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Mg^{2+} sensitizes K_{ATP} channels to inhibition by DIDS: dependence on the sulphonylurea receptor subunit

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1 ATP-sensitive potassium channels (K_{ATP} channels) consist of pore-forming Kir6.x subunits and of sulphonylurea receptors (SURs). In the absence of Mg^{2+}, the stilbene disulphonate, DIDS, irreversibly inhibits K_{ATP} channels by binding to the Kir subunit. Here, the effects of Mg^{2+} on the interaction of DIDS with recombinant K_{ATP} channels were studied in electrophysiological and [^{3}H]-glibenclamide binding experiments.

2 In inside-out macropatches, Mg^{2+} (0.7 mM) increased the sensitivity of K_{ATP} channels towards DIDS up to 70 fold ($IC_{50}=2.7\ \mu M$ for Kir6.2/SUR2B). Inhibition of current at DIDS concentrations $\geqslant 10\ \mu M$ was irreversible.

3 Mg^{2+} sensitized the truncated Kir6.2Δ26 channel towards inhibition by DIDS only upon coexpression with a SUR subunit (SUR2B). The effect of Mg^{2+} did not require the presence of nucleotides.

4 [^{3}H]-glibenclamide binding to SUR2B(Y1206S), a mutant with improved affinity for glibenclamide, was inhibited by DIDS. The potency of inhibition was increased by Mg^{2+} and by coexpression with Kir6.2.

5 In the presence of Mg^{2+}, DIDS inhibited binding of [^{3}H]-glibenclamide to Kir6.2/SUR2B(Y1206S) with $IC_{50}=7.9\ \mu M$ by a non-competitive mechanism. Inhibition was fully reversible.

6 It is concluded that the binding site of DIDS on SUR that is sensed by glibenclamide does not mediate channel inhibition. Instead, Mg^{2+} binding to SUR may allosterically increase the accessibility and/or reactivity of the DIDS site on Kir6.2. The fact that the Mg^{2+} effect does not require the presence of nucleotides underlines the importance of this ion in modulating the properties of the K_{ATP} channel.

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Keywords: K_{ATP} channels; magnesium ion; sulphonylurea receptor subtypes; DIDS; stilbene disulphonates; patch-clamp; inwardly rectifying K^+ channels; [^{3}H]-glibenclamide

Abbreviations: AMP-PCP, β-γ-methylene-adenosine 5′-triphosphate; DIDS, 4,4′-diisothiocyanato-stilbene-2,2′-disulphonic acid; GBC, glibenclamide; HEK cells, human embryonic kidney cells; K_{ATP} channels, ATP-sensitive K^+ channels; Kir, inward rectifier potassium channel; NBF, nucleotide binding fold; SUR, sulphonylurea receptor

Introduction

ATP-sensitive K^+ (K_{ATP}) channels are closed by ATP and opened by MgADP (and other nucleoside diphosphates); hence, these channels link the metabolic state of the cell to membrane potential and cellular excitability. In addition to their regulation by intracellular nucleotides, K_{ATP} channels are the target of important drugs, the sulphonylureas exemplified by glibenclamide and the potassium channel openers such as levcromakalim and pinacidil (Ashcroft & Ashcroft, 1990). K_{ATP} channels are composed of two types of subunits, the weakly inwardly rectifying K^+ channels of the family Kir6.x and the sulphonylurea receptors (SURs), arranged in a hetero-octameric complex $(Kir6.x/SURx)_4$ (Aguilar-Bryan *et al*., 1995; Sakura *et al*., 1995; Clement *et al*., 1997; Shyng & Nichols, 1997). The inhibition of K_{ATP} channels by ATP is mediated by the Kir subunit (Tucker *et al*., 1997) whereas the activation by MgADP is mediated by SUR (Nichols *et al*., 1996; Gribble *et al*., 1997).

SURs are members of the ATP-binding cassette proteins. They have two nucleotide binding folds (NBFs) with NBF_1 binding ATP and NBF_2 mediating the activation of the channel by ADP in Mg^{2+}-dependent manner (Nichols *et al*., 1996; Gribble *et al*., 1997); in addition, SURs carry the binding sites for the sulphonylureas and the openers (Aguilar-Bryan *et al*., 1995; Sakura *et al*., 1995; Hambrock *et al*., 1998; Schwanstecher *et al*., 1998). Kir6.x and SURx each are encoded by two genes; this and alternative splicing of the SUR gene products lead to subtypes of SUR and Kir6 (reviewed in Aguilar-Bryan & Bryan, 1999). Different combinations of Kir6.x and SURx are the basis for the tissue-specific differences of K_{ATP} channels with Kir6.2/SUR1 being the channel in the pancreatic β-cell and neurons,

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Kir6.2/SUR2A in cardiomyocytes and skeletal muscle cells and Kir6.x/SUR2B in smooth muscle myocytes.

Stilbenes like DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulphonic acid), at high concentrations ($\geqslant 100$ μM), inhibit anion transporters and channels (Cabantchik *et al*., 1978) and activate KvLQT1/Isk channels (Busch *et al*., 1994; Abitbol *et al*., 1999). In the low μM range, DIDS acts as an antagonist at P_2-purinoceptors (Ralevic & Burnstock, 1998). Noting structural similarities of DIDS with sulphonylureas, Furukawa *et al*. (1993) investigated the effect of DIDS on K_{ATP} channels in guinea-pig ventricular myocytes. They found that DIDS, applied to the intracellular side of the membrane and in the absence of Mg^{2+}, inhibited the channel with $IC_{50} = 71$ μM in an irreversible manner (Furukawa *et al*., 1993). The reactive SCN groups of DIDS are known to covalently modify cysteine and lysine residues of proteins (Gatto *et al*., 1997). Again in the absence of Mg^{2+} and using the truncated K_{ATP} channel Kir6.2Δ36 (which exhibits channel activity in the absence of SUR (Tucker *et al*., 1997)), Proks *et al*. (2001) showed that DIDS interacted with the Kir6.2 subunit. They also showed that ATP protected against (irreversible) DIDS block suggesting interaction of the ATP and the DIDS sites of Kir6.2. Interestingly, coexpression of Kir6.2 with SUR2A reduced both the rate and extent of channel block as compared to Kir6.2/SUR1, indicating that the sensitivity of the channel towards inhibition by DIDS was modulated by the SUR subunit (Proks *et al*., 2001).

We have found that purinergic antagonists such as phloxin B and DIDS inhibit binding of the opener [^{3}H]-P1075 to SUR2B; in the presence of 3 mM MgATP, the IC_{50} value of DIDS was 1.6 μM (Russ *et al*., 2000), i.e. a potency more than 40 fold higher than that observed for channel block in the absence of Mg^{2+}. This raised the question whether the presence of Mg^{2+} (or MgATP) affected the block of K_{ATP} channels by DIDS and whether the binding of DIDS to SUR was in any way transduced into modulation of channel activity. Here we present electrophysiological and radioligand binding studies designed to answer these questions. Since the effects of Mg^{2+} were most pronounced at the Kir6.2/SUR2B channel, experiments were focused on this channel.

Methods

Recombinant K_{ATP} channels, mutations, cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured as described previously in minimum essential medium containing glutamine, supplemented with 10% foetal bovine serum and 20 μg ml^{-1} gentamycin (Hambrock *et al*., 1998). Cells were transfected with the pcDNA 3.1 vector (Invitrogen, Karlsruhe, Germany) containing the coding sequence of rat SUR1 (GenBank accession number X97279), murine SUR2A, SUR2B (GenBank accession numbers: D86037 and D86038; Isomoto *et al*., 1996) or SUR2B(Y1206S). Cell lines stably expressing these proteins were isolated as described previously (Hambrock *et al*., 1998).

The mutants SUR2B(Y1206S) (Hambrock *et al*., 2001), Kir6.2(G334D) (Drain *et al*., 1998) and a truncated form of Kir6.2, which lacks the C-terminal 26 amino acids (Kir6.2Δ26) were prepared as described by Hambrock *et al*. (2001).

For patch clamp and some binding experiments, cells were transiently transfected with Kir6.2 (GenBank accession number D50581; Inagaki *et al*., 1996) and SURx at a molar plasmid ratio of 1 : 1 using LipofectAMINE and OptiMEM (Invitrogen) as described previously (Hambrock *et al*., 1998). In cotransfections used for electrophysiological experiments, the pEGFP-C1 vector (Clontech, Palo Alto, CA, U.S.A.), encoding for green fluorescent protein, was added for identification of transfected cells. Cells were allowed to express transfected DNA for 48 h before use in electrophysiological experiments (Russ *et al*., 1999).

Patch-clamp experiments

The patch-clamp technique was used in the inside-out configuration as described by Hamill *et al*. (1981). Transfected HEK cells showing green fluorescence were chosen. Patch pipettes were drawn from borosilicate glass capillaries (GC 150T, Harvard Apparatus, Edenbridge, U.K.) and heat polished using a horizontal microelectrode puller (Zeitz, Augsburg, Germany). Bath and pipette were filled with a high K^+-Ringer solution containing (in mM): KCl, 142; NaCl, 2.8; $MgCl_2$, 1; $CaCl_2$, 1; D(+)-glucose, 11; 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES), 10 titrated to pH 7.4 with NaOH. After filling with buffer, pipettes had a resistance of 1–1.5 MΩ. After excision of the patch, the pipette was moved in front of a pipe with a high K^+-EGTA-Ringer solution containing (in mM): KCl, 143; MgCl, 0.85; $CaCl_2$, 1; ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 5; D(+)-glucose, 11; HEPES, 10 titrated to pH 7.2 with NaOH. If ATP was added, the concentration of free Mg^{2+} was kept constant at 0.7 mM. The Mg^{2+}-free solution consisted of (in mM): KCl, 145; ethylenediaminetetraacetic acid (EDTA), 5; HEPES, 10 and D(+)-glucose, 11. For the bath solution at pH 6, 2-(N-morpholino)-ethanesulphonic acid (MES, 10 mM) was used as the buffering ion. All responses were normalized to the current prior to DIDS application. Patches were clamped to −50 mV. All experiments were done at a room temperature of 22°C.

Data were recorded with a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA, U.S.A.) using a MacLab interface and the Chart software (AD Instruments, Castle Hill, Australia). Signals were filtered at 200 Hz, digitized online at 100 Hz, and stored on a Power Macintosh 8200/120 computer for later analysis.

Membrane preparation and [^{3}H]-glibenclamide binding experiments

For cells stably expressing SUR2B(Y1206S) alone, the antibiotic geneticin was withdrawn from the culture medium 1 week prior to membrane preparation; cells transiently expressing Kir6.2/SUR2B(Y1206S) were harvested 2–3 days after transfection. Membranes were prepared as described (Hambrock *et al*., 1998). In brief, cells were centrifuged for 5 min at $500 \times g$ and 4°C and lysed by addition of ice-cold hypotonic buffer containing (in mM): HEPES, 10; EGTA, 1 at pH 7.4. The lysate was centrifuged at $10^5 \times g$ and 4°C for 60 min and the resulting membrane pellet was resuspended in

a buffer containing (in mM) HEPES, 5; KCl, 5; NaCl, 139; at pH 7.4 and 4°C at a protein concentration of ~1.5–3.0 mg protein ml^{-1} and frozen at −80°C. Protein concentration was determined according to Lowry *et al*. (1951) using bovine serum albumin as the standard.

The interaction of DIDS with SUR was studied in [^{3}H]-glibenclamide competition or saturation experiments. Membranes (final protein concentration 100–150 μg protein ml^{-1}) were added to the incubation buffer (NaCl 139 mM, KCl 5 mM, HEPES 10 mM) supplemented with [^{3}H]-glibenclamide (0.5–2.5 nM for competition experiments), Mg^{2+} (0/0.7/2.2 mM), EDTA (1/0/0 mM) and ATP (0/0/1 mM) at 22°C for 45 min or at 37°C for 15 min. Incubation was stopped by diluting, in triplicate 0.3 ml aliquots into 8 ml of quench solution (50 mM tris-(hydroxymethyl)-aminomethane, 154 mM NaCl, pH 7.4) at 0°C and filtration over Whatman GF/B filters. Nonspecific binding was determined in the presence of 100 μM unlabelled P1075 and ranged from 22–32% of total binding. Non-transfected HEK 293 cells possess endogenous low affinity glibenclamide sites which are not sensitive to P1075; however, P1075 completely displaces specific glibenclamide binding (=glibenclamide binding to mutant SUR2B) (Hambrock *et al*., 2001).

Data analysis

Data are shown as mean±s.e.mean. Concentration dependencies were analysed by fitting the logistic form of the Hill equation,

$$y = 100 - A(1 + 10^{n\,(px-pK)})^{-1}, \quad (1)$$

to the data. Here A denotes the extent of the effect (amplitude); n (=n_H) is the Hill coefficient, x the concentration of the compound under study and K (=IC_{50}) the midpoint of the curve with px=−logx and pK=−logIC_{50}. For the analysis of biphasic curves, the superposition of two logistic terms was used and Hill coefficients were set to 1 if not stated otherwise.

In case of competitive inhibition, the IC_{50} value of the binding inhibition curve was corrected for the presence of the competing radioligand, L, according to Cheng & Prusoff (1973), to give the inhibition constant, K_i:

$$K_i = IC_{50}(1 + L\,K_L^{-1})^{-1}, \quad (2)$$

where K_L is the equilibrium dissociation constant of the radioligand, L. The Cheng-Prusoff correction was generally less than 2.

Saturation experiments were analysed according to the equation,

$$B_{TOT} = B_{MAX}L(L + K_D)^{-1} + aL. \quad (3)$$

Total binding (B_{TOT}) is the sum of specific and nonspecific binding. Here, B_{MAX} (fmol mg^{-1} protein) denotes the concentration of specific binding sites in the preparation, K_D is the equilibrium dissociation constant, L the concentration of the radioligand and a the proportionality constant describing nonspecific binding as a linear function of L. Nonspecific binding was determined as described above. Specific binding was also plotted in the Scatchard presentation (Scatchard, 1949).

Fitting of equations to data was performed according to the method of least squares using the SigmaPlot6.1 programme (SPSS Science, Chicago, IL, U.S.A.). Errors in the parameters derived from fit to a single curve were estimated using the univariate approximation (Draper & Smith, 1981) and assuming that amplitudes and pIC_{50} values are normally distributed (Christopoulos, 1998). These were then averaged and pIC_{50} values±s.e.mean were converted to IC_{50} values with the 95% confidence interval in parentheses. Significance of differences between parameters obeying the normal distribution was determined using the two-tailed unpaired Student's *t*-test.

Chemicals

4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), glibenclamide, MgATP and β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) were from SIGMA (Deisenhofen, Germany). The pinacidil analogue, P1075 was a gift from Leo Pharmaceutical (Ballerup, Denmark). DIDS was dissolved in bath solution or incubation buffer and protected from light with aluminium foil and used within 3 h. Glibenclamide and P1075 were dissolved in dimethyl sulphoxide/ethanol (1 : 1) and further diluted with incubation buffer; the final solvent concentration was always below 0.3%. The reagents and media used for cell culture and transfection were from Life Technologies (Eggenstein, Germany).

Results

Interaction with recombinant K_{ATP} channels

Figure 1 shows the effects of DIDS on the Kir6.2/SUR2B channel in recordings from inside-out patches. Upon patch excision into a Mg^{2+} (0.7 mM)-containing nucleotide-free solution, the current underwent rapid run-down (Figure 1A,B). Addition of ATP (1 mM) induced complete channel block and, upon washout, a large 'refreshment' of the current was observed. This cycle was repeated and application of DIDS (10 μM) inhibited the current by ~90% within 90 s (Figure 1A). Upon washout of the agent with 1 mM ATP, the inhibition proved essentially irreversible. At 100 μM DIDS, inhibition was almost complete within 10 s (Figure 1B). In the absence of Mg^{2+} (5 mM EDTA, no extra Mg^{2+}), run-down was greatly slowed down and inhibition by DIDS (10 μM) was small (~8% after 90 s, Figure 1C). At 100 μM, DIDS inhibited the current by ~40% in an irreversible manner; in addition, channel block by ATP (1 mM) was decreased (Figure 1D). These data show that presence of Mg^{2+} strongly sensitized the Kir6.2/SUR2B channel to the inhibitory effect of DIDS.

The inhibition kinetics of the Kir6.2/SUR2B channel by DIDS were often biphasic, comprising a rapid first component (which was essentially complete after 10 s) and a slower component (Figure 2). The contribution of the rapid phase increased with increasing DIDS concentration and, in the presence of Mg^{2+}, much lower concentrations of DIDS

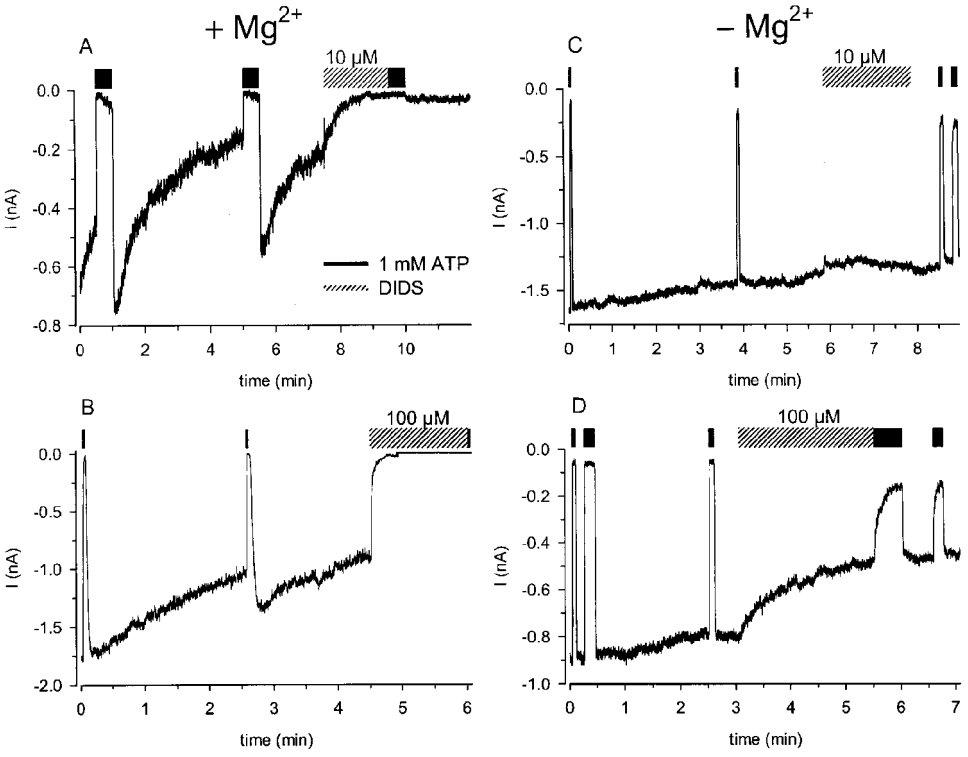

Figure 1 Recordings from inside-out patches showing the inhibition of K_{ATP} (Kir6.2/SUR2B) currents by DIDS in the presence (A,B) and absence (C,D) of Mg^{2+}. Holding potential was −50 mV and temperature 22°C. 1 mM ATP, 10 μM (A,C) or 100 μM (B,D) DIDS were applied as indicated.

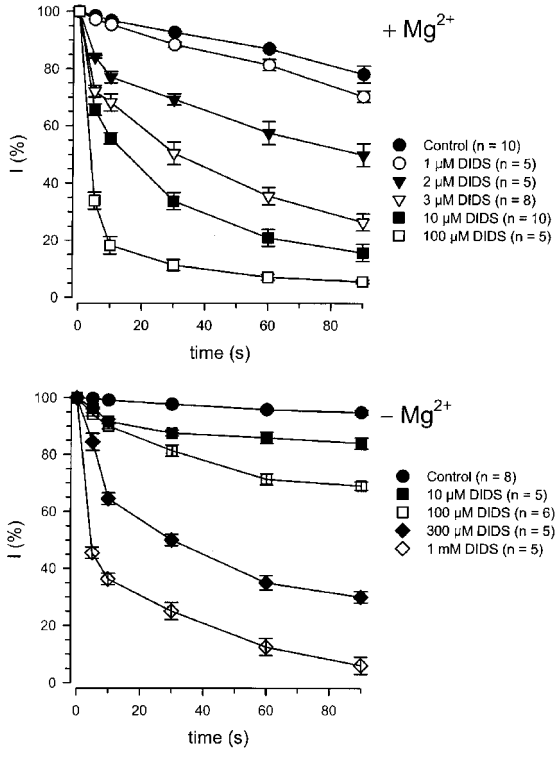

Figure 2 Time-dependent inhibition of the Kir6.2/SUR2B channel by DIDS in the presence and absence of Mg^{2+}. Note the biphasic nature of most inhibition kinetics. Patches with little run-down were selected and responses were normalized with respect to the current before drug application. Data are means ± s.e.mean.

were required to reach an equivalent level of inhibition. Qualitatively similar observations were made with the other recombinant K_{ATP} channels containing SUR2A or SUR1. Evaluating the inhibition of the K_{ATP} currents in the presence and absence of Mg^{2+} at 10 s and 90 s after DIDS application, concentration-inhibition curves were constructed. Figure 3 illustrates the inhibition curves for the three channels after 90 s DIDS application; the fitting parameters are listed in Table 1 together with those from the inhibition curves after 10 s. It is seen that the sensitivity of all channel combinations was increased by Mg^{2+}; however, the effect was strongest with SUR2B. Therefore, further studies were concentrated on Kir6.2/SUR2B.

Proks and colleagues have reported that inhibition of various K_{ATP} currents by DIDS (0.7 mM, in the absence of Mg^{2+}) was irreversible (Proks *et al.*, 2001). Figure 1A suggested that this may hold true also for the low concentrations of DIDS required to inhibit the Kir6.2/SUR2B channel in the presence of Mg^{2+} and, during up to 4 min washout, no significant recovery from DIDS block (10 μM, 90 s, $+Mg^{2+}$) was observed ($n=10$, data not shown).

In the [^{3}H]-glibenclamide binding studies presented below, a SUR2B mutant with increased affinity for glibenclamide was used (SUR2B(Y1206S); Hambrock *et al.*, 2001). No differences in channel characteristics between wild type and mutant SUR2B (with the exception of the sensitivity to glibenclamide) have been observed so far (Hambrock *et al.*, 2001; 2002). However, it seemed adequate to repeat the key experiments on the Kir6.2/SUR2B response to DIDS with the mutant. Figure 4A shows that in the presence of Mg^{2+},

DIDS (30 μM, applied for 90 s) produced almost complete current inhibition (97±1%, $n=5$). In the absence of Mg^{2+}, inhibition was much less pronounced (35±5%; $n=5$; Figure 4B). These results agree well with the data for the wild type (Figure 3A). In addition, inhibition persisted in both cases during alternating application of MgATP (1 mM ATP, 30 s) and control buffer (30 s) for up to 7 min (Figure 4). These experiments demonstrated that the mutation did not alter the effect of Mg^{2+} on channel inhibition by DIDS.

Investigation into the mechanism of the Mg^{2+} effect

The effect of Mg^{2+} on the sensitivity of K_{ATP} channels to DIDS could be mediated by Mg^{2+} binding to Kir or SUR. In order to decide between these possibilities, experiments were performed using a truncated Kir6.2 (Kir6.2Δ26), which shows channel activity in the absence of SUR, whereas Kir6.2 does not (Tucker *et al*., 1997). The truncated channel expressed poorly and only small currents were obtained (Figure 5A,B). DIDS (30 μM) was only weakly effective and inhibition (up to 50%) was the same regardless of the presence or absence of Mg^{2+} ($n=5$, each). Coexpression of Kir6.2Δ26 with SUR2B greatly improved expression and restored the Mg^{2+} effect (Figure 5C,D): In the presence of Mg^{2+}, DIDS (3 μM) inhibited the Kir6.2Δ26/SUR2B channel by 62±4% ($n=4$); in the absence of Mg^{2+}, inhibition by DIDS (300 μM) was 88±3% ($n=4$). Together, these experiments showed that the presence of SUR was required for Mg^{2+} effect and the truncation of Kir6.2 *per se* was not responsible for the absence of the Mg^{2+} effect on the Kir6.2Δ26 channel.

Next, the question was addressed whether the effect of Mg^{2+} required ATP and/or ADP bound to SUR. Although DIDS was applied in the absence of nucleotides, MgATP and/or MgADP may have remained tightly bound to SUR during exposure of the channel to nucleotide-free solutions. In order to eventually displace such nucleotides, the Kir6.2/SUR2B channel was kept in a Mg^{2+}-free solution (5 mM EDTA) and then exposed to the nonhydrolysable ATP-analogue AMP-PCP (1 mM) for 2 min; this manoeuvre induced immediate channel closure (Figure 6A). After washout of AMP-PCP, DIDS (10 μM) was applied in the presence of Mg^{2+}, producing almost complete and irreversible inhibition of the current (Figure 6A; in six experiments, current decrease was 83±3%). As a control, the K_{ATP} channel opener P1075 (at the saturating concentration of 0.1 μM and in the presence of Mg^{2+}) was applied in the continued presence of AMP-PCP (Figure 6B). P1075 was unable to open the channel, suggesting that

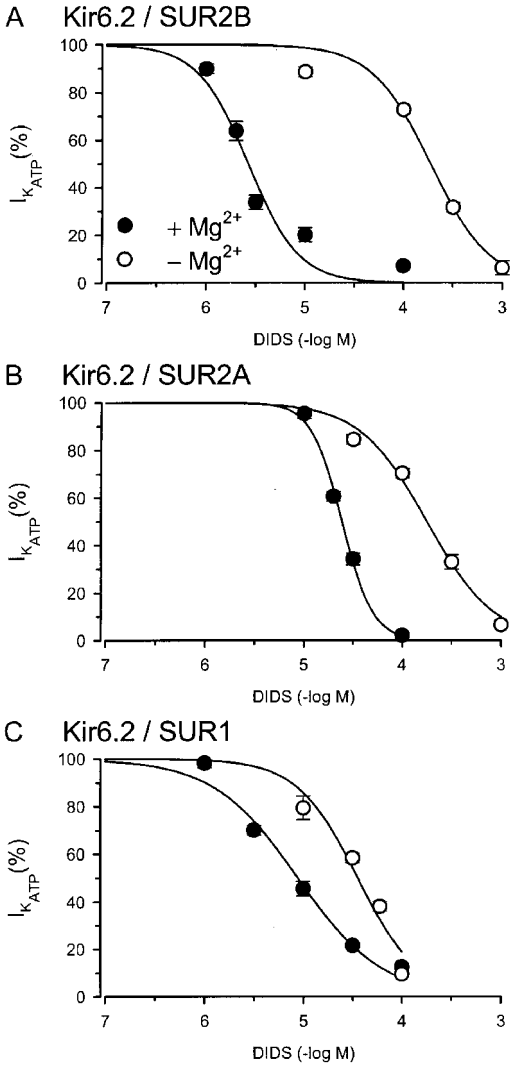

Figure 3 Concentration-dependent inhibition of Kir6.2/SUR2B (A), Kir6.2/SUR2A (B) and Kir6.2/SUR1 (C) currents by DIDS in the presence and absence of Mg^{2+}. Currents, measured 90 s after DIDS application, were expressed as percentage of control current prior to DIDS application (% I_{KATP}). Current inhibition was corrected for run down determined in control experiments (*c.f.* controls in Figure 2). Data were analysed using the Hill equation and assuming complete inhibition at saturation. Parameters are listed in Table 1.

Table 1 Inhibition of recombinant K_{ATP} channels by DIDS

		$+Mg^{2+}$ (0.7 mM)		$-Mg^{2+}$		
K_{ATP} channel		*IC_{50}* (μM)	*n_H*	*IC_{50}* (μM)	*n_H*	r
Kir6.2/SUR2B	10 s	14 (9,21)	0.8±0.1	600 (470,750)	1.1±0.2	43 (26,71)
	90 s	2.7 (2.1,3.5)	1.7±0.4	190 (140,240)	1.5±0.3	70 (48,103)
Kir6.2/SUR2A	10 s	35 (28,44)	2.4±0.6	550 (390,770)	1.1±0.2	16 (10,24)
	90 s	24 (23,26)	2.8±0.3	180 (150,210)	1.3±0.1	7.3 (6.0,8.9)
Kir6.2/SUR1	10 s	17 (12,25)	0.8±0.1	51 (37,70)	1.6±0.5	3.0 (1.8,5.0)
	90 s	8.8 (6.8,11.5)	1.0±0.1	36 (27,48)	1.4±0.3	4.0 (2.7,6.0)

Listed are the IC_{50} values (with confidence intervals in parentheses) and Hill coefficients (n_H) from inhibition curves obtained at 10 and 90 s after application of DIDS; *r* denotes the Mg^{2+}-induced shift of the inhibition curve: $r = IC_{50}\ (-Mg^{2+})/IC_{50}\ (+Mg^{2+})$. Comparison of the pIC_{50} values in the presence and absence of Mg^{2+} by the two-tailed unpaired *t*-test showed that the difference was significant in each case ($P<0.05$). Conditions were as in Figures 1–3; the fits extrapolated to 100% inhibition at saturation.

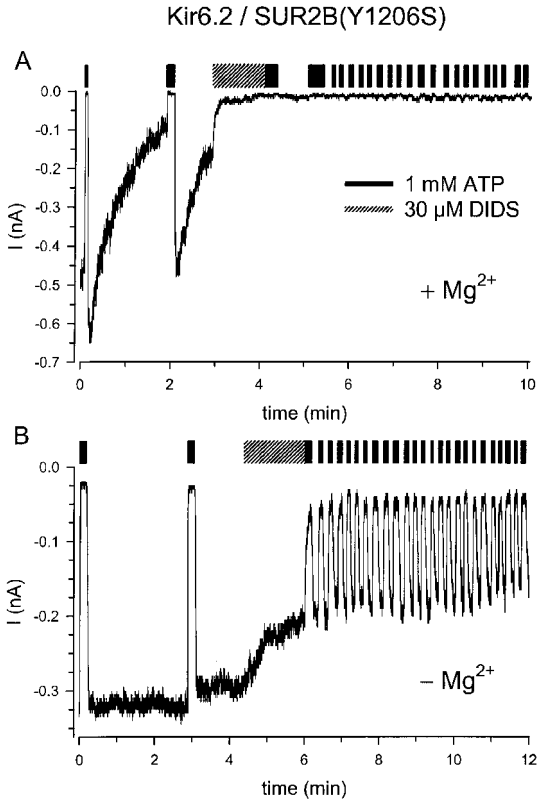

Figure 4 Inhibition of K_{ATP} (Kir6.2/SUR2B(Y1206S)) currents by DIDS showing Mg^{2+} sensitization and irreversibility of DIDS-induced block. Experimental conditions were as in Figure 1. ATP (1 mM) and DIDS (30 μM) were applied as indicated.

no significant amounts of MgATP/MgADP had remained bound to the channel ($n=5$). Together, these experiments showed that the potentiating effect of Mg^{2+} did not require the presence of hydrolysable adenine nucleotides bound to SUR.

Interaction of DIDS and MgATP at K_{ATP} channels

In the absence of Mg^{2+}, ATP (3 mM) has been shown to afford protection of Kir6.2Δ36 and Kir6.2/SUR1 channels against irreversible inhibition by DIDS (0.7 mM; Proks *et al*., 2001). It was therefore of interest to examine if this was true also for the Kir6.2/SUR2B channel in the presence of Mg^{2+}. The results of these experiments are summarized in Table 2.

MgATP (30 μM) completely protected against the irreversible block by DIDS (10 μM). Protection by 100 μM MgATP against 30 μM DIDS was only partial but total by 1 mM MgATP. With increasing DIDS concentration (0.1 or 0.3 mM), protection by MgATP (1 mM) was partial or absent. These experiments show that MgATP protected against DIDS also in the presence of Mg^{2+} and that protection depended on the concentration ratio of ATP : DIDS.

In order to learn more about the interaction between ATP and DIDS at Kir6.2, we studied the effects of the mutation G334D which exhibits about 1000× lower sensitivity to ATP, presumably due to a reduction in ATP binding (Drain *et al*., 1998). When Kir6.2(G334D) was coexpressed with SUR2B, only small currents were obtained; therefore, experiments were performed with the Kir6.2(G334D)/SUR1 channel which gave better currents. In agreement with Drain *et al*. (1998), the channel was insensitive to ATP (1 mM, not

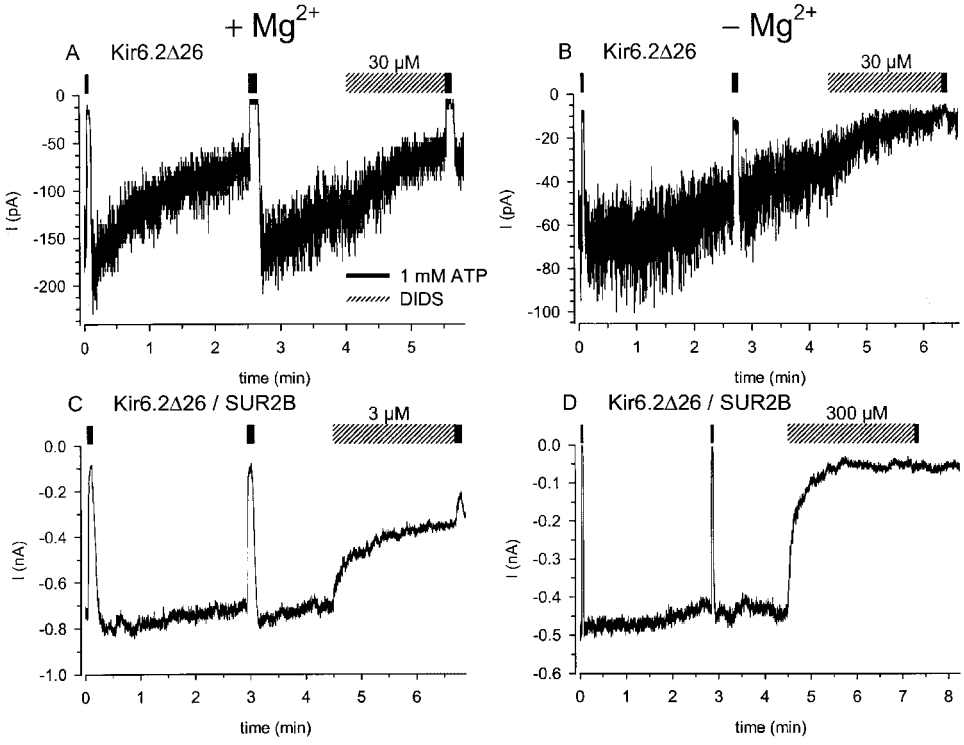

Figure 5 Inhibition of Kir6.2Δ26-containing K_{ATP} channels by DIDS in the presence and absence of Mg^{2+}. Mg^{2+} did not alter inhibition of the Kir6.2Δ26 channel (A,B) but sensitized the Kir6.2Δ26/SUR2B channel for inhibition by DIDS (C,D). Conditions were as in Figure 1. DIDS (3, 30 or 300 μM) and ATP (1 mM) were applied as indicated by the bars.

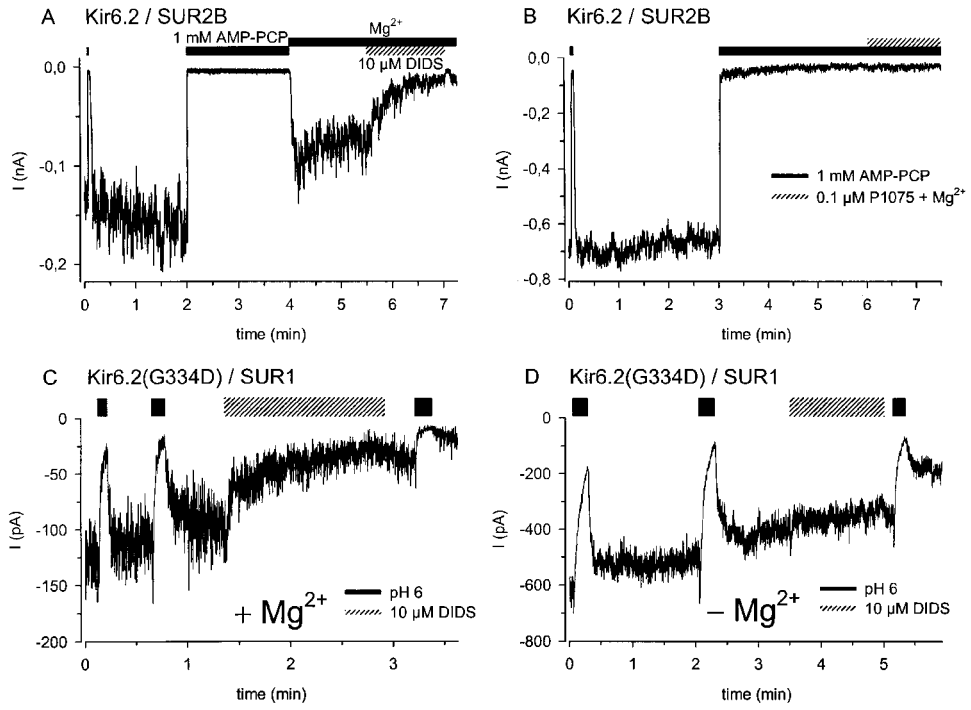

Figure 6 Effects of AMP-PCP and of the mutation Kir6.2(G334D) on the inhibition of K_{ATP} currents by DIDS. Upper panels: AMP-PCP. (A) After exposure of the channel to AMP-PCP in the absence of Mg^{2+} for 2 min, the enhancement of DIDS block by Mg^{2+} was preserved. (B) The channel opener P1075 (0.1 μM, in the presence of Mg^{2+}) was unable to open the channel in the continued presence of AMP-PCP (see text). Lower panels: Channels containing the ATP-insensitive subunit Kir6.2(G334D) can still be sensitized to DIDS by Mg^{2+} (C,D). Conditions were as in Figure 1. Pipe solutions were applied as indicated by the bars.

Table 2 Protection by MgATP of the Kir6.2/SUR2B current against irreversible DIDS block

MgATP (mM)	*DIDS* (μM)	*% recovery in (n) exp.*	*Protection*
0	10	16±3 (10)	–
0.03	10	69±2 (4)	complete
0.1	30	41±9 (4)	partial
1	30	72±2 (4)	complete
1	100	23±2 (5)	partial
1	300	0 (4)	–

MgATP was applied 30 s prior to and during DIDS application (90 s) and 30 s thereafter. Currents were not corrected for run-down. Data are means±s.e.mean from (*n*) experiments.

shown); however, channel activity was greatly reduced by acidification to pH 6 (Figure 6C,D). In the presence of Mg^{2+}, DIDS (10 μM) inhibited the current by 70±3% ($n=5$). In the absence of Mg^{2+}, no block was observed (Figure 6; $n=4$) whereas at 100 μM, DIDS inhibited this channel almost completely by 95±1% ($n=4$, not illustrated). These data are similar to those observed with the native channel (Figure 3C). It is concluded that the mutation G334D did not interfere with the Mg^{2+}-dependent channel inhibition by DIDS.

Radioligand binding experiments: interaction of DIDS with SUR2B(Y1206S)

The electrophysiological experiments in Figure 5 had shown that the Mg^{2+} effect required the presence of SUR and earlier experiments using the opener, [^{3}H]-P1075, as the radioligand had shown that DIDS interacted with SUR2B (Russ *et al*., 2000). In order to study the Mg^{2+}-dependence of this interaction, [^{3}H]-glibenclamide was chosen as the radioligand instead of [^{3}H]-P1075, since the latter requires the presence of Mg^{2+} and ATP for high affinity binding to SUR2 (Hambrock *et al*., 1998; 1999; Schwanstecher *et al*., 1998) whereas glibenclamide does not (Hambrock *et al*., 2001; 2002). To obtain sufficient binding, the mutant SUR2B(Y1206S), which exhibits higher affinity for glibenclamide, was used.

Figure 7A illustrates the inhibition of [^{3}H]-glibenclamide binding by DIDS. In the absence of Mg^{2+}, the inhibition curve was monophasic with an IC_{50} value of 60 μM (Table 3, 22°C). In the presence of Mg^{2+}, the competition curve was flattened (Hill coefficient = 0.74). Two component analysis revealed a smaller component with high affinity ($IC_{50,1}=1.4$ μM) and a second component with $IC_{50,2}=39$ μM (Table 3). Analogous experiments were performed also with SUR2B(Y1206S) coexpressed with Kir6.2 (Figure 7B). In the absence of Mg^{2+}, the inhibition curve was monophasic with an IC_{50} of 39 μM, i.e. similar to that found with mutant SUR2B in the absence of Kir6.2. In the presence of Mg^{2+}, the curve was essentially monophasic (Hill coefficient 0.89±0.05) with an IC_{50} value of 7.9 μM. This showed that coexpression with Kir6.2 slightly altered the interaction of (mutant) SUR2B with DIDS, once Mg^{2+} was present. One also notes that in the presence of Mg^{2+}, there is reasonable agreement between the IC_{50} value determined in the binding assay (7.9 μM) and that of DIDS blocking the Kir6.2/SUR2B channel (2.7 μM).

In order to see whether DIDS inhibited glibenclamide binding by a competitive mechanism, [^{3}H]-glibenclamide binding to SUR2(Y1206S) coexpressed with Kir6.2 was measured in saturation experiments in the presence of Mg^{2+} and the absence and presence of DIDS (10 μM). The results are illustrated in Figure 8A and the parameters are listed in Table 4. It is seen that DIDS decreased the number of glibenclamide sites leaving the K_D unchanged, i.e. inhibition was of a pure non-competitive type. Surprisingly, however, the mechanism of inhibition depended on assay conditions. Analogous experiments were performed with SUR2B (Y1206S) expressed alone, albeit in the absence of Mg^{2+} and at 37°C, the standard temperature for binding assays in this laboratory. Under these conditions, DIDS (20 μM) left the number of glibenclamide sites unchanged but shifted the K_D of [^{3}H]-glibenclamide binding curve from 4.5 to 15 nM, compatible with a competitive mechanism of inhibition (Figure 8B, Table 4). Analysing the rightward shift of the K_D value induced by DIDS according to Cheng-Prusoff (eqn (2), which is based on competition of the two ligands for a common binding site) one estimates for DIDS a K_i value of 9.6 (4.8,19) μM, which is in reasonable agreement with the value obtained from the DIDS inhibition curve under these conditions (K_i = 17 (15,19) μM, Table 3). One also notes that the (apparent) competition between DIDS and glibenclamide means that under these conditions the interaction of DIDS with SUR2B(Y1206S) is reversible.

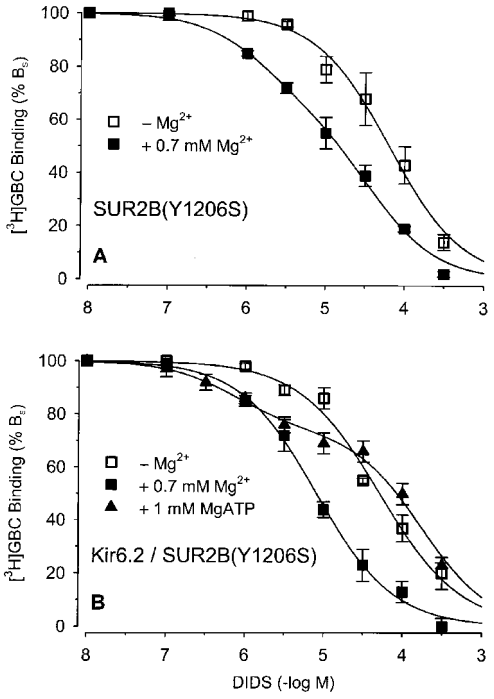

Figure 7 Inhibition by DIDS of [^{3}H]-glibenclamide binding to (A) SUR2B(Y1206S) alone and (B) coexpressed with Kir6.2: Effects of Mg^{2+} (0.7 mM) and MgATP (1 mM). Data are means ± s.e.mean from 3–4 experiments performed at 22°C. The logistic equation (eqn (1)) for one or two binding components was fitted to the individual inhibition curves and averaged parameters are listed in Table 2. [^{3}H]-glibenclamide concentration was 1 nM and 100% specific binding corresponded to 209 ± 14/229 ± 29 fmol mg^{-1} protein in the absence/presence of Mg^{2+}, respectively (mutant SUR2B); in case of coexpression, specific binding was ($-Mg^{2+}$/$+Mg^{2+}$/MgATP) 172 ± 65/152 ± 32/176 ± 13 fmol mg^{-1} protein, respectively. This showed in particular that Mg^{2+} did not affect specific glibenclamide binding. Data in the absence of Mg^{2+} (no Mg^{2+} added, 5 mM EDTA) are corrected for a DIDS-induced decrease in nonspecific binding.

Reversibility of inhibition may not hold in the experiments using the Kir6.2/SUR2B(Y1206S) complex in the presence of Mg^{2+}. Under these conditions, inhibition was unsurmountable (Figure 8A) and this could reflect an irreversible interaction of DIDS with the Kir/SUR complex. To test this possibility, membranes were incubated in the presence of DIDS (30 μM) or solvent, then strongly diluted into [^{3}H]-glibenclamide containing solution (1 : 17 fold, to induce dissociation of DIDS from SUR in case of a reversible interaction), and the association kinetics were monitored. Figure 9 shows that glibenclamide binding to complex preincubated with DIDS reached ~90% of the control level. This indicated that the interaction of DIDS with the complex was entirely reversible, the slight 10% loss in binding reflecting inhibition by the DIDS concentration (1.8 μM) remaining after dilution. Figure 9 also shows that the kinetics of glibenclamide binding to complex preincubated with DIDS were significantly slower than that in the absence of DIDS (half-times ~4.0 min (DIDS) vs 2.7 min (control)), indicating that dissociation of DIDS from the complex was rate-limiting.

Table 3 Inhibition of [^{3}H]-glibenclamide binding to SUR2B(Y1206S) by DIDS

SUR	*Temp.* (°C)	*IC50 and* K_i (μM)[1]/*Amplitude* (% B_s)[2] *DIDS* $-Mg^{2+}$	$+Mg^{2+}$ (0.7 mM)	*MgATP* (1 mM)	K_D (nM)[3] GBC $\pm Mg^{2+}$/*MgATP*
SUR2B(Y1206S)	22	60 (32,110)	1.4 (0.61,3.6)/38 ± 12[4] 39 (25,62)/62 ± 12[4]	n.d.[5]	0.89/n.d.[5]
Kir6.2/SUR2B(Y1206S)	22	39 (30,52)	7.9 (5.2,12)[6]	1.1 (0.98,1.3)/33 ± 3 200 (170,210)/67 ± 3[7]	0.46/0.99
SUR2B(Y1206S)	37	27 (23,31) *17 (15,19)*	n.d.[5]	5.1 (3.2,8.0)/100[8]	4.1/3.9

[1]Since DIDS is not always competitive with glibenclamide IC_{50} values are given; in case of competitivity (Table 4), K_i *values* are also listed in italics. The correction factor for the conversion of IC_{50} values for DIDS into K_i values (where appropriate) lies between 1.5 and 2. Hill coefficients were not different from 1 if not stated otherwise. [2]Monophasic curves: A = 100%; B_S, specific binding. [3]The K_D values of glibenclamide (GBC) were not affected by Mg^{2+}. Values at 22°C were determined for this study, values at 37°C are from Hambrock *et al.* (2002) and Table 4. [4]The two-component fit was significantly better than the fit of the Hill equation (eqn (1)) which gave an IC_{50} value of 13 (10,17) μM and a Hill coefficient of 0.74 ± 0.1. Comparison of models was made using the Minimum Akaike Information Criterion as described (Quast & Mählmann, 1982). [5]n.d.; not determined. [6]Hill coefficient was 0.89 ± 0.05 indicating slight heterogeneity; however, the curve could not be broken down into two components. [7]Hill coefficient of low affinity component was n_H = 1.6 ± 0.2. [8]Hill coefficient was 0.84 ± 0.03 indicating (slight) heterogeneity; fit of two-component model not possible.

Since MgATP protected against the inhibition of the Kir6.2/SUR2B channel by DIDS it was examined whether the nucleotide (in the presence of Mg^{2+}) affected also the interaction of DIDS with mutant SUR2B. To facilitate comparison with the electrophysiological experiments, [^{3}H]-glibenclamide-DIDS inhibition experiments were performed at 22°C and using SUR2B(Y1206S) coexpressed with Kir6.2. In the presence of MgATP (1 mM), the inhibition curve was biphasic with 33% of glibenclamide binding being inhibited with $IC_{50,1} = 1.1$ μM and the remaining 67% with $IC_{50,2} = 200$ μM (Figure 7B, Table 3). As shown above, the corresponding DIDS inhibition curve in the presence of Mg^{2+} alone, was (essentially) monophasic with $K_i = 7.9$ μM (Figure 7B). Hence, MgATP induced a heterogeneity in the DIDS sites or in their coupling to the glibenclamide site shifting one part towards ~7 fold higher and the other to 25 fold lower affinity (Table 3). Qualitatively similar observations were made at 37°C for mutant SUR2B expressed alone (Table 3). In the absence of Mg^{2+}, the inhibition curve was monophasic with an IC_{50} value of 27 μM; MgATP induced a 5 fold leftward shift ($IC_{50} = 5.1$ μM) and a slight heterogeneity (Hill coefficient 0.84 ± 0.03).

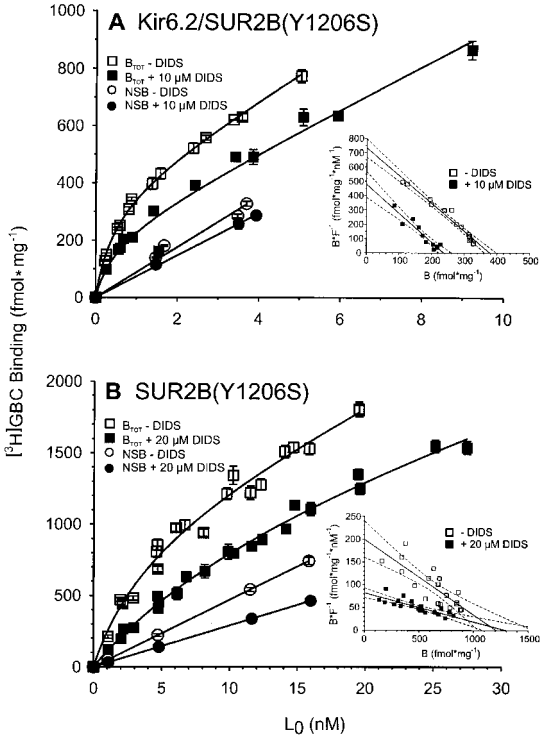

Figure 8 [^{3}H]-glibenclamide saturation binding in the absence and presence of DIDS. (A) Kir6.2/SUR2B(Y1206S) in the presence of Mg^{2+} (0.7 mM) at 22°C. Data show total (B_{TOT}) and nonspecific binding (NSB) in the absence and presence of DIDS (8 μM). The fit of eqn (3) to B_{TOT} gave the B_{MAX} and K_D values listed in Table 4. The inset shows the data in the Scatchard transformation to highlight non-competitive inhibition of glibenclamide binding by DIDS. Data are pooled from three experiments; binding experiments ± DIDS were performed in parallel. (B) [^{3}H]-glibenclamide binding to SUR2-B(Y1206S) in the absence of Mg^{2+}, at 37°C, and in the absence and presence of DIDS (20 μM). Note the competitive appearance of the inhibitory effect of DIDS.

Discussion

It has previously been shown that, in the absence of Mg^{2+}, high concentrations of DIDS irreversibly inhibited K_{ATP} channels by binding to Kir6.2 (Furukawa *et al*., 1993; Proks *et al*., 2001). This study describes two new findings: First, Mg^{2+} sensitizes the K_{ATP} channel for the blocking action of DIDS in a nucleotide independent manner and, second, as sensed by [^{3}H]-glibenclamide, the interaction of DIDS with (mutant) SUR2B is modulated by Mg^{2+}.

Mg^{2+} modulation of DIDS block

In the absence of Mg^{2+}, the sensitivity of the recombinant K_{ATP} channels (Kir6.2/SURx) to inhibition by DIDS

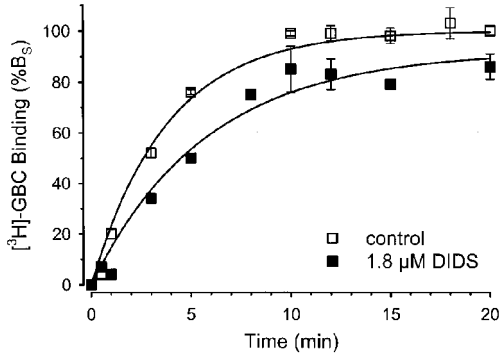

Figure 9 Kinetics of [^{3}H]-glibenclamide binding to Kir6.2/SUR2B (Y1206S) after preincubation with DIDS. Membranes (3.9 mg protein ml^{-1}) were preincubated with DIDS (30 μM) or solvent in the presence of Mg^{2+} (0.7 mM) for 45 min at 22°C. At time 0, membranes were diluted 1 : 17 into [^{3}H]-glibenclamide (2 nM) containing solution and the binding kinetics were measured (final DIDS concentrations 0 and 1.8 μM). Data are means from three experiments. The fit of an exponential to the data gave time constants of 0.26 ± 0.02 min^{-1} (control) and 0.17 ± 0.03 min^{-1} (DIDS), corresponding to half-times of 2.7 and 4.0 min, respectively.

Table 4 Effect of DIDS on [^{3}H]-glibenclamide saturation binding in the absence of ATP

Receptor	*Temp.* (°C)	Mg^{2+} (mM)	*DIDS*[1] (μM)	K_D (GBC) (nM)	B_{MAX} (pmol mg^{-1})	K_i (DIDS)[2] (μM)
Kir6.2/SUR2B(Y1206S)	22	0.7	0	0.46 (0.44,0.48)	0.37 ± 0.04#	
			10	0.48 (0.28,0.83)	0.24 ± 0.01#	not applicable
SUR2B(Y1206S)	37	0	0	4.5 (3.1,6.1)#	1.1 ± 0.1	
			20	15 (12,18)#	1.2 ± 0.1	9.6 (4.8,19)

Respective experiments in the absence and presence of DIDS were done in parallel and parameters were obtained by analysing individual experiments ($n = 2-4$) according to eqn (1). # Denotes a significant difference ($P < 0.01$) between the respective parameters in the presence and absence of DIDS. [1]DIDS concentration chosen in the range of the respective IC_{50} value (Table 3). [2]K_i values for DIDS were calculated according to eqn (2) for each saturation experiment separately and then averaged.

decreased with the rank order SUR1 > SUR2A ~ SUR2B (Table 1, values at 90 s). The rank order SUR1 > SUR2A agrees with the observation of Proks *et al.* (2001) and the IC_{50} value of 180 μM for the Kir6.2/SUR2A channel is in reasonable agreement with that determined by Furukawa *et al.* (1993) for the K_{ATP} channel in guinea-pig cardiocytes (73 μM). These values are in the range of inhibition constants observed for various anion transporter and channels (Furukawa *et al.*, 1993). The presence of physiological concentrations of Mg^{2+} increased the sensitivity towards DIDS in a manner depending on the SUR subtype with the most dramatic effect occurring with the Kir6.2/SUR2B channel: Here the IC_{50} value of DIDS was shifted by Mg^{2+} to 70 fold lower concentrations and the rank order of sensitivity towards DIDS was now SUR2B > SUR1 > SUR2A.

This dependence on the SUR subtype raised the possibility that Mg^{2+} exerted its effect by binding to SUR. More direct evidence for this possibility came from the observation that Mg^{2+} did not affect the inhibition by DIDS of the Kir6.2Δ26 channel, which forms a channel in the absence of SUR (Tucker *et al.*, 1997); however, sensitivity to Mg^{2+} was restored by coexpression with SUR2B. These experiments exclude the possibility that, similar to EDTA, Mg^{2+} forms a complex with DIDS, thereby changing the chemical nature of the blocker. Instead, they show that the presence of SUR is required for Mg^{2+} to be effective. It seems plausible to assume that Mg^{2+} binds to SUR to elicit its effect; the ability of Mg^{2+} to affect DIDS binding to SUR2B(Y1206S) in the absence of Kir6.2 supports this hypothesis. However, more work like mutating the conserved Asp residue in the Walker B motif of the NBFs (Nichols *et al.*, 1996) or other residues possibly involved in coordinating Mg^{2+} (Gaudet & Wiley, 2001; Urbatsch *et al.*, 2000a,b) must be done to definitively prove this point.

Another point of interest is that Mg^{2+} did not require the presence of a hydrolysable nucleotide like ATP or ADP in order to affect channel inhibition by DIDS. This is again matched by the results of the binding studies (Table 3). Comparison with sulphonylureas shows similarities and differences: Similar to DIDS, the potency of tolbutamide in blocking the cardiac K_{ATP} channel is increased by Mg^{2+} in the absence of nucleotide (Miyamura *et al.*, 2000); however, unlike in the case of DIDS, Mg^{2+} alone does not affect glibenclamide binding to SUR2B(Y1206S) (Hambrock *et al.*, 2002) and the presence of the Mg-nucleotide complex is required to inhibit binding. A further point is that in the presence of Mg^{2+}, exposure of the channel even to low concentrations of DIDS ($\geqslant$10 μM) led to irreversible inhibition and that a sufficiently large excess of MgATP afforded protection.

Interaction of DIDS with SUR

It was shown earlier that DIDS inhibits binding of [^{3}H]-P1075 to SUR2B with an IC_{50} value of 1.6 μM (3 mM MgATP, 37°C; Russ *et al.*, 2000). Here we showed that DIDS inhibited [^{3}H]-glibenclamide binding to (mutant) SUR2B in a manner dependent on Mg^{2+} and on coexpression with Kir6.2. With mutant SUR alone, Mg^{2+} induced a leftward shift and a flattening of the DIDS inhibition curve. This means that Mg^{2+} produced an apparent heterogeneity in the binding sites for DIDS by inducing a new class of sites with higher affinity (>40 fold) and leaving the other part essentially unchanged. Coexpression with Kir6.2 reduced this heterogeneity and reduced the Mg^{2+} shift to a factor of ~5. In the presence of MgATP, the effect of coexpression was even more prominent: Whereas for mutant SUR2B alone MgATP induced a ~5 fold leftward shift of a homogenous inhibition curve (data at 37°C), it induced strong heterogeneity in the binding curve of the Kir/SUR complex (Table 3).

The biphasic inhibition curve for mutant SUR2B in the presence of Mg^{2+} and the fact that the relationship between DIDS and glibenclamide has a competitive or a non-competitive appearance depending on the assay conditions (Figure 8) suggested that SUR harbours more than one binding site for DIDS. However, these complexities may also be interpreted assuming that SUR expressed alone forms tetramers (Löffler-Walz *et al.*, 2002; Hambrock *et al.*, 2002) and that site-site interactions between subunits occur. More importantly, the inhibition of [^{3}H]-glibenclamide binding to the Kir6.2/SUR2B(Y1206S) complex by DIDS (30 μM) was entirely reversible with a half-time of 4 min.

Since binding of DIDS and glibenclamide to mutant SUR2B was measured at 22 and 37°C (Table 3), one obtains a rough estimate of the thermodynamics of binding. The K_i values of DIDS ($-Mg^{2+}$) did not change much with temperature, indicating a negligible change in free enthalpy (ΔH) for DIDS binding to SUR2B(Y1206S). In view of the two negative charges carried by DIDS this is surprising. In contrast, the K_D values of glibenclamide binding strongly decreased with decreasing temperature. From the two values in Table 3 one estimates $\Delta H \sim -8.5$ kcal mol^{-1} for this interaction in good agreement with the value determined for glibenclamide binding to rat brain cortex and heart (-10.5 and -13.5 kcal mol^{-1} respectively; Gopalakrishnan *et al.*, 1991).

Does DIDS binding to SUR mediate channel block?

In the absence of Mg^{2+}, the IC_{50} value of DIDS binding to Kir6.2/SUR2B(Y1206S) was ~5x lower than the IC_{50} value for block of the Kir6.2/SUR2B channel. This difference can be explained by assuming that there are four equal and independent binding sites for DIDS and that occupation of all of them is required for channel block (Dörschner *et al.*, 1999; Russ *et al.*, 1999). However, preliminary experiments with other SUR subtypes show discrepancies of 10× (SUR1) and 20× (mutant SUR2A; Quast, unpublished results), rendering this explanation unlikely. More importantly, Proks *et al.* (2001) have demonstrated that in the absence of Mg^{2+}, DIDS blocked recombinant K_{ATP} channels by binding to the Kir6.2 subunit at a site interacting with, but different from the ATP site. Our data with the Kir6.2Δ26 channel and the Kir6.2(G334D) mutant confirm and extend these results. One has to conclude that, in the absence of Mg^{2+}, binding of DIDS to SUR is not transduced into channel inhibition.

In the presence of Mg^{2+}, the binding studies involving the Kir/SUR complex and the electrophysiological experiments gave several concordant results. First, Mg^{2+} moved both inhibition curves leftwards and the IC_{50} values were similar (7.9 vs 2.7 μM). Second, MgATP (1 mM) shifted most of the

binding sites to low affinity (200 μM) which is compatible with the decreasing protection afforded against increasing concentrations of DIDS (30, 100, 300 μM) in the electrophysiological experiments. However, the fact that binding of DIDS (30 μM) to the channel complex as seen by [^{3}H]-glibenclamide was entirely reversible whereas channel inhibition was irreversible clearly shows that the binding site seen by radioligand is not the one which mediates channel inhibition. Therefore one is left with the tentative model that Mg^{2+}, probably by binding to SUR, allosterically affects the binding site for DIDS on Kir6.2, rendering it more accessible and/or more reactive. This allosteric effect depends on the SUR subtype. Obviously, this model could be tested using some of the mutants recently described by Proks *et al.* (2001).

In conclusion, we have shown here that Mg^{2+} sensitizes the K_{ATP} channel for the blocking action of DIDS in a manner dependent on the SUR subtype. The fact that Mg^{2+} was effective in the absence of nucleotides highlights its role in signal transduction in K_{ATP} channels.

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