Potassium channel openers require ATP to bind to and act through sulfonylurea receptors

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K_{ATP} channels are composed of a small inwardly rectifying K⁺ channel subunit, either K_{IR}6.1 or K_{IR}6.2, plus a sulfonylurea receptor, SUR1 or SUR2 (A or B), which belong to the ATP-binding cassette superfamily. SUR1/K_{IR}6.2 reconstitute the neuronal/pancreatic β -cell channel, whereas SUR2A/K_{IR}6.2 and SUR2B/K_{IR}6.1 (or K_{IR}6.2) are proposed to reconstitute the cardiac and the vascular-smooth-muscle-type K_{ATP} channels, respectively. We report that potassium channel openers (KCOs) bind to and act through SURs and that binding to SUR1, SUR2A and SUR2B requires ATP. Non-hydrolysable ATP-analogues do not support binding, and Mg²⁺ or Mn²⁺ are required. Point mutations in the Walker A motifs or linker regions of both nucleotide-binding folds (NBFs) abolish or weaken [³H]P1075 binding to SUR2B, rendering reconstituted SUR2B/K_{IR}6.2 channels insensitive towards KCOs. The C-terminus of SUR affects KCO affinity with SUR2B ~ SUR1 > SUR2A. KCOs belonging to different structural classes inhibited specific [³H]P1075 binding to SUR2B in a monophasic manner, with the exception of minoxidil sulfate, which induced a biphasic displacement. The affinities of KCO binding to SUR2B were 3.5–8-fold higher than their potencies for activation of SUR2B/K_{IR}6.2 channels. The results establish that SURs are the KCO receptors of K_{ATP} channels and suggest that KCO binding requires a conformational change induced by ATP hydrolysis in both NBFs.

Keywords: ATP-sensitive potassium channel/K_{IR}6.2/potassium channel openers/SUR

Introduction

Potassium channel openers (KCOs) comprise a structurally diverse group of drugs with a broad spectrum of potential therapeutic applications (e.g. hypoglycaemia, hypertension, arrhythmias, angina pectoris, asthma; Lawson, 1996). These drugs (e.g. diazoxide, pinacidil, levromakalim and minoxidil sulfate) exert their effects on secretory cells, neurones, vascular and non-vascular smooth muscle, and on cardiac and skeletal muscle by opening ATP-sensitive

potassium channels (K_{ATP} channels), thus shifting the membrane potential towards the reversal potential for potassium and reducing cellular electrical activity (Edwards and Weston, 1993). K_{ATP} channels are assembled with an octameric stoichiometry (SUR/Kir6.x)₄ from two structurally distinct subunits, an inwardly rectifying potassium channel subunit (K_{IR}6.1 or K_{IR}6.2) forming the pore and a regulatory subunit, a sulfonylurea receptor (SUR), belonging to the ATP-binding cassette (ABC) superfamily with multiple transmembrane domains and two nucleotide-binding folds (NBFs) (Aguilar-Bryan *et al.*, 1995, 1998; Inagaki *et al.*, 1995a, 1996; Isomoto *et al.*, 1996; Clement *et al.*, 1997; Yamada *et al.*, 1997). Three isoforms of SURs have been cloned, a high-affinity sulfonylurea receptor, SUR1, and two low-affinity receptors, SUR2A and SUR2B (Aguilar-Bryan *et al.*, 1995; Inagaki *et al.*, 1996; Isomoto *et al.*, 1996). The latter are splice products of a single gene, differing only in their C-terminal 42–45 amino acids (Chutkow *et al.*, 1996; Isomoto *et al.*, 1996; Aguilar-Bryan *et al.*, 1998). SUR1/K_{IR}6.2 have been proposed to reconstitute the neuronal/pancreatic β -cell- (Inagaki *et al.*, 1995a), SUR2A/K_{IR}6.2 the cardiac- (Inagaki *et al.*, 1996; Okuyama *et al.*, 1998) and SUR2B/K_{IR}6.1 (or K_{IR}6.2) the vascular smooth muscle-type K_{ATP} channels (Isomoto *et al.*, 1996; Yamada *et al.*, 1997).

Whereas a truncated form of K_{IR}6.2 was not activated by KCOs (Tucker *et al.*, 1997), SUR isoforms confer different KCO sensitivities when co-expressed with K_{IR}6.2 (Inagaki *et al.*, 1995a, 1996; Isomoto *et al.*, 1996) suggesting that the KCO receptor sites reside on SURs and that the C-terminus might be critical for KCO binding. However, K_{IR}6.1 expressed alone in *Xenopus* oocytes has been reported to be activated by diazoxide (Inagaki *et al.*, 1995b), arguing for a direct effect of KCOs on the inward rectifier subunit. The present results establish that KCOs bind to and act through SURs, that binding requires ATP and both NBFs, and that the C-terminus of SURs affects binding affinity with SUR2B ~ SUR1 > SUR2A.

Results

KCOs bind to SUR2 isoforms but not to inward rectifiers

Indirect evidence suggests that KCOs act through SURs and that ATP is required for both their binding and action (Dunne *et al.*, 1987; Niki and Ashcroft, 1991; Schwanstecher *et al.*, 1991, 1992; Bray and Quast, 1992; Quast *et al.*, 1993; Dickinson *et al.*, 1997; Löffler-Walz and Quast, 1998). Consistent with this idea, in the absence of ATP, no specific binding of the tritiated pinacidil analogue P1075 was detected in membranes from COS-7 cells transiently expressing SUR subunits (Figure 1). Addition of ATP (100 μ M), however, produced strong specific binding (maximum binding capacity, $B_{\max}$ = 20–

Fig. 1. Specific [3 H]P1075 binding to K_{ATP} channel subunits. Binding was assessed to membranes from COS-7 cells transiently expressing mouse $K_{IR}6.1$ ($K_{IR}6.1$), human $K_{IR}6.2$ ($K_{IR}6.2$), rat SUR2A (SUR2A), human SUR2B (SUR2B), rat SUR2A with the C-terminus of hamster SUR1 (SUR2/ct1) or rat SUR2A with the C-terminus of human SUR2B (SUR2/ctB). Incubations contained 3 nM [3 H]P1075, 1 mM of free Mg^{2+} , 5–50 μ g of membrane protein and either no ATP or 100 μ M ATP. Specific binding was defined by the addition of 100 μ M pinacidil and is shown as % of total binding. Maximum binding capacity (B_{max}) of the SUR2 isoforms in the presence of 100 μ M ATP ranged from 20 to 50 pmol per mg membrane protein.

50 pmol/mg membrane protein, $n = 30$ independent transfections) to both membranes with SUR2A and SUR2B. Membranes with $K_{IR}6.1$ or $K_{IR}6.2$ (devoid of SURs) did not show specific P1075 binding in either the presence or absence of ATP (Figure 1).

Binding requires hydrolysable nucleoside triphosphates and divalent cations

[3 H]P1075 binding to SUR2B was half-maximally stimulated at 6 μ M ATP with a Hill coefficient close to 1 (Figure 2A). ADP and other hydrolysable nucleotides (GTP, GDP, UTP, UDP, ITP, CTP) imitated the action of ATP. However, ADP was not effective when the ATP concentration was kept low by the hexokinase reaction and therefore nucleoside diphosphates probably act indirectly via enzymatic nucleoside triphosphate formation. The non-hydrolysable ATP analogues, AMP-PNP and AMP-PCP (up to 10 mM), failed to reproduce the effect. However, AMP-PNP abolished [3 H]P1075 binding induced by 100 μ M ATP when present in excess (IC_{50} value of 1.2 ± 0.1 mM; $n = 4$) suggesting that ATP hydrolysis is necessary for KCO binding. This idea is strengthened by the requirement for Mg^{2+} or Mn^{2+} , and the weak effectiveness of Ca^{2+} , Zn^{2+} and Sr^{2+} (Figure 2B), a pattern characteristic of other ATP hydrolases.

KCO binding to SUR1

The affinity of [3 H]P1075 for SUR1 was too weak to allow direct detection of binding using filtration assays; thus KCO binding to SUR1 was measured indirectly through the allosteric inhibition of high-affinity sulfonylurea binding (Schwanstecher *et al.*, 1991, 1992). Displacement of [3 H]glibenclamide ($K_D = 0.55 \pm 0.03$ nM, $B_{max} = 51 \pm 0.5$ pmol/mg membrane protein, $n = 30$ independent transfections) by diazoxide or pinacidil was weak in the absence of nucleotides (Figure 2C, open symbols), but was enhanced by ATP (Figure 2C, closed symbols), yielding apparent dissociation constants of 140 μ M or 480 μ M, respectively (Table I). The effect of ATP on KCO binding to SUR1 was similar to that determined for SUR2B (compare Figure 2A with 2D); both effects

Fig. 2. Specific KCO binding to SURs requires ATP plus divalent cations. Binding assays were carried out with membranes from COS-7 cells expressing wild-type human SUR2B (A and B) or hamster SUR1 (C and D). (A) Effect of MgATP on [3 H]P1075 (3 nM) binding to SUR2B ($EC_{50} = 6 \pm 1$ μ M, Hill coefficient = 1.03). (B) P1075 binding to SUR2B requires divalent cations. (C) Effect of diazoxide (circles) or pinacidil (squares) on [3 H]glibenclamide binding (0.3 nM) to hamster SUR1 in the absence (open symbols) or presence of 100 μ M MgATP (closed symbols). * $p < 0.05$ for comparison with the corresponding value in the absence of ATP. Values for IC_{50} and Hill coefficient: 210 ± 19 μ M, 0.90 (diazoxide + MgATP; ●); 750 ± 62 μ M, 0.98 (pinacidil + MgATP; ■). (D) Effect of MgATP on diazoxide binding to SUR1 ($EC_{50} = 0.9 \pm 0.2$ μ M, Hill coefficient = 0.92). Diazoxide (%) is the percentage of ATP-dependent displacement of [3 H]glibenclamide by 280 μ M diazoxide (vertical line in C) as defined in the Materials and methods. Non-specific binding was defined by addition of 100 μ M pinacidil (A and B) or 100 nM glibenclamide (C and D), and amounted to less than 2% of total maximal binding.

required Mg^{2+} or Mn^{2+} , and were not supported by non-hydrolysable ATP analogues or by ADP in the presence of hexokinase.

Pharmacological characterization of SUR2B

Competition-binding experiments, in the presence of MgATP, were performed to characterize the properties of the P1075 binding site on SUR2B. Unlabelled P1075, pinacidil, levcromakalim or diazoxide induced monophasic inhibition curves with Hill coefficients close to 1 and dissociation constants of 12 nM, 0.14 μ M, 0.38 μ M or 14 μ M, respectively (Figure 3A; Table I). Interestingly, minoxidil sulfate induced a biphasic inhibition with apparent K_D s of 0.50 μ M and 0.77 mM. MgADP or MgGDP (up to 2 mM) did not significantly displace P1075 binding (Figure 3A).

To assess the functional relevance of the KCO site on SUR2B the ability of the openers to stimulate reconstituted SUR2B/ $K_{IR}6.2$ channels was tested. In the presence of MgATP all of the drugs stimulated K_{ATP} channel activity (Figure 3B and C) in inside-out patches from COS-7 cells transiently co-expressing SUR2B and $K_{IR}6.2$ with EC_{50} values of 45 nM (P1075), 0.68 μ M (pinacidil), 3.1 μ M (levcromakalim) or 58 μ M (diazoxide). These values correspond with the concentrations of these drugs reported to be half-maximally effective on K_{ATP} channels from

Fig. 3. Binding affinities for SUR2B and potencies of KCOs to activate SUR2B/ $\text{K}_{\text{IR}}6.2$ channels. (A) $[^3\text{H}]\text{P1075}$ (3 nM) displacement assays were carried out with membranes from COS-7 cells expressing wild-type human SUR2B. All incubations contained 100 μM MgATP, 1 mM free Mg^{2+} and displacing drugs or nucleoside diphosphates as indicated. The IC_{50} values (half-maximal inhibitory concentrations) and Hill coefficients are: 15 ± 2 nM, 1.0 (P1075; ●); 0.16 ± 0.01 μM , 0.94 (pinacidil; ○); 0.45 ± 0.03 μM , 0.91 (levcromakalim; LC, ■); 16 ± 2 μM , 0.90 (diazoxide; □). Minoxidil sulfate (MS; ▲) induced a biphasic displacement with $\text{IC}_{50(1)} = 0.62 \pm 0.2$ μM and $\text{IC}_{50(2)} = 0.96 \pm 0.2$ mM. (B) Levcromakalim-induced activation of SUR2B/ $\text{K}_{\text{IR}}6.2$ channels transiently expressed in COS-7 cells. Representative current recorded from an inside-out patch at -50 mV. Inward currents are shown as downward deflections. Free Mg^{2+} was maintained at 1 mM in all solutions. The patch was exposed to 1 mM MgATP and increasing levcromakalim concentrations as indicated by the line above the record. (C) Potencies of KCOs to activate recombinant SUR2B/ $\text{K}_{\text{IR}}6.2$ channels. Channel activation was recorded in inside-out patches as shown in B. Results are expressed as percentages of currents induced by maximally effective drug concentrations. The EC_{50} values (half-maximally effective concentrations) and Hill coefficients are: 45 ± 10 nM, 1.5 (P1075; ●); 0.68 ± 0.15 μM , 1.3 (pinacidil; ○); 3.1 ± 1.3 μM , 1.3 (levcromakalim, LC, ■); 58 ± 11 μM , 2.1 (diazoxide; □). Results shown as mean $\pm$ SEM ($n = 4$ –5). ATP and/or the KCOs did not show a significant effect on single channel current amplitudes.

mesenteric vein (Russell *et al.*, 1992; Quayle *et al.*, 1995) or coronary arteries (Xu and Lee, 1994), arguing that SUR2B is the KCO receptor of vascular smooth muscle. None of the KCOs tested was capable of enhancing the activity of SUR2B/ $\text{K}_{\text{IR}}6.2$ -channels inhibited by either

AMP-PNP (1 mM, presence of Mg^{2+}) or free ATP (1 mM, absence of Mg^{2+}), which was consistent with the finding that neither of these conditions supports KCO binding to SUR2B.

The C-terminus of SURs is critical for KCO binding

To analyse the importance of the SUR C-terminus for KCO binding we engineered plasmids that express chimeric proteins based on a common 1503 amino acid rat SUR2 'backbone' to minimize interspecies variability. We assessed the affinities of P1075, pinacidil, levcromakalim and diazoxide for rat SUR2A and two chimeras containing either the C-terminal 42 residues of hamster SUR1 (SUR2/ct1) or human SUR2B (SUR2/ctB; Figure 1). The dissociation constants of the two chimeras did not differ significantly from those observed for human SUR2B, whereas the affinities for binding to rat SUR2A were 3–5-fold lower (Table I).

ATP activation of KCO binding requires two intact NBFs

SURs are members of the ATP-binding cassette (ABC) superfamily with two folds (NBFs) for binding and hydrolysis of nucleotides (Aguilar-Bryan *et al.*, 1995, 1998; Inagaki *et al.*, 1996). These folds have the Walker A and B motifs and a connecting linker region (Higgins, 1992; Aguilar-Bryan *et al.*, 1998). The Walker A motif, -gly-x-gly-x-gly-lys-(ser/thr)-, forms part of the nucleotide-binding pocket, with the conserved lysine interacting with the β - and γ -phosphates of ATP (Saraste *et al.*, 1990; Higgins, 1992). Mutation of this residue significantly reduces or abolishes hydrolytic activity in other ATPases (e.g. P-glycoprotein). The linker region with the consensus motif, -leu-ser-gly-gly-gln-, is thought to be involved in transducing conformational changes resulting from ATP hydrolysis (Ames *et al.*, 1992). Mutation of the highly conserved Walker A lysine (K711R or K1352R) in either NBF of human SUR2B eliminated ATP activation of KCO binding, whereas binding was weakened, but not abolished by mutations in the linker regions (G809D or G1446R; Figure 4A). As indicated by displacement studies, the mutations did not alter the affinity for P1075 binding but decreased the B_{max} (maximal number of KCO binding sites). Loss of KCO binding caused by defective processing was ruled out since the mutations did not significantly diminish K_{ATP} channel activity in the absence of nucleotides. Whereas channels reconstituted with the K/R mutations (K711R or K1352R) could not be activated by 30 μM pinacidil (1–5% of activation observed in wt SUR2B/ $\text{K}_{\text{IR}}6.2$ channels; $n = 4$ each), the mutations in the linker regions (G809D or G1446R) showed reduced activation (42–59% of wt activation; $n = 4$ each; traces for the mutations in NBF1 shown in Figure 4B). Consistent with results reported for SUR1 (Gribble *et al.*, 1997), the K/R mutations revealed a slightly increased sensitivity towards the inhibitory action of ATP (Figure 4B). None of the mutations studied induced a significant effect on the single-channel current amplitude.

Discussion

Evidence for a conformational change in SURs, that requires MgATP and controls KCO binding

This study is the first to present direct evidence for an effect of ATP on KCO binding to SURs. The results show

Table 1. Dissociation constants for binding of KCOs to sulfonylurea receptor isoforms

KCO	SUR2B	SUR2/ct1	SUR2/ctB	SUR2A	SUR1
P1075	12 ± 2 nM	9 ± 1 nM	10 ± 1 nM	46 ± 2 nM ^a	1.02 ± 0.07 mM
pinacidil	135 ± 11 nM	140 ± 6 nM	87 ± 4 nM	404 ± 19 nM ^a	0.48 ± 0.04 mM
levromakalim	380 ± 31 nM	630 ± 32 nM	390 ± 15 nM	1880 ± 85 nM ^a	> 0.5 mM
diazoxide	14 ± 2 µM	18 ± 1 µM	19 ± 1 µM	76 ± 2 µM ^a	0.14 ± 0.01 mM

[³H]P1075 (3 nM, SUR2 isoforms) or [³H]glibenclamide (0.3 nM, hamster SUR1) displacement assays were carried out with membranes from COS-7 cells transiently expressing human SUR2B (SUR2B), rat SUR2A with the C-terminus of hamster SUR1 (SUR2/ct1), rat SUR2A with the C-terminus of human SUR2B (SUR2/ctB), rat SUR2A (SUR2A) or hamster SUR1 (SUR1), and K_D values were calculated from competition curves as described previously (Schwanstecher *et al.*, 1991). Results shown as mean ± SEM from 4–6 independent displacement curves. ^a $p < 0.05$ for the comparison with SUR2/ctB.

Fig. 4. Effects of mutations in conserved motifs of both NBFs on [³H]P1075 binding to SUR2B and activation of SUR2B/K_{IR}6.2 channels by pinacidil. (A) Binding assays were carried out with membranes from COS-7 cells expressing wild-type or mutant human SUR2B. All incubations contained 3 nM [³H]P1075, 100 µM MgATP and identical amounts of membrane protein (50 µg/ml). Specific binding is given as the percentage of binding to the wild-type (wt). (B) Currents were measured from wild-type or mutant channels in inside-out patches from COS-7 cells expressing human SUR2B and K_{IR}6.2. Free Mg²⁺ was maintained at 1 mM in all solutions. Patches were exposed to 1 mM MgATP and 30 µM pinacidil as indicated by the bars above the traces.

that SURs are the KCO receptors of K_{ATP} channels and that KCO binding to SUR1, SUR2A and SUR2B requires ATP. The observations are consistent with the idea that ATP binding and presumably hydrolysis at both NBFs

induces a conformational change in SUR that greatly increases its affinity for KCOs. Specifically: (i) non-hydrolysable ATP analogues, or ADP in the presence of an ATP capturing system, do not stimulate KCO binding; (ii) divalent cations are required and have a rank-order effectiveness (Mg = Mn > Zn > Ca > Sr) characteristic for ATPase activity of other ABC proteins (e.g. MalK and P-glycoprotein; al-Shawi and Senior, 1993; Morbach *et al.*, 1993; Urbatsch *et al.*, 1994); (iii) ATP, GTP, CTP and ITP are all effective, a nucleoside triphosphate specificity typical for other traffic ATPases (Higgins, 1992; Gadsby *et al.*, 1995; Senior *et al.*, 1995a); and (iv) point mutations in either NBF, which affect nucleotide binding or hydrolysis in other ABC proteins, block the stimulatory effect of ATP on KCO binding.

SUR's role in regulation of K_{ATP} channel activity

There is a strong correlation between KCO binding and the activation of mutant SUR2B/wild-type K_{IR}6.2 channels which establishes that the KCO binding sites on SURs are functionally relevant receptor sites. Mutants without binding activity (K711R and K1352R) are not activated by pinacidil, whereas G809D and G1446R show both impaired [³H]P1075 binding and pinacidil activation.

Both NBFs are required for KCO binding. Similarly, ATP hydrolysis at both NBFs is necessary for the transport activity of P-glycoprotein and other ABC proteins, including STE6 (Higgins, 1992). This analogy suggests that transition from the non-binding to the KCO-binding state might reflect a step which is critical for SUR's role in the regulation of K_{ATP} channel activity. Consistent with this idea, point mutations in the NBFs of SUR1 affected channel activation by both KCOs and nucleoside diphosphates (Gribble *et al.*, 1997; Shyng *et al.*, 1997), and were shown to cause persistent hyperinsulinemic hypoglycemia of infancy (PHHI; Bryan and Aguilar-Bryan, 1997).

We infer that ATP binding and/or hydrolysis at both NBFs might be required to put SUR into a 'sensitive' conformational state that can bind both KCOs and nucleoside diphosphates. Alternatively this state might be induced by the interaction of ATP with either of the two NBFs, which could operate in alternating catalytic cycles similar to the proposed model for P-glycoprotein (Senior *et al.*, 1995b; Senior and Gadsby, 1997).

It has been suggested that KCOs and nucleoside diphosphates exert channel activation via interaction with the same receptor site on SUR1 (Shyng *et al.*, 1997). However, neither MgADP nor MgGDP (up to 2 mM) affect ATP-stimulated P1075 binding to SUR2B, arguing against this model.

Both NBFs are essential for KCO binding and action

The requirement of both NBFs for KCO binding to SUR2B suggests a re-interpretation of some of the SUR1 NBF mutation data. For example, the substitution of an alanine for the conserved lysine in the Walker A motif in NBF1 (K719A) abolished diazoxide-induced activation of SUR1/ $K_{IR}6.2$ channels, whereas substitution of a methionine at the equivalent position in NBF2 (K1384M) had a more subtle effect, eliminating activation by diazoxide in the presence of low (10 μ M), but not high (100 μ M) [ATP] (Gribble *et al.*, 1997). This led to the interpretation that NBF1, but not NBF2, was essential for channel activation by KCOs. However, by analogy with the results seen with SUR2B, substitution of a lysine for an arginine in either NBF of SUR1 (K719R and/or K1384R) eliminates diazoxide binding and induces a complete loss of activation of SUR1/ $K_{IR}6.2$ channels (results not shown). On the other hand, substitution of a methionine in NBF2 of SUR1 (K1384M) does not reduce KCO binding at saturating ATP, but decreases the affinity for ATP (M.Schwanstecher *et al.*, unpublished data). Thus differential activation of SUR1_{K1384M}/ $K_{IR}6.2$ channels by diazoxide at two ATP concentrations reflects a subtle decrease in the affinity of NBF2 for ATP, rather than providing the information that NBF2 is not important for KCO-induced channel activation.

The Hill coefficients for ATP stimulation of KCO binding to SUR1 and SUR2B were close to 1, suggesting that both NBFs have similar affinities for ATP with K_D s in the low μ M range (EC_{50} for SUR1 = 1 μ M and for SUR2B = 6 μ M). Alternatively, analogous to a model proposed for CFTR (Senior and Gadsby, 1997), one of the two NBFs might have a much higher affinity, with ATP stimulation of KCO binding 'seeing' only the 'low'-affinity site. We infer that at least one NBF has a K_D for ATP in the micromolar range. Consistent with this conclusion, the binding and photolabelling of SUR1 with micromolar concentrations of 8-azido ATP has been demonstrated (Ueda *et al.*, 1997). This labelling was impaired by mutations in NBF1, but not NBF2, leading to the proposal that ATP interacts solely with the first NBF. However, these results are also compatible with a sequential interaction of ATP with both NBFs where binding of ATP to NBF2 requires prior ATP binding (and/or hydrolysis) in NBF1.

Evidence for distinct KCO receptor sites on SURs

In rat heart membranes, minoxidil sulfate did not completely displace [³H]P1075 binding and it has been suggested that this might be due to different receptor isoforms in the membrane preparation (Löffler-Walz and Quast, 1998). We observed biphasic displacement by minoxidil sulfate in membranes expressing SUR2B only, indicating that this reflects an intrinsic property of SUR2B. The stoichiometry of KCO binding to SUR is not yet clear. Our displacement data are consistent with either a single KCO-binding site per SUR or multiple non-interacting sites. Thus the simplest explanation of biphasic displacement would be two KCO sites per SUR, with minoxidil sulfate being the only drug tested which binds differentially to both sites. Alternatively, biphasic displacement could be explained by binding of minoxidil sulfate

to a distinct site on SUR2B coupled with negative allosterism to the P1075 site.

Role of the C-terminus in KCO binding

The affinities of P1075, pinacidil, levromakalim and diazoxide were 4–5-fold lower for rat SUR2A than for human SUR2B, or a chimera containing the rat SUR2 backbone and the C-terminal 42 amino acids of human SUR2B (SUR2/ctB). This demonstrates that the C-terminus affects KCO affinity and explains the lower potencies of these compounds at cardiac versus vascular K_{ATP} channels. Interestingly, the C-terminal 42 amino acids of SUR1 were not sufficient to confer low affinity to the SUR2 backbone (SUR2/ct1; Table I) indicating that the C-terminus of SURs is not the only determinant of KCO affinity. The C-terminus of SUR2B is more similar to that of SUR1 than that of SUR2A (30/42 identical residues versus 15/42) and thus the sequence comparison is consistent with the finding that KCO affinity of the SUR2/ct1 chimera does not differ significantly from that of SUR2B. It will be of interest to identify which C-terminal residues are responsible for lower KCO affinity of SUR2A.

Stoichiometry of KCO-induced channel activation

The potencies of KCOs to activate recombinant SUR2B/ $K_{IR}6.2$ channels were 3.5–8-fold lower than their binding affinities (Figure 3). Since K_{ATP} channels are octamers with four SUR and four K_{IR} subunits, this rightward shift may indicate that channel opening requires occupation of multiple KCO receptor sites per channel. This idea is supported by the finding that Hill coefficients for channel activation were > 1 (1.3–2.1; Figure 3C). Pinacidil affinity for SUR2B was not changed by co-expression with $K_{IR}6.2$ or by shifting [ATP] from 100 μ M to 1 mM (either in membranes with SUR2B alone or SUR2B + $K_{IR}6.2$; results not shown) suggesting that the lower potencies do not reflect reduced affinity caused by association with $K_{IR}6.2$ or the higher [ATP] in the patch clamp experiments (Figure 3). The observed rightward shift is consistent with significantly lower potencies of KCOs to induce ⁸⁶Rb⁺ efflux versus binding affinities described for aortic rings (Quast *et al.*, 1993).

Conclusions

This study provides a new insight into the mode of action of KCOs. We establish that these drugs bind to and act through SURs and present evidence that binding requires a conformational change mediated by ATP binding and presumably hydrolysis in both NBFs. Identification of SURs as KCO receptors provides a basis for the development of tissue selective compounds. The importance of the C-terminus suggests that it is part of the KCO-binding pocket, the localization and characterization of which might facilitate drug design.

Materials and methods

Materials

[³H]P1075 (specific activity 116 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). Levromakalim was from SmithKline Beecham (Harlow, UK). All other chemicals and drugs were

obtained from sources described elsewhere (Schwanstecher *et al.*, 1992, 1994). Stock solutions of KCOs were prepared in ethanol and dimethyl sulfoxide (1:1) with a final solvent concentration in the media <0.5%.

Molecular biology

Mutant constructs of SUR2B were prepared by sequential overlap extension polymerase chain reactions (PCR). Chimeras were constructed by substituting the complementary DNA coding for the C-terminal 42 amino acids of rat SUR2A by the corresponding sequences from hamster SUR1 (SUR2/ct1) or human SUR2B (SUR2/ctB). The resulting products were subcloned into the PECE vector and sequenced to verify the point mutations or chimeric constructs before transfection.

Binding experiments

Transfections and membrane preparations were performed as described previously (Schwanstecher *et al.*, 1992; Clement *et al.*, 1997). Briefly, COS-7 cells cultured in DMEM HG (10 mM glucose), supplemented with 10% fetal calf serum (FCS), were plated at a density of 5×10^5 cells per dish (94 mm) and allowed to attach overnight. pECE-SUR (200 µg) and/or pCMV6c-K_{IR} (200 µg) complementary DNA were used to transfect 10 plates. For transfection the cells were incubated 4 h in a Tris-buffered salt solution containing DNA (5–10 µg/ml) plus DEAE-dextran (1 mg/ml), 2 min in HEPES-buffered salt solution plus dimethyl sulfoxide (10%) and 4 h in DMEM-HG plus chloroquine (100 µM). Cells were then returned to DMEM-HG plus 10% FCS. Membranes were prepared 60–72 h post-transfection as described previously (Schwanstecher *et al.*, 1992). For binding experiments resuspended membranes (final protein concentration 5–50 µg/ml) were incubated in Tris-buffer (50 mM, pH 7.4) containing either [³H]P1075 (final concentration 3 nM, non-specific binding defined by 100 µM pinacidil) or [³H]glibenclamide (final concentration 0.3 nM, non-specific binding defined by 100 nM glibenclamide) and other additions as shown in the figures. The free Mg²⁺ concentration was kept close to 1 mM unless indicated otherwise (Figure 2B). Incubations were carried out for 1 h at room temperature and were terminated by rapid filtration through Whatman GF/B filters. Diazoxide binding to SUR1 was determined by an indirect assay based on the displacement of [³H]glibenclamide as follows: Diazoxide (%) = $(A-B)/(A-C) \times 100$, where $A = [^3H] (Dz) / [^3H] (control)$, $B = [^3H] (Dz + [ATP]) / [^3H] ([ATP])$ and $C = [^3H] (Dz + [ATP]_{MAX}) / [^3H] ([ATP]_{MAX})$. [³H] is the amount of [³H]glibenclamide specifically bound in the absence of further additions (control) or presence of 280 µM diazoxide (Dz) and/or [ATP]; [ATP]_{MAX} = 100 µM.

Electrophysiology

Transfections were performed as described above with the following modification. COS cells were plated at a density of 8×10^4 cells per dish (35 mm). pECE-human SUR2B complementary DNA (20 µg) and pCMV6c-human K_{IR}6.2 complementary DNA (20 µg) were mixed and used to transfect six 35 mm plates. Experiments in the inside-out configuration of the patch-clamp technique were performed at room temperature as described previously (Schwanstecher *et al.*, 1994). Membrane patches were clamped at −50 mV. The intracellular bath solution contained (mM) 140 KCl, 2 CaCl₂, 1.0 free Mg²⁺, 10 EGTA, 5 HEPES (pH 7.3) and the pipette solution 146 KCl, 2.6 CaCl₂, 1.2 MgCl₂ and 10 HEPES (pH 7.4).

Data

Data analysis and statistics were performed as described (Schwanstecher *et al.*, 1992, 1994). Results shown as mean ± SEM ($n = 4-15$).

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