hydrolysis of PIP_2 and that the ability of MgATP to refresh channel activity involves re-formation of the phospholipid (Hilgermann & Ball, 1996). It has been shown that wortmannin, an inhibitor of phosphatidylinositol 3- and 4-kinases, blocked the MgATP-dependent recovery (Xie et al., 1999). However after the MgATP-dependent recovery was blocked by wortmannin, PIP_2 still reactivated the channels. These results support the view that MgATP-dependent recovery may involve membrane lipid phosphorylation rather than protein phosphorylation, and that synthesis of PIP_2 or PIP_3 may upregulate Kir6.2 channels. Mutations of two positively charged amino acid residues at the C-terminus of Kir6.2 reduced the activating effects of phospholipids, suggesting involvement of this region in the activation (Fan & Makielski, 1997).

Endosulfine is a Potential Endogenous Regulator of K_{ATP} Channels

Screening of peptide fractions from brain for their ability to displace [^{3}H]-glibenclamide from brain or β-cell membranes (Virsolvy-Vergine et al., 1992) led to the isolation of two peptides, α- and β-endosulfine, able to interact with glibenclamide binding sites and to stimulate insulin secretion (Peyrollier et al., 1996; Virsolvy-Vergine et al., 1996). α-Endosulfine, M_r 23 kDa, has been cloned (Heron et al., 1998) and found to be highly similar to a protein kinase A regulated phosphoprotein of unknown function known as ARPP-19. α-Endosulfine is widely expressed. The recombinant protein, expressed

in bacteria competes with binding of [^{3}H]-glibenclamide to SUR1 with an EC_{50} of ~1 μM, inhibits cloned β-cell K_{ATP} channels with a similar efficacy and stimulates insulin secretion from MIN6 β-cell at a similar concentration in the absence of glucose (Heron et al., 1998). That endosulfine may be an endogenous regulator of β-cell channels is an attractive idea. However, this remains to be established.

G-Proteins Can Regulate K_{ATP} Channels

When tested in inside-out patches, Gα-i1 increased the activity of SUR/-Kir6.2 and SUR2A/Kir6.2 channels by 200 and by 30%, respectively (Sanchez et al., 1998). Conversely, Gα-i2 had no effect on the activity of SUR1/Kir6.2 channels, but increased the activity of SUR2A/Kir6.2 channels by 30%. No effects of the βγ subunits from either G(i1) or G(i2) on the single channel activity were observed. The molecular basis for these effects is unknown.

Leptin May be a Physiological Regulator of K_{ATP} Channels

Leptin, the product of the *ob* gene (Zhang et al., 1994), has been shown to inhibit insulin release in vitro (Emilsson et al., 1997; Kieffer et al., 1997) via activation of β-cell K_{ATP} channels (Harvey et al., 1997; Kieffer et al., 1997). There is evidence that the effect involves tyrosine kinase phosphorylation since the effect can be mimicked by inhibitors of tyrosine kinases and blocked by a tyrosine phosphatase inhibitor (Harvey & Ashford, 1998*b*). Moreover wortmannin and LY294002 prevented leptin activation of K_{ATP} channels, indicating that the effect of leptin may involve stimulation of phosphoinositide 3-kinase and hence may be mediated by PIP_2 (Harvey & Ashford, 1998*a*). Wortmannin also prevented activation of K_{ATP} channels by a tyrosine kinase inhibitor (Harvey & Ashford, 1998*b*). Paradoxically, insulin, whose actions involve activation of phosphoinositide 3-kinase (Shepherd, Withers & Siddle, 1998), did not mimic the effect of leptin on the β-cell K_{ATP} but rather reversed its effect, producing a complete block if applied before leptin (Harvey & Ashford, 1998*a*). This effect of insulin was specific for leptin, as diazoxide still activated K_{ATP} channels following prior exposure to insulin.

Phosphorylation Regulates the Activity of K_{ATP} Channels

K_{ATP} channels are modulated by hormones and neurotransmitters (De Weille et al., 1989; Wellman, Quayle & Standen, 1998). Both Kir6.2 and SUR1 contain consensus sites for phosphorylation by protein kinase A (PKA) and protein kinase C. Exogenous protein kinase A catalytic subunit has been shown to activate K_{ATP} channels (Ribalet, 1989) whereas activators of protein kinase C have been reported to lead to channel closure (Wollheim et al., 1988). Phosphorylation of the β-cell K_{ATP} channel by PKA has been studied in detail (Béguin et al., 1999). The PKA consensus sequences of human SUR1 and Kir6.2 were mutated to determine which potential phosphorylation sites could be phosphorylated. Ser372 of Kir6.2 and Ser1571 of SUR1 were both phosphorylated by PKA in homogenates of *Xenopus* oocytes expressing wild-type or mutant Kir6.2 and SUR1. The PKA site in Kir6.2 could also be phosphorylated in intact COS cells transfected with wild-type or mutant K_{ATP} channels subunits after Gs-coupled receptor (adrenaline, PACAP or GIP) stimulation or by direct PKA activation with forskolin or 3-isobutyl-1-methylxanthine. In contrast Ser1571 of SUR1 was already phosphorylated under basal conditions and could not be further phosphorylated. Functional studies showed that phosphorylation of PKA increased K_{ATP} channel activity whereas phosphorylation of SUR1 affected the basal channel properties by decreasing burst duration, interburst interval and open probability and also increased channel expression at the plasma membrane. Although the data show that tonic phosphorylation of K_{ATP} channels may be of considerable importance in channel characteristics, whether *changes* in PKA phosphorylation are physiologically important seems unclear. Stimulation of PKA is invariably associated with increase of insulin secretion (Ashcroft, 1994) whereas activation of K_{ATP} channels would be expected to lead to a decreased rate of secretion.

K_{ATP} Channels Contain Targeting Signals to Regulate Assembly and Transit to the Plasma Membrane

Although Kir6.2 fails to express K-channel activity in mammalian cells in the absence of SUR1 (Inagaki et al., 1995*a;* Sakura et al., 1995), a C-terminally truncated Kir6.2 is active (Tucker et al., 1997). These observations have been rationalized by studies on the trafficking of K_{ATP} channel subunits to the membrane in *Xenopus* oocytes (Zerangue et al., 1999). A novel endoplasmic reticulum (ER) retention signal, the three amino acid motif RKR, was discovered in the C-terminal portion of Kir6.2. Replacement of these residues by alanines allowed Kir6.2 to reach the surface in the absence of SUR1. The C-terminus of Kir6.2 also contains a dileucine motif that has been found to play a role in endosomal targeting (Trowbridge, Collawn & Ho, 1993). Mutation of the dileucine sequence did not result in surface expression but when combined with a mutation in the RKR motif led to a 5-fold increase in appear-

ance of Kir6.2 at the plasma membrane (Zerangue et al., 1999). This finding explains why Kir6.2ΔC36 which lacks both motifs shows much greater surface expression that Kir6ΔC26 in which only the RKR sequence is missing. SUR1 was also found to contain an RKR sequence located in the cytosolic region between TM11 and NBD1 (Zerangue et al., 1999). Mutation of this sequence resulted in a marked increase in expression of SUR1 at the cell surface in the absence of Kir6.2. In the absence of SUR1 Kir6.2 was found to have a strong dominant negative effect on surface expression of Kir6.2ΔC implying that Kir6.2 traps Kir6.2ΔC in a complex that does not reach the plasma membrane. The magnitude of the effect was consistent with that predicted for a dominant negative subunit in a tetramer. Thus Kir6.2 channels form tetramers in the absence of SUR1 but the RKR motif prevents their expression at the cell surface. The triple tandem fusion protein SUR1 ~ $(Kir6.2)_2$ will form an incomplete complex containing only 2 SUR1 subunits per Kir6.2 tetramer. Such an incomplete complex does not express at the cell surface unless free SUR1 is also present. This failure of incomplete complexes to reach the surface was also found to be attributable to the RKR motifs, with that in SUR1 playing a more important role than that in Kir6.2.

Despite these results, however, a number of studies have observed significant trafficking of individual K_{ATP} channel subunits to the plasma membrane in the absence of the other subunit. In a study with GFP-tagged Kir6.2 expressed in COS cells there was a similar amount of fluorescence at the plasma membrane whether or not SUR1 was co-expressed, and SUR1-GFP could also be visualized at the surface in the absence of Kir6.2 (Makhina & Nichols, 1998). Expression of active channels was also observed in EK293 cells transfected with Kir6.2; however expression was increased greatly by co-expression with SUR1 (John et al., 1998). The RKR signal does not seem to be effective in Sf9 cells since expression of glibenclamide binding activity using baculovirus was not increased by co-infection with Kir6.2; indeed a moderate decrease was observed (Mikhailov & Ashcroft, 2000).

A further signal affecting trafficking of K_{ATP} channels has been identified. The C-terminus of SUR1 has been shown to possess a positive signal composed of a dileucine and a downstream phenylalanine whose deletion severely impaired surface expression of K_{ATP} channels in transfected COS cells (Sharma et al., 1999). The mutations $SUR1_{L1566A}$ and $SUR1_{F1574A}$ essentially abolished surface expression of K_{ATP} channels. Photolabeling studies showed that C-terminally truncated SUR1 can assemble with Kir.2 but fails to traffic into the Golgi and plasma membrane.

The location of these trafficking signals on K_{ATP} channel subunits is indicated in Fig. 6.

Heteromeric and Homeric Interactions are Involved in Assembly of K_{ATP} Channels

There is growing evidence on regions of K_{ATP} channel subunits which may interact to form the active channel. Since the fusion protein SUR1-Kir6.2 leads to active K_{ATP} channels (Clement et al., 1997; Inagaki et al., 1997; Shyng & Nichols, 1997) it seems clear that in the native protein there is close juxtaposition of the C-terminus of SUR1 and the N-terminus of Kir6.2. Using an in vitro protein–protein interaction assay it has been demonstrated that the two intracellular domains of Kir6.2 mutually interact (Tucker & Ashcroft, 1999). A highly conserved region within the N-terminus is responsible for this interaction and a mutation within this region (G40D) which disrupts the interaction severely interferes with the ability of Kir6.2 to form a functional K_{ATP} channel.

There is evidence for a direct interaction between Kir6.2 and SUR1. From a mixture of Kir6.2 and SUR1 in vitro-translated proteins, and from COS cells transfected with both channel subunits, a Kir6.2-specific antibody co-immunoprecipitated Kir6.2 and SUR1 (Lorenz et al., 1998). Kir6.2ΔC37 also co-immunoprecipitated with SUR1, suggesting that the distal carboxy terminus of Kir6.2 is unnecessary for subunit association. However, a co-immunoprecipitation approach in HEK293 cells stably transfected with SUR1 and Kir6.2 suggested that a domain in the C-terminus of Kir6.2 (amino acids 208–279) was involved in biochemical interaction with SUR1 (Giblin, Leaney & Tinker, 1999). The domain, while necessary, was not sufficient, however, and full reconstitution of K_{ATP} channels required a largely intact N- and C-terminus. A requirement for a proximal C-terminal domain of Kir6.2 has also been observed for association of Kir6.2 and SUR2A (Lorenz & Terzic, 1999). Using a baculovirus system, enhanced glibenclamide binding activity was observed when Kir 6.2 was co-expressed with N- and C-terminal SUR1 half-molecules, again indicating interaction between SUR1 and Kir6.2 (Mikhailov & Ashcroft, 2000). The C-terminal domain of SUR1, however, does not appear to play a major role in this interaction since the increased glibenclamide binding elicited by Kir6.2 was not reduced when NBD2 was deleted from the C-terminal SUR1 half-molecule.

A study on K_{ATP} channels in *Xenopus* oocytes co-expressing SUR1 and wild-type or mutant Kir6.2 showed that N-terminal deletions of Kir6.2 did not affect the intrinsic properties of Kir6.2 but abolished block by sulfonylurea (Reimann et al., 1999*b*). Site-directed mutagenesis suggested that hydrophobic residues in Kir6.2 may be involved in this effect. These data suggest that the N-terminus of Kir6.2 plays a role in coupling sulfonylurea binding to SUR1 to closure of the Kir6.2 pore.

There is evidence for an interaction of NBD1 and

NBD2 (Mikhailov & Ashcroft, 2000). NBD1 expressed in sF9 cells as a GFP fusion protein was distributed throughout the cell. After co-expression of NBD1-GFP with the C-terminal half of SUR1, NBD1-GFP was localized near the plasma membrane. This effect disappeared when NBD2 was deleted from the C-terminal fragment indicating strong interaction between NBD1 and NBD2. Co-expression of NBD1-GFP with Kir6.2 did not localize NBD1-GFP to the plasma membrane suggesting a lack of strong interaction between Kir6.2 and NBD1.

In view of the strong evidence for association of 4 Kir6.2 and 4 SUR1 in the active channel it is of interest that the isolated purified NBD1 domain of SUR1 alone has been reported to form a tetramer (Mikhailov & Ashcroft, 2000), suggesting that homomeric interactions between NBD1 domains participate in channel assembly.

The available evidence on these homo- and heteromeric interactions is summarized in Fig. 6.

Transcriptional Regulation of K_{ATP} Channel Genes is Important for Specific Channel Expression

The genes encoding human Kir6.2 and SUR1 have been cloned and shown to lie adjacent to one another on chromosome 11p15.1. The gene for SUR1 consists of 39 exons spanning approximately 100 kb of genomic DNA whereas the gene for Kir6.2 consists of a single intron-free exon (Aguilar-Bryan et al., 1998). The genes are closely spaced with only 4.5 kilobases separating the 3′ end of SUR1 and the 5′ end of Kir6.2 (Aguilar-Bryan et al., 1995). Transcriptional regulation of the two genes is important for correct tissue-specific expression and to ensure that equal amounts of the two subunits are produced. A channel containing one (out of four) Kir6.2 subunits not coupled to an SUR1 subunit has no activity (Clement et al., 1997; Inagaki et al., 1997) so an excess of Kir6.2 would produce inactive K_{ATP} channels containing four Kir6.2 but less than four SUR1 subunits. The upstream DNA sequences of both genes have been cloned and shown to drive expression of a reporter gene in pancreatic β-cells (Ashfield & Ashcroft, 1998). Deletion analysis showed that the minimum promoter sequence for SUR1 comprised a short (173 bp) 5-sequence. In contrast, over 900 bp of the Kir6.2 upstream sequence was required for high level expression. Both promoters resemble those for ubiquitously expressed housekeeping genes as they lack a TATA box in the minimal promoter regions, are GC-rich and contain multiple SP1 sites (GGGCGG). Both promoters contain E-boxes (CANNTG) known to be important for insulin gene regulation which bind a family of helix-loop-helix proteins e.g., neuroD. Both promoters also contain AP2 sites suggesting possible regulation of transcription by protein kinase A and/or protein kinase C. In addition the SUR1 and Kir6.2 promoters contain potential binding sites for a number of tissue-specific factors including Pdx-1, Pax6, HNF3, and PEA3. The most important elements regulating SUR1 promoter activity are likely to be the two SP1 sites, the G-box (MAZ/Pur-1 binding site) and the three E-boxes in the minimal promoter sequence (Ashfield & Ashcroft, 1998). Mobility shift assay showed interaction of the SP1 transcription factor with the proximal promoter region of the mouse SUR1 gene (Hernández-Sánchez et al., 1999). Although the Kir6.2 promoter requires sequences up to 1 kb 5′ of the gene, the proximal 300 bp contains an Alu repeat and its removal results in increase of activity (Ashfield & Ashcroft, 1998). The region important for Kir6.2 promoter activity lies therefore upstream of the Alu repeat and contains several SP1 sites, G-boxes and E-boxes. Which transcription factors are important in vivo has yet to be determined. However in mouse β-cells SUR1 and Kir6.2 mRNA levels are both down-regulated by exposure to glucocorticoid (Hernández-Sánchez et al., 1999).

Studies on the cell specificity of K_{ATP} gene promoter activity (Ashfield & Ashcroft, 1998) showed that the promoter sequences described are not completely tissue-specific, as SUR1 and Kir6.2 are not normally expressed in liver or epithelium and yet the promoters drove reporter gene expression in cell lines derived from these tissues. There must therefore be sequences distal to the core promoters that are required for full tissue specificity, such as enhancer or silencer elements. As SUR1 and Kir6.2 are not expressed in exactly the same tissues, the two promoters are unlikely to share the same elements.

Mutations of K_{ATP} Channel Subunits Occur in Neonatal Hyperinsulinemia

Neonatal hyperinsulinism (HI) is a disease characterized by inadequate suppression of insulin secretion in the presence of severe hypoglycemia (Dunne et al., 1999; Glaser, Landau & Permutt, 1999). Mutations in both Kir6.2 and SUR1 have been shown to result in HI (Thomas et al., 1995; Thomas, Ye & Lightner, 1996; Nestorowicz et al., 1997; Aguilar-Bryan & Bryan, 1999). β-Cells from patients with HI and SUR1 mutations that result in C-terminal deletions have been shown to lack functioning K_{ATP} channels (Kane et al., 1996; Dunne et al., 1997*b*). One reason for the failure of such truncated SUR1 molecules to produce functional channels is the absence of the positive C-terminal trafficking signal described above (Sharma et al., 1999). A number of SUR1 point mutations spread throughout the molecule have been found to result in channels that have reduced sensitivity to activation by MgADP (Shyng et al., 1998). Mutation of a conserved residue in NBD2 (R1420C), found in a Japanese HI patient, was shown to result in an

impairment of the stabilization by MgATP of ATP binding to NBD1 (Tanizawa et al., 1999).

The β-Cell also Contains Intracellular K_{ATP} Channels

Sulfonylureas stimulate insulin secretion in a Ca^{2+}-dependent manner from permeabilized β-cells by a mechanism in which plasma membrane K_{ATP} channels cannot be involved. (Tian, Johnson & Ashcroft, 1998). Moreover exocytosis can be stimulated by sulfonylureas applied intracellularly during whole-cell patch recording at a fixed intracellular Ca^{2+} concentration and this effect is blocked by diazoxide (Barg et al., 1999). These observations suggested that there may be sulfonylurea receptors playing a role in insulin secretion located elsewhere than in the plasma membrane K_{ATP} channels. The stimulatory action of sulfonylureas in whole cell patch recordings could also be antagonized by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid which blocks Cl^--channels and by tamoxifen which inhibits the multidrug resistance (mdr) protein- and volume-regulated Cl^- channels. Neither of these drugs is particularly specific. However the action of sulfonylureas was also blocked by intracellular application of an antibody raised against a 170 kDa mdr. Immunocytochemistry and Western blot analyses showed that the antibody recognizes a 65-kDa protein in the secretory granules. In contrast to results reported for zymogen granules which showed that a 65 kDa mdr1 gene product constituted a sulfonylurea receptor (Braun, Anderie & Thévenod, 1997), the β-cell protein did not bind sulfonylureas and was distinct from SUR1 (or similar protein) also present in the granules. It was concluded (Barg et al., 1999) that sulfonylureas have a direct effect on exocytosis of insulin via binding to an SUR in the secretory granules that is coupled to a 65-kDa mdr-like protein, resulting in the activation of a granular Cl^- conductance.

An additional site of action of sulfonylureas is a mitochondrial K_{ATP} channel (mitoK_{ATP}) first identified in liver (Inoue et al., 1991). This K-channel was blocked both by ATP and by glibenclamide (Garlid, 1996; Szewczyk, 1996) and can be activated by KCOs (Szewczyk, Wójcik & Nalecz, 1995; Garlid et al., 1996). MitoK_{ATP} channel activity has been reconstituted in liposomes and planar lipid membranes containing partially purified mitochondrial inner membrane proteins (Paucek et al., 1992). The reconstituted channel had a unit conductance at saturating $[K^+]$ of about 30 pS. K^+ flux was inhibited with high affinity by ATP and ADP in the presence of divalent cations and by glibenclamide in the absence of divalent cations. The nucleotide-binding site(s) face the cytosol (Yarov-Yarovoy et al., 1997). Binding studies with mitochondria have shown the existence of a single class of low affinity glibenclamide binding sites (Szewczyk et al., 1997). The molecular nature of the mitochondrial SUR has not been established. However there is evidence from immunofluorescence and immunogold staining that the mitochondrial inner membrane contains Kir6.1 (Suzuki et al., 1997). The mitoK_{ATP} channel has only been studied so far in heart and liver (Szewczyk, 1998). Whether a mitoK_{ATP} channel is present in the β-cell and if so what role it plays remains to be established.

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